Embryonic exposure to soil samples from a gangue stacking area induces thyroid hormone disruption in zebrafish

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HIGHLIGHTS

- Soil leachate from coal gangue stacking area affects the development of zebrafish.
- Soil leachate exposure alters the expression of HPT axis regulating genes.
- Soil leachate exposure attenuates the THs levels of zebrafish embryos.

GRAPHICAL ABSTRACT
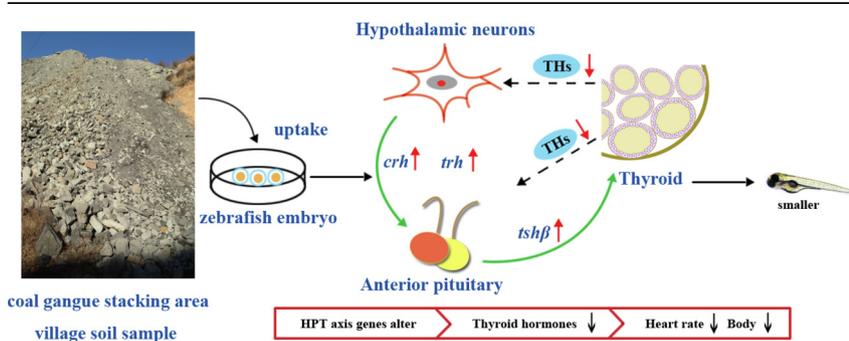
ABSTRACT

The total accumulative stockpiles of gangue from long-term coal mining exceed 1 billion tons and occupy 182 square kilometers, and 50 million tons of additional gangue are generated per year in Shanxi, a major energy province in China. The objective of this study was to examine whether exposure to village soils affected by gangue stacking would disrupt thyroid hormone system homeostasis and eventually affect endocrine system and development, using zebrafish (*Danio rerio*) as a model organism. The zebrafish embryos were exposed to village soil leachates at 0, 1:9, 1:3 and 1:1 from 1 to 120 h postfertilization (hpf), and the sample caused a dose-dependent increase in the mortality and malformation rate, and decrease in the heart rate, hatching rate and body length of zebrafish larvae. Importantly, the soil leachate alleviated the whole-body triiodothyronine (T3) and thyroxine (T4) levels at higher concentrations, and altered the expression of the hypothalamic-pituitary-thyroid (HPT) axis-regulating genes *crh, trh, tshβ, nis, tg, nkx2.1, pax8, hhex, ttr, dio1, dio2, ugt1ab, trα,* and *trβ* and the PAH exposure-related genes *ahr2* and *cyp1a*. These findings highlight the potential risk of thyroid hormone disruption and developmental toxicity from soil samples around coal gangue stacking areas.

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1. Introduction

Coal gangue is a kind of black-gray rock that is associated with coal seams during the coal formation process and has a lower combustion value than raw coal. Coal gangue is discharged from coal exploitation and washing, accounting for 10%−15% of raw coal production, depending on the changes in geological and mining

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conditions (Liu and Liu, 2010). The cumulative reserves of coal gangue in China reach total 4.5 billion tons, with an annual increase of 659 million tons (National Development and Reform Commission, China, 2012), and the reserves occupy approximately 65,000 ha of land (Li et al., 2010). Coal gangue hills are prone to spontaneous combustion and heat release during the stacking process, resulting in an increase in the temperature of the coal gangue hills, accelerating its physical and chemical weathering process and discharging harmful heavy metals, radioactive elements, polycyclic aromatic hydrocarbons (PAHs) and other organic pollutants into the surface soil. These pollutants may permeate the deep soil and contaminate groundwater through the leaching process, and eventually causing ecological fragilities and health risks (Fan et al., 2013). As reported, PAHs in the sediments of the Tennessee Valley and the Cumberland River Basin in the United States, contaminated by coal mining activities, caused adverse effects on mussels (Wang et al., 2013a). Moreover, 15 PAHs (221−432 μg/kg) have been detected in the tissues of winter wheat collected from coal combustion areas (Tian et al., 2018). The average concentrations of 16 PAHs in the surface water and groundwater were 426.98 and 381.20 ng/L, respectively, in the Heshan coal district of Guangxi, South China (Huang et al., 2016b). These findings indicate that residents living in coal gangue stacking areas might pose direct or indirect exposure to the pollutants through food chains. Therefore, it is critical to investigate the potential health risks of residents in the regions.

