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Contamination profiles and health impact of benzothiazole and its derivatives in $PM_{2.5}$ in typical Chinese cities

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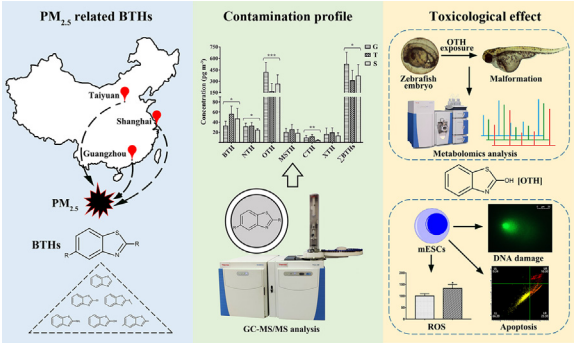
HIGHLIGHTS

- BTHs were detected in 26 outdoor $PM_{2.5}$ samples from Guangzhou and Taiyuan, China.
- OTH was the predominant compound among the $PM_{2.5}$-bound BTHs in the three regions.
- OTH and the outdoor exposure risk of toddlers were much higher than that of adults.
- OTH has potential developmental and reproduction toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Although benzothiazole and its derivatives (BTHs) are considered emerging contaminants in diverse environments and organisms, little information is available about their contamination profiles and health impact in ambient particles. In this study, an optimized method of ultrasound-assisted extraction coupled with the selected reaction monitoring (SRM) mode of GC-EI-MS/MS was applied to characterize and analyze $PM_{2.5}$-bound BTHs from three cities of China (Guangzhou, Shanghai, and Taiyuan) during the winter of 2018. The total BTH concentration (ΣBTHs) in $PM_{2.5}$ samples from the three cities decreased in the order of Guangzhou > Shanghai > Taiyuan, independently of the $PM_{2.5}$ concentration. Despite the large variation in concentration of ΣBTHs in $PM_{2.5}$, 2-hydroxybenzothiazole (OTH) was always the predominant compound among the $PM_{2.5}$-bound BTHs and accounted for 50–80% of total BTHs in the three regions. Results from human exposure assessment and toxicity screening indicated that the outdoor exposure risk of $PM_{2.5}$-bound BTHs in toddlers was much higher than in adults, especially for OTH. The developmental and reproduction toxicity of OTH was further explored in vivo and in vitro. Exposure of mouse embryonic stem cells (mESCs) to OTH for 48 h significantly increased the intracellular reactive oxygen species (ROS) and induced DNA damage and apoptosis via the functionally activating p53 expression. In addition, the growth and development of zebrafish embryos were found to be severely affected after OTH treatment. An overall metabolomics study was conducted on the exposed zebrafish larvae. The results indicated that exposure to OTH inhibited the phenylalanine hydroxylation reaction, which further increased the accumulation of toxic phenylpyruvate and acetylphenylalanine in zebrafish. These findings provide important insights into the contamination profiles of $PM_{2.5}$-bound BTHs and emphasize the health risk of OTH.

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1. Introduction

Benzothiazole and its derivatives (BTHs) are a group of heterocyclic aromatic compounds with a 1,3-thiazole ring fused to a benzene ring. The immense utility of BTHs is directly linked to their vast range of physicochemical properties and biological activities, which makes them useful for applications including vulcanization accelerators in rubber production, fungicides in leather production, corrosion inhibitors, and ultraviolet (UV) light stabilizers (Avagyan et al., 2015; Gill et al., 2015; Khan et al., 2016; Kloepfer et al., 2004; Kloepfer et al., 2005; Liao et al., 2018; Luongo et al., 2016; Ni et al., 2008; Reddy and Quinn, 1997). The production of BTHs has increased significantly in the past few years. However, growing production has also led to the increased release of BTHs and risk of their widespread accumulation in the environment.

