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Research paper

$PM_{2.5}$ disrupts thyroid hormone homeostasis through activation of the hypothalamic-pituitary-thyroid (HPT) axis and induction of hepatic transthyretin in female rats 2.5

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ABSTRACT

Fine particulate matter ($PM_{2.5}$), a ubiquitous environmental pollutant, has been indicated to affect thyroid hormone (TH) homeostasis in women, but the detailed mechanism behind this effect remains unclear. The objective of this study was to evaluate the roles of the hypothalamic-pituitary-thyroid (HPT) axis and hepatic transthyretin in the thyroid-disrupting effects of $PM_{2.5}$. Sprague Dawley rats were treated with $PM_{2.5}$ (0, 15 and 30 mg/kg) by passive pulmonary inhalation for 49 days; and recovery experimental group rats were dosed with $PM_{2.5}$ (30 mg/kg) for 35 days, and no treatment was done during the subsequent 14 days. $PM_{2.5}$ was handled twice a day by passive pulmonary inhalation throughout the study. After treatment, pathological changes were analyzed by performing haemotoxylin and eosin staining, measuring levels of THs and urine iodine (UI) in serum, plasma, and urine samples using enzyme-linked immunoabsorbent assay, and expression of proteins in the hypothalamus, pituitary, thyroid, and liver tissues of rats were analyzed by immunohistochemistry and Western blotting. The levels of oxidative stress factors, such as reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (Gpx), and nuclear factor-kappa B (NF-κB) in female rats' plasma were also evaluated by ELISA. The results of these analyses revealed that $PM_{2.5}$ treatment induced pathologic changes in rat thyroid and liver characterized by increased follicular cavity size and decreased amounts of follicular epithelial cells and fat vacuoles, respectively. Serum levels of triiodothyronine, thyroxine, and thyroid stimulating hormone were significantly decreased, plasma NF-κB level was increased and plasma redox state was unbalanced (enhanced ROS, MDA and Gpx levels; reduced SOD activities) in female rats treated with $PM_{2.5}$ ($P < 0.05$). $PM_{2.5}$ treatment suppressed the biosynthesis and biotransformation of THs by increasing sodium iodide symporter, thyroid transcription factor 1, thyroid transcription factor 2, and paired box 8 protein expression levels ($P < 0.05$). Additionally, thyroid stimulating hormone receptor and thyroid peroxidase levels were significantly decreased ($P < 0.05$). Both thyrotropin releasing hormone receptor and thyroid stimulating hormone beta levels were enhanced ($P < 0.05$). Moreover, transport of THs was inhibited due to reduced protein expression of hepatic transthyretin upon treatment with $PM_{2.5}$. In summary, $PM_{2.5}$ treatment could perturb TH homeostasis by affecting TH biosynthesis, biotransformation, and transport, affecting TH receptor levels, and inducing oxidative stress and inflammatory responses. Activation of the HPT axis and altered hepatic transthyretin levels therefore appear to play a crucial role in $PM_{2.5}$-induced thyroid dysfunction.

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1. Introduction

Thyroid disease can occur during all stages of life, from fetuses and children to young and middle-aged people as well as the elderly (Wang et al., 2020). In the past 30 years, the incidence of thyroid cancer has been increasing yearly all over the world, and the incidence of thyroid cancer in China specifically has also been on the rise (Sui et al., 2020). According to the data from the American Cancer Institute, a survey from 2009 to 2011 shows that the average annual diagnosis of thyroid cancer is about 100000 new cases in men and 129000 in women. A population survey in South Korea confirmed that thyroid cancer has the highest incidence among all female malignant tumors, including breast cancer (Jung et al., 2015). In Italy, thyroid cancer is the second most common malignancy in women under 45 years of age (Dal Maso et al., 2011). Population-based studies have shown that the incidence of thyroid cancer is greater in women than men (Parkin et al., 2010). Therefore, the study of development of thyroid disease in women deserves a great deal of attention.

In recent years, significant environmental pollution problems have become increasingly prominent, seriously affecting and restricting the health of society as a whole. Particulate matter 2.5 ($PM_{2.5}$), a main component of air pollutants, has a large specific surface area and strong adsorption capacity due to its small inner diameter and lengthy settling time. $PM_{2.5}$ can attach a large number of toxic substances and go deep into the alveolar area where it can enter the circulatory system, thus affecting all organ systems. Therefore, the impact of increased $PM_{2.5}$ exposure on human health has attracted a great deal of attention in recent years. *In vivo* and in vitro studies initially focused on the role of $PM_{2.5}$ exposure in cardiovascular disease (Weichenthal et al., 2014) and chronic pulmonary diseases (Yin et al., 2017), but more recently, the effects of $PM_{2.5}$ on thyroid function have begun to receive more attention.

Thyroid hormones (THs), such as thyroxine (T4) and triiodothyronine (T3), play a key role in regulating the growth and development of many organ systems. Therefore, a variety of symptoms, such as fatigue, hair loss, and diminished memory capacity, can be the result of slight variations in thyroid hormone homeostasis (Liu et al., 2015a). Recently, increasing studies from humans and animals have showed that the thyroid function is very sensitive to disruption by $PM_{2.5}$ (Janssen et al., 2017). In 2017, a birth cohort study in Belgium found that, after adjusting for other confounding factors, exposure to $PM_{2.5}$ in the third trimester in areas with severe air pollution reduced TSH levels in cord blood (Janssen et al., 2017). In 2018, a cohort study in the United States showed that prenatal $PM_{2.5}$ exposure is associated with higher neonatal serum total thyroxine (TT4) concentrations (Howe et al., 2018). In 2019, a prospective Chinese birth cohort study found that early exposure of pregnant women to $PM_{2.5}$ and its six main constituents- organic matter (OM), black carbon (BC), sulfate (SO4(2-)), nitrate (NO3(-)), ammonium (NH4(+)), and soil dust was negatively correlated with free thyroid hormone (FT4) levels in maternal serum. Maternal FT4 may play a role in the effects on birth weight observed with exposure to $PM_{2.5}$ (Wang et al., 2019). A 2019 study in Shanghai found that prenatal air pollution exposure, especially $PM_{2.5}$ exposure in early pregnancy, was significantly inversely associated with FT4 levels (Zhao et al., 2019). In 2020, a recent population study in London found that exposure to higher levels of $PM_{2.5}$ during pregnancy increased risk of premature delivery through perturbation of TH levels (Smith et al., 2020). Although more studies have begun to focus on how $PM_{2.5}$ exposure disrupts thyroid function and TH homeostasis in women, the mechanisms by which $PM_{2.5}$ exposure affects TH levels remain unclear. Studies have shown that $PM_{2.5}$ exposure can activate the hypothalamus-pituitary (HPT) axis (Qiu et al., 2018) by regulating the function of the hypothalamus and pituitary (Xu et al., 2016). Exposure to $PM_{2.5}$ via inhalation affected hypothalamic monoamine and corticotrophin releasing hormone levels in lean and obese rats. $PM_{2.5}$ exposure most likely activates the paraventricular nucleus (PVN) of the hypothalamus, which may be closely related to the activation of the HPT axis (Balasubramanian et al., 2013). Other possible mechanisms may be associated with changes in levels of TH receptors (Lee et al., 2007) and transthyretin (TTR) (Ishihara et al., 2003), which were observed upon exposure to similar environmental pollutants in other studies both in vitro and in vivo. Furthermore, a recently published study (Riggs et al., 2020) suggested that another possible mechanism involved in the observed disturbance of TH metabolism may be associated with increased levels of oxidative stress and inflammation following exposure to $PM_{2.5}$, which could also cause activation of the HPT axis.