Normal thyroid function is essential for several biological processes, including cognitive (Taylor et al., 2014), cardiovascular (Cappola et al., 2019), skeletal muscle (Salvatore et al., 2014), neurodevelopmental (Dingemans et al., 2011) and immune system functions (Fabris et al., 1995). Some studies have shown that several environmental contaminants have been recognized as endocrine-disrupting compounds (EDCs) that interfere with the hypothalamus-pituitary-thyroid (HPT) axis, leading to thyroid disruption and dysfunction (Calsolaro et al., 2017). The HPT axis plays crucial roles in the growth and development of vertebrates and is responsible for hormone synthesis, secretion, transport and metabolism and the maintenance of normal physiological hormone concentrations (Zhang et al., 2018). Altered HPT axis function usually indicates endocrine and developmental effects (Spachmo and Arukwe, 2012). The prolonged occupational exposure to coal dust resulted in a significant increase in serum thyroid stimulating hormone (TSH) levels in coal mine workers compared to the non coal-exposed population, which interfered with the HPT axis (Tumane et al., 2015). The total prevalence of subclinical thyroid disease in the Xuzhou mining area in China is as high as 52.14% (Zhang et al., 2010). Exposure to coal-water extract significantly inhibited the synthesis of thyroid hormones (triiodothyronine (T3) and thyroxine (T4)) in Buffalo rats (Gaitan et al., 1993). However, little is known about the impacts of coal gangue stacking on thyroid function and even the development.

The development of the endocrine system can be easily visualized in real-time during the whole period of early development in zebrafish (Jacobs et al., 2018). An additional advantage of zebrafish embryos is that they are highly homologous to humans, and the thyroid system of zebrafish is similar to that of mammals and amphibians in many aspects (Segner, 2009). These properties make zebrafish a suitable model for investigating the thyroid system in vivo, which can provide valuable information for humans. In the present study, we used zebrafish (Danio rerio) as the model organism and investigated the thyroid-disrupting potential of village soil samples contaminated by coal gangue stacking, including the developmental toxicity, thyroid hormones (THs) contents, and gene transcription levels associated with the HPT axis.

2. Materials and methods

2.1. Study area and soil sample collection

In this study, a coal gangue hill of the Qinxin coal mine in Qinyuan County was selected as the study area. Qinyuan County is located in the southeast portion of Shanxi Province, at 36°34′12.24″N and 12°8′55.67″E, and is the key coal production base in Shanxi Province. We collected village soil samples in the vicinity of the coal gangue stacking area. The soil sampling site included 3 subsamples, and for each, the surface soil was removed, and 0−20 cm of topsoil was collected, packaged and moved to the laboratory under natural drying conditions. All samples were sieved through a 100-mesh sieve to obtain particle sizes of less than 0.15 mm in diameter. Following the preprocessing of the samples, the soil samples were stored at 4 °C for further analysis.

2.2. Soil leachate preparation

To 100 g of village soil samples was added 100 mL of ultrapure water (1 g/mL), and the mixture was vigorously shaken for 3 d. Then, the mixture stood overnight followed by centrifugation at 3000 rpm for 10 min to obtain soil leachate. The soil leachate was filtered using sterilizing filters to prevent microbiological contamination in the exposure experiment, stored in a sealed PTFE (poly tetra fluoroethylene) bottle and maintained in darkness at 4 °C.

2.3. Zebrafish maintenance and embryo/larva exposure

Adult AB-type zebrafish (6 months old) were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Fertilized and developmentally normal embryos were immediately collected after fertilization (≤2 h postfertilization, hpf) and washed with E3 culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$, pH 7.4) to remove debris and excrement. The embryos were randomly distributed into petri dishes with 50 mL soil leachate solution at various concentrations (1:9, 1:3 or 1:1); E3 culture medium was used for control groups and for the dilution of soil leachate. Each treatment group consisted of 6 replicates, and each petri dish contained 100 eggs. The embryos were held at 28 ± 0.5 °C in a humidified incubator with a 14 h:10 h light: dark cycle, the dead embryos were removed, and 50% of the exposure solution was renewed with fresh solution daily.

