



Liver Function of Male Rats Exposed to Manganese at Different Time Points

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Abstract

As an essential trace element in the human body, manganese (Mn) is involved in many important biochemical reactions. However, excessive exposure to manganese can cause multiple systematic damages to the body. This study aims to investigate the effects of manganese exposure on serum hepatic enzymes in male rats at different time points. After adaptive feeding for 7 days, male Sprague–Dawley (SD) rats were injected intraperitoneally with 30 mg/kg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ once a day for 21 days at zeitgeber time point 2 (ZT2), ZT8, ZT14, and ZT20, respectively. We found that short-term repeated exposure to manganese caused slower body weight gain and increased relative liver and spleen weight index in male rats at different time points. Moreover, serum total bile acid (TBA) increased while aspartate aminotransferase (AST) decreased at ZT2, ZT8, and ZT20. Cholinesterase (ChE) decreased at ZT2 and ZT20, lactic dehydrogenase (LDH) decreased at ZT2, ZT14, and ZT20, and acid phosphatase (ACP) decreased at ZT2 and ZT14. Alkaline phosphatase (ALP) decreased at ZT2, ZT14, and ZT20, but increased at ZT8. Alanine amino transferase (ALT) decreased at ZT2 and ZT20, but increased at ZT8. There was a negative correlation between relative liver weight index with AST, ACP, ALP, and LDH, while a positive correlation with TBA. However, relative spleen weight index had a positive correlation with relative liver weight index and TBA, while a negative correlation with ALT, AST, ACP, ALP, LDH, and ChE. Our study shows that the injury of liver function is caused by short-term repeated manganese exposure at different time points. The time effect should be considered in manganese toxicity evaluation.

Keywords Manganese · Time toxicity · Liver function injury · Serum hepatic enzymes · Short-term repeated exposure

Introduction

Manganese (Mn) is an essential trace element in the human body. As a structural component, an activating factor, or cofactor of various enzymes in animals, manganese is involved in many important biochemical reactions [1]. However, excessive exposure to manganese can cause multiple systematic damages to the body. Manganese enters the body and deposits in the liver through binding to mitochondria. The liver is the main accumulation and target organ of manganese, only second to the brain [2]. Manganese excessive exposure could cause slight congestion in the hepatic central vein and adjacent sinus tubules, a focal necrotic area composed of denatured hepatocytes and inflammatory cells, disappearance of mitochondrial extracorporeal

membrane and crest in hepatocytes, degranulation of endoplasmic reticulum, karyopyknosis, cholestasis, and tissue biochemical changes [3]. Among the indices indicating liver function damage, alanine amino transferase (ALT), aspartate aminotransferase (AST), acid phosphatase (ACP), and alkaline phosphatase (ALP) are the most widely used markers at present. Total bile acid (TBA) is an important indicator of hepatocyte damage and liver dysfunction [4]. In addition, manganese can also activate acetylcholinesterase (AChE) to decrease the content of acetylcholine (ACh) in the cholinergic system [5].

The physiological and behavioral activities of most organisms are affected by circadian rhythm [3], which leads to different toxic reactions of experimental animals to the same dose of chemicals. Different exposure times can cause different degrees of toxic reaction. It may even cause a phenomenon called all or none, that is highly sensitive at one time point while no reaction at another time point. For example, oxidative stress indices in carp gills changed differently on the 15th, 30th, and 45th days of chlorpyrifos treatment [6]. IL-1 β , IL-4, and IL-6 were elevated significantly with the increase of time after middle and high ammonia exposure, while had no

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significant changes among different time points after low ammonia exposure [7]. Therefore, the time toxicity of exogenous chemicals is a key issue, especially in the toxicity study of heavy metals [8].

In order to explore the time toxicity of excessive manganese exposure, animals were treated with excessive manganese at different time points in 24 h of a day under the same conditions. The liver, spleen, and serum enzymes of liver function were detected to measure the difference of manganese treatment among different time points. Our study aims to provide a scientific basis for toxicity evaluation and prevention of manganese.