A growing body of research has shown that $PM_{2.5}$ exposure can disrupt thyroid function in women, while some possible mechanisms have been elucidated to explain this effect, the specific changes in biosynthesis, biotransformation and transport of THs following exposure to $PM_{2.5}$ remain unknown. Thus, the specific effects of $PM_{2.5}$ exposure on THs homeostasis still need to be explored and clarified. Therefore, the purpose of this study was to determine how $PM_{2.5}$ exposure induces TH homeostasis disorder and identify possible mechanisms. We propose the hypothesis that $PM_{2.5}$ exposure may disturb the biotransformation, transport, and biosynthesis of THs in vivo. Additionally, we further explored the hypothesis that the HPT axis may play a key role in $PM_{2.5}$-induced disruption of TH homeostasis by interacting with TH synthesis-related proteins and receptors, as well as promoting a disordered redox state, induction of nuclear factor-kappa B, and alterations in hepatic transthyroxine levels.

2. Materials and methods

2.1. Animal studies

Thirty-two healthy SPF female Sprague-Dawley rats with an average weight of 60 ± 20 g after weaning were purchased from SPF (Beijing) Biotechnology Co., Ltd (License key: SCXK (Jing) 2019-0010). The rats were housed in a temperature (22 ± 0.5°C), humidity (50 ± 5%), and light (12 h cycle) controlled room for 5 days, with free access to food and water.

After adaptation for 5 days, rats were randomly divided into 4 groups (n=8/dose group): Control group (C, 0 mg/kg), Medium-dose $PM_{2.5}$ group (M, 15 mg/kg), High-dose $PM_{2.5}$ group (H, 30 mg/kg) and Recovery-dose $PM_{2.5}$ group (R, 30 mg/kg, dosed for 35 days (d) and no treatment for 14 d). Rats were observed twice daily, and general health parameters were recorded (fear of cold, lack of energy, fatigue, anorexia, constipation, etc.). All rats were dosed twice a day by passive pulmonary inhalation according to our published methods (Jin et al., 2016) for 49 d with $PM_{2.5}$, which was collected from the atmospheric sampling station using a large flow $PM_{2.5}$ particulate sampling device (TE-6070 American TISCH) located at Xinxiang Medical University, China. $PM_{2.5}$ was dissolved in saline solution at 0 (control), 15, and 30 mg/kg body weight; this dosing regimen was designed according to doses utilized in other published animal (Kodavanti et al., 2003) and human (Chen et al., 2017) studies. All rat body weights were measured at the end of each week. The age of the animals upon initiation of treatment and the duration of treatment were based on the recommendations of the U.S. EPA Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC).

The research personnel involved in this study were systematically and specifically trained before conducting experiments, including regarding methods of anesthesia, placement, and execution. The ultimate goal of all training was to alleviate any unnecessary pain for the experimental rats. All animal experiments were performed in accordance with the Institute of Zoology Animal and Medical Ethics Committee of Xinxiang Medical University as well as the current Chinese legislation, in addition to international standards (NIH publications No 80-23 revised 1996).

2.2. Sample collection and processing

Rats were sacrificed under sodium pentobarbital anesthesia after 49 d of treatment. Blood samples were obtained from the abdominal aorta before the rats were sacrificed and prior to blood clotting. Serum was obtained by centrifugation of the blood at 3000 rpm for 15 min in a refrigerated centrifuge (H1650-W, Eppendorf) and then immediately stored at -80°C until further analysis. Thyroid, liver, brain, and pituitary tissue samples were rapidly weighed, frozen in liquid nitrogen, and stored at -80°C until use.

2.3. Haemotoxylin and eosin (H&E) staining and histological evaluation

Following $PM_{2.5}$ treatment, the rat thyroids and livers were dissected, fixed with 4% paraformaldehyde solution, routinely sampled, dehydrated, paraffin-embedded, prepared (4 μm thick), and stained with H&E for microscopic examination. After scanning with a panoramic scanner (3D HISTECH Pannoramic250 , made in Hungary), thyroid and liver lesions were observed and described.

2.4. Enzyme-linked immunoabsorbent assay (ELISA)

The levels of serum and plasma total T4 (TT4), total T3 (TT3), TSH, as well as urine iodine (UI) levels were determined using ELISA kits (Shanghai Future Industrial Co. Ltd., China) according to the manufacturer's instructions. The serum and urine samples, which were left at room temperature for 2 h, were directly assayed after centrifuging at 3000 rpm for 20 min. The plasma samples were also directly assayed after centrifuging at 3000 rpm for 20 min under heparin anticoagulation. No significant cross-reactivity or interference was observed. All samples and standards were run in duplicate and detected on a BioTek instrument (Perkin Elmer Singapore Pte. Ltd., Singapore), and the standard curve was used for data analysis.

In addition, the levels of plasma oxidative stress factors (ROS, MDA, SOD, and Gpx) in female rats were determined according to the manufacturer's instructions (Shanghai Future Industrial Co. Ltd., China) and the analytical method described previously (Kobayashi et al., 2001; Kuthan et al., 1986; Rotruck et al., 1973). Moreover, the levels of plasma nuclear factor-kappa B (NF-κB) were determined using enzyme-linked immunoabsorbent assay (ELISA) kit (Shanghai Future Industrial Co. Ltd., China) by following the manufacturer's instructions.

2.5. Immunohistochemical staining analysis

Sections of thyroids and livers were fixed in 4% paraformaldehyde, embedded in paraffin and sliced into 4 μm thick sections. After a series of conventional procedures, the sections were incubated with either NIS antibody (bs-0448R, dilution 1:100), TPO antibody (bs-23884R, dilution 1:100), TTF1 antibody (bs-0826R, dilution 1:100), TG antibody (bs-0291R, dilution 1:200), PAX8 antibody (bs-1201R, dilution 1:100), or TTR antibody (bs-0152R, dilution 1:100) overnight at 4°C. Antibodies were purchased from Beijing Biosynthesis Biotechnology Co., LTD. China. Secondary antibodies were applied for 15 min at room temperature, and sections were rinsed in PBS. Peroxidase-conjugated streptavidin was applied for 15 min, followed by diaminobenzidine (DAB) substrate for 10 min and hematoxylin for 5 min. Immunohistochemical micrographs were taken with an Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Areal density analysis was used to evaluate the immunohistochemical results: higher areal density indicated a higher positive expression level. Areal density=IOD/AREA. Integral optical density (IOD) and pixel area of (AREA) of positive expression in the tissue sections were analyzed using Image Pro-Plus 6.0 software.

2.6. Western blot analysis

Twenty milligrams of liver, thyroid, hypothalamus and pituitary were homogenized in 200 μl of cell lysis buffer (RIPA, G2002, Servicebio) for Western blotting (Wuhan Servicebio Technology Co. LTD., China) supplemented with 2 μl PMSF (100 mM, G2008, Servicebio). Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Biotech Inc., China). Equal amounts of protein (30 μg) were electrophoresed through 12% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were incubated overnight at 4°C with NIS polyclonal antibody (bs-0448R, dilution 1:500), TPO polyclonal antibody (bs-23884R, dilution 1:500), TTF1 polyclonal antibody (bs-0826R, dilution 1:500), FOXE1 polyclonal antibody (bs-0446R, dilution 1:500), PAX8 polyclonal antibody (bs-1201R, dilution 1:500), TSHR polyclonal antibody (bs-0460R, dilution 1:500), TRHR polyclonal antibody (bs-16719R, dilution 1:500), TSHβ polyclonal antibody (A14523, Wuhan Aptech Biotechnology Co. LTD., China, dilution 1:500), TTR polyclonal antibody (bs-0152R, dilution 1:100), GAPDH (GB12002, Wuhan Servicebio Technology Co. LTD., China, dilution 1:500), or β-actin (GB12001, Wuhan Servicebio Technology Co. LTD., China, dilution 1:3000) overnight at 4°C. Antibodies were purchased from Beijing Biosynthesis Biotechnology Co. LTD., China. Then, membranes were incubated with an HRP-conjugated secondary antibody for 1 h at 37°C. An ECL reaction solution was used to visualize the protein present in the membrane. A scanner (V300, EPSON) was used to scan and archive the film, and the color was organized using Image-Pro Plus (Adobe Photoshop, Adobe). The optical density of target bands was then analyzed using gray level analysis software (AlphaEase FC, Alpha Innotech).