2.4. Developmental toxicity test

In our study, the developmental toxicity endpoint data consisted of mortality, malformation, hatching rate, heart rate and body length data. The experiment began at 24 hpf, and the number of dead, hatched and malformed individuals in each group was observed at 24-h intervals under a stereomicroscope (OLYMPUS, SZX2-ILLT, Japan). The mortality and hatching rate for each replicate were obtained by using the number of dead and hatched individuals divided by the total number of embryos of each replicate (100). The malformation rate is the number of deformities divided by the number of survivors. Moreover, the type of malformation, including yolk sac edema (YE), pericardial edema (PE), spinal curvature (SC), tail malformation (TM) and eye malformation (EM), was recorded. In addition, 30 randomly chosen zebrafish larvae per replicate at 72, 96 and 120 hpf were randomly chosen using ImagePro Plus software (Media Cybernetics) to measure the body length from the anterior part of the head to the end of the body axis. Under a stereomicroscope, the heart rate (beats/30 s) was

measured with a stopwatch.

2.5. Thyroid hormone assays

The way of extracting TH contents in the whole body were performed based on a method described in the previous study (Zhang et al., 2018), 200 zebrafish embryos or larvae (120 hpf) of each replicate were homogenized and extracted with an ice-cold phosphate buffer (g/mL = 1:4) with pH 7.4. Thereafter, the extract was homogenized on ice for 5 min using intermittent sonic oscillation followed by strong vortexing for another 10 min. Later, after centrifugation at $3500 \times g$ for 20 min at 4 °C, the supernatants were transferred and analyzed. Whole-body T4 and T3 levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) test kits (EIAab Science Co., Ltd.,Wuhan, China) following the manufacturer's instructions. All the detection levels of T3 and T4 were within the detection limits of the manufacturer's instructions (78.0−5000 pg/mL for T3 and 0.19−12.5 ng/mL for T4).

2.6. Quantitative real-time PCR assay

For gene transcription analysis, the 120 hpf larvae (30 larvae per replicate) were randomly harvested. Total RNA was extracted using TRIzol Reagent (TaKaRa, China) and first-strand complementary DNA (cDNA) was synthesized using a reverse transcription kit (TaKaRa, China) according to the manufacturer's instructions. Moreover, the mRNA expression levels of several regulating genes of the HPT axis were analyzed. The relative quantification of the mRNA expression of these genes was determined by a qTOWER 2.2 real-time PCR instrument (Analytik Jena AG, Jena, Germany). Based on the good average expression stability of β-actin in zebrafish tissues, β-actin was chosen as the internal reference gene in the present study (Tang et al., 2007; Yun et al., 2017; Xu et al., 2019). The sequences of each gene-specific primer are provided in Table 1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out for the 120 hpf zebrafish larvae and to quantify the expression of TG and TTR. Briefly, 100 larvae for each replicate were homogenized in ice-cold phosphate buffer (g/mL = 1:3) with pH 7.4. The homogenates were centrifuged at $12,000 \times g$ for 10 min at 4 °C and supernatants were collected. The expression of TG and TTR were determined using corresponding ELISA kits (Jianglai, Shanghai, China) according to the manufacturer's instructions, respectively.

Table 1
Primer sequences for the genes tested in the present study.

Gene	Forward primer	Reverse primer
β-actin	GTTGGTATGGGACAGAAAG	GGCGTAACCCTCGTAGAT
crh	TTCGGGAAGTAACCACAAGC	CTGCACTCTATTCGCCTTCC
trh	CACACAGATGGAGGAGCAGA	AGCAGCATCAGGTAGCGTTT
tshβ	CAGATCCTCACTTCACCTACC	GCACAGGTTTGGAGCATCTCA
tg	TCTGAGCAACACCGACTT	CAGCGATGTATTGACCCT
nis	GGTGGCATGAAGGCTGTAAT	GATACGGGATCCATTGTTGG
nkx2.1	AGGACGGTAAACCGTGTCAG	CACCATGCTGCTCGTGTACT
pax8	CCTGTCTGCCATTTCGTC	CGTCTGGTGGAGGGTTAG
hhex	TGTGGTCTCCGTTCATCCAG	TTTGACCTGTCTCTCGCTGA
ttr	GCACAACTTGATCACGGAGC	TGTGGTGTACGAGAAAGGGC
dio1	GTTCAAACAGCTTGTCAAGGACT	AGCAAGCCTCTCCTCCAAGTT
dio2	TTCTCCTTGCCTCCTCAGTG	AGCCACCTCCGAACATCTTT
ugt1ab	CCACCAAGTCTTTCCGTGTT	GCAGTCCTTCACAGGCTTTC
tra	CAATGTACCATTTCGCGTTG	GCTCCTGCTCTGTGTTTTCC
trβ	TGGGAGATGATACGGGTTGT	ATAGGTGCCGATCCAATGTC
ahr2	CTACTTGGGCTTCCATCAGTCG	GTCACTTGAGGGATTGAGAGCG
cyp1a	AGGACAACATCAGACACATCACCG	GATAGACAACCGCCCAGGACAGAG

