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ORIGINAL ARTICLE

Oxidative stress parameters and histopathological lesions in the liver and kidneys of manganese intoxicated rats

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ABSTRACT

The aim of the research was to verify the theory of the free radical interaction of manganese compounds by assessing the activity of the enzymes: catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) in an experimental rat model of exposure to Mn. Forty five male Buffalo rats were divided into: control, group I (MnCl2; 5 mg/kg b.w.) and groUp II (MnCl2; 7.5 mg/kg b.w.). The activity of the enzymes: CAT, GPx, GR and GST activity were determined in the supernatants of the kidneys and liver. The histopathological alterations were evaluated in tissue sections. In rats intoxicated with Mn (group I and II) the decreased CAT and GR activities was observed in the liver, with a significantly lower the all enzymes activities measured in the kidneys. The the lowest CAT, GPx, GR and GST activity were observed in the kidneys of group II rat. An examination of hepatic tissues revealed moderate (group I) and severe (group II) histopathological lesions, with a insignificant lesions oserved in the renal tissues. It has been shown that manganese intoxication has cytotoxic activity in the kidneys and liver, causing an decrease in antioxidant enzymes activity. The observed histopathological changes in the liver tissue suggest sensitivity to oxidative stress in this organ. In the kidneys, despite a significant weakening of the antioxidant defense, insignificant histopathological lesions were found, which suggests a different defense mechanism independent of the antioxidant enzymatic defense.

KEY WORDS: manganese; oxidative stress; rats; antioxidant enzymes; histopathological lesions; liver; kidneys

Introduction

Manganese (Mn) is an essential trace metal found in all tissues, essential for the proper metabolism of amino acids, lipids, proteins and carbohydrates in vivo [1, 2]. While Mn deficiency is extremely rare in humans, toxicity from overexposure to Mn is more common, especially in mining and welding workers who are chronically exposed to aerosols and dusts containing Mn [3, 4]. Chronic Mn poisoning in humans causes permanent neurodegenerative damage in the nigrostriatal area, resulting in Parkinson's-like syndrome [3, 5, 6, 7, 8].

Therefore, most of the research related to Mn has focused on its neurotoxicity, but not on its effects on other organs. Experimental models and studies of the human brain suggest that manganese exposure is related to oxidative stress, which may play an important role in neuronal degeneration [5, 9]. However, apart from its evident neurotoxicity, Mn affects several other target organs, especially those prone to the accumulation of Mn and possibly damaged as a result of the deposition. The main organ involved in the metabolism of Mn is the liver, which controls the redistribution of excess Mn to specific tissues/organs and hepatobiliary clearance [10]. Chronic exposure to Mn causes the liver to overflow, which transports Mn to other organs [11]. However, little research has been done on the adverse effects of Mn on the liver as well as on the kidneys. Ponnapakkam et al. Reported that male rats consuming Mn in the amount of 87 mg/kg b.w. there is glomerular glazing, glomerulonephritis and a very common nephrolithiasis [12]. Chronic nephropathy was observed when rats were administered 200 mg Mn/kg b.w / day for 2 years [13].

Until now, the mechanism of the toxicity of manganese remains unclear. Since the manganese outer electron

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shell can donate up to seven electrons, Mn can assume 11 different oxidation states. Different oxidation states result in the formation of Mn species with different ion size and reactivity [14]. As a metal ion, Mn is toxic due to the increase in the formation of reactive oxygen species (ROS) and by-products of catecholamine oxidation [15]. Probably the basis of most changes generated by this element is oxidative stress, quite well studied in the nervous and respiratory systems, but very poorly understood in the case of the effect of this element on other tissues. The aim of the research is to try to determine the mechanism of manganese toxicity, in particular, to verify the theory of the free radical effect of compounds of this element on the liver and kidneys.

Methods

Research on animals based on the consent of the 1st Local Ethical Committee for Experiments on Animals of the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław, opinion no. 55/03 and 43/05.

45 male Buffalo rats were used in the experiments. Before starting the experiment, each animal was weighed. The average weight of the rat was 174±18.1 g. The animals were divided into three groups (15 each) based on the manganese dose they received, according to the following rule: animals receiving 0.9% NaCl solution (saline) constituted a control group; rats administered an aqueous solution of $MnCl_2$ at the dose of 5 mg/kg b.w. Mn^{2+} ions, included in group I; animals treated with an aqueous solution of $MnCl_2$ at a dose of 7.5 mg/kg b.w. Mn^{2+} ions were included in group II.

All solutions were administered to the rats by the intraperitoneal route, 3 times a week for two months. The administered volume of the solutions was always constant, approximately 1.0 mL (depending on the weight of the animal). The total dose of manganese administered and absorbed by each animal (assuming 100% availability of Mn2+ ions administered by the intraperitoneal route) was: 0 mg in the control group, 120 mg/kg b.w. in group I and 180 mg/kg b.w. in group II.