Materials and Methods

Reagents

MnCl₂·4H₂O purity greater than 99% was purchased from Sigma (St. Louis, MO, USA). ELISA kits for ALT, AST, ACP, ALP, LDH, TBA, and ChE detection were purchased from Lingnan Biological Products (Guangxi, China).

Experimental Animals and Feeding

The experimental animals used are of SPF grade and provided by the Experimental Animal Center of Guilin Medical University (the animal certificate number is SCXK (Guangxi) 2013/0001). The conditions of the animal feeding room were as follows: light/darkness (12 h:12 h), temperature (24 ± 1) °C, humidity (55 ± 10)%. The animals were free to drink and eat during the whole experiment period. The animals were managed in agreement with the criteria defined in the NIH publication (no. 85-23, revised in 1985), and the experimental protocol was approved by the Ethics Committee for Animal Care of Guilin Medical University (no. GLMC201603027).

Zeitgeber Time

The natural time is converted to zeitgeber time (ZT). The starting time of light is defined as ZT0 (equivalent to 7:00 of Beijing time). ZT2, ZT8, ZT14, and ZT20 in this study are equivalent to 9:00, 15:00, 21:00, and 3:00 of Beijing time, respectively.

Short-Term Repeated Toxicity Test

Ninety-six healthy adult Sprague–Dawley male rats had an initial weight of 251.06 ± 21.52 g. After 1 week of adaptive feeding, the rats were randomly divided into 8 groups. There were four ZT points, ZT2, ZT8, ZT14, and ZT20. Each ZT point had two groups, one control group and one manganese

group. Distilled water and 30 mg/kg MnCl₂·4H₂O were injected intraperitoneally at the corresponding ZT time point once a day for 21 days. When the experiments were finished, the animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) at the same ZT time point. After the animals were asleep, they were killed by cervical dislocation, and 5 mL of whole blood was obtained from each animal. The blood samples were kept at room temperature for 1 h and then centrifuged in separate serum at 3000 rpm for 10 min. The serum was stored at –80 °C for detecting the liver function enzymes within 2 weeks.

Detection of Liver Function Enzymes

The activities of serum ALT, AST, ACP, ALP, LDH, TBA, and ChE were detected by ELISA kits according to the manufacturer's protocol of a 7170A automatic biochemical analyzer (Olympus, Japan).

Statistical Analysis

All the data was analyzed on SPSS 16.0. Two samples *t* test, factorial analysis, and correlation analysis were carried out, and *P* < 0.05 was considered statistically significant.