2.8. Statistical analysis

Statistics were carried out using SPSS 23.0. The analysis of normality and homogeneity of variances of all data were evaluated by Kruskal-Wallis test and Levene's test, respectively. If data met the normality and homogeneity assumptions, using one-way analysis of variance (ANOVA) followed by an Fisher's least significant difference (LSD) test to analyze the differences among the groups compared to the control. If groups did not show homogeneity of variance by Levene's test, they were log-transformed before further statistical analysis. Statistically significant differences were established at $p < 0.05$. All data are presented as the means ± standard error (SE) and figure generation were conducted using Origin 9.1.

3. Results

3.1. Developmental changes in zebrafish embryos

After exposure for 120 h, soil leachate sample affected developmental parameters in a concentration-dependent manner (Fig. 1A−D). The mortality rates in the 1:3 and 1:1 treatment groups significantly increased and reached 3.62- and 3.98-fold of the control, respectively. The heart rate decreased with the increase of exposure concentration. Moreover, the hatching time delayed following the exposure at higher concentrations, and the hatching rate significantly decreased functioning as the exposure concentration. And the body length was obviously inhibited at differently developmental windows.

The malformation rate significantly increased in the 1:3 and 1:1 treatment groups and reached 4.27- and 7.68-fold of control (Fig. 2C). Among the exposure groups at high concentrations, the degree of malformation with severe morphological changes also significantly increased. As shown in Fig. 2A-B, there was single deformity, such as cyclopia, the inconsistent development of the left and right eyes, and spinal curvature in groups at low concentrations; however, the superposition of multiple malformations in zebrafish larvae occurred in the group at high concentrations, even four types of malformations (YE, PE, TM and EM) occurring in one fish.

### 3.2. Whole-body thyroid hormone levels

After 120 h exposure, the whole-body thyroid hormone levels in zebrafish larvae significantly decreased (Fig. 3). The total T3 levels significantly attenuated to 0.69-fold and 0.50-fold of control in the 1:3 and 1:1 treatment groups, respectively. The total T4 level also showed a decreasing trend in the 1:3 and 1:1 exposure groups.