Currently, BTHs are considered emerging contaminants and have been shown to be ubiquitous in the air (Simcox et al., 2011; Wan et al., 2016; Wang et al., 2013a), water (Kong et al., 2015; Reddy and Quinn, 1997), sediment (Dsikowitzky et al., 2014; Karthikraj and Kannan, 2017; Reddy and Quinn, 1997), soil (Speltini et al., 2016), food (Fedrizzi et al., 2007) and organisms (Asimakopoulos et al., 2013b; Wang et al., 2015). Therefore, possible risks and hazards to human health need to be considered and addressed. Aside from ingestion exposure, the inhalation pathway of BTHs has attracted growing interest because of reports in the last few years that BTHs are present in both indoor and outdoor air environments (Maceira et al., 2018; Wan et al., 2016; Wang et al., 2013a). In addition, two studies have recently been published estimating the concentration of BTHs in $PM_{2.5}$ samples collected from urban road dust and port areas. The median $\sum$BTHs concentration was found to be highest in $PM_{2.5}$ (26.6 mg g^{-1}), followed by PM_{10} (22.0 mg g^{-1}) and total suspended particles (TSP) (0.68 mg g^{-1}) in road dust, and the mean concentration in the port was 2.08 ng m^{-3}, with the most predominant contaminants being 1-H-benzothiazole (average concentration: 0.57 ng m^{-3}), 2-methylbenzothiazole (average concentration: 0.52 ng m^{-3}) and 2-chlorobenzothiazole (average concentration: 0.41 ng m^{-3}) (Nunez et al., 2020; Zhang et al., 2018). $PM_{2.5}$ can carry toxic pollutants and pass through the lung into the body's circulatory system. Exposure to $PM_{2.5}$ may be associated with a variety of diseases, such as cardiovascular and respiratory diseases, diabetes, neurological diseases and deep vein thromboses (Chen et al., 2019; Kloog et al., 2015; Lin et al., 2017; Qi et al., 2020; Zanobetti et al., 2014). Thus, a clear understanding of the contamination profiles of BTHs in $PM_{2.5}$, especially in densely populated megacities, is important to assess possible health hazards. However, current research aimed at monitoring $PM_{2.5}$-bound BTHs is mainly focused on limited areas and few data are available regarding the regional and/or global scale distribution of $PM_{2.5}$-bound BTHs.

Since 1985, the bioaccumulation of BTHs has been reported in various human samples. The highest geometric mean concentration of BTHs was found in urine from Japan (22.7 μg L^{-1}) (Asimakopoulos et al., 2013a; Asimakopoulos et al., 2013b; Ferrario et al., 1985; Li et al., 2017; Wang et al., 2015). Therefore, identifying the toxicological effect of BTHs is critical for the environment and especially human health. Studies of the adverse effects of BTHs in vitro using human cell lines have revealed that BTHs can induce genotoxicity and cytotoxicity (Liao et al., 2018; Ozpinar et al., 2020; Ye et al., 2014). Hornung et al. (2015) reported that thyroid hormone activity was modulated by 2-SH-BTH, 2-NH_2-BTH, 2-OH-BTH, and 2-Me-S-BTH in a porcine thyroid gland. Other in vivo studies have indicated that the acute toxicity of BTHs varies from animal to animal and the major adverse effects originate from chronic exposure by inhibiting thyroxine release, increasing the activities of phase I metabolic and conjugation enzymes and inducing several kinds of tumors (Hornung et al., 2015; National Toxicology, 1988; Seo et al., 2000). In general, current toxicological research on BTHs is still at an initial stage and further work is needed to examine the more specific effects of BTHs. In addition, the toxic mechanisms of BTHs have not yet been fully elucidated.

This study aimed to establish a set of analysis methods for environmental monitoring, risk screening and toxicity exposure assessment in order to (a) identify the pollution characteristics of BTHs in urban $PM_{2.5}$, (b) screen potential risks of the pollutants, and (c) study the toxicity mechanisms both in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and reagents

The target standards used in this study were 1H-benzothiazole (BTH), 2-chlorobenzothiazole (CTH), 2-hydroxybenzothiazole (OTH), 2-(methylthio)-benzothiazole (MSTH), 2-aminobenzothiazole (NTH) and 2,5-dimethyl-1H-benzothiazole (XTH). Information on the target samples is given in the Supplementary material (Table S1). BTH and CTH were obtained from Sigma-Aldrich (St. Louis, MO, USA). XTH was acquired from the Tokyo Chemical Industry (Shanghai, China), and OTH, NTH and MSTH were purchased from CNW (ANPEL Laboratory Technologies Inc., Shanghai, China). Hexamethylbenzene (HMB) and benzotriazole-d_4 (BTR-d_4) were supplied by Dr. Ehrenstorfer (Augsburg, Germany) and Toronto Research Chemicals (Toronto, Canada), respectively. Acetone, dichloromethane and methanol were acquired from Merck (Munchen, Germany). Anhydrous sodium sulfate and silica gel (70–230 mesh) were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.2. $PM_{2.5}$ samples preparation