2.7. Statistical analysis

SPSS 21.0 (Beijing Stats Data Mining Co. Ltd, Beijing, China) software was used for all statistical analyses. All experiments were performed at least in triplicate. Values are presented as mean ± standard deviation (SD). The statistical difference between control and $PM_{2.5}$-treated group was compared by Student's *t*-test. When multiple comparison tests for different dose groups were conducted, variance of homogeneity was examined using the Analysis of variance (ANOVA) and the significance of inter-group differences were analyzed using Dunnett's Test or Bonferroni post hoc test. Heterogeneous data was analyzed using Kruskal-Wallis Test and the significance of inter-group differences between the control and treated groups were assessed using Dunn's Rank Sum Test. The statistical was set at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. PM 2.5 treatment altered the body weight (BWs) of treated rats

To evaluate the toxic effects induced by long-term exposure to $PM_{2.5}$ on the growth status of female rats, the body weights (BWs) of rats treated with $PM_{2.5}$ were recorded every week (Fig. 1). As shown in Fig. 1, the BWs in the $PM_{2.5}$-treated groups over the course of 7 weeks were significantly decreased when compared with the control group at the relevant time points ($P < 0.05$).

3.2. PM 2.5 exposure elevated the relative liver and thyroid weights of treated rats

To assess the effects of $PM_{2.5}$ exposure on the livers and thyroids of female rats, the relative liver and thyroid weights of $PM_{2.5}$-treated rats were calculated. As shown in Table S1, the relative liver and thyroid weights of female rats were all significantly increased in the $PM_{2.5}$-treated groups compared with the control group at 7 weeks ($P < 0.05$), and there was a decreasing trend in the recovery group with regard to liver weight when compared with the high-dose group at 49 d, as well as a non-significant increasing trend in the recovery group thyroid weights at 49 d.

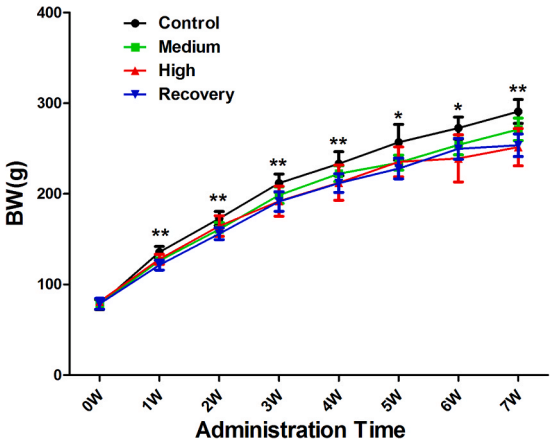

Fig. 1. Effects of $PM_{2.5}$ treatment on body weight (BW) of rats at each time point and dose. Data are expressed as the mean ± standard deviation (SD). *Significantly different from the control ($P < 0.05$); **Significantly different from the control ($P < 0.01$). C: (Control group, normal saline for 49 d); M: (Medium-dose group, 15 mg/kg for 49 d); H: (High-dose group, 30 mg/kg for 49 d); R: (Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d).

3.3. *PM 2.5 exposure decreased serum and plasma levels of thyroid hormones*

To analyze TH homeostasis following exposure to $PM_{2.5}$, serum and plasma levels of thyroid hormones were examined by ELISA. As shown in Fig. 2, THs in serum exhibited a decreasing trend up to 49 d. In this study, serum levels of TT3, TT4 and TSH in the high- and recovery-$PM_{2.5}$ dose groups were all decreased compared to the control at 49 d ($P < 0.05$) (Fig. 2A–C). However, rat serum TT3 and TSH levels in the recovery-dosed group showed a non-significant increasing trend compared with the high $PM_{2.5}$-dosed group at 49 d. In addition, ELISA results indicated that $PM_{2.5}$ treatment decreased plasma TT4 levels (Fig. 2E) when compared with the control group at 49 d ($P < 0.05$).

3.4. *PM 2.5 treatment induced alterations in levels of oxidative stress factors and nuclear factor-kappa B (NF-κB) in female rat plasma*

To evaluate the effects of $PM_{2.5}$-induced changes in oxidative stress and inflammatory responses on thyroid hormone homeostasis, the levels of plasma oxidative stress factors (ROS, MDA, SOD, and Gpx) and NF-κB in female rats were analyzed. As shown in Table S2, changes in the levels of plasma oxidative stress factors (enhanced ROS, MDA and Gpx levels; reduced SOD activities) in female rats were induced upon prolonged exposure to $PM_{2.5}$ ($P < 0.01$). In addition, we observed increased NF-κB levels in the $PM_{2.5}$-dosed groups compared with the control group ($P < 0.01$). However, decreased MDA, Gpx and NF-κB levels and

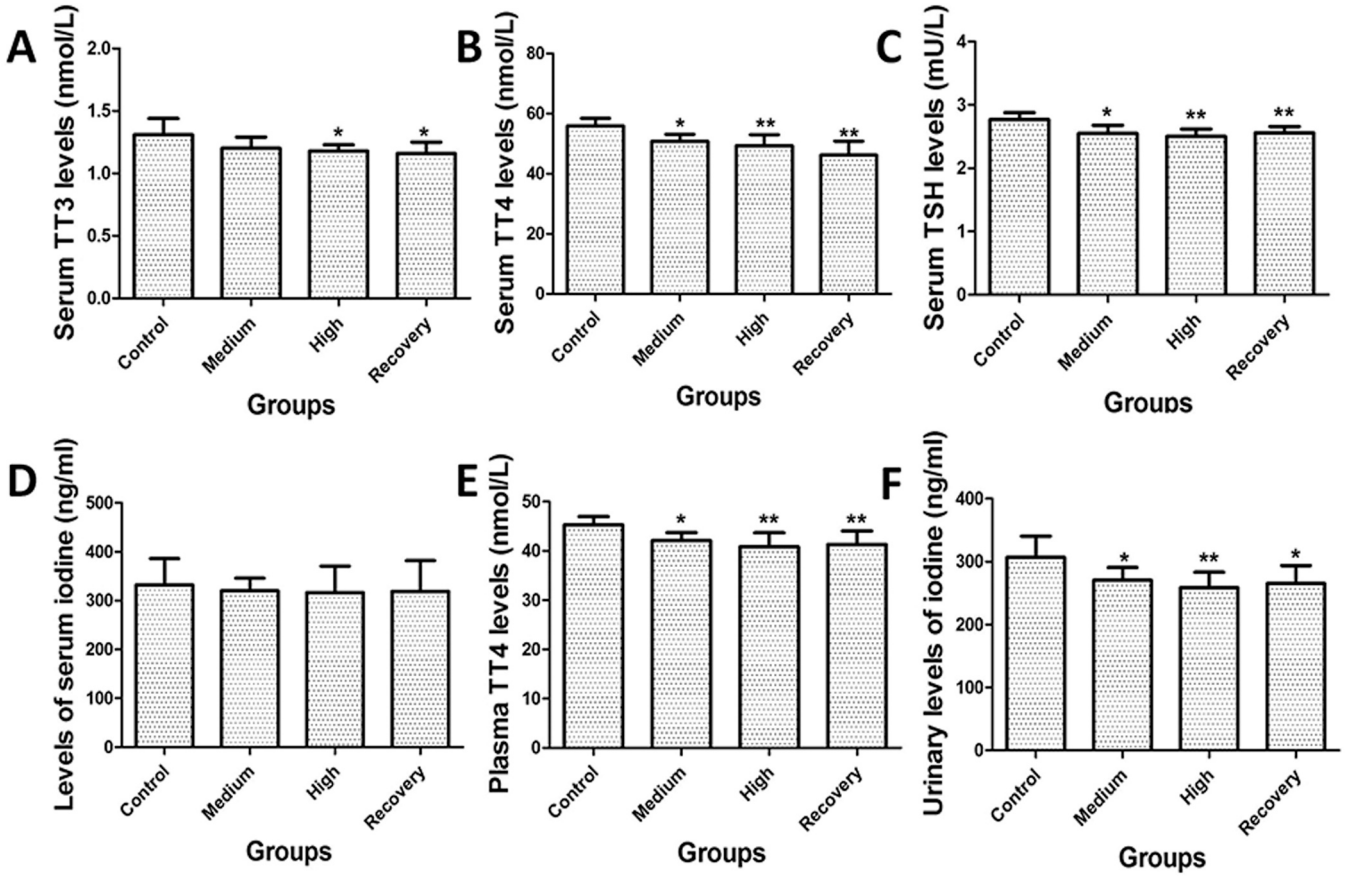

Fig. 2. Effects of $PM_{2.5}$ treatment on thyroid hormone and urine iodine levels. Rats (n = 8/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. C: (Control group, normal saline for 49 d); M: (Medium-dose group, 15 mg/kg for 49 d); H: (High-dose group, 30 mg/kg for 49 d); R: (Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d). A-C: Serum levels of thyroid hormones; D: Serum level of urine iodine; E: Plasma levels of thyroid hormone; F: Levels of urine iodine. Error bars represent the standard deviation. Significant difference: *$P < 0.05$ versus control.