3.3. HPT axis gene expression in zebrafish larvae

Following the exposure of the leachate sample, several genes involved in the regulation, transport, binding and metabolism of THs were affected in zebrafish larvae 120 hpf (Fig. 4). The expression of corticotrophin-releasing hormone (crh) and thyrotropin-releasing hormone (trh) slightly upregulated (1.23- and 1.34-fold of control) in the 1:1 treatment group. And the levels of the thyroid stimulating hormone beta (tshβ), thyroglobulin (tg), sodium/iodide symporter (nis), NK2 homeobox1 (nkx2.1), paired box protein 8 (pax8), hematopoietically expressed homeobox (hhex) and trans-thyretin (ttr) significantly increased to 1.36-, 2.16-, 1.55-, 1.61-, 1.63-, 2.16- and 1.48-fold of control, respectively, in the 1:1 treatment group. After exposure to 1:3 and 1:1 leachate samples, the expression of the deiodinase type 1 (dio1) gene significantly increased to 1.81- and 3.40-fold of control, and the uridine
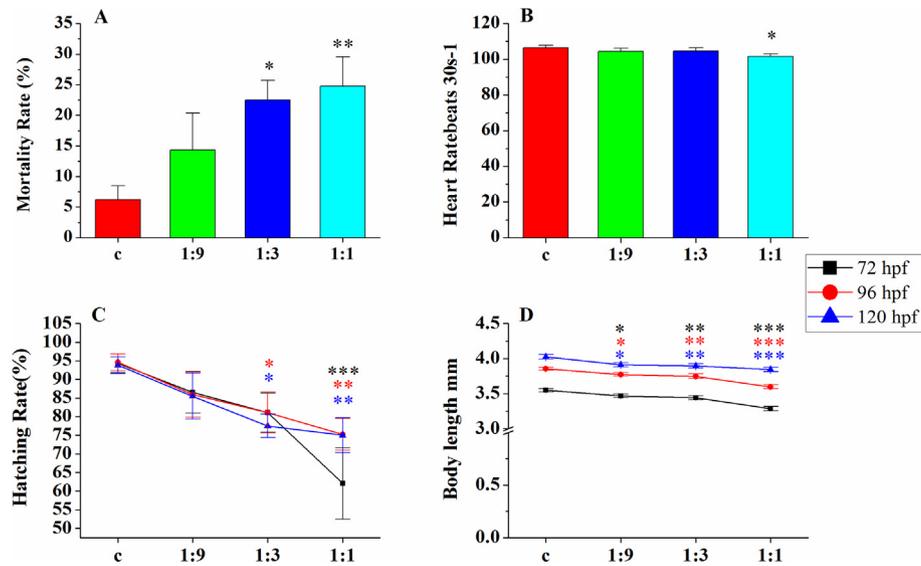
Fig. 1. Effects of village soil leachate from gangue stacking area on the developmental parameters of zebrafish embryos. (A) Mortality rate, (B) heart rate, (C) hatching rate, and (D) body length. Values are expressed as the mean ± SE of six replicate samples and analyzed by one-way ANOVA followed by an LSD test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the control group.
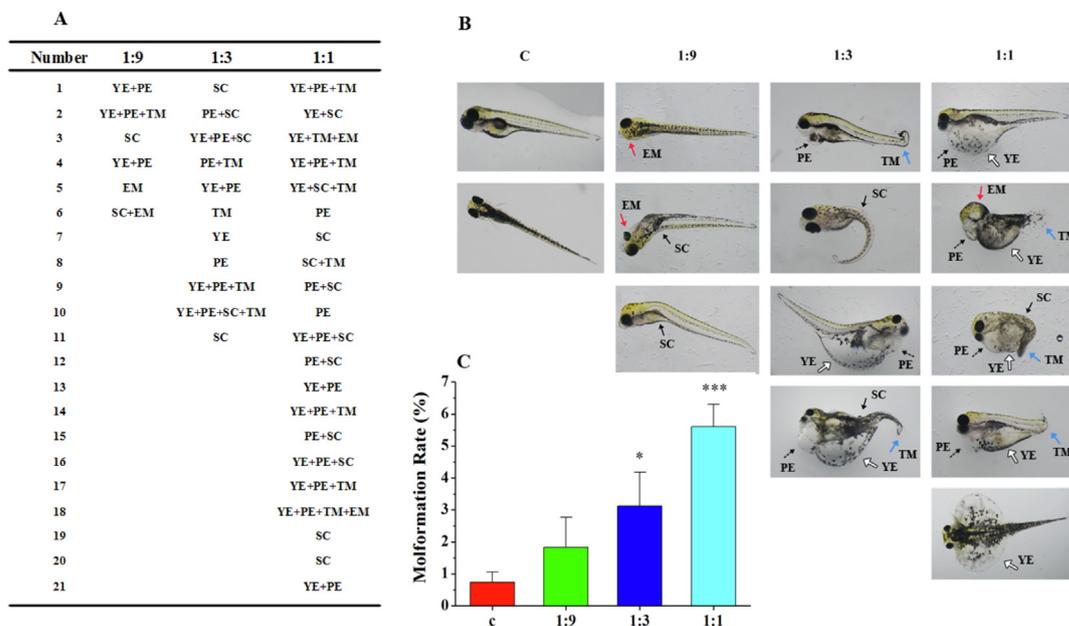
Fig. 2. Effects of village soil leachate from gangue stacking area on the morphology of zebrafish larvae. (A) Malformation degree of zebrafish larvae. (B) Developmental abnormalities observed in zebrafish larvae. White arrows indicate yolk sac edema (YE); broken arrows indicate pericardial edema (PE); black arrows indicate spinal curvature (SC); blue arrows indicate tail malformations (TM); and red arrows indicate eye malformations (EM). (C) Malformation rate. The results are the means ± SE of six replicate samples. *$p < 0.05$, ***$p < 0.001$ versus the control group.

diphosphate glucuronosyltransferase (*ugt1ab*) gene increased to 1.57- and 1.86-fold of control. The level of *deiodinase type 2* (*dio2*) gene upregulated to 1.47-fold of control after exposure to 1:1 soil leachate, and the transcription of the *thyroid hormone receptor α* (*trα*) and *thyroid receptor β* (*trβ*) genes remained unchanged (Fig. 4C). Similarly, the expression of the *aryl hydrocarbon receptor* (*ahr2*) markedly increased to 1.48-fold of control after exposure to 1:1 leachate sample, and the *cytochrome P4501A* (*cyp1*a) gene significantly upregulated to 1.57- and 2.98-fold of control in the 1:3 and 1:1 treatment groups, respectively (Fig. 4D).