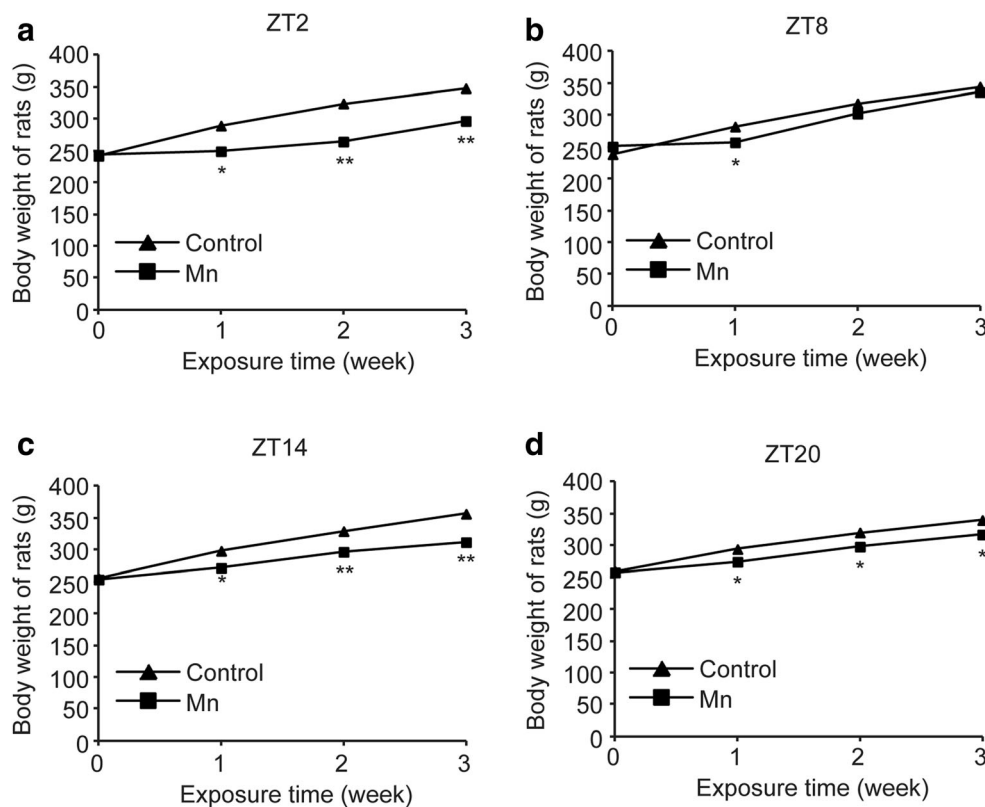
Results

Body Weight Gain of Male Rats After Short-Term Repeated Manganese Exposure at Different Time Points

We first measured the body weight of the male rats after manganese exposure. As shown in Fig. 1, the body weight gain of rats was slower after manganese exposure than that of the control group at all time points, suggesting that the 30-mg/kg manganese exposure used in this experiment could induce toxic reactions in male rats. However, at ZT8 time point, there was no significant difference in body weight gain between manganese and the control group at the second and third week of exposure time (*P* > 0.05, Fig. 1b). Compared with the control group, the body weight gain was decreased after manganese exposure for 1 week, 2 weeks, or 3 weeks at ZT2, ZT14, and ZT20 time points (*P* < 0.05).

We also performed a factorial analysis on the effects of manganese and exposure time points on body weight gain of male rats. As shown in Table 1, the interaction between manganese and exposure time points on body weight gain of male rats was not significant (*P* > 0.05).

Fig. 1 Body weight gain of male rats after manganese exposure at different ZT points. **a** Body weight gain of male rats after manganese exposure at ZT2 point. **b** Body weight gain of male rats after manganese exposure at ZT8 point. **c** Body weight gain of male rats after manganese exposure at ZT14 point. **d** Body weight gain of male rats after manganese exposure at ZT20 point. * $P < 0.05$ and ** $P < 0.01$ based on Student's t test compared with the control



Relative Organ Weight Index of Male Rats After Short-Term Repeated Manganese Exposure at Different Time Points

The relative organ weight indices are reliable indicators of toxic organ damage, so we also calculated the relative organ weight indices of the male rats after short-term repeated manganese exposure. Especially, the relative liver and spleen weight indices of rats were increased after exposed to manganese at all time points (Fig. 2), suggesting that the liver and spleen of rats may have hyperemia, edema, or hyperplasia. Factorial analysis showed that there was a significant interaction between manganese and exposure time points on the relative spleen weight index of rats ($P < 0.05$, Table 2), while no interaction effect on the relative liver weight index ($P > 0.05$).

Serum Enzymes of Male Rats After Short-Term Repeated Manganese Exposure at Different Time Points

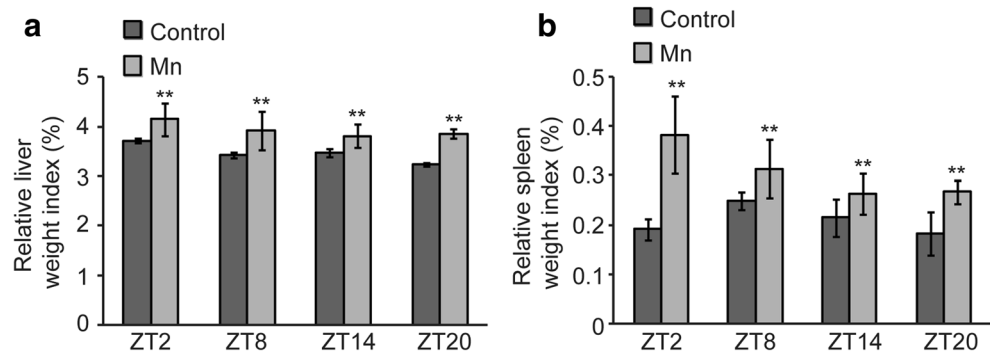
Liver enzymes are important indications of toxic effects on the hepatocytes. As shown in Fig. 3, short-term repeated manganese exposure caused changes in serum enzymes, but the changes were different at different time points of manganese exposure. In Fig. 3a, the ALT activity was decreased at ZT2 and ZT20, but increased at ZT8 and no significant change at ZT14. In Fig. 3b, the AST activity was decreased at ZT2, ZT8, and ZT20, but no significant change at ZT14. In Fig. 3c, the ACP activity was reduced at ZT2 and ZT14, while had no significant change at ZT8 or ZT20. In Fig. 3d, the activity of ALP was reduced at ZT2, ZT14, and ZT20, while increased at