increased SOD and ROS activities were also present in the recovery-$PM_{2.5}$ dose group when compared with high-$PM_{2.5}$ dose group at 49 d ($P < 0.01$).

3.5. PM 2.5 exposure reduced iodine levels in the serum and urine of treated rats

To examine the nutritional status of iodine in female rats, urine iodine levels were measured by ELISA. In this study, serum iodine levels displayed a non-significant decreasing trend in the high (319.15 ± 62.77) and recovery (316.36 ± 54.11) $PM_{2.5}$-dose groups when compared with the control group (332.21 ± 54.01) at 49 d (Fig. 2D). However, the levels of iodine in urine were significantly lower in the high $PM_{2.5}$-treated group compared with the control group at 49 d ($P < 0.05$) (Fig. 2F).

3.6. PM 2.5 exposure led to histopathologic changes in rat thyroid and liver tissues

H&E staining was used to evaluate histological changes in the thyroid and liver tissues of $PM_{2.5}$-treated rats. A large number of abnormal changes were observed in the thyroid tissues of the $PM_{2.5}$-treated groups: the follicular cavity were increased and filled with a large amount of dyed gelatin (Fig. 3A–D represent the thyroid). In the medium-dose group, a few follicular epithelial cells appear to have been shed and lost, a small number of immersed macrophages were present, and colloid staining of the follicle was lighter (Fig. 3B and C). More inflammatory cells were also observed in the stroma (Fig. 3C). Increased levels of macrophages and inflammatory cells were observed as well (Fig. 3D). Histopathological changes in the liver were also studied via H&E staining analysis at 49 d. Histopathological changes in the livers of $PM_{2.5}$-treated rats were characterized by increased fat degeneration, fat drops, empty lipocytes, and the presence of apoptotic cells in the high-dose group when compared with the control group at 49 d after exposure to $PM_{2.5}$ (Fig. 3A–D represent the liver). Many of the observed histopathological changes, such as increased follicular cavity size and increased numbers of macrophages and inflammatory cells were present in the thyroids and livers of $PM_{2.5}$-induced recovery-dose group rats as well as in the high-dose group (Fig. 3D).

3.7. PM 2.5 exposure disturbed thyroid-related protein and receptor levels in vivo

To determine the effects of $PM_{2.5}$ treatment on the transport of THs and TH-related receptor levels, protein expression levels of NIS, TTF1, FOXE1, TSHR, TPO, and PAX8 were analyzed by WB. The protein expression levels of thyroid NIS, TTF1, FOXE1, and PAX8 in the $PM_{2.5}$-dosed groups were significantly increased at 49 d ($P < 0.01$), while significant increases were observed in the recovery-dose group at 49 d when compared with the high-dose group ($P < 0.05$). However, the levels of thyroid TSHR and TPO protein expression were decreased in all $PM_{2.5}$-treated groups at 49 d ($P < 0.05$) (Fig. 4). IHC analysis also revealed a statistically significant increase in the levels of thyroid NIS, TTF1, and PAX8 protein expression in all $PM_{2.5}$-treated groups at 49 d when compared with the control ($P < 0.05$) (Fig. 5 and Fig. S1). IHC analysis further indicated that thyroid TG levels were increased in the high-dose group at 49 d ($P < 0.05$) (Fig. 5 and Fig. S1). Moreover, thyroid-related protein and receptor levels in the recovery-dose group showed a reverse trend when compared the high-dose group.

3.8. PM 2.5 exposure affected expression of proteins related to the hypothalamus-pituitary-thyroid (HPT) axis

To elucidate the role of the HPT axis in $PM_{2.5}$-induced TH disequilibrium, hypothalamus TRHR and pituitary TSHβ protein expression levels were analyzed. $PM_{2.5}$ exposure resulted in significantly increased

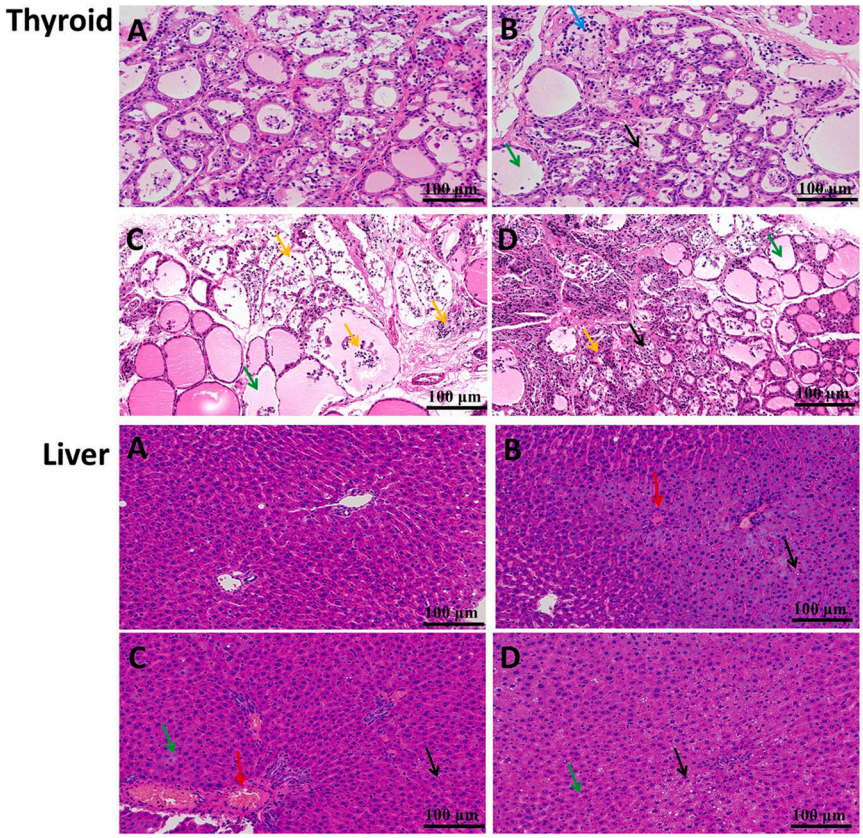

Fig. 3. Effects of $PM_{2.5}$ treatment on the histology of thyroids and livers. H&E staining was conducted to assess histological changes in the thyroid and liver. Rats (n=6/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. A: Control group, normal saline for 49 d; B: Medium-dose group, 15 mg/kg for 49 d; C: High-dose group, 30 mg/kg for 49 d. D: Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d. For the thyroid: black arrow denotes a few follicular epithelial cells shed and disappear; blue arrow denotes a small number of immersed macrophages; green arrow denotes lightened colloid stain in the follicle; yellow arrow denotes inflammatory cells in the stroma. For the liver: black arrow denotes empty lipocytes; red arrow denotes apoptotic cells; green arrow denotes pronounced fat degeneration, fat drops. Magnification: 200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