After the completion of the exposure, each animal was weighed on the day of sacrifice. Animals from the control group weighed 258±26.8 g on average, animals from group I weighed an average of 248±23.9 g, and rats from group II had a mean body weight of 256±18.2 g. There were no statistically significant differences between the body weights of the animals from the individual groups.

After weighing, the rats were sacrificed according to the adopted procedure. Each rat received ketamine intramuscularly, which made the animal anesthetized, and then the cervical vertebrae dislocated. After the animals were sacrificed, the body cavities were opened for the preparation of the following organs: liver and both kidneys. The organs, after dissection and cleaning of unnecessary tissues, were rinsed in cold (4 °C) saline solution in order to rinse out any residual blood and quickly cool the organs down. The organs were then divided into two pools: for biochemical examination and for histopathological examination. The organs intended for histopathological examination were fixed in a buffered 4% formaldehyde solution. Tissues intended for biochemical tests were frozen quickly and stored at −81 °C.

Before starting the biochemical analysis, the frozen tissues were prepared, at 4 °C, with homogenates in phosphate buffered saline with 0.5% Triton X100 detergent. The resulting homogenates were centrifuged at 10.000 rpm for 10 minutes at 4 °C. After separating the supernatant, the protein concentration (in g/L) and the activities of catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase were determined. The values obtained were converted into gram of protein, and reported in units of IU/g of protein.

Determination of the activity of antioxidant enzymes

Catalase

The catalase (CAT) activity was determined by the spectrophotometric method [16]. Measurements were made using a PowerVave XS ELISA reader (BioTek, USA) at a wavelength of 550 nm. Calibration was performed using a series of standard solutions of formaldehyde at concentrations of 8–512 µmol/L. Activity calculations were made based on the assumption that 1 IU of catalase is the amount of enzyme that oxidizes 1 µmol of substrate per minute under the conditions of the method. The obtained results were converted into IU/g with correction for the determined protein concentration in the tested material. The markings were made twice. The method variation coefficient was 6.8%.

Glutathione reductase

Glutathione reductase (GR) determinations were made on diluted PBS tissue homogenates with 0.1% bovine serum albumin. GR activity was determined at 37°C in accordance with the instructions for the Bioxytech kit. GR activity was determined by the kinetic-spectrophotometric method, and the readings were made using the Technicon RA-XTTM System Technicon® biochemical analyzer (Technicon Instruments Corporation, USA) at 340 nm. The results were converted into U/g of protein in the homogenate, after prior determination of its concentration in the tested material. The coefficient of variation for these determinations was 4.21%.

Glutathione peroxidase

The activity of glutathione peroxidase (GPx) was also determined by the kinetic-spectrophotometric method. The Bioxytech kit was used for the analysis. The measurement was made at 37°C with a Technicon RA-XTTM System Technicon® Analyzer (Technicon Instruments Corporation, USA) at a wavelength of 340 nm. The results were converted into U/g protein taking into account the determined protein concentrations in the tissue homogenates and the dilution of the samples. The coefficient of variation for this method of determination was 1.82%.

Glutathione S-transferase

The activity of glutathione S-transferase (GST) was determined using the kinetic-spectrophotometric method according to the instructions provided with the kit from Cayman Chemical Company (USA). The determinations were made at 37 °C with the Technicon RA-XTTM System Technicon® biochemical analyzer (Technicon Instruments Corporation, USA) at 340 nm. The obtained results were converted into U/g protein, taking into account the protein concentration in the tissue homogenates and corrected for dilution of the samples. The determined coefficient of variation for this method of determining GST activity was 2.73%.

Total protein concentration determination

The concentration of total protein in the tissue homogenates was determined by the pyrogallol red method using the reagent kit from Sentiel Diagnostics (Italy). The determination was carried out by direct colorimetric method at 37 °C using the Technicon RA-XTTM System Technicon® biochemical analyzer (Technicon Instruments Corporation, USA) at a wavelength of 600 nm. The determined coefficient of variation for this method was 2.07%.

Histopathological preparations

Tissues fixed in formaldehyde solution were thoroughly rinsed in water, and then chemically treated and embedded in paraffin. The embedded tissue pieces were cut on a microtome, transferred to a glass slide, and stained with hematoxylin and eosin. The fixed preparations were assessed at 40, 100 and 400 times magnification.

Statistical analyses

The results obtained on the basis of average values of the activity of each enzyme per gram of total protein. The STATISTICA 11.0 program was used for statistical calculations. The compliance of the distributions of variables with the normal distribution was checked using the Shapiro-Wilk test. Student's t-tests with correction for small groups were used for comparisons between groups. Significance levels $p<0.05$ were considered statistically significant.

Results

Liver

The liver is an organ that plays a leading role in the biotransformation of manganese. This organ is enzymatic rich, with a particularly high activity of glutathione S-transferase and catalase. Catalase activity was significantly changed in the livers of animals intoxicated with manganese chloride. In the control group, the activity of this enzyme was at the level of 59.83±10.04 IU/g of protein. Animals exposed to lower doses of manganese (group I) had a 26.4% reduction in the activity of catalase in the liver compared to the control group ($p<0.005$). The activity of CAT in the livers of this group of animals was 44.05±8.87 IU/g of protein. An even greater decrease in activity was noted in the livers of animals intoxicated with higher doses of manganese (group II). In this group of animals there was a decrease in activity by 28.4% compared to the control group ($p<0.0005$), ie to the value of 42.87±9.03 IU/g protein (Figure 1a).