Table 1 Factorial analysis on the effects of manganese and exposure time points on body weight gain of male rats

Source	1 week		2 weeks		3 weeks	
	<i>F</i>	<i>P</i> value	<i>F</i>	<i>P</i> value	<i>F</i>	<i>P</i> value
Adjusted model	4.933	<i>0.000</i>	5.470	<i>0.000</i>	4.992	<i>0.000</i>
Intercept	9.41E+03	<i>0.000</i>	8.77E+03	<i>0.000</i>	9.80E+03	<i>0.000</i>
Mn	2.154	0.100	1.760	0.161	1.317	0.275
ZT	25.474	<i>0.000</i>	24.809	<i>0.000</i>	22.566	<i>0.000</i>
Mn*ZT	0.499	0.684	2.253	0.088	2.154	0.100

Italicized values indicate significance
ZT, zeitgeber time; Mn, manganese

Fig. 2 Relative organ weight index of male rats after exposure at different ZT points. **a** Relative liver weight index of male rats after exposure at different ZT points. **b** Relative spleen weight index of male rats after exposure at different ZT points. ** $P < 0.01$ based on Student's *t* test compared with the control



ZT8. In Fig. 3e, the LDH activity was higher after manganese exposure than that of the control group at ZT2, ZT14, and ZT20, while there was no difference between the two groups at ZT8. In Fig. 3f, the TBA was increased at ZT2, ZT8, and ZT20, while no significant change at ZT14. In Fig. 3g, the activity of ChE was lower in the manganese exposure group than that of the control group at ZT2 and ZT20, while there was no difference between the two groups at ZT8 or ZT14.

As shown in Table 3, the short-term repeated manganese exposure resulted in a change in serum liver function enzymes. Factorial analysis showed that there was a significant interaction between manganese and exposure time points on these enzymes, except ChE ($P < 0.05$).

Correlation Analysis of Serum Enzymes and Relative Organ Weight Index of Male Rats After Short-Term Repeated Manganese Exposure at Different Time Points

We did correlation analysis on the above serum enzymes and the relative liver or spleen weight index after short-term repeated manganese exposure at different time points. As shown in Table 4, there was a positive correlation between the relative liver weight index and the relative spleen weight index of male rats ($r = 0.442$, $P < 0.05$). The relative liver weight index was negatively correlated with ALT, ALP, ACP, and LDH ($r = -0.458$, -0.358 , -0.264 , and -0.321 , $P < 0.05$), while

positively correlated with TBA ($r = 0.757$, $P < 0.01$). The relative spleen weight index was negatively correlated with ALT, AST, ALP, ACP, LDH, and ChE ($r = -0.376$, -0.509 , -0.577 , -0.260 , -0.509 , and -0.293 , $P < 0.05$), while positively correlated with TBA ($r = 0.277$, $P < 0.01$).

