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Manganese exposure caused reproductive toxicity of male mice involving activation of GnRH secretion in the hypothalamus by prostaglandin E2 receptors EP1 and EP2

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ABSTRACT

Exposure to manganese (Mn) can cause male reproductive damage and lead to abnormal secretion of sex hormones. Gonadotropin-releasing hormone (GnRH) plays an important role in the neuromodulation of vertebrate reproduction. Astrocytes can indirectly regulate the secretion of GnRH by binding paracrine prostaglandin E_2 (PGE_2) specifically to the EP1 and EP2 receptors on GnRH neurons. Prior studies assessed the abnormal secretion of GnRH caused by Mn exposure, but the specific mechanism has not been reported in detail. This study investigated the effects of Mn exposure on the reproductive system of male mice to clarify the role of PGE_2 in the abnormal secretion of GnRH in the hypothalamus caused by exposure to Mn. Our data demonstrate that antagonizing the EP1 and EP2 receptors of PGE_2 can restore abnormal levels of GnRH caused by Mn exposure. Mn exposure causes reduced sperm count and sperm shape deformities. These findings suggest that EP1 and EP2, the receptors of PGE_2, may be the key to abnormal GnRH secretion caused by Mn exposure. Antagonizing the PGE_2 receptors may reduce reproductive damage caused by Mn exposure.

1. Introduction

The clinical evaluation of the infertile male is comprised of a patient's history, physical exam, laboratory studies, and select diagnostic studies aiming to identify pathophysiology which adversely affects reproductive function. An infertile patient's reproductive potential is often linked to medical comorbidities, medication use, and past surgeries. Yet, with the infer tile couple, we must also consider environmental factors as significant contributors. As spermatogenesis is more sensitive to environmental contaminants when compared to their female counterparts, this decline may be due to exposures to toxins from industrial, agricultural, and by-products of other technological advancements (Mima et al., 2018). Mn is abundant in the environment and can be found in air, soil, and water (Mima et al., 2018). Due to its natural characteristics, Mn is widely used in mining (Mutti et al., 1996), smelting (Cowan et al., 2009; Jiang et al., 2007), welding (Bowler et al., 2011; Racette et al., 2012) and other industries. Mn is also used as a gasoline antiknock agent (Lyznicki et al., 1999). The Mn content in the atmosphere in cities or regions with Mn industry is between 0.00013 and -0.0003 mg/m^3, which is 13–30 times the background value. The concentration of Mn in the atmosphere near Mn mines, gas stations, and main roads is higher, 358, 0.22–0.76, and 0.0087–0.82 mg/m^3, respectively. Welders who inhale more than 0.15 mg/m^3 of Mn for more than 10 years can experience neurobehavioral symptoms such as decreased sensitivity. Although it is an essential trace element, excessive Mn exposure can cause male reproductive dysfunction, such as impotence, loss of libido, decrease in sperm counts, and sperm dis-viability (Emara et al., 1971; Mena et al., 1967; Wu et al., 1996). This study adds to the several reports on the effects of Mn on the reproductive system in humans with some reports suggesting that when men are exposed to Mn for a longer period of time they may show signs of loss of libido and impotence and impaired fertility (Pizent et al., 2012). Animal experiments also confirmed Mn's reproductive toxicity. Mn can not only retard sexual development but also cause reduction in sperm counts and sperm motility of mice despite normal testicular histology (Kogevinas et al., 1998; Ponnapakkam et al., 2003a). Studies demonstrated that the mechanism of Mn-caused reproductive disorder involved oxidative injury (Sharpe, 2001), apoptosis of spermatogenic cells (Osada and Treen, 2013), and mitochondrial damage (Seeburg et al., 1987)), among others. However, the mechanism remains unclear.

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Abbreviations

AA	Arachidonic acid
DMSO	Dimethyl sulphoxide
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
HPGs	hypothalamic - pituitary - gonadal axis
LH	Luteinizing hormone
Mn	Manganese
mRNA	Messenger RNA
PGE_2	Prostaglandin E_2
T	Testosterone

Among Mn-induced reproductive disorders, abnormal secretion of sex hormones is important. The balance of sex hormones in the human body depends on the regulation of the hypothalamic-pituitary-gonadal (HPG) axis. GnRH is a neuropeptide that plays an essential role in the neural regulation of vertebrate reproduction (Osada and Treen, 2013). It is secreted from the hypothalamus in a periodic pulsatile manner to stimulate the secretion of LH and FSH by the anterior pituitary cells (Seeburg et al., 1987). LH can bind with LHRH receptors on Leydig cells in the testes to promote the secretion of T. This is the well-known HPG axis. An elevated serum T concentration causes negative feedback to GnRH, FSH, and LH (Ullah et al., 2014). Mn accumulates in the hypothalamus (Deskin et al., 1981). Therefore, the HPG axis may play an important role in Mn-induced male reproductive dysfunction. Mn's mechanism causing abnormal GnRH secretion should be further studied.

PGE_2 is involved in GnRH secretion. PGE_2 is one of the prostanoids synthesized from arachidonic acid that is produced from membrane phospholipids by a phospholipase A_2 (Clasadonte et al., 2011a, 2011b) . Harms et al. (1973) verified that the injection of PGE_2 into the third ventricle of rat brain induced the release of LH into the general circulation. This is consistent with the experimental conclusions of Eskay et al. (1975) and Ojeda et al. (1975). Hiney et al. (2016) administered 10 mg/kg of $MnCl_2$ to rats and found that the PGE_2 serum increased. We speculated that PGE_2 may play a key role in Mn-caused GnRH secretion alteration. However, the precise effect and relative mechanism have yet to be clarified. PGE_2 binds to the EP receptor. EP is a G protein-coupled receptor with seven transmembrane domains. It is divided into four subtypes: EP1, EP2, EP3, and EP4 (Milatovic et al., 2011). GnRH neurons mainly express EP1 and EP2 (Milatovic et al., 2011). Experiments demonstrating that GnRH neurons express EP1 receptors in vivo and that the EP1 agonist 17-phenyl-trinor PGE_2 promotes GnRH release in the GnRH-producing neuronal cell line, GT1-1 cells in vitro (Ojeda et al., 1988). The excitatory effect of PGE_2 on GnRH neuronal activity was selectively mimicked by the EP2 receptor agonist butaprost previously shown to promote GnRH release in the GnRH-producing neuronal cell line, GT1-7 (Ojeda et al., 1988). Thus, whether EP1 and EP2 receptors of PGE_2 play a role in Mn-induced GnRH secretion abnormalities requires further study.