There was no statistically significant change in glutathione peroxidase activity in the livers of animals intoxicated with manganese. In the control group of

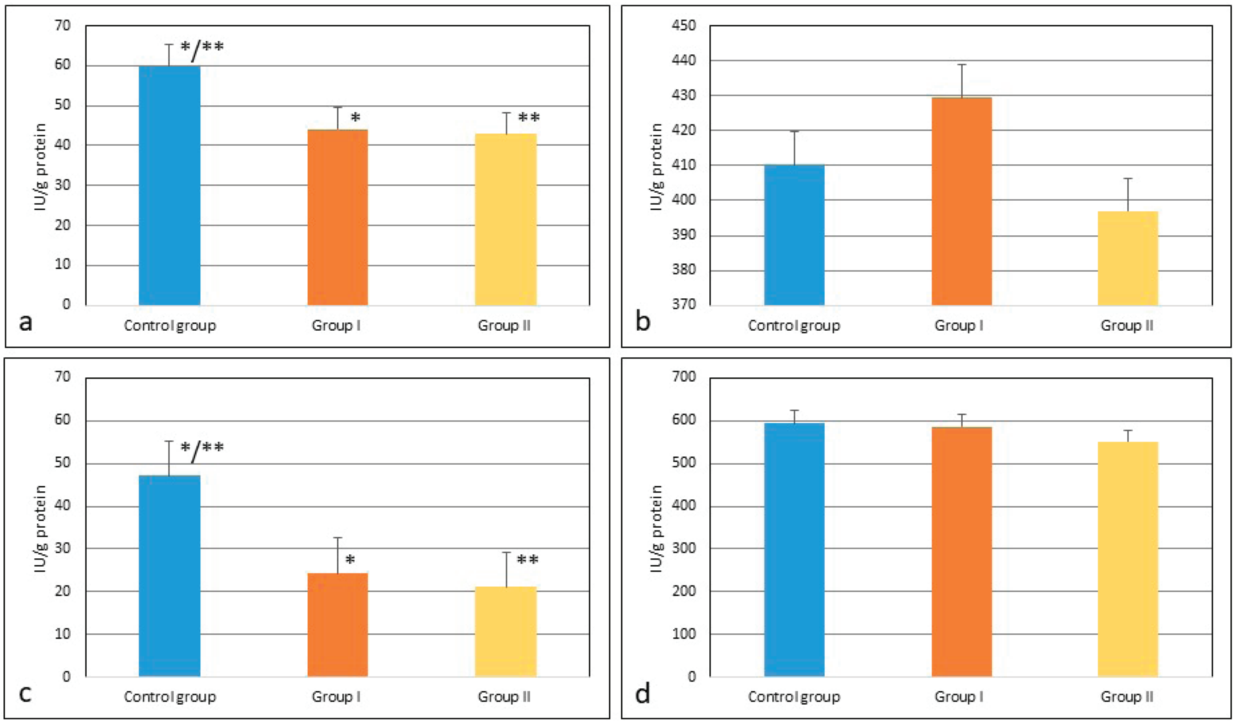

Figure 1. Enzyme activity in livers (in IU/g protein): a. Catalase; b. glutathione peroxidase; c. Glutathione reductase; d. Glutathione S-transferase. * group I / control group $p<0.005$ ** group II / control group $p<0.0005$

animals, the GPx activity in this organ was 410.2 65.8 IU/g of protein. In the group of animals exposed to lower doses of Mn, the activity of glutathione peroxidase increased to 429.4±42.0 IU/g of protein, while in the livers of animals exposed to higher doses of manganese, the activity of this enzyme decreased to 397.0±43.7 IU/g protein (Figure 1b).

The activity of GR in the livers of animals from the control group was 47.13±23.91 IU/g of protein. Animals exposed to lower doses of manganese, as compared to the control group, had a decreased GR activity in the livers by 48.4% (p<0.05), ie to the value of 24.34±13.24 IU/g of protein. In animals exposed to higher doses of Mn (group II), the decrease in glutathione reductase activity was even greater. Compared to the control group, GR activity was reduced by 55.1% to a value of 21.18±14.52 IU/g protein (p<0.005) (Figure 1c).

The activity of GST in the livers changes only slightly under the influence of manganese. In the control group, the activity of glutathione S-transferase was 593.8±64.1 IU/g of protein. In both groups of animals exposed to manganese compounds, a statistically insignificant decrease in the activity of this enzyme was observed. In group I, the GST activity decreased only slightly and amounted to 585.0±58.3 IU/g of protein. In the livers of animals exposed to higher doses of Mn (group II), the activity of glutathione S-transferase was 550.3±44.3 IU/g of protein (Figure 1d).