Discussion

Mammals produce circadian rhythms under the regulation of the biological clocks from the superior optic chiasma, nucleus of the inferior colliculus, and peripheral tissues, which regulate most biological, physiological, and behavioral functions of the body. Because of circadian rhythms, the sensitivity to the same poison can be different after exposure at different time points in a day. Middle and high ammonia could cause impaired immune function and mitochondrial integrity in the broilers bursa of Fabricius, compared with low ammonia treatment [9]. Broiler chickens exposed to 8 ppm hydrogen sulfide had significantly decreased average daily gain and body weight at 0–3 weeks, but there were no differences at 4–6 weeks with 12 ppm hydrogen sulfide exposure [10]. Therefore, circadian rhythms and time impact are of great importance in the study of toxicology [11]. It is necessary to analyze the toxicity of chemicals not only from the dose but also from the time point of view.

On the one hand, the change of body weight is a comprehensive indicator for the toxic effects of the body exposed to chemicals. In the 2-year POUNDS Lost randomized clinical trial, perfluoroalkyl substances exposure caused that participants lost an average of 6.4 kg of body weight during the first 6 months and subsequently regained an average of 2.7 kg of body weight during the period of 6–24 months [12]. Chronic pyocyanin administration was found to induce sustained reductions in body weight and fat mass of C57BL/6J mice [13]. Our results showed that the body weight gain of male rats was lower after short-term repeated manganese exposure than that in the control group at different time points, especially at the ZT2 time point, suggesting a toxic reaction was induced by 30 mg/kg manganese in male rats. On the other hand, the relative organ weight index is a sensitive indicator to evaluate

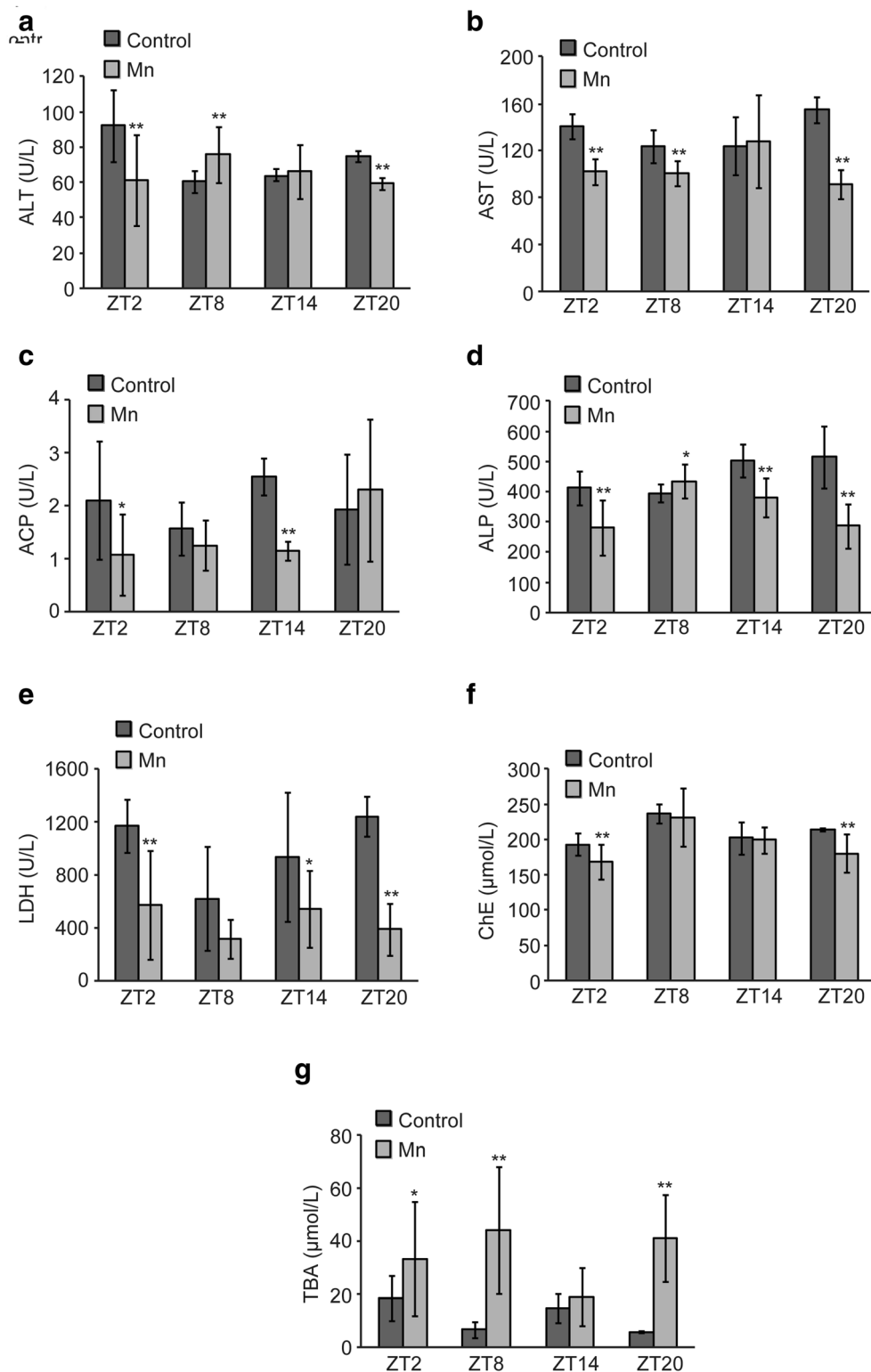
Table 2 Factorial analysis on the effects of manganese and exposure time points on relative liver or spleen weight index of male rats