### 3.4. TG and TTR contents in zebrafish larvae

After 120 h exposure, the leachate sample significantly elevated the expression of TG, and reached 1.30- and 1.42-fold of control in the 1:3 and 1:1 treatment groups, respectively; and elevated the level of TTR in 1:1 treatment group (1.2-fold of control) (Fig. 5).

4. Discussion

Environmental pollutants that cause the interruption of TH
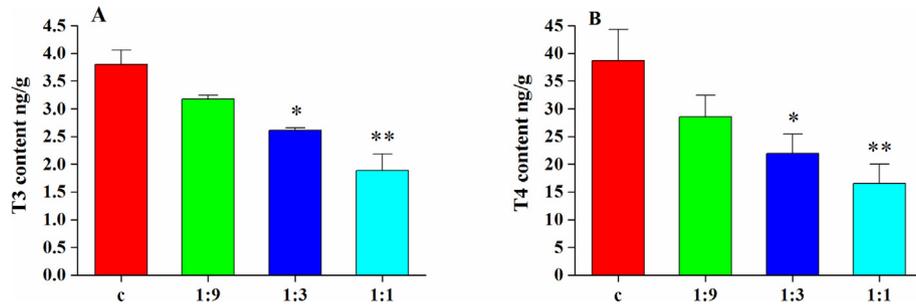
Fig. 3. Effects of village soil leachate from gangue stacking area on the whole-body total T3 (A) and T4 (B) levels in zebrafish larvae. The results are the means ± SE of six replicate samples. *$p < 0.05$, **$p < 0.01$ versus the control group.
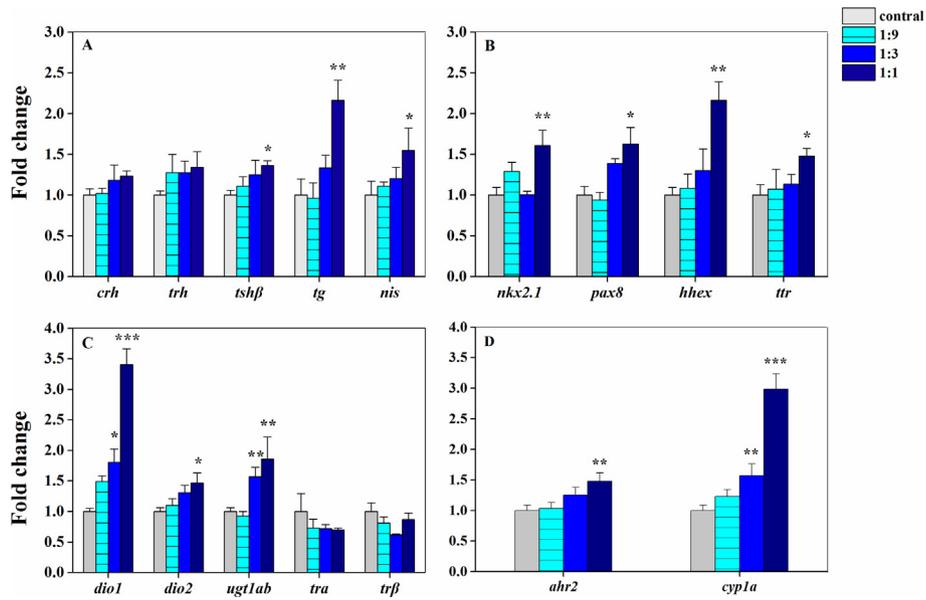
Fig. 4. Effects of village soil leachate from gangue stacking area on the mRNA expression of HPT axis-related genes in zebrafish larvae. The results are the means ± SE of six replicate samples. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus the control group.
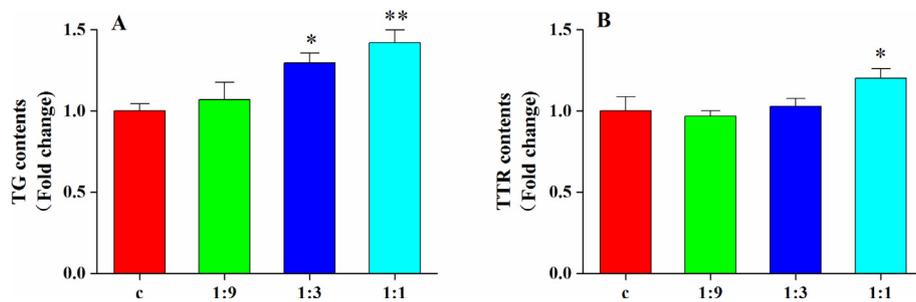
Fig. 5. Effects of village soil leachate from gangue stacking area on the levels of TG (A) and TTR (B) in zebrafish larvae. The results are the means ± SE of six replicate samples. *$p < 0.05$ and **$p < 0.01$ versus the control group.