As mentioned above, the liver plays an essential role in the metabolism of manganese. On the one hand, this organ may be particularly sensitive to the action of the element, and on the other hand, it is characterized by significant regenerative potential – this means that in the livers of experimental animals, a large histopathological differentiation can be expected.

Passive hyperemia, secondary to the death of violent animals, was observed in each of the groups. In the livers of animals exposed to lower doses of manganese (group I), rather sparse infiltrates consisting of lymphoid cells and neutrophils were noted,with a marked tendency to form small clusters – abscesses. In the livers of rats from group II, the changes were much more extensive, with the formation of massive abscesses, mainly subcapsular, with visible necrosis of hepatocytes and necrotic hemorrhages separated by a demarcation line composed of neutrophils. Moreover, disseminated eosinophilic infiltrates were observed in the liver parenchyma (Table 1).

Table. 1. Histopathological lesions in the livers.

lesions	Control group	Group I	Group II
Passive congestion	+	+	+
Eosinophils infiltrates	–	–	+
Neutrophils infiltrates	–	+	+++
Features of marginal necrosis	–	+	++
Subcapsular abscesses	–	–	+++

Legend: (-) no lesions; (+) current lesion; (++) very marked intensification of lesions; (+++) severe lesions

Kidneys

The kidneys play an essential role in removing unnecessary metabolites from the body.

In the case of manganese, however, they are not so important, because this element is removed primarily in the faeces. The kidneys contain a very high concentration of a protein with a strong ability to bind divalent metals, including manganese, which may be important in the effect of Mn on this organ. In the biochemical tests of kidneys of animals exposed to manganese, statistically significant changes in the activity of all determined enzymes were observed.

In the control group, the CAT activity was 84.67±12.10 IU/g of protein. In the kidneys of animals exposed to manganese at a dose of 5 mg/kg b.w. (Group I) there was a decrease in the activity of this enzyme by 29% compared to the control group (p<0.005), to the value of 60.15±12.76 IU/g of protein. Administering manganese in rats at the dose of 7.5 mg / kg b.w. (Group II) reduced the catalase activity by as much as 44.8%, to the level of 46.77±6.66 IU/g of protein (p<0.0005). The change in catalase activity in group II was so significant that the difference in CAT activities between groups II and I was also statistically significant (p<0.005) (Figure 2a).

The activity of glutathione peroxidase in kidney of rats from the control group was 628.83±61.56 IU/g of protein. In both groups of animals exposed to manganese, a decrease in GPx activity was observed. In the kidneys of rats from group I, the activity of glutathione peroxidase was reduced by 15.6%, to the value of 530.66±37.58 IU/g of protein (p<0.001). Group II animals had a reduced GPx activity in the kidney by 18.6% to a level of 512.20±32.90 IU/g protein (p<0.0005) (Figure 2b).

The activity of GR in the kidneys was significantly reduced after exposure of the animals to manganese compounds. In the control group of animals, kidney glutathione reductase activity was 158.70±24.93 IU/g of protein. GR activity in kidneys of animals exposed to lower doses of manganese (group I) was lower than in the control group by 25.1% and amounted to 122.01±14.49 IU/g of protein (p<0.05). Higher doses of manganese induced even more severe disturbances, expressed as a decrease in glutathione reductase activity in the kidneys of group II rats by 59.3% (p<0.0005). GR activity in a group of rats intoxicated with manganese at the dose of 7.5 mg/kg b.w. was 80.49 10.75 IU/g. A statistically significant difference was also found between the glutathione reductase activities in the kidneys of the animals of groups I and II (p<0.001) (Figure 2c).

Although the kidneys also carry out the biotransformation process (albeit to a much lesser extent than the liver), the activity of glutathione S-transferase in this organ is not high. In the control group of animals, the GST activity in the kidneys was 66.46±15.51 IU/g of protein. The influence of manganese on the activity of glutathione S-transferase in the kidney is very clear. In the group of animals exposed to the dose of 5 mg Mn/kg b.w. glutathione S-transferase activity was reduced by 35.3% to the level of 43.02±16.32 IU/g of protein (p<0.005). Decrease in