Source	Liver		Spleen	
	<i>F</i>	<i>P</i> value	<i>F</i>	<i>P</i> value
Adjusted model	21.424	0.000	24.151	0.000
Intercept	2.73E+04	0.000	2.84E+03	0.000
Mn	13.892	0.000	9.986	0.000
ZT	112.249	0.000	100.741	0.000
Mn*ZT	1.59	0.198	11.784	0.000

Italicized values indicate significance

ZT, zeitgeber time; Mn, manganese

Fig. 3 Serum enzymes of male rats after manganese exposure at different ZT points. **a** ALT of male rats after manganese exposure at different ZT points. **b** AST of male rats after manganese exposure at different ZT points. **c** ACP of male rats after manganese exposure at different ZT points. **d** ALP of male rats after manganese exposure at different ZT points. **e** LDH of male rats after manganese exposure at different ZT points. **f** ChE of male rats after manganese exposure at different ZT points. **g** TBA of male rats after manganese exposure at different ZT points. * $P < 0.05$ and ** $P < 0.01$ based on Student's *t* test compared with the control



the physiological functions of organs. It is calculated by the percentage of organ weight in the body weight, and it can show the growth of organs in a broader way. The organ weight and the relative organ weight index are commonly used to measure the functional state and development process of

animals [14]. In short-term and subchronic toxicity tests, the relative organ weight index can directly indicate whether organs have hyperplasia, congestion, edema, or atrophy. The increase of the relative organ weight index shows organ hyperemia, edema, hyperplasia, or hypertrophy, while the

Table 3 Factorial analysis on the effects of manganese and exposure time points on hepatic enzymes of male rats

Source	ALT		AST		ALP		ACP		LDH		TBA		ChE	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Adjusted model	6.659	0.000	13.049	0.000	17.785	0.000	5.604	0.000	13.937	0.000	11.768	0.000	10.607	0.000
Intercept	1.93E+03	0.000	3.28E+03	0.000	2.99E+03	0.000	4.03E+02	0.000	4.81E+02	0.000	2.26E+02	0.000	6.35E+03	0.000
Mn	2.904	0.040	1.904	0.135	7.992	0.000	3.014	0.035	6.965	0.000	1.990	0.122	18.904	0.000
ZT	5.164	0.026	53.001	0.000	56.820	0.000	11.710	0.001	66.333	0.000	57.206	0.000	10.616	0.002
Mn*ZT	10.473	0.000	11.480	0.000	13.568	0.000	5.050	0.003	3.209	0.027	7.146	0.000	2.072	0.110

Italicized values indicate significance

ZT, zeitgeber time; Mn, manganese; ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminase; ALP, alkaline phosphatase; ACP, acid phosphatase; LDH, lactic dehydrogenase; TBA, total bile acid; ChE, cholinesterase

decrease of the relative organ weight index indicates organ atrophy or degenerative changes [15, 16]. A previous study found that oridonin could increase the relative weights of spleen and bursa, the number of proliferation peripheral blood T and B lymphocytes in broiler chickens at days 14, 28, and 42 of treatment [17]. Our results showed that the relative liver/spleen weight index was increased after short-term repeated manganese exposure at different time points, and there was a correlation between the relative liver/spleen weight index and hepatic enzymes. Therefore, as the target organs of short-term repeated manganese exposure, the liver and spleen may have congestion, edema, hyperplasia, or hypertrophy, as well as liver function.