synthesis have attracted much attention (Kim et al., 2016; Zhang et al., 2017, 2018). To determine the possible endocrine disruption risks following exposure to the contaminated soil adjacent to coal gangue, we used developing zebrafish as a model and assessed the thyroid dysfunction after exposing the zebrafish to soil leachate at various concentrations.

The main harmful organic components in coal gangue have been identified as PAHs (Yun et al., 2017). Given their excellent stability, PAHs are prone to accumulate and be preserved in soils for a long

time once they are discharged into the surrounding environment, which has a significant impact on the health of the residents living in the vicinity of a coal gangue stacking area (Tong et al., 2018). Several studies have indicated that extremely high levels of PAHs have been detected in the leachate from abandoned coal gangue piles, as well as in the surrounding soil and groundwater. Along a stream, PAHs have even polluted 1800 m away from the nearby coal gangue dumps (Sun et al., 2009; Wang et al., 2009). Previous studies from our laboratory have shown that the coal gangue hill of

the Qinxin coal mine, a long-abandoned colliery, has caused serious PAH pollution in the downstream village soil environment. The total content of 18 PAHs reached 4133.58 ng/g in the soil samples closest to the villages, significantly higher than the natural PAH concentration of 1−10 ng/g in the soil (Yun et al., 2017). Previous studies have shown that exposed to tailing pond sediments (a representative mixture of PAHs and alkylated PAHs) for 31 d caused 100% mortality of walleye (*Sander vitreus*) embryos at 0.04−1.0 g/L, as well as yolk sac edema, pericardial edema, spinal malformations, craniofacial deformities and decreased length simultaneously (Raine et al., 2017). Similarly, our study found prominent growth delay and decreased body length, slight decreased heart rate of zebrafish larvae, and multiple malformations, such as YE, PE, SC, TM and EM.

THs have been considered essential for the transition of zebrafish from larvae to juveniles, including the formation of scales and pigmentation (Brown, 1997). T4 and T3, as the main products of the thyroid gland, play pivotal roles in the growth and development of teleost fish (Power et al., 2001). Here, a significant decline in whole-body T4 and T3 levels was observed in zebrafish larvae after soil leachate exposure (Fig. 3). Consistently, a previous report showed the reduced plasma T3 and T4 levels in yellow perch (*Perca flavescens*) samples from the mining region lakes of Rouyn-Noranda, Québec (Levesque et al., 2003). These findings suggest that the village soil sample close to the coal gangue stacking area caused body growth retardation through thyroid disruption in zebrafish larvae.

The secretion of *crh, trh* and *tshβ*, which are regarded as important regulatory factors for the HPT axis, is triggered by changes in circulating TH concentrations; therefore, their transcription levels can be used to evaluate whether environmental chemicals are likely to disturb thyroid function (De Groef et al., 2006; Wang et al., 2013b). In the present study, we observed the elevation of the *crh* and *trh* gene expression following soil leachate exposure. Previous evidence indicated that prolonged occupational exposure to coal dust caused a significant increase in the serum TSH levels of coal mine workers (Tumane et al., 2015). Here the significant upregulation of the *tshβ* gene may be attributed to the negative feedback mechanism of the zebrafish pituitary in response to the decreased T3 and T4 levels.

It has been reported that NIS is a transmembrane glycoprotein that transfers sodium and iodide to thyroid follicular cells through basolateral plasma, and TG is the protein precursor of THs (Li et al., 2016) stored in the thyroid follicular cavity, which can be used by the thyroid gland to produce THs. Since *nis* and *tg* are involved in TH synthesis, alterations in the transcription levels of *nis* and *tg* have been used as convenient and sensitive markers to detect thyroid activity during fish development (Dohan and Carrasco, 2003; Yan et al., 2012; Zhang et al., 2018). Normally, the bioactivity of *nis* and the synthesis of *tg* are regulated by *tsh*. Therefore, upregulation of *tsh* expression can lead to an increase in *nis* and *tg* mRNA levels (Li et al., 2016). Consistently, exposure to soil leachate significantly increased the mRNA expression of *nis* and *tg* and the protein expression of TG (Fig. 5A). Also, we examined several other genes involved in thyroid gland development and differentiation during embryogenesis, such as *nkx2.1, pax8* and *hhex* (Sun et al., 2018), and found significant upregulation following soil leachate treatment at higher concentrations. This upregulation of these genes may stimulate thyroid development to compensate for the decline in T4 levels (Huang et al., 2016a).