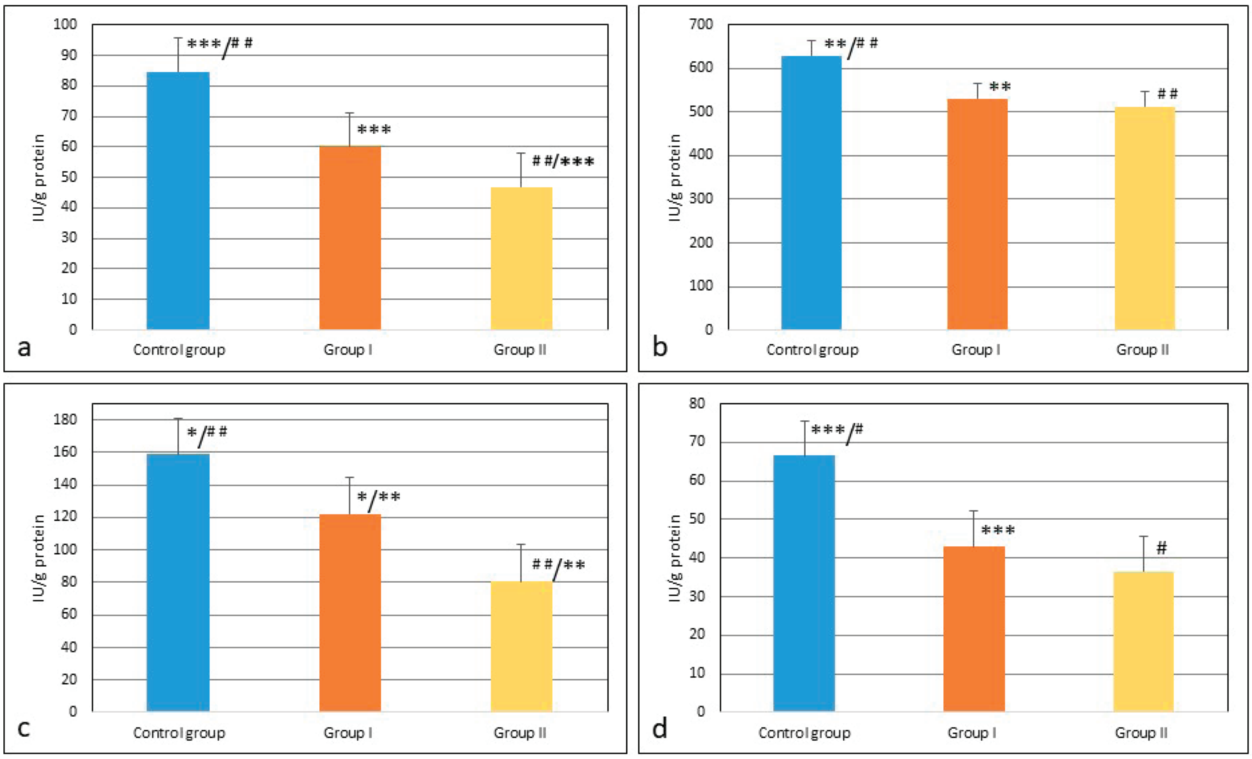

Figure 2. Enzyme activity in kidneys (in IU/g protein): a. Catalase; b. glutathione peroxidase; c. Glutathione reductase; d. Glutathione S-transferase. * $p<0.05$; ** $p<0.001$; *** $p<0.005$; # $p<0.0001$; ## $p<0.0005$

GST activity in kidneys of animals exposed to manganese at a dose of 7.5 mg/kg b.w. was 45% ($p<0.0001$). The GST activity in the kidney homogenates of group II animals was 36.56±11.15 IU/g protein (Figure 2d).

Despite profound biochemical disturbances in the kidneys of animals exposed to manganese, no significant histopathological lesions were found in this organ. The kidneys appear to be particularly protected against the effects of manganese. It seems that metallothionein may play a protective function. This phenomenon is discussed in more detail later in the study.

As in the case of the liver, the common lesion in the kidneys for all groups of animals was passive hyperaemia. No pathologies were found in the kidneys of the animals from group I. In the kidneys of animals from group II, only one preparation showed small, diffuse infiltrates from lymphoid cells (Table 2).

Correlation of the activity of antioxidant enzymes

In order to assess the mutual influence of the activity of individual enzymes on each other, the Persona linear correlation coefficients were determined between the variables, which were the activities of individual enzymes in organs. The results obtained are presented below.

The activity of the measured antioxidant enzymes in the liver parenchyma was characterized by a significant, positive correlation. Catalase activity positively correlated with the activity of glutathione reductase ($r^2=1.00$) and glutathione S-transferase ($r^2=0.70$). The activity of glutathione S-transferase positively correlated with the activity of glutathione peroxidase ($r^2=0.68$) and glutathione reductase ($r^2=0.74$). There was no relationship between the activities of the other enzymes (Table 3).

Table. 2. Histopathological lesions in the kidneys.

lesions	Control group	Group I	Group II
Passive congestion	+	+	+
Minor infiltrates of lymphoid cells	–	–	+/–

Legend: (-) no lesions; (+/-) current lesion in only one preparation; (+) current lesion

Table. 3. Pearson linear correlation coefficients for enzyme activity in livers.

	CAT	GPx	GR	GST
CAT		– 0.04	1.00	0.70
GPx			0.01	0.68
GR				0.74

Legend: CAT - catalase; GPx - glutathione peroxidase; GR - Glutathione reductase; GST- glutathione S-transferase

Table. 4. Pearson linear correlation coefficients for enzyme activity in the kidney.