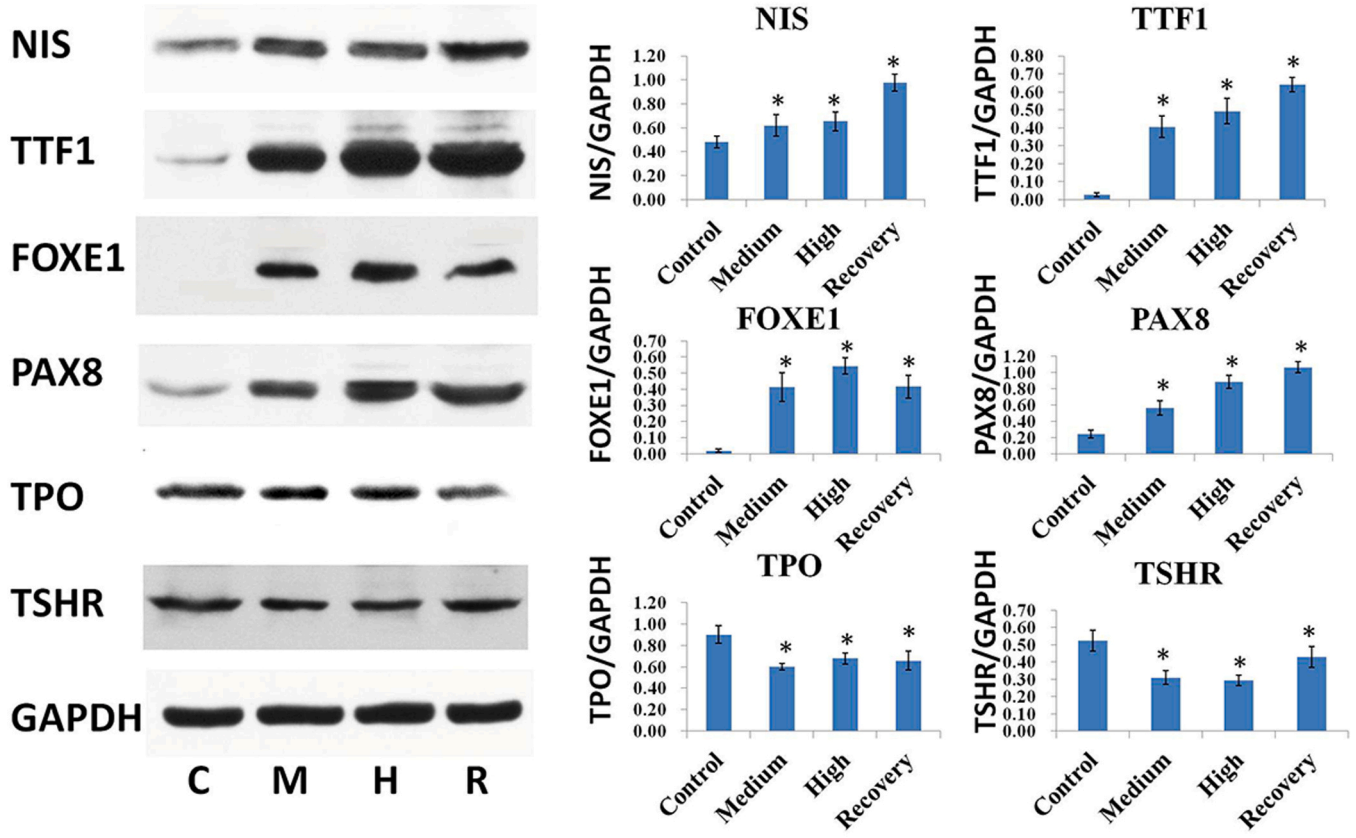

Fig. 4. Effects of $PM_{2.5}$ treatment on thyroid-related protein and receptor levels. Protein expression levels of thyroid NIS, TTF1, FOXE1, PAX8, TPO, and TSHR were assessed by Western blot. Rats (n = 5/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. C: (Control group, normal saline for 49 d); M: (Medium-dose group, 15 mg/kg for 49 d); H: (High-dose group, 30 mg/kg for 49 d); R: (Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d). The housekeeping gene GAPDH was used as an internal positive control. The relative expression of target proteins was calculated using the gray value ratio of the target protein band and the reference protein band (AlphaEase FC software). Error bars represent the standard deviation. Significant difference: $^*P < 0.05$ versus control. NIS: Sodium iodide symporter, TTF1: Thyroid transcription factor1, FOXE1: Thyroid transcription factor 2, PAX8: paired box 8, TPO: Thyroid peroxidase, TSHR: Thyroid stimulating hormone receptor.

protein expression of hypothalamus TRHR in the medium- and high-dose groups at 49 d. When $PM_{2.5}$ exposure increased, however, such expression seemed to increase in the recovery group compared with the high-dose group (Fig. 6). In addition, $PM_{2.5}$ exposure also induced significantly increased expression of pituitary TSHβ at 49 d. However, increased TSHβ protein expression was also present in the recovery group following exposure to $PM_{2.5}$ at 49 d (Fig. 6).

3.9. *PM $_{2.5}$ exposure induced protein expression of hepatic TTR*

To determine effect of $PM_{2.5}$ exposure on the transport of THs, the areal density and protein levels of TTR were both analyzed. As shown in Fig. 7, IHC analysis revealed that liver TTR levels exhibited a dose-dependent decrease, and the protein expression of liver TTR was also significantly decreased after exposure to $PM_{2.5}$ for 49 d ($P < 0.05$). Compared to the control group, a significantly increased level of TTR was present in the high-dose group, with the areal density (IOD/AREA) decreasing by 72.7% ($P = 0.012$), while the areal density of TTR in the control group showed a 3.7-fold ($P < 0.001$) increase over the high-dose group (IHC results shown in Fig. 7).

Compared to the control group, a significantly declined level of TTR protein was observed in the medium-dose group, with protein expression decreasing by 37.2% ($P = 0.018$), while protein expression of TTR in the high-dose group exhibited a 1.6-fold ($P < 0.001$) decrease when compared to the control. In addition, the protein level of liver TTR in the control group displayed a remarkable increase when compared to the high-dose group (WB results shown in Fig. 7).

4. Discussion

The current study explored the roles of the HPT axis and hepatic transthyroxine in $PM_{2.5}$-mediated perturbation of TH homeostasis. The experimental findings indicate the presence of histological changes in the thyroid and liver (Fig. 3) with $PM_{2.5}$ treatment, as well as the decline of urine iodine levels (Fig. 2F), and circulating TH levels were significantly decreased (Fig. 2A-C, E). A recent study by Wang et al. Wang et al. (2019) indicated that maternal exposure to $PM_{2.5}$ in humans could reduce maternal FT4 levels. There are several possible mechanisms for these effects, including that $PM_{2.5}$ treatment could influence TH homeostasis, including the biosynthesis, biotransformation, transport, receptor levels, and metabolism of molecules relevant to TH homeostasis, via activation of the HPT axis, and that $PM_{2.5}$ treatment might drive the downregulation of THs through modulation of hepatic transthyroxine levels, which would induce oxidative stress and inflammatory responses in vivo.