2. Materials and methods

2.1. Reagents

Mn chloride ($MnCl_2{\cdot}4H_2O$) was obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). SC-51322 and AH6809 were purchased from Cayman Chemical, Inc. (Ann Arbor, MI, USA). EP1 and EP2 rabbit antibodies were bought from Bioss Biotechnology Co., Ltd. (Beijing, China). GAPDH rabbit antibody was purchased from Proteintech Group, Inc. (Rosemont, IL, USA). GnRH-1 mouse antibody was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). PGE_2 rabbit antibody was bought from Absin Bioscience, Inc. (Shanghai, China). Alexa Fluor 488-labeled goat anti-rabbit secondary antibody and Cy3-labeled goat anti-mouse secondary antibody were provided by Beyotime Institute of Biotechnology (Shanghai, China). HRP-labeled goat anti-rabbit IgG (H + L) was bought from Beyotime Institute of Biotechnology (Shanghai, China). Local chemical suppliers provided the other chemicals. All of the chemicals had the highest grade available.

2.2. Animals

This study was conducted on Kunming mice (25 ± 2 g weight at the beginning of the experiment; N = 112) purchased from the Laboratory Animal Center of China Medical University, Shenyang, China (SPF grade, certificate no. SCXK 2013-0001). Among them, 48 mice were castrated. The others were uncastrated. The mice were housed in plastic cages in a climate-controlled animal room (temperature, 24 °C ± 1 °C, humidity, 55% ± 5%) with a 12 h light/dark cycle. Food and water were freely available. The animal experiment was conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications no. 8023, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

A total of 48 of the mice were castrated. The remainder were sham operated (Hashimoto et al., 2016). All were housed individually. All of the operative procedures were conducted under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneal injection). An incision was made in the abdominal. The testes and epididymis were removed following seminal duct ligation. Gentamicin 20000 U was intramuscularly injected daily for three days after surgery. The wound healing of the mice was observed. Their breeding environment was the same as the uncastrated mice.

2.3. Treatment and tissue collection

Sixty-four uncastrated mice were randomly divided into eight groups with eight animals each: a control group, 12.5, 25, and 50 mg/kg $MnCl_2$-treated groups, an SC-51322 intervention group (SC-51322 + 50 mg/kg $MnCl_2$), an AH6809 intervention group (AH6809 + 50 mg/kg $MnCl_2$), a DMSO vehicle group (DMSO + 50 mg/kg $MnCl_2$), and a DMSO vehicle group (DMSO). The volume of administration was 5 mg/kg body weight. The intraperitoneal injection was administered every day for 2 weeks. The intervention group was intraperitoneally injected with the corresponding intervention agent 2 h before Mn treatment. The intervention agent was administered twice a week.

The forty-eight castrated mice were randomly divided into six groups with eight animals each: a control group, a 50 mg/kg $MnCl_2$-treated group, an SC-51322 intervention group (SC-51322 + 50 mg/kg $MnCl_2$), an AH6809 intervention group (AH6809 + 50 mg/kg $MnCl_2$), a DMSO dissolvent group (DMSO + 50 mg/kg $MnCl_2$), and a DMSO dissolvent group (DMSO). The intraperitoneal injection was administered every day for 2 weeks. The intervention group was intraperitoneally injected with the corresponding intervention agent 2 h before Mn treatment. The intervention agent was administered twice a week.

Blood was collected from all of the mice after they were anesthetized. The collected serum was stored at −20 °C for later detection. We immediately separated the testes and epididymides. The testes weight and caudal epididymides weight of each mouse were recorded. The caudal epididymides of each mouse were finely minced with small scissors in 2 ml of normal saline. The suspension was dispersed and filtered to expel large tissue fragments to prepare a sperm suspension

for sperm count and sperm abnormality experiments. The left testis was soaked in 10% paraformaldehyde for paraffin embedding to observe the pathological changes and two hypothalami of the normal and castrated mice were soaked in 10% paraformaldehyde for paraffin embedding to use in immunofluorescence double-staining experiments. The total RNA and protein were then extracted from the hypothalamus of the other mice in each group to determine the EP1, EP2 mRNA, and protein levels.

2.4. Evaluations of sperm count and sperm shape abnormalities

A drop of sperm suspension was placed on a red blood cell counter to cover the coverslip. After 2 min of rest, the total number of sperm was counted using a high-power microscope (400×) according to the red blood cell counting method. The number of sperm in the five squares were counted and denoted as R. The number of sperm in each epididymal sperm suspension was calculated according to the formula R × 50000 = the number of sperm (million/ml). The sperm suspension after incubation at 37 °C was filtered through a double-sided mirror paper. One drop of suspension was placed on the side of a clean glass slide, which was dried and placed in methanol for 5 min. After drying, it was stained with a 2% aqueous eosin solution for 1 h (Takeda et al., 2016). The sperm morphology was examined under a high-power microscope (400×). Each mouse was examined for 500 intact sperm and the sperm deformity rate was calculated. Sperm malformations mainly manifested in the head, tail, and neck. Amorphous deformities and double-tailed spermatozoa also occurred (Goud et al., 1987).

2.5. HE staining

After the testis were removed and fixed in 4% paraformaldehyde, they were serially dehydrated in graded ethanol and xylene. The tissues were paraffin-embedded and sectioned to 5 μm thick. Regular hematoxylin and eosin staining was conducted for morphological observation with a microscope (Olympus AX-70) (Zhang et al., 2018).

2.6. Western blotting analysis of EP1 and EP2

The total protein was extracted from the hypothalamus using RIPA buffer (10 mM Na_2HPO_4, pH 7.2, 150 mM of NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) containing protease inhibitors (1 mM of phenylmethylsulfonyl fluoride, 0.2 m Ml, 10-phenanthroline, 10 μg/ml of pepstatin A, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 10 mM of benzamidine). The protein concentrations were determined using BCA reagent. Equal amounts of protein (40 μg) were separated via 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA). The PVDF membranes were blocked overnight at 4 °C in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% bovine serum albumin fraction V. The membranes were then rinsed in PBS 3 times for 5 min each and incubated with primary EP1 (1:500), EP2 (1:300), and GAPDH (1:2000) antibody in TBST for 2 h at room temperature. The secondary antibodies were applied at a dilution of 1:2000. Bands were visualized using an ECL kit. To quantify the staining, densitometric analysis was conducted using Flurchen V2.0 Stand Alone software. The relative intensity of each protein sample was normalized to GAPDH (Li et al., 2018).

2.7. Total RNA isolation and quantitative real-time PCR analysis of EP1 and EP2

Total mRNA was extracted from the hypothalamus of the mice in each group. The tissues were homogenized in RNAiso Plus. After a 5 min of incubation at room temperature (20 °C), chloroform was added for phase separation. The upper aqueous phase was collected and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellets were washed once with 75% ethanol, air-dried, and re-dissolved in RNase-free water. The RNA solution's absorbance was determined using a nanophotometer (Implen, Munich, Germany) at 260 and 280 nm, respectively. The A_{260}/A_{280} ratios were between 1.8 and 2.1. First-strand cDNA was synthesized from 3 μg of total RNA by reverse transcriptase using PrimeScript RTEnzyme Mix I (TaKaRa, Kyoto, Japan) and oligo (dT) primers (TaKaRa, Japan) according to the manufacturer's protocol. Real-time quantitative PCR (RT-PCRq) was conducted via a SYBR Premix Ex Taq II kit (TaKaRa, Japan) using an ABI 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Two microliters of template cDNA were added to the final 20 μl volume of reaction mixture. Real-time PCR cycle parameters included 30 s at 90 °C followed by 40 cycles of denaturation at 90 °C for 3 s, annealing at 60 °C for 34 s, and elongation at 72 °C for 20 s. The specific sets of primer sequences for EP1, EP2, and β-actin used in this study are shown in Table 1. Expressions of the selected genes were normalized to β-actin gene, which was used as an internal housekeeping control. For the relative quantification of the tested EP1 and EP2, we used the comparative CT method ($^{\Delta\Delta}$CT). All of the real-time PCR experiments were conducted in triplicate and data were expressed as the mean of at least three independent experiments (Yang et al., 2015).

Table 1
Primer sequences used for the amplification of each gene in this study.

Name	Oligo	Primer sequence predicted
EP1	Sense prime	5′-ACGATGTGGAAATGGTGGG-3′
	Anti-sense primer	5′-CGTACAGCCAGAAAGAGTGG-3′
EP2	Sense prime	5′-ACCATCACCTTCGCCATATG-3′
	Anti-sense primer	5′-GAGGTCCCACTTTTCCTTTAGG-3′
β-actin	Sense prime	5′-ACCTTCTACAATGAGCTGCG-3′
	Anti-sense primer	5′-CTGGATGGCTACGTACATGG-3′

2.8. Serum hormone test

The collected serum was stored at −20 °C until it was used to estimate the hormonal levels. Enzyme-linked immunosorbent assays were used to determine the GnRH, PGE_2, FSH, LH, and T levels in the serum specimens. The ELISA kit was provided by Mei Mian (Jiangsu, China) (Majeed et al., 2019).

2.9. Double immunofluorescent staining of EP1, EP2, and GnRH

At 24 h after the final injection, the mice were terminally anesthetized and perfused with 4% paraformaldehyde. The brains were removed and post-fixed in the same fixative overnight and then replaced with 50% alcohol for 1 h, followed by 70% alcohol 3 times for 1 h, and then replaced with 100% alcohol for 1 h. After treatment with the alcohol solution, the tissue was embedded in paraffin. We prepared and inspected 5 μm paraffin sections. All of the sections were first blocked with 10% goat serum for 1 h at room temperature to avoid non-specific staining. The sections were incubated with primary antibodies of both GnRH (anti-mouse, 1:500) and EP1 (anti-rabbit, 1:100) or EP2 (anti-rabbit, 1:100) at 4 °C overnight. Then the sections were incubated with FITC-labeled anti-rabbit secondary antibody (1:1000) and Cy3-labeled donkey anti-mouse secondary antibody (1:1000) for 1 h at room temperature. The sections were again washed with PBS three times prior to counterstaining with DAPI for 10 min. After three washes again, the sections were covered with a microscopic glass with antifade polyvinylpyrrolidone mounting medium for further study. Images were captured and the immunofluorescent signal intensity was assessed using a fluorescence microscope (Leica, Tokyo, Japan) (Isidro et al., 2015).

3. Result

The changes in the mice body weight were recorded every week.

After two weeks of Mn treatment, the weight of the mice in each group increased. However, there was no significant difference in body weight between the castrated Mn-treated groups and the uncastrated Mn-treated groups ($p > 0.05$). There were no significant differences in both testicular and epididymal organ coefficients ($p > 0.05$).

3.1. Sperm count and sperm deformity of mice

The sperm count results (Fig. 1) showed that the number of sperm in the Mn-treated groups significantly decreased compared to the control group ($p < 0.01$). The number of sperm in the EP1 and EP2 receptor antagonist groups was significantly increased compared with the 50 mg/kg Mn-treated group ($p < 0.05$). The results of the eosin-stained sperm smears (Fig. 2) showed a small amount of deformed sperm in the 50 mg/kg Mn-treated group. The results of the control group, EP1, and EP2 receptor antagonist groups demonstrated no obvious abnormal sperm morphology.

3.2. Testicular morphology

The tissue regularity was assessed in each testes, deep nuclear staining, and clear nuclear membrane group (Fig. 3). The cross-sections of the seminiferous tubules were closely arranged. The spermatogonia and spermatocytes at different stages of development were arranged in an orderly manner.

3.3. Protein level and mRNA expression of EP1 and EP2

We also studied the changes in the protein levels and mRNA expression of EP1 and EP2. The results were divided into two parts, protein and mRNA changes in the uncastrated Mn-treated mice and castrated Mn-treated mice. Fig. 4 (a), (b), (c), and (d) show the changes in EP1 and EP2 protein levels and mRNA expression in the uncastrated Mn-treated mice, respectively. Fig. 4 (e), (f), (g), and (h) demonstrate the changes of EP1 and EP2 protein levels and mRNA expression in the castrated Mn-treated mice, respectively. The results indicate that the protein levels of EP1 and EP2 in the uncastrated Mn-treated groups increased after Mn treatment ($p < 0.01$). The protein levels of EP1 and EP2 were significantly lower in the antagonist SC-51322 + 50 Mn and AH6809 + 50 Mn groups than in the 50 mg/kg Mn-treated group ($p < 0.01$). The mRNA expression of EP1 and EP2 increased compared with the control group after Mn exposure ($p < 0.01$), and the increase in EP1 was more obvious. The mRNA expression of EP1 and EP2 were significantly lower in the antagonist SC-51322 + 50 Mn and AH6809 + 50 Mn groups than in the 50 Mn-treated group ($p < 0.01$). The protein level of EP1 increased after castration ($p < 0.01$).

3.4. Serum sex hormone levels

The changes in the serum hormone levels after Mn exposure are shown in Table 2. The GnRH, FSH, LH, and PGE_2 hormone levels increased significantly in the 50 mg/kg $MnCl_2$ group compared with the control group ($p < 0.01$). The levels of serum T decreased significantly in the 50 mg/kg $MnCl_2$ group compared with the control group ($p < 0.01$). The levels of GnRH, FSH, and LH in the intervention group (SC-51322 and AH6809) decreased significantly compared with the 50 mg/kg $MnCl_2$ group ($p < 0.05$ and $p < 0.01$). The levels of PGE_2 and T in the intervention group (SC-51322 and AH6809) increased compared with the 50 mg/kg $MnCl_2$ group ($p < 0.05$ and $p < 0.01$). As shown in Table 3, the serum GnRH, FSH, LH, and PGE_2 levels increased compared with the control group after castration. The levels of GnRH, FSH, and LH, in the intervention group (SC-51322 and AH6809) decreased significantly compared with the 50 mg/kg $MnCl_2$ group ($p < 0.05$ and $p < 0.01$). PGE_2 in the intervention group (SC-51322 and AH6809) increased significantly compared with the 50 mg/kg $MnCl_2$ group ($p < 0.05$, $p < 0.01$).

3.5. Colocalization of the EP1, EP2 and GnRH in the hypothalamus

To identify the colocalization between the EP1, EP2, and GnRH levels in the hypothalamus after the mice were exposed to different Mn doses, we conducted double immunofluorescence staining to investigate their relationship. After double immunofluorescence staining, the hypothalamus was examined with both combinations of immunofluorescence markers for GnRH and EP1 or EP2. As shown in Fig. 5, the results demonstrated that GnRH (red) and EP1 (green) immunofluorescence expression increased after Mn exposure and the number of neurons with co-expression of GnRH and EP1 obviously increased in the 50 mg/kg $MnCl_2$ group. As shown in Fig. 6, the results indicated that GnRH (red) and EP2 (green) immunofluorescence expression were not significantly different from the control group after Mn exposure.

4. Discussion

The World Health Organization has defined infertility as the third most prevalent disease that affects human health after cancer and cardiovascular disease. A recent meta-analysis by Levine et al. (2017) identified a 50% decline in sperm counts in Western men over the last 40 years. Mn is a type of metal element widely found in nature and an essential trace element in the human body. Mn is widely used in battery manufacturing, electric welding, smelting, and gasoline production. However, excessive exposure to Mn can cause damage to the body, mainly in occupational chronic Mn poisoning (Sriram et al., 2015). Wirth et al. (2007) examined the serum Mn levels in 200 infertile clinical patients and found that patients with high Mn serum levels had a higher risk of low sperm motility and low sperm concentrations. The influence of Mn as a widely distributed heavy metal on male reproductive development cannot be ignored.

Sperm count is an important observational endpoint for studying the effects of environmental factors on male reproductive health (Linder et al., 1992). Mn can accumulate in the testes and damage the brain-pituitary-testicular axis, causing increased oxidative stress in the brain, pituitary, testes, and epididymis, reducing antioxidant enzyme activity and glutathione levels, sperm count, and quality (Adedara et al., 2017; Liu et al., 2013). This is important for promoting sexual organ development and spermatogenesis and maintaining secondary sexual characteristics. Abnormal T levels can lead to obstruction of

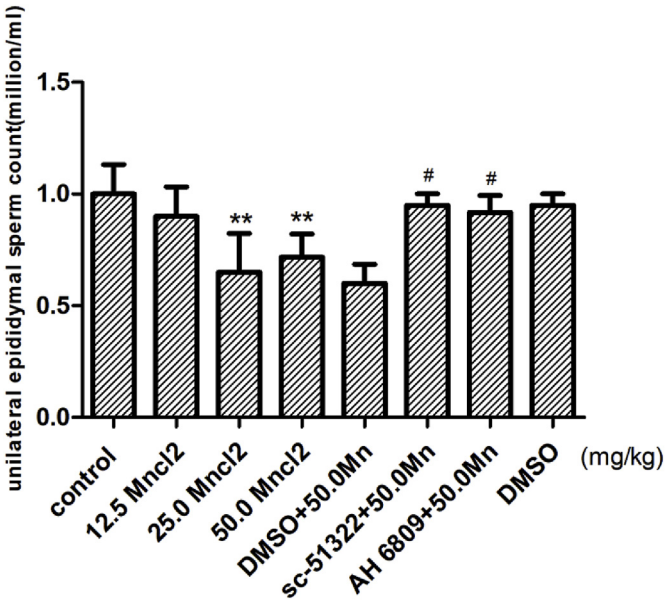

Fig. 1. The change of sperm count after Mn exposured. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the 50 mg/kg Mn-treated group.

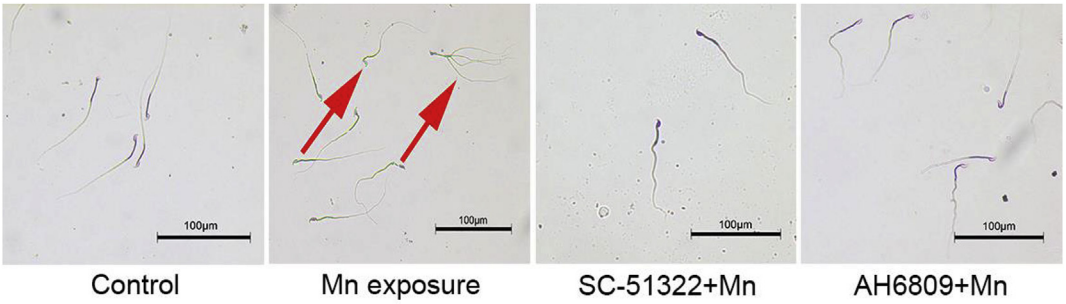

Fig. 2. The change of sperm shape after Mn exposured. Two-tailed and neck deformity of sperm (marked with the red arrowhead) occurred in the 50 mg/kg Mn-treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

spermatogenesis and imbalance of hormone levels in the body and induce diseases such as delayed hypogonadism, osteoporosis, and hemorrhoids in middle-aged men (Baas and Kohler, 2016). Our study examined the serum T levels, sperm count, and sperm morphology of male mice after Mn exposure. The results demonstrated that there were significant decreases in the sperm count after treatment with 25 mg/kg and 50 mg/kg of $Mncl_2$. In the Mn-treated group, the normal sperm morphology changed, and double-tailed spermatozoa and neck deformities appeared. The T levels in the Mn-treated group were lower than those in the control group. This indicated that excessive manganese exposure can cause damage to the male reproductive system. The results were similar to previous studies (Baas and Kohler, 2016). Prior studies also reported changes in testosterone hormone levels in manganese workers (Ou et al., 2018). This may confirm that excessive manganese exposure can affect male reproductive health. To further verify the injury to the male reproductive system caused by Mn exposure, we examined the pathological changes in the testicular tissue. The results showed there were no significant pathological changes in the Mn-treated group. Consistent results in prior research demonstrated that excessive exposure to Mn significantly reduced sperm counts and sperm motility despite normal testicular histology in CD-1 mice (Ponnapakkam et al., 2003a). However, different results were reported previously that suggested that $MnCl_2$ exposure in rats caused pathological injury of the testes with cell loss, abnormal structure, and irregular arrangement (Ponnapakkam et al., 2003b). The reasons may be related to differences in the Mn doses and exposure times. In summary, the accumulation of Mn may damage the male reproductive system.

Mn can penetrate the blood-brain barrier and accumulate in the hypothalamus (Aschner, 2000; Garcia et al., 2006; Pine et al., 2005). GnRH is a decapeptide hormone secreted by the hypothalamus and one of the most important hormones in animal reproduction. Under normal circumstances, GnRH is secreted from the hypothalamus into the pituitary portal system to the anterior pituitary and binds to receptors on pituitary gonadotropin cells to promote LH and FSH in the anterior pituitary (synthesis and secretion of FSH), which is often called the hypothalamic-pituitary-gonadal axis (Jin and Yang, 2014). Mature male differentiation, reproductive organ maturation, sex hormone secretion, and spermatogenesis are mediated by various reproductive hormones. If the body's reproductive system is damaged, the dynamic balance of the hypothalamic-pituitary-gonadal axis is broken, and the level of sex hormones in the body also changes. The GnRH level is a vital indicator of damage to the reproductive system. Studies found that Mn can directly stimulate pre-pubertal GnRH and LH secretion (Jin and Yang, 2014). Mn^{2+} promotes maturation and glial cell communication by upregulating IGF-1 and COX-2 and promotes increased neurosecretory activity, including GnRH, leading to precocious puberty development (Hiney et al., 2011). GnRH and LH secreted by the hypothalamus and pituitary can promote testes to secret testosterone, regulating the male reproductive system. A negative feedback to the hypothalamus can occur when T hormone secretion is too high or too low to stabilize

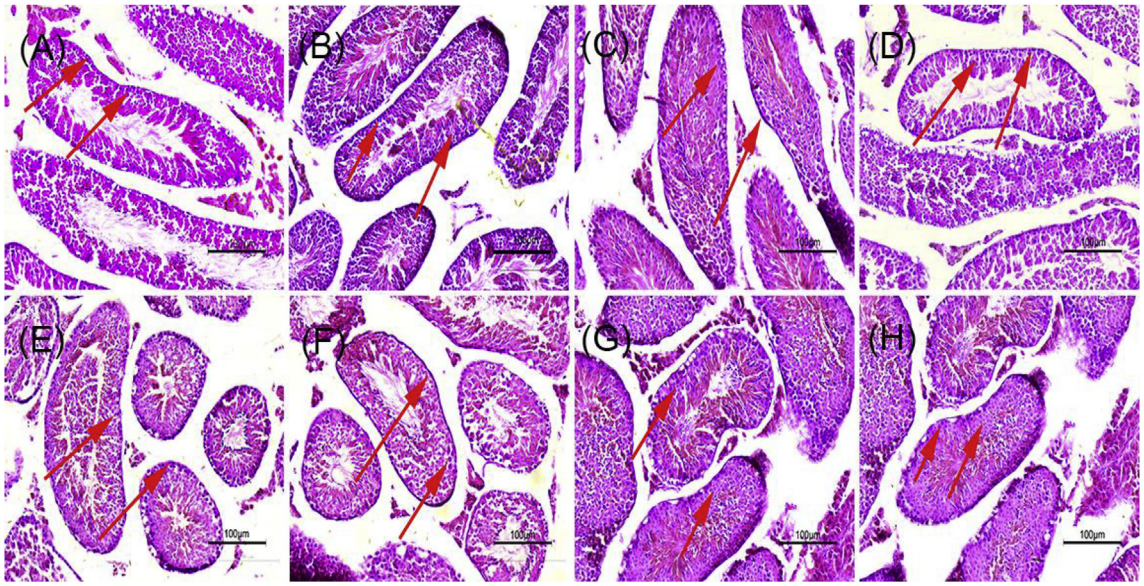

Fig. 3. Light microphotographs showed the pathological changes of testis tissues after they were exposed to different dosages of Mn. A, Control; B, 12.5 mg/kg $MnCl_2$; C, 25 mg/kg $MnCl_2$; D, 50 mg/kg $MnCl_2$; E, SC-51322 + 50 mg/kg $MnCl_2$; F, AH6809 + 50 mg/kg $MnCl_2$; G, DMSO +50 mg/kg $MnCl_2$; H, DMSO. The sections were stained with HE staining. Magnification was ×400 in each group, respectively.

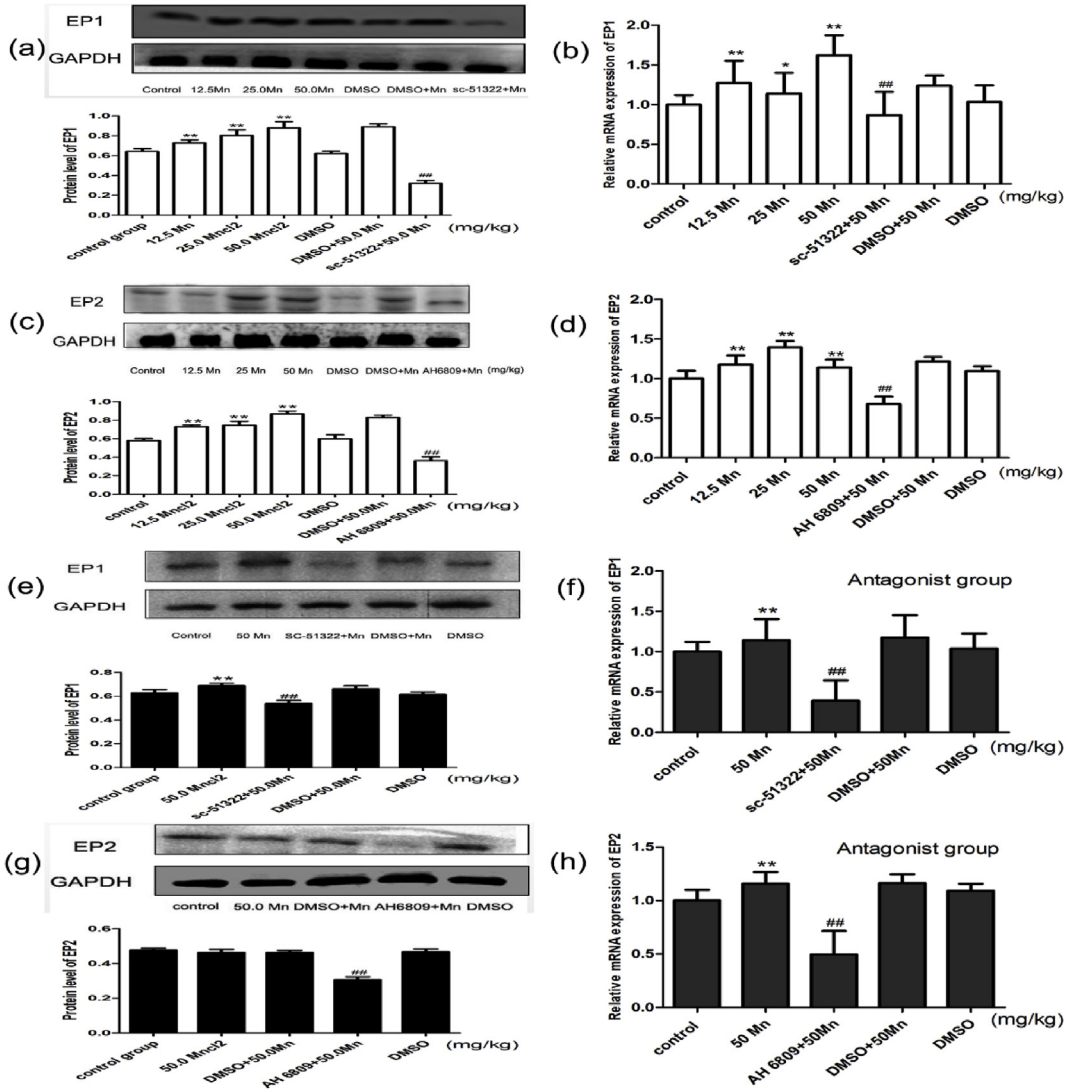

Fig. 4. The protein level of EP1 and EP2 in hypothalamus of uncastration group are shown in (a) and (c) after Mn exposure. The protein level of EP1 and EP2 in hypothalamus of castration group are shown in (e) and (g) after Mn exposure. The effect of Mn on real-time PCR analysis of the EP1 and EP2 in the hypothalamus of uncastration group are shown in (b) and (d). The effect of Mn on real-time PCR analysis of the EP1 and EP2 in the hypothalamus of castration group are shown in (f) and (h). Data are presented as the mean ± SEM for four animals in each group. *p < 0.05; **p < 0.01 compared with control group; #p < 0.05, ##p < 0.01 compared with 50 mg/kg $MnCl_2$ group.

the balance of the hypothalamic-pituitary-gonadal axis. To investigate the reasons for the changes in T hormone levels, we detected the serum levels of GnRH, FSH, and LH in mice after Mn exposure. The results showed that the serum GnRH, FSH, and LH levels increased after Mn exposure. Is this in T levels related to GnRH regulation after Mn infection? Previous research examined the level of serum FSH, LH, and T in 251 welders and 100 age-matched control workers. The results showed that the welders' serum FSH and LH levels were significantly higher and their serum T levels were lower than those of the control group (Kim et al., 2007). Animal experiments also confirmed that

Table 2
Changes of serum related hormone levels.

Group	GnRH (ng/L)	FSH (U/L)	LH (pg/ml)	T (nmol/L)	PGE_2 (ng/L)
control	55.75 ± 7.17	8.47 ± 0.55	1559.70 ± 63.44	172.11 ± 13.70	38.21 ± 3.06
12.5$MnCl_2$	60.48 ± 3.86	9.09 ± 0.83	1670.30 ± 80.09*	150.21 ± 10.59	52.15 ± 14.57
25 $MnCl_2$	62.12 ± 6.38*	9.21 ± 0.44	1911.00 ± 51.36**	134.53 ± 14.87**	53.40 ± 18.50*
50 $MnCl_2$	67.99 ± 4.51**	11.49 ± 1.38**	2264.50 ± 90.63**	119.38 ± 10.83**	61.57 ± 9.48**
DMSO + 50$MnCl_2$	60.94 ± 4.65	9.75 ± 1.28	1982.23 ± 127.46	118.25 ± 17.23	53.88 ± 3.89
SC-51322 + 50$MnCl_2$	42.00 ± 2.44$^{\#\#}$	4.39 ± 0.40$^{\#\#}$	1625.79 ± 128.83$^{\#\#}$	168.41 ± 18.02$^{\#\#}$	74.46 ± 8.46
AH6809 + 50$MnCl_2$	44.74 ± 6.16$^{\#\#}$	4.52 ± 0.53$^{\#\#}$	1575.43 ± 115.10$^{\#\#}$	151.97 ± 21.17$^{\#}$	80.71 ± 17.42$^{\#}$
DMSO	51.81 ± 3.82	8.97 ± 0.76	1496.13 ± 147.60	170.70 ± 26.00	36.28 ± 6.83

Data are mean ± SD, n = 8 each.
*P < 0.05, **P < 0.01 denote statistical significance compared with control group.
$^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 denote statistical significance compared with 50 $MnCl_2$ group.

Table 3
Changes of serum related hormone levels in Castrated mice.

Group	GnRH (ng/L)	FSH (U/L)	LH (pg/ml)	PGE_2 (ng/L)
control	47.96 ± 11.15	7.70 ± 0.44	1566.07 ± 51.64	38.30 ± 5.37
50 $MnCl_2$	60.21 ± 12.46*	10.20 ± 1.02**	1901.78 ± 68.10**	56.86 ± 9.84**
DMSO + 50$MnCl_2$	44.25 ± 5.21	10.16 ± 1.01	1848.21 ± 237.21	45.51 ± 6.11
SC-51322 + 50$MnCl_2$	34.32 ± 5.68##	5.60 ± 1.06##	1619.64 ± 170.67##	71.67 ± 6.67#
AH6809 + 50$MnCl_2$	32.25 ± 2.50##	6.42 ± 0.45#	1666.07 ± 71.99#	76.48 ± 12.33##
DMSO	44.00 ± 6.78	7.82 ± 0.43	1675.00 ± 66.75	39.17 ± 4.51

Data are mean ± SD, n = 8 each.
*$P < 0.05$, **$P < 0.01$ denote statistical significance compared with control group.
#$P < 0.05$, ##$P < 0.01$ denote statistical significance compared with 50 $MnCl_2$ group.

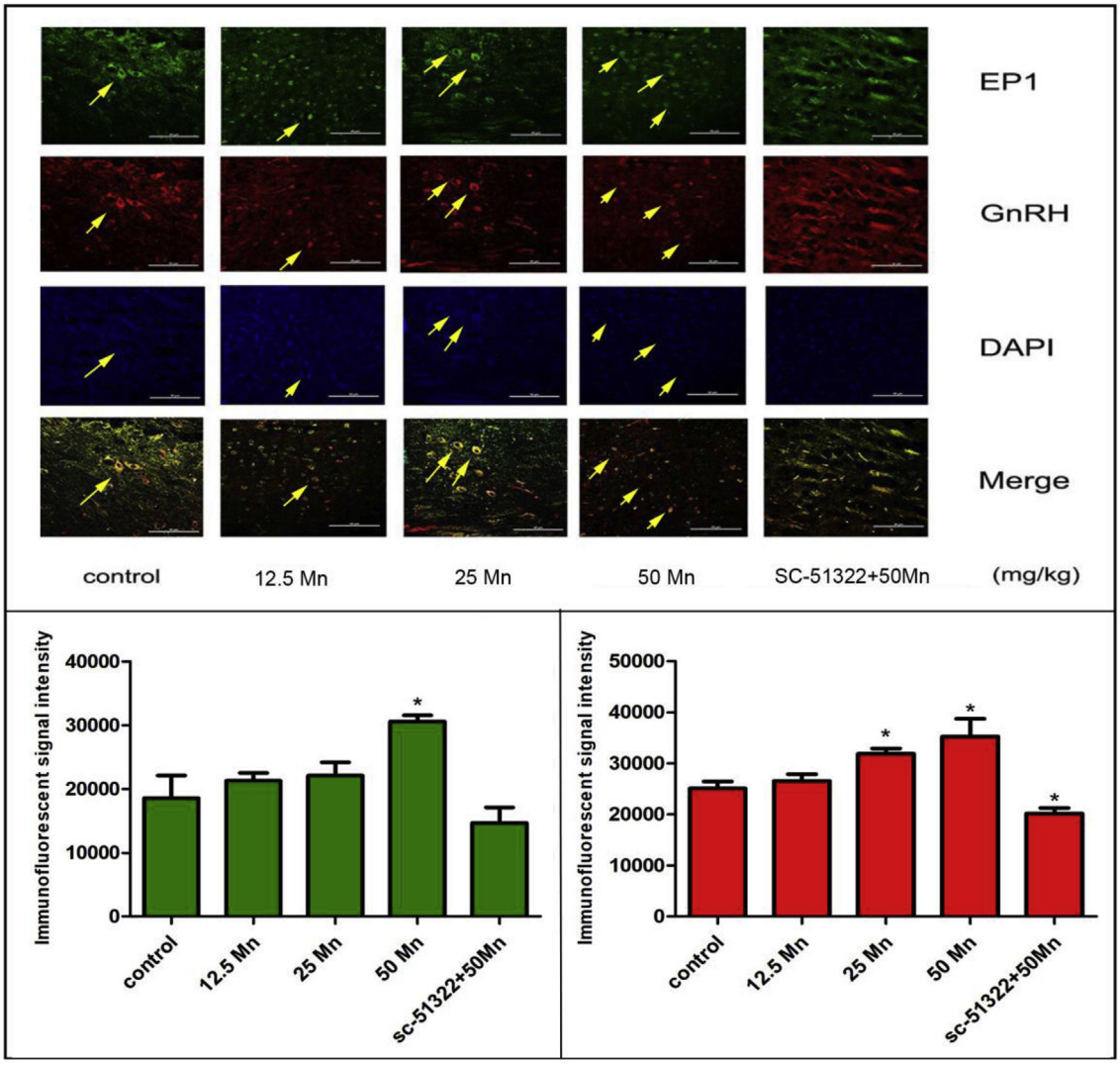

Fig. 5. The double immunofluorescent staining of the EP1 and GnRH in the hypothalamus. It identified DAPI (blue), GnRH (red) and EP1 (green) immunofluorescent expressions. The magnification was set at ×400. The immunofluorescent signal intensity of EP1, GnRH and DAPI in the hypothalamus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

environmental Mn can act on the hypothalamus to cause prepubertal GnRH secretion in rats (Sharif et al., 2013). Experiments determined that Mn exposure can increase the level of hypothalamic GnRH hormone and decrease the level of T hormone. We analyzed previous research to elucidate the reasons that may cause the T level to decrease. One cause may be due to Mn acting on the testes and affecting T secretion. Another reason may be the negative feedback regulation from the hypothalamic-pituitary-gonadal axis. As previously reported in the literature, Leydig cells express negative feedback in the pituitary and/or hypothalamus through the production of T, thereby maintaining the balance of the HPG axis. To eliminate negative feedback from T secreted by testes from the HPG axis, we conducted bilateral testicular removal on male mice, blocking the negative feedback of T from the testis on the GnRH at the beginning of the HPG axis. The results showed that although the levels of serum GnRH, FSH, and LH were lower than those in the uncastrated mice, they were still higher than those in the control group after castration. Therefore, excluding the negative feedback from T, the increase in the serum GnRH, FSH, and LH levels may

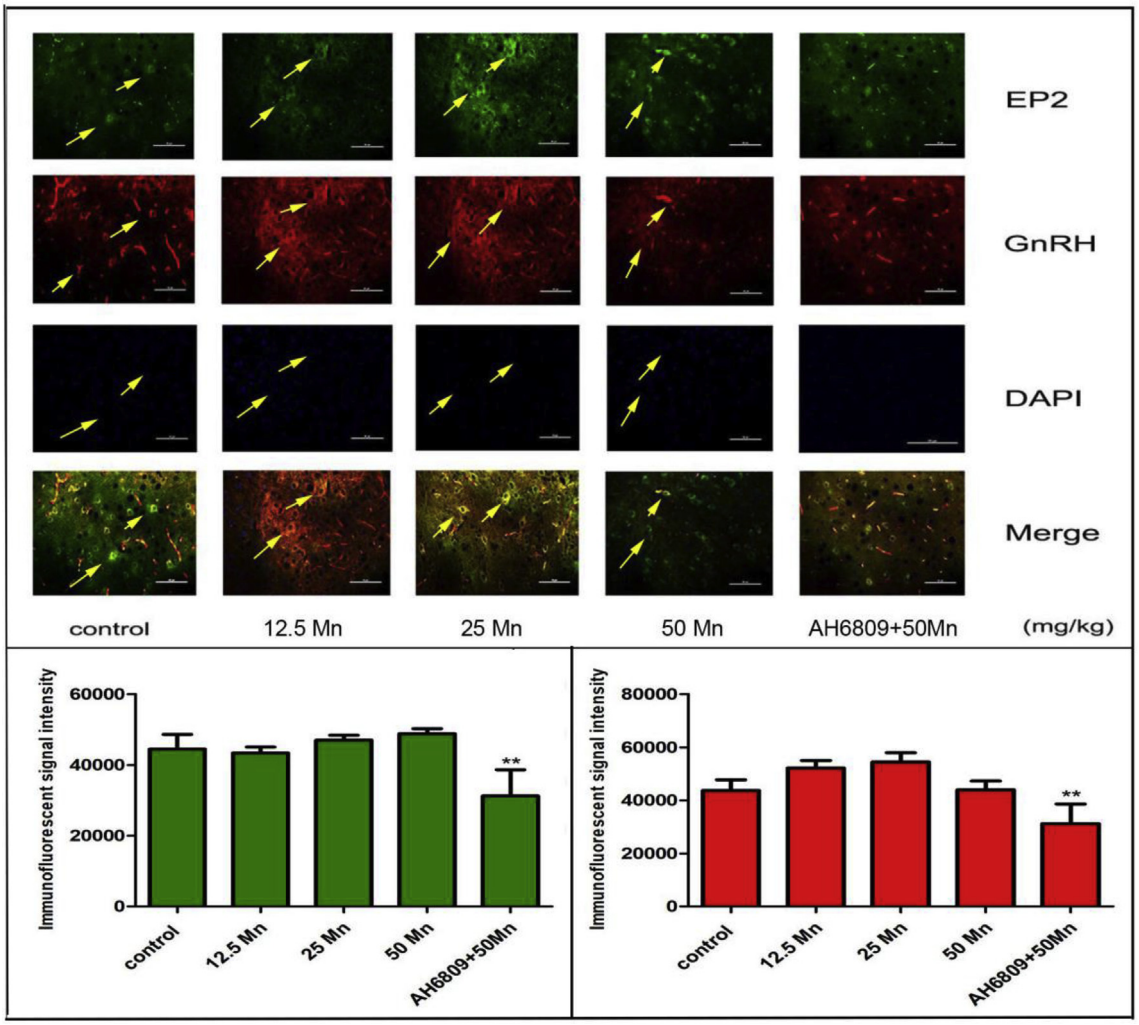

Fig. 6. The double immunofluorescent staining of the EP2 and GnRH in the hypothalamus. It identified DAPI (blue), GnRH (red) and EP2 (green) immunofluorescent expressions. The magnification was set at ×400. The immunofluorescent signal intensity of EP2, GnRH and DAPI in the hypothalamus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

have been due to Mn exposure.

How does Mn exposure change GnRH hormone levels? There may be many reasons. We analyzed previous research and found that astrocyte paracrine prostaglandin E2 may be an influencing factor. It mainly regulates animal reproduction by regulating astrocytes to affect the activity and secretion of GnRH neurons (Srivastava et al., 2013). Hypothalamic astrocytes catalyze the production of PGE_2 mainly by cyclooxygenase-2 (COX-2), an important rate-limiting enzyme in the synthesis of PGE_2 (Font-Nieves et al., 2012). PGE_2 acts on the two major parts of the hypothalamus, the cell bodies of the GnRH neurons in the preoptic area and the nerve endings of the GnRH neurons in the hypothalamic nodules and median ridges. By in vitro culture of hypothalamic explants, PGE_2 can effectively promote GnRH release from GnRH nerve endings in the median uplift (Ojeda and Campbell, 1982). PGE_2 induces GnRH release from GnRH neurons, possibly by activating EP1 receptors and regulating intracellular Ca^{2+}. PGE_2 can also act on the cell bodies of GnRH neurons and regulate their electrophysiological activities. PGE_2 binds to EP2 receptor on the cell body and activates the cAMP/PKA signaling pathway to affect the release of GnRH, delaying animal puberty and disturbing the reproductive function of adult animals (Fiebich et al., 2001). GnRH is secreted by the hypothalamus and is the initial link of the HPG axis (Clasadonte et al., 2011a, 2011b). Astrocytes located in the hypothalamus regulate GnRH secretion by paracrine PGE_2 binding to EP1 and EP2 receptors on GnRH neurons (Milatovic et al., 2011). Previous studies found that NE stimulation of LHRH release requires an intermediate role in PGE_2, and PGE_2-induced LHRH release may be mediated by glial cells. Ojeda et al. (Rage et al., 1997) reported that the release of serum FSH and LH decreased after blocking the synthesis and secretion of PGE_2. This was similar to our experimental results, indicating that PGE_2 plays an important role in the LH secretion process. The two antagonists of the PGE_2 receptors EP1 and EP2 (SC-51322 and AH6809) were used in our study to verify the role of EP1 and EP2 in Mn-induced GnRH secretion abnormalities. The serum GnRH levels were elevated in the normal and castrated mice after Mn exposure, but were significantly reduced after antagonizing EP1 and EP2 receptors. Previous studies also reported the same results and found elevated serum GnRH levels in rats after Mn exposure (Zeng et al., 2013). Naor et al. (2007) studied the expression of EP1 and EP2 receptors in GnRH neurons using dual-fluorescence confocal microscopy. The results suggested that PGE_2 may have a direct effect on gonadotropin function. Kobayashi and Narumiya (Naor et al., 2007) also found that knocking out mouse EP2 receptors will affect mouse fertilization. To further validate the relationship between the EP1 and EP2 receptors and GnRH neurons, our study examined changes in the protein levels expressed by both receptors and mRNA after Mn exposure and colocalization of EP1 and EP2 receptors and GnRH neurons. As the dose of Mn increased, the protein levels and mRNA expression of EP1 and EP2 increased. This result is consistent with an increase in serum

GnRH levels. After administering antagonists of the two receptors (EP1 and EP2), the reproductive function indicators of the mice also changed. After the intervention of EP1 and EP2 receptor antagonists, the serum level of T was significantly higher than in the 50 mg/kg Mn-treated group and the sperm count also relatively increased.

In summary, after Mn exposure, the number of sperm and T levels in mice decreased. GnRH secretion abnormalities are thought to play an important role in Mn-induced male reproductive dysfunction, and PGE_2 may be a key factor affecting GnRH secretion. In this experiment, the serum GnRH levels decreased after Mn exposure, and after antagonizing PGE_2 receptors EP1 and EP2, the T levels and sperm counts significantly increased compared with the high Mn-treated group. This indicates that the receptors EP1 and EP2 of PGE_2 play a role in GnRH secretion abnormalities and reproductive toxicity caused by Mn exposure. However, our research had some limitations. Since some humans are exposed to Mn for long periods of time each day, time-dependent responses to Mn exposure should also be assessed.

Credit author statement

Yu Deng; Fengdi Wu; Yanan Liu: Conceptualization, Methodology, Software. Fengdi Wu; Xinxin Yang: Data curation, Writing- Original draft preparation. Haibo Yang: Visualization, Investigation. Yu Deng; Bin Xu; Wei Liu; Zhaofa Xu: Supervision. Xinxin Yang: Software, Validation. Fengdi Wu: Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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