	CAT	GPx	GR	GST
CAT		0.98	0.98	0.99
GPx			0.92	1.00
GR				0.94

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Strong positive correlation coefficients between the activities of all enzymes were observed in the kidneys of animals exposed to manganese. Catalase activity was strongly positively correlated with glutathione peroxidase (r^2=0.98), glutathione reductase (r^2=0.98) and glutathione S-transferase (r^2=0.99). The activity of glutathione peroxidase was strongly positively correlated with glutathione reductase (r^2=0.92) and glutathione S-transferase (r^2=1.00). The activity of glutathione reductase also strongly positively correlated with the activity of glutathione S-transferase (r^2=0.94) (Table 4).

The study of the correlation of enzyme activity shows that:

- the activity of catalase, glutathione peroxidase and reductase strongly positively correlated with the activity of glutathione S-transferase,
- the activity of catalase was strongly positively correlated with the activity of glutathione peroxidase in the kidneys and glutathione reductase in the liver and kidneys,
- glutathione peroxidase activity strongly positively correlated with GR activity in kidneys.

Discussion

In the present study, exposure to manganese was carried out by administering to the animals by the intraperitoneal route of manganese (II) chloride solutions. After the manganese got through from the peritoneum to the blood, in the first stage it goes through the portal system to the liver, from where it is distributed to other organs of the body only later. Due to the fact that the liver is the organ which receives the „shock concentration" of manganese, and is the main organ biotransforming this metal, it could be expected that intraperitoneal exposure to manganese in the liver would cause the greatest biochemical and histopathological changes.

In the presented study, it was observed that exposure to manganese causes a decrease in catalase activity in organs showing the physiologically highest activity of this enzyme, i.e. in the kidneys and livers. A phenomenon different from that observed was described by the team of Czeczot et al., who exposed isolated rat hepatocytes to increasing doses of cadmium [17]. These authors presented a time-dose-effect relationship consisting in increasing changes in the activity of antioxidant enzymes depending on the administered dose and exposure time. Czeczot et al. showed that short-term exposure to cadmium leads to an increase in the activity of catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase. This change in enzyme activity was probably secondary to the increased radicalogenesis. A different behavior of catalase, i.e. a decrease in its activity, was described in the case of long-term exposure of hepatocytes to cadmium ions. The described results indicate differentiated activity of catalase after exposure to oxidative stress – with short-term exposure its activity increases, with longer exposure it decreases. In the conducted studies, the exposure of animals to manganese lasted 2 months, so a decrease in catalase activity could be expected, which was also observed, and in line with the results presented by Czeczot et al [17].

When considering the reasons for the decrease in catalase activity in the livers and kidneys of manganese exposed animals, several mechanisms should be considered. One of the reasons for the decrease in the activity of this enzyme may be the direct interaction of manganese ions on enzyme proteins. Such a mechanism is proposed, among others, by Jena et al. [18]. A slightly different position is taken by Qato and Maines, who claim that the decrease in CAT activity is to be associated with manganese-induced disturbance of heme metabolism in the brain [19]. The heme and hemoprotein synthesis is particularly high in the liver and kidneys, where a significant decrease in catalase activity has been observed. Another concept of the influence of manganese on the activity of catalase can be derived from the research of the Casalino et al. [20]. Casalino et al. conducted research on the mechanisms of cadmium influence on the activity of antioxidant enzymes in the liver and kidneys of rats. They found that the activity of catalase could be reduced for two reasons, ie reducing its synthesis and blocking the enzyme's active site. The reduction in catalase synthesis is related to the effect of cadmium on the binding of divalent iron to the prosthetic group of catalase [20]. This phenomenon, seen with cadmium, can also be extended to other transition metals, including manganese. After all, it has been described that manganese has a particularly strong influence on the absorption, and thus the availability, of iron. Chronic exposure to manganese leads to a decrease in iron absorption, and thus to a decrease in the available pool of this element, which in turn causes a decrease in the synthesis of ferroproteins, including catalase. Such a phenomenon was observed in the presented study. A second mechanism leading to a decrease in catalase activity may be related to blocking the active site. In the case of cadmium, the inhibition of CAT is associated with the binding of Cd2+ ions to the imidazole residue of His-74, which consequently leads to the loss of the catalase's ability to degrade hydrogen peroxide [20]. The described mechanism is very interesting, but it is difficult to relate it to the effect of manganese on catalase, as no such research has been found in the available literature.

It should be noted that the conducted experiment was based on chronic exposure to a compound with potential pro-oxidative properties. The radicalogenesis intensified by manganese should lead to the gradual depletion of cellular defense mechanisms, which in turn should be reflected not only in a decrease in the concentration of low molecular weight antioxidants, but also in a decrease in the activity of enzymes removing free radicals. Such a phenomenon has been observed with regard to catalase in the liver and kidney, as well as glutathione peroxidase in the kidney.

Catalase is also important in inflammatory processes and protection against apoptosis [21, 22, 23]. Acute inflammation shows an increase in catalase activity and is rather of a defensive role [24]. However, in the case of long-term oxidative stress, a decrease in the activity of this enzyme is observed [25], as in the present study.