TTR is an important TH carrier protein and associated with the thyroid axis in fish (Morgado et al., 2009; Liang et al., 2015). In our study, the elevation of the mRNA and protein levels of TTR may provide a compensatory effort in response to decreased TH levels.

TTR is an important T3 binding protein in teleost fish and amphibian that is responsible for transporting THs to various peripheral tissue targets (Yamauchi et al., 1999; Power et al., 2000). Meanwhile, when in combination with TTR, the metabolic elimination of free THs from circulation can be delayed (Kim et al., 2016).

Upregulation of the *dio1* gene in zebrafish after the soil leachate exposure (Fig. 4C) may reflect a compensatory effort against the decrease in T3 levels. Two types of *deiodinases*, *dio1* and *dio2*, play a crucial role in the regulation of peripheral and circulating TH levels in teleosts. Studies have indicated that *dio1* has a major influence on iodine recovery and TH removal (Tang et al., 2015). Moreover, T4 is converted into the more active T3 as catalyzed by DIO2, generally in the euthyroid state (Bianco and Kim, 2006; Murk et al., 2013). Previous studies have shown that the mRNA expression of *deiodinases* could serve as a sensitive biomarker to detect TH alterations in vertebrates exposed to environmental pollutants (Coimbra et al., 2005; Dong et al., 2014; Zhang et al., 2018). In this study, upregulation of *dio2* promoted the transcription of T4 into T3 and led to a decrease in T4 levels. As a compensatory mechanism, the induction in the transformation of T4 into T3 might further induced the upregulation of *dio1* gene expression and contributed to the degradation of the elevated T3 content and help to maintain TH homeostasis in plasma (Zhai et al., 2014).

UGT enzymes have been reported to play a key role in the inactivation and excretion of many exogenous and endogenous compounds, including T4. The induction of UGT can enhance glucuronidation and promote the elimination of T4, which eventually leads to a decrease in whole-body T4 in zebrafish (Kim et al., 2016; Zhang et al., 2018). Several reports have indicated that a decrease in T4 levels is accompanied by an increase in the expression of UGT1ab, a member of the UGT protein family, after exposure to different environmental pollutants (Yu et al., 2010; Chen et al., 2012; Zhai et al., 2014). Consistent with these previous studies, significant upregulation of *ugt1ab* mRNA expression in fish larvae was also observed following exposure to soil leachate in our study. It is implicated that an increase in the expression of *ugt1ab*, which played an important role in the study of thyroid hormone disruption induced by soil leachate exposure, might lead to a decrease in T4 levels.

The induction of CYP1A has been considered a biomarker of PAH exposure (Goksoyr, 1995; Whyte et al., 2000; Sehonova et al., 2019), here the significant induction of the transcriptional levels of the *cyp1a* and *ahr2* genes in zebrafish larvae meeted our expectations. Both AhR1 and AhR2 are orthologs of AhR in fish, and each of them has different functions. AhR2 has more abundant transcripts than AhR1, and AhR2 appears to have a considerable function in xenobiotic metabolism (Hahn et al., 1997; Hahn, 2002; Karchner et al., 2002). In our present study, the transcriptional changes in *cyp1a* and *ah*r2 might be attributed to the PAH composition in the soil samples around coal gangue stacking areas.

5. Conclusion

In the present study, the village soil leachate from coal gangue stacking area caused a dose-dependent increase in the mortality and malformation rate, and decrease in the heart rate, hatching rate and body length of zebrafish larvae. Importantly, the soil leachate alleviated the whole-body T3 and T4 levels at higher concentrations, and altered the expression of the HPT axis-regulating genes *crh, trh, tshβ, nis, tg, nkx2.1, pax8, hhex, ttr, dio1, dio2, ugt1ab, trα,* and *trβ* and the PAH exposure-related genes *ahr2* and *cyp1a*. These findings highlight the potential risk of thyroid hormone disruption and developmental toxicity from soil samples around coal gangue stacking areas.

Acknowledgements

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