2.2.1. Sample collection

$PM_{2.5}$ samples were obtained in the winter (November 2018 to January 2019) from Guangzhou (South China), Shanghai (East China) and Taiyuan (North China). $PM_{2.5}$ samplers were installed on the roofs of buildings located in South China Normal University (Guangzhou, China), Fudan University (Shanghai, China) and Shanxi University (Taiyuan, China). There were no major industrial pollution sources near the sampling locations, and therefore the samples were assumed to reflect the overall urban air conditions. Medium-volume air samplers (Laoying Co. Ltd., Qingdao, China) were used to collect samples for 24 h at a flow rate of 100 L min^{-1} at the Guangzhou and Taiyuan sampling sites. High-volume air sampler (Tianhong Environmental Protection Industry Co. Ltd., Wuhan, China) were applied to collect samples in Shanghai for 24 h at a flow rate of 1.05 m^3 min^{-1} on quartz microfiber filters (QMF, 203 mm × 254 mm). After sampling, the QMFs were wrapped in aluminum foil and stored at −20 °C until testing. In this study, eight to ten winter $PM_{2.5}$ samples were collected at each of the three locations for quantitative analysis. Detailed information of the sampling can be found in Table S2.

2.2.2. Sample extraction

Ultrasound-assisted solvent extraction (USAE) was used as an extraction method due to its high efficiency, wide adaptability and convenience (Avagyan et al., 2015; Westerholm, 2013). Before extraction, each QMF sample was cut into pieces and placed into a brown glass tube with 100 ng of BTR-d_4, 1 g of anhydrous sodium sulfate and 30 mL of ethyl acetate, and then ultrasonically extracted for 20 min. The experiment was repeated by adding 20 mL of ethyl acetate, and the solutions were combined twice. All supernatants were concentrated to about 1 mL (40 °C, 150–800 MPa) on a rotary evaporator.

2.2.3. Sample purification

To reduce matrix effects during detection, a packed column purification method was adopted. The column was packed from the bottom to the top with glass wool, 2 g of activated silica gel (pre-heated at 100 °C for half an hour) and 1 g of anhydrous sodium sulfate. Above all, the packed column was pretreated with three aliquots of 5 mL n-hexane. The above extracts were added to the column and BTHs were eluted with a 40 mL mixture of acetone and dichloromethane (1:4, v/v). The eluent was nearly evaporated to dryness by rotary evaporation (40 °C, 150–800 MPa) and purging with a gentle stream of nitrogen purge.

After adding 5 ng of HMB (instrument standard), the eluent was reconstituted in 100 μL acetone and transferred to a 250 μL inner lining pipe for GC–MS/MS analysis.

2.3. Quantitative GC–MS/MS analysis

The analysis was operated on a TSQ 8000 Evo gas chromatograph mass spectrometer (Thermo Scientific, USA) equipped with a Thermo TRACE™ 1300 gas chromatograph, an electron ionization (EI) source, triple quadropole analyzer and automatic injector (GC-EI-MS/MS). A Thermo TG-5MS capillary column (Thermo Scientific, USA) was applied to the separation. The detailed chromatographic and mass spectrometric conditions of GC-EI-MS/MS can be found in the "Supplementary Material". Table S3 shows the optimal quantitative/qualitative ions and retention times of the target BTHs in the GC-EI-MS/MS analysis. The total ion chromatograms of the BTHs at a concentration of 1 μg mL^{-1} are shown in Fig. 1a.