Both the disturbances in catalase activity and the interaction of manganese on the cell itself can lead to its death. Some studies indicate that Mn disrupts the mitochondria level, leading to increased generation of reactive oxygen species. ROS can lead to apoptosis or necrosis [26, 27]. Programmed cell death, in this case related to manganese exposure, is also associated with an increase in the number of intranuclear DNA breaks, activation of JNK and p38 proteins (the so-called stress-activated kinases) or activation of caspase-3 [27, 28, 29].

Mitochondrial dysfunction leading to cell death may be related to two other mechanisms:

1. Intracellularly, manganese very quickly flows from the cytoplasm into the mitochondria, causing calcium to accumulate in these organelles. This phenomenon is related to the inhibition of both the sodium-dependent and the sodium-independent pump by manganese [30],
2. Manganese also causes disorders of oxidative phosphorylation by inhibiting both mitochondrial F1-ATPase [30] and complex I [31], as a consequence of which there is a decrease in ATP production [32, 33].

Disruptions in iron metabolism, discussed above in the presentation of the influence of manganese on catalase activity, are also associated with inflammatory processes. As it is known, the transporter-1 of divalent metals (DMT1) is responsible for the proper management of metallic elements at the cell level. Some researchers argue that DMT1 is also responsible for the toxicity of manganese. Expression of DMT1 is regulated at many levels, incl. at the stage of transcription, translation and post-translational processes. Such complex regulation is necessary to keep the cell safe by regulating the level of essential ions in the cell. This applies to a situation when a stress factor appears, e.g. exposure to manganese [34, 35], or the appearance of an inflammatory process with the release of a number of pro-inflammatory cytokines, e.g. interleukin I beta, IFNγ or TNFα alpha . All of these factors induce the induction of the factor NF-κB which stimulates the transcription of the DMT1 variant 1B [36, 37].

Changes in DMT1 expression have a profound effect of accumulation of iron and other divalent metals in the central nervous system during times of stress or inflammation. This phenomenon correlates with observations of abnormal Fe accumulation in many neurodegenerative disorders, e.g. Friedrich's ataxia, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease and vertical diseases [38, 39, 40].

Glutathione peroxidase, like catalase, removes hydrogen peroxide. It also has the ability to remove lipid hydroxides produced in lipid peroxidation processes. In the presented study, a decrease in the activity of glutathione peroxidase in the kidneys was observed. These changes significantly expose the cell to damage caused by reactive oxygen species. It has been proven that both the high activity of GPx and the high concentration of reduced glutathione protect cells against oxidative damage [41]. The presented results may suggest that the manganese toxicity may be due to not only a disturbance in the catalase activity, but also a decrease in the activity of glutathione peroxidase, leading to a decrease in the concentration of reduced glutathione. Such a mechanism was also postulated by Ivakhnenko et al. [42].

It has been proved that metallothioneins (MT), in particular the MT-1 and MT-2 isoforms protect cells not only against heavy metals, but also essential metals, but those found in the cell in increased concentration (e.g. as a result of intoxication). Metallothionein, in addition to the described function, has the ability to „scavenge" free radicals, and thus supports the enzymatic antioxidant defense system. The protective role of MT against the toxic effects of manganese may also be demonstrated by the fact that exposure to this metal leads to increased synthesis of this protein [43, 44].

In addition to metallothionein, reduced glutathione plays an important role in protecting the cell against free radicals. The correct amount of reduced glutathione in the cell is maintained by the action of glutathione reductase. The activity of glutathione reductase, like that of other antioxidant enzymes, is induced by reactive oxygen species. In the presented studies, a decrease in the activity of GR in livers and kidneys was observed.

In a study by Jadhav et al., rats were subchronic exposed to a mixture of eight metals (As, Cd, Pb, Hg, Cr, Ni, Mn, Fe) in doses corresponding to the concentrations determined in contaminated water in various areas of India. It has been noticed that a shorter exposure leads to an increase in the activity of all antioxidant enzymes (SOD, CAT, GPx, GR) in erythrocytes, while longer administration of contaminated water causes a decrease in the activity of these enzymes with a simultaneous increase in the concentration of lipid peroxidation products [45].

The activity of glutathione reductase may also be reduced as a result of direct interaction of Mn2+ ions on this enzyme. A decrease in glutathione reductase activity in the GR-Mn (II) system was observed in one of the authors' own studies, which described a decrease in glutathione reductase activity in liver homogenates in vitro after exposure to increasing concentrations of $Mn^{2}+$, in the concentration range of 0.5–5 g $Mn^{2}+$/L [46]. A similar phenomenon of glutathione reductase inhibition under the influence of other transition metals (zinc and nickel) and calcium ions was described by Tandogan and Ulusu [47]. Although the concentration of reduced glutathione in the tissues of the exposed animals was not determined, on the basis of the obtained results it can be assumed that this concentration was reduced, which is a consequence of the increased formation of free radicals.

A reduction in the concentration of reduced glutathione in tissues exposed to manganese has been reported in the literature, e.g. by Santos et al. [48]. The reduction in the concentration of this active thiol is the result of consuming it in antioxidant defense. Such a phenomenon was observed by the Park and Park, who described that

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the decrease in the concentration of reduced glutathione was accompanied by increased radicalogenesis and an increase in the concentration and expression of inflammatory cytokine genes, such as IL-6 and IL-8, as well as an increase in caspase-3 activity [49].