Thyroid hormones have many biological functions, including promoting growth, regulating metabolism, and improving organ activity. The formation of thyroxine requires six processes, including synthesis, storage, iodization, reabsorption, decomposition, and release. First, TH biosynthesis and storage occur in the follicular lumen in follicular epithelial cells, which synthesize the TH precursor thyroglobulin (TG) in the rough endoplasmic reticulum. TG can take part in TH biosynthesis due to the existence of iodine. Second, follicular epithelial cells can absorb iodine from the blood, and iodine is activated by thyroid peroxidase (TPO). Iodide uptake and storage is controlled by the sodium

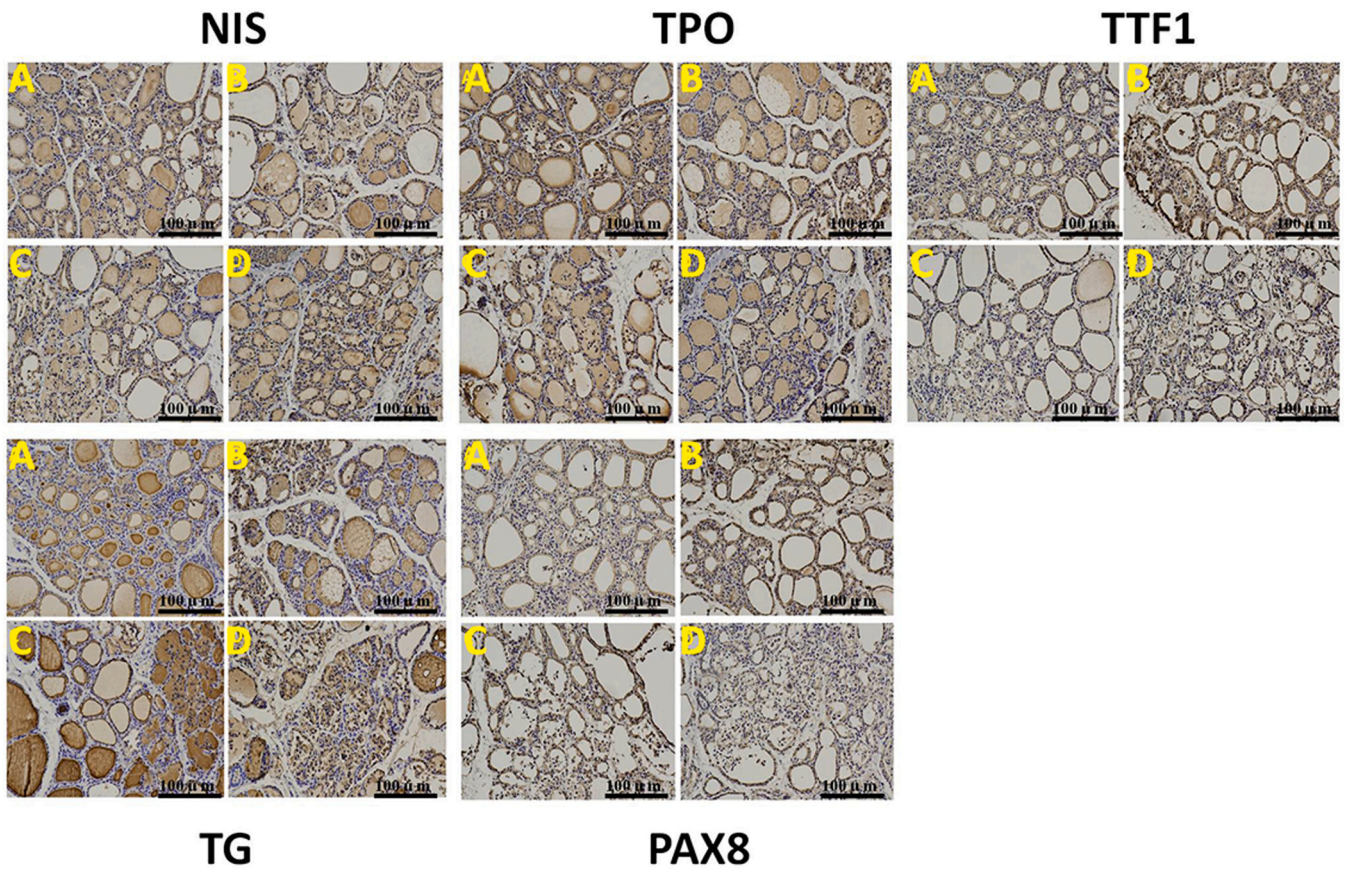

Fig. 5. Effect of $PM_{2.5}$ treatment on the expression of thyroid hormone-related proteins. Protein expression levels of thyroid NIS, TPO, TTF1, TG, and PAX8 were assessed by immunohistochemical analysis. Rats (n=5/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. The development of brown and yellow color in the tissue sections indicated positive expression of the relevant target protein. A: Control group, normal saline for 49 d; B: Medium-dose group, 15 mg/kg for 49 d; C: High-dose group, 30 mg/kg for 49 d. D: Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d. NIS: Sodium iodide symporter, TPO: Thyroid peroxidase, TTF1: Thyroid transcription factor1, TG: Thyroglobulin, PAX8: paired box 8. Magnification: 200×.

iodide symporter (NIS) (Bizhanova and Kopp, 2009; Guo et al., 2015). Next, activated I^- enters the follicular cavity and binds to TG, forming iodized TG, which is then absorbed by follicular epithelial cells to form glial vesicles under the role of thyroid stimulating hormone (TSH) secreted by the pituitary gland. TSH functions in the control of thyroid structure and metabolism (Ikeda et al., 2017; Shih et al., 2018). Finally, the glial vesicles fuse with lysosomes, and iodinated thyroglobulin is decomposed by hydrolases to form T4 and T3 (Kohn et al., 2001). In the present study, the elevated levels of NIS (Fig. 4 and Fig. 5) observed following $PM_{2.5}$ exposure could be responsible for concentrating iodide within thyroid follicular cells (Chen et al., 2016). Decreased levels of TPO (Fig. 4 and Fig. 5) in the $PM_{2.5}$-treated group inhibited I^- transport and iodization, and further reduced circulating TH levels. The reduced levels of urine iodine (Fig. 2F) observed following $PM_{2.5}$ exposure in this study further indicated that iodine levels were insufficient, resulting in severe thyroid dysfunction. In addition, we also found that TG levels were significantly increased following $PM_{2.5}$ treatment (Fig. 5), further indicating that iodination of Tyr residues failed due to a shortage of iodine, resulting in decreased T4 and T3 levels (Figs. 2A and 2B). This will decrease the levels of TSH and further stimulate iodinated TG in the follicular lumen of the thyroid (Lin, 2008). The increased pituitary TSHβ (Fig. 6) and thyroid TG levels (Fig. S1D) observed following $PM_{2.5}$ exposure in this study provide further evidence to support this phenomenon. Taken together, these findings indicate that $PM_{2.5}$ treatment could interfere with the synthesis of TH-related proteins, thus reducing TH levels. Transcription factors, such as TTF1 and TTF2 (FOXE1), are related to development of the thyroid gland and are crucial for the maintenance of the thyroid differentiation phenotype (Huang et al., 2011). These transcription factors can bind to and activate the promoter of thyroid specific genes such as TG, TPO, and TSHR (Shimura et al., 1995). In the current study, $PM_{2.5}$ exposure significantly increased levels of TTF1 and FOXE1 (Fig. 4), leading to activation of thyroid specific genes such as TG, but not TPO and TSHR (Fig. 4 and Fig. 5), resulting in I_2 deficiency and a subsequent decrease in TH levels. PAX8 encodes a member of the paired box (PAX) family of transcription factors (Huang et al., 2016). Members of this gene family typically encode proteins that contain a paired box domain, an octapeptide, and a paired-type homeodomain. This protein is involved in thyroid follicular cell development and expression of thyroid-specific genes. Mutations in this gene have been associated with thyroid dysgenesis, thyroid follicular carcinomas, and atypical follicular thyroid adenomas (Ozcan et al., 2011). The increased levels of PAX8 (Fig. 4) detected after $PM_{2.5}$ exposure indicated that thyroid follicular cell development was enhanced following $PM_{2.5}$ exposure, and this finding is consistent with the histological changes we observed in the thyroid (Fig. 3).