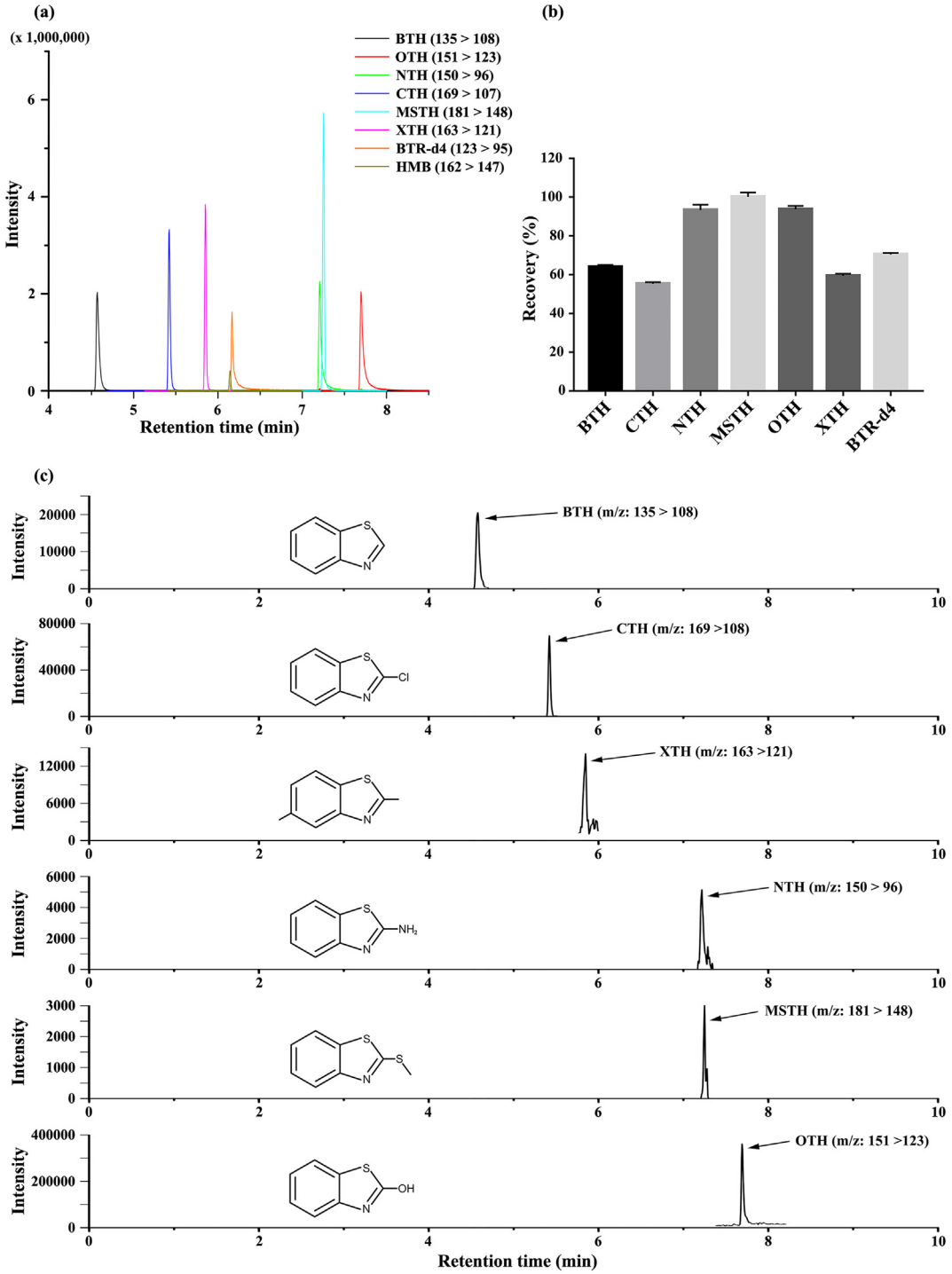

Fig. 1. Test of the analytical methods used in this experiment. (a) Extracted ion chromatograms of BTHs (1 μg mL^{-1}), BTR-d_4 (1 μg mL^{-1}) and HMB (50 ng mL^{-1}) obtained with the SRM mode of GC-EI-MS/MS. (b) Recoveries of BTHs (1 μg mL^{-1}) obtained by the combination of USAE and packed column purification. (c) Extracted ion chromatograms of BTHs with the SRM mode in real atmospheric $PM_{2.5}$ samples.

2.4. Analytical methods and quality control

Signal-to-noise ratios of 3 and 10 were assumed to correspond to the detection limit and limit of quantification, respectively. Instrument detection limits (IDLs) were determined as below