Reduced glutathione may arise not only as a result of „regeneration" of the form oxidized by GR, but also de novo in the so-called the γ-glutamyl cycle. In the case of the influence of manganese on the γ-glutamyl cycle, a rather unusual phenomenon was observed, in which complexes of this metal, unlike other metals, e.g. Hg, Pt, increase the activity of γ-glutamyl peptidase [50, 51, 52]. The increase in γ-glutamyl peptidase activity should lead to an increase in the concentration of reduced glutathione, however, this effect is reduced as a result of a decrease in GR activity.

The observed reduction in the activity of glutathione reductase has serious consequences, as its deficiency prevents the regeneration of oxidized glutathione, thus exposing cells to the increased effects of free radicals.

The enzyme supporting the proper functioning of the above-described enzymes is glutathione S-transferase, the role of which in reducing the effects of oxidative stress is reduced to the coupling of toxic products of macromolecular oxidation with reduced glutathione. Casalino et al. were observed changes in glutathione S-transferase activity in the livers of rats exposed to a single intraperitoneal dose of manganese (II) chloride (2 mg/kg b.w.). Researchers reported an increase in GST activity by 36% 24 hours after the administration of Mn2+ ions. Interestingly, no similar changes were reported after in vitro exposure of hepatocyte cytosol incubated for 10 minutes with a 40 μM manganese (II) chloride solution [53].

In our study, a decrease in glutathione S-transferase activity under the influence of manganese was observed in the kidneys.The low activity of glutathione reductase indicates the cell's inability to regenerate glutathione, therefore the conjugation of oxidized metabolites must also be impaired. This may explain the low glutathione S-transferase activity observed in this organ.

The presented study describes a number of enzymatic disorders related to the exposure of the rat's organism to manganese compounds. The changes in activity applied to practically every enzyme determined. The consequence of such serious disorders should be cellular damage, visible in histopathological examinations.

In the presented histopathological study, inflammatory changes in the liver were observed. In the case of animals from group I (exposed to a lower dose of Mn), only disseminated inflammatory infiltrates from lymphoid cells and neutrophils were observed, which were located in the area of the triads and in the marginal area of the organ. In group II, the changes were very intense, with extensive areas of hepatocyte necrosis and the production of abscesses, which were located mainly subcapsularly.

In the available literature, there are few old papers discussing the influence of manganese on the histopathological picture of the liver. Śmiałek et al. Observed the presence of coarse-grained cytoplasm in hepatocytes, loosening of the trabecular system, blurring of hepatocyte outlines and different size of cell nuclei [54]. The changes identified in the present study were definitely more extensive and of a different nature than the changes described by the cited authors. It is difficult to clearly indicate the cause of this phenomenon, but perhaps it depends on the route of administration – in the present case, intraperitoneal, in the case of the above examination – intravenous. The differences found were most likely not related to the dose administered, as in our case the animals received a lower dose than in the studies of the cited authors.

On the other hand, Khan et al. reported massive, periportal necrotic changes in hepatocytes with periportal hemorrhages and bile duct epithelial hyperplasia in animals intoxicated with manganese. Khan et al. also suggest that the changes found are secondary to the oxidative damage to cell membranes by manganese-induced ROS [55]. The type of changes described in the present study, and the changes identified by Khan et al., were observed not only in rats, but also in rabbits and humans exposed to this element [56, 57].

Surprising results were obtained when analyzing the influence of manganese on histopathological lesions in the kidneys. No changes were found on microscopic examination, except in a single case where only small, diffuse infiltrates from lymphocytes were observed. The lack of histopathological changes was accompanied by significant changes in the activity of all tested antioxidant enzymes. Some other authors have also observed kidney damage in animals exposed to manganese [58], some have only found discrete interstitial lymphocytic infiltrates in the kidney cortex of animals exposed to manganese. These researchers also described damage and exfoliation of epithelial cells of the collecting tubules in the medullary part [54]. Completely different data are presented by Ponnapakkam et al., who exposed animals to high doses of Mn in drinking water. These authors observed lesions of the type of tubular nephropathy, interstitial nephritis, sclerosis and glomerulonephritis, and nephrolithiasis in exposed animals [12].

The lack of histopathological lesions in the kidney, combined with severe oxidative disorders, suggests that this organ must have defense mechanisms independent of antioxidants. Perhaps such a function is fulfilled by metallothionein, which is present in significant amounts in the kidneys (Ruiz-Lopez 2006). The observed phenomenon is very interesting and worth exploring in further studies.

Conclusions

In the presented results, both the histopathological and biochemical studies, the severity of the disorders was related to the administered dose of manganese. Therefore, all the research presented in this paper is in line with the theory of the pro-oxidative effect of manganese ions on organisms.

Data Availability Statement. *The data that support the findings of this study are available from the corresponding author upon reasonable request.*

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