Normally, the hypothalamus can regulate the secretion of pituitary TSH by releasing thyrotropic hormone releasing hormone (TRH) under the regulation of the central nervous system, which will stimulate thyroid cells to secrete T4 and T3. Therefore, the interaction between THs and their combinative receptors (TSHR and TRHR) plays a critical role in regulating the physiology and function of the thyroid by stimulating the release of THs (Shih et al., 2018; De Miguel et al., 2005). The diminished TSHR (Fig. 4) and elevated TRHR protein levels (Fig. 6) observed following $PM_{2.5}$ exposure in vivo further indicated that TSHR and TRHR take effect on $PM_{2.5}$-induced TH homeostasis disorder. This negative feedback effect on the pituitary weakens when the concentration of T4

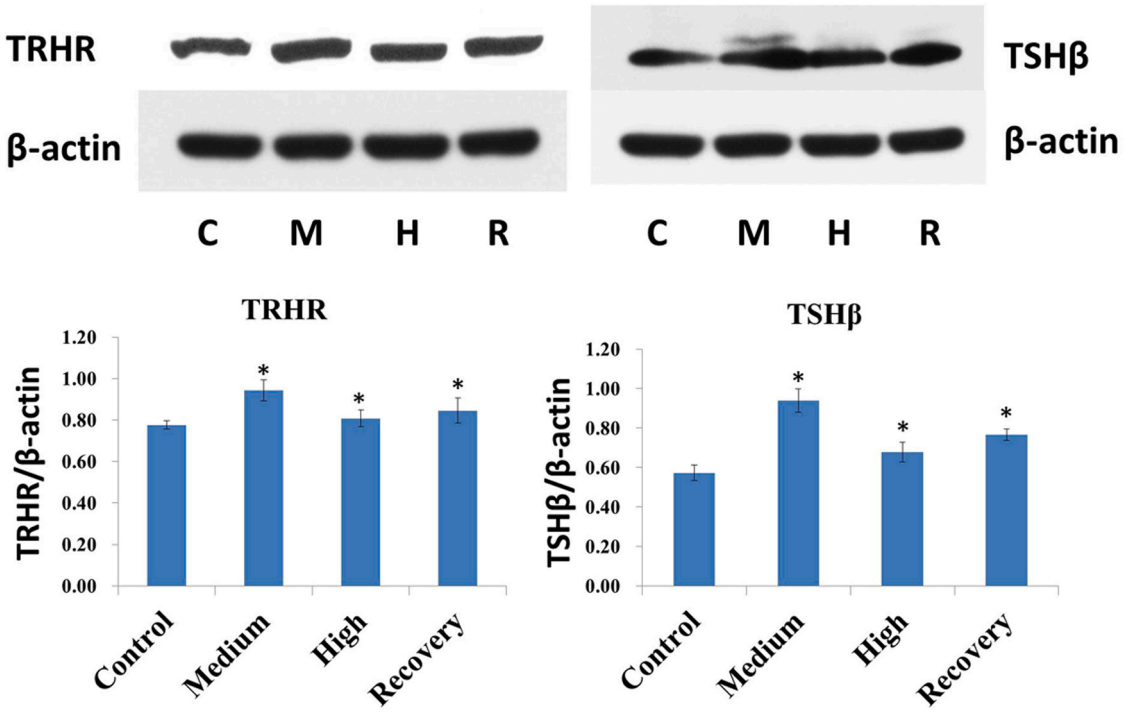

Fig. 6. Effects of $PM_{2.5}$ treatment on the protein expression of hypothalamus thyrotropin-releasing hormone receptor (TRHR) and pituitary thyroid stimulating hormone beta (TSHβ). Protein expression levels of hypothalamus TRHR and pituitary TSHβ were assessed by Western blot. Rats (n = 5/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. C: (Control group, normal saline for 49 d); M: (Medium-dose group, 15 mg/kg for 49 d); H: (High-dose group, 30 mg/kg for 49 d); R: (Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d). The housekeeping gene β-actin was used as an internal positive control. The relative expression of each target protein was calculated using the gray value ratio of the target protein band and the reference protein band (AlphaEase FC software). Error bars represent the standard deviation. Significant difference: $^*P < 0.05$ versus control.

and T3 in the blood decreases. At this time, increased TSHβ secretion (Fig. 6) promotes the secretion of T4 and T3, and the negative feedback effect of the HPT axis can help maintain relatively constant levels of thyroid hormone secretion. To further elucidate the relationship between enhanced TRHR and activation of the HPT axis, a recovery group intervention was also utilized in the in vivo study. When the HPT axis was activated, TRHR expression was elevated; when the HPT axis was not affected by $PM_{2.5}$, TRHR levels subsequently declined. Decreasing levels of TSHR would result in the separation of TSHR and TSH, further suppressing the biosynthesis of THs in the thyroid of female rats. In this study, T3 and T4 levels in serum and plasma were reduced; however, TSH levels in serum were not increased to compensate for the reduction in TH levels, suggesting there was perturbation of the HPT axis negative feedback system. However, Lee et al. found that TRα1 expression levels, as a TH homeostasis modulator, were elevated in rats with hyperthyroidism after dibutyl phthalate (DBP) exposure, which is not consistent with the findings of this study (Lee et al., 2007). This discrepancy may be due to the different chemical properties of $PM_{2.5}$ and DBP. These observations demonstrate how affecting TH-related receptors and the subsequent HPT axis activation can disturb TH homeostasis. Both Hallgren and Liu's studies on other environmental pollutants came to similar conclusions (Hallgren et al., 2001; Liu et al., 2012).

Since iodization requires H_2O_2 as a substrate during the process of THs synthesis, thyroid follicular cells continuously produce ROS to promote the synthesis of THs (Song et al., 2007; Denef et al., 1996). In this study, the enhanced levels of ROS and MDA induced by $PM_{2.5}$ treatment in female rats suggested that oxidative stress could affect the synthesis of THs and further disrupt TH homeostasis. While studying the effect of oxidative stress on the synthetic function of THs, it was found that (Nadolnik et al., 2008) oxidative stress could also stimulate the iodization effect of TG, inhibit the iodization of iodide ions in the thyroid gland, and reduce effective uptake. The increased levels of ROS and MDA (Table S2) observed in this study with $PM_{2.5}$ treatment could therefore be responsible for elevating TG levels (Fig. S1) and decreasing iodine levels (Fig. 2). These results are consistent with the above mentioned results regarding regulation of TG and iodine levels in the body. On the contrary, studies have shown that thyroid hormone disorders can produce more ROS, causing oxidative stress and further damage to the body (Lampka et al., 2006). Disruption of TH homeostasis in our study induced an unbalanced plasma redox state (enhanced ROS, MDA and Gpx levels; reduced SOD activities) (Table S2) in female rats following $PM_{2.5}$ treatment, and this finding is consistent with the results of other studies (Panda and Kar, 1998). Oxidative stress also can regulate the HPT axis by not only reducing negative feedback regulation of TRH and TSH through THs, but also stimulating the sympathetic nervous system to promote thyroid activity (Ando and Fujita, 2012). Therefore, the activation of the HPT axis observed after $PM_{2.5}$-induced oxidative stress in rats is important for maintaining balanced thyroid hormone metabolism.

Sorensen et al. found that personal exposure to particles in moderate concentrations can induce oxidative stress and the adverse effect of $PM_{2.5}$ on the body was mediated by oxidative stress (Sørensen et al., 2003). The lysosome participates in the regulation of secretory processes, such as the degradation of TG to active thyroxine. The increased TG levels (Fig. S1D) observed in our study also indicated that oxidative stress was activated upon exposure to $PM_{2.5}$. While stimulating the production of ROS (Table S2), $PM_{2.5}$ treatment also appears to inhibit antioxidant activity in vivo, such as inhibiting the activity of SOD in cells (Table S2).