The activity of serum hepatic enzymes can change upon liver injury from harmful chemicals [18, 19]. Manganese exposure has been shown to increase or decrease these serum hepatic enzymes. Hoseini et al. found that ALT, AST, and ALP of fish exposed to 60, 150, and 300 mg/L manganese were higher than that in the control group [20]. However, Tutkun et al. showed that ALT and AST were lower in the manganese-exposed population than that of the non-exposed group [21]. Our results showed both increased and decreased

serum hepatic enzymes after short-term repeated manganese exposure at different time points, such as the ALT activity was decreased at ZT2 and ZT20, while increased at ZT8 and had no significant change at ZT14. And, the LDH activity was increased after manganese exposure at ZT2, ZT14, and ZT20, while had no change at ZT8. Moreover, there was a significant interaction between manganese and exposure time points for these enzymes, except ChE.

ChE is a non-specific esterase with a half-life of only 10 days, which can only be synthesized in liver. When liver function is impaired, the amount of ChE is reduced in hepatocytes and resulting in a significant activity decrease of serum ChE [22]. Manganese is a choline-like substance that can affect the synthesis of AChE and promote ACh accumulation to affect the delivery of neurotransmitters. The low dose of manganese activated AChE, but the high dose of manganese inhibited the activity of ChE in manganese-exposed workers [23, 24]. This study found that the activity of serum ChE was reduced upon short-term repeated manganese exposure at ZT2 and ZT20, suggesting serum ChE was more sensitive to liver function injury of male rats induced by manganese at ZT2 and ZT20.

The production and metabolism of bile acids are closely related to liver. The detection of TBA can directly indicate the function of liver secretion, synthesis, and metabolism, as well as the degree of liver injury [25, 26]. Serum TBA is easy to increase upon hepatocyte lesions, so the level of serum TBA can reflect the injury of liver parenchyma. TBA is thought to be a good indicator of hepatocyte injury [27, 28]. Our study detected an increase of serum TBA caused by short-term repeated manganese exposure only at ZT2, ZT8, and ZT20 time points. Moreover, the change of serum TBA was under the interaction between manganese and exposure time points. These results suggested that serum TBA was sensitive to liver function injury induced by short-term repeated manganese exposure at ZT2, ZT8, and ZT20 time points in male rats.

In conclusion, short-term repeated manganese exposure can lead to liver injury in male rats, which is shown by slower body weight gain, increased relative liver weight index, and deregulated serum hepatic enzymes. Moreover, the change of serum hepatic enzymes is different upon manganese exposure

Table 4 Correlation analysis between hepatic enzymes and relative liver or spleen weight index of male rats

Index	Relative liver weight index	Relative spleen weight index
ALT	0.124	-0.376**
AST	-0.458**	-0.509**
ALP	-0.358**	-0.577**
ACP	-0.264*	-0.260*
TBA	0.757**	0.277**
LDH	-0.321**	-0.509**
ChE	-0.120	-0.293**
Relative liver weight index	-	0.442**
Relative spleen weight index	0.442**	-

* $P < 0.05$

** $P < 0.01$

at different zeitgeber time, indicating a time toxicity induced by manganese on liver function. Therefore, when evaluating the toxicity of manganese, it may be necessary to consider the effect of zeitgeber time.

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Compliance with Ethical Standards The animals were managed in agreement with the criteria defined in the NIH publication (no. 85-23, revised in 1985), and the experimental protocol was approved by the Ethics Committee for Animal Care of Guilin Medical University (no. GLMC201603027).

Conflict of Interest The authors declare that they have no conflict of interest.

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