Nuclear factor (NF)-kB proteins are a family of transcription factors that regulate the transcription of a variety of genes involved inflammation responses (Morgan and Liu, 2011). The oxidative pathway can activate the NF-κB signaling pathway, which induces the production of many inflammatory cytokines (Hughes et al., 2005). Exposure of human respiratory epithelial cells to atmospheric PM activates a series of protein phosphorylation events that induces the translocation of IκB from

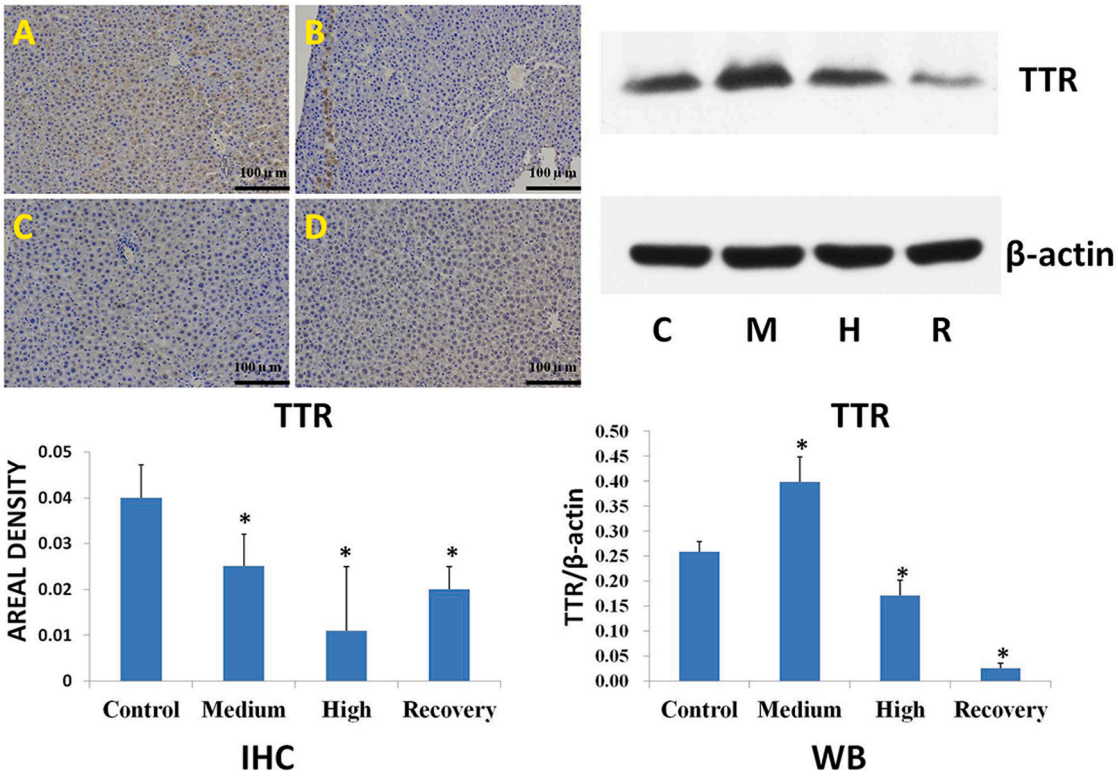

Fig. 7. Effects of $PM_{2.5}$ treatment on the protein expression levels of hepatic transthyretin (TTR). Rats (n = 5/group) were treated with $PM_{2.5}$ by passive pulmonary inhalation for 49 d. C: (Control group, normal saline for 49 d); M: (Medium-dose group, 15 mg/kg for 49 d); H: (High-dose group, 30 mg/kg for 49 d); R: (Recovery-dose group, 30 mg/kg for 35 d and no treatment for 14 d). The housekeeping gene β-actin was used as an internal positive control. The relative expression of each target protein was calculated using the gray value ratio of the target protein band and the reference protein band (AlphaEase FC software). Error bars represent the standard deviation. Significant difference: *$P < 0.05$ versus control. IHC: immunohistochemical analysis; WB: Western blot analysis. Magnification: 200×.

the activated NF-κB dimer, which then enters the nucleus and induces the expression and transcription of IL-8 and other related inflammatory factor genes (Silbajoris et al., 2011). Inflammatory factors can also promote oxidative stress in cells, leading to an increase in the production of ROS, thereby causing mitochondrial damage (Ahmadian et al., 2017). In this study, NF-κB levels were increased in the $PM_{2.5}$-dosed groups (Table S2), indicating that oxidative stress in cells caused by $PM_{2.5}$ could induce ROS-dependent activation of NF-κB and result in mitochondrial damage. A study by Riggs et al. Riggs et al. (2020) also suggested that exposure to $PM_{2.5}$ could cause impaired vascular function resulting from increased oxidative stress and inflammation.

Transthyroxine protein (TTR) is a carrier protein that transports thyroid hormones in the plasma and cerebrospinal fluid (Cheek et al., 1999). Meanwhile, THs are metabolized predominantly in the liver and are excreted into bile. As a result, the hepatic-endocrine axis plays a vital role in regulating TH homeostasis (Qatanani et al., 2005). In the current study, IHC analysis indicated that the areal density of TTR was significantly decreased after $PM_{2.5}$ exposure (Fig. 7). In addition, WB results also showed that TTR levels were significantly decreased (Fig. 7), which would contribute to the inhibition of T3 and T4 production in serum. This effect may be associated with PM2.5-induced liver damage, which is characterized by changes in liver weight and pathology. These results are in accordance with many previous studies regarding similar environmental endocrine disruptors. Fisher et al. Fisher et al. (2006) found that the HPT axis perturbation could be attributed to hepatic thyroxine changes caused by PCB126 exposure. Liu et al. Liu et al. (2015b) also showed that di-(2-ethylhexyl) phthalate (DEHP) exposure significantly inhibited the biological effects of THs on target sites due to the reduction of THs level in serum induced by decreased TTR levels (Ishihara et al., 2003). Moreover, a study also revealed that *p,p*'-DDE exposure could disturb TTR expression levels, and then further reduce the TH content in rat serum (Liu et al., 2011). Therefore, our present study finds that $PM_{2.5}$-induced alterations in hepatic TTR levels could perturb TH transport, indicating another key mechanism driving disruption of TH homeostasis following $PM_{2.5}$ treatment.

5. Conclusions

We designed an in vivo study to examine the mechanism driving the high morbidity and mortality rates associated with thyroid diseases that developed due to exposure to ambient fine particulate matter ($PM_{2.5}$) among different populations. We found that $PM_{2.5}$, an ambient air pollutant, exhibits thyrotoxicity in a dose- and time-dependent manner. A 7 week in vivo study suggested that $PM_{2.5}$ exposure could reduce circulating thyroid hormone levels in rat serum and plasma by disturbing TH biosynthesis, biotransformation, and transport, altering levels of related TH receptors, and inducing oxidative stress and inflammatory responses, ultimately further activating the HPT axis and inducing production of hepatic transthyretin. These alterations are

Table 1
The relative liver and thyroid weights of $PM_{2.5}$-treated rats (means ± SD, n=8).

Groups	Thyroid/weight (mg/100 g)	Liver/weight (g/100 g)
C	6.56 ± 0.74	2.81 ± 0.12
M	6.85 ± 2.03	3.21 ± 0.25**
H	9.20 ± 1.20*	3.28 ± 0.24**
R	10.23 ± 1.40**	3.12 ± 0.11**

* $P < 0.05$ compared to group C.
** $P < 0.01$ compared to group C. C: (Control group, normal saline for 49d); M: (Middle-dose group, 15 mg/kg for 49d); M: (High-dose group, 30 mg/kg for 49d); R: (Recovery-dose group, 30 mg/kg for 35d and no treatment for 14d). SD, standard deviation.

likely key for determining the mechanism of $PM_{2.5}$-induced thyroid toxicity, and further studies can help improve understanding of how $PM_{2.5}$ exerts thyroid-disrupting effects. Table 1.

Ethics approval and consent to participate

All experimental protocols and procedures were approved by the Animal Care and Use Committee of Xinxiang Medical University (Xinxiang, China).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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CRediT authorship contribution statement

Xinwen Dong: Conceptualization, Writing - original draft, Writing - review & editing. **Sanqiao Yao:** Data curation. **Haibin Li:** Formal analysis. **Li Zhang:** Formal analysis, Software, Supervision, Validation. **Zhichun Li:** Methodology. **Jing Jiang:** Project administration. **Fengquan Zhang:** Resources, Software, Supervision, Validation. **Jie Xu:** Software, Supervision, Validation. **Weidong Wu:** Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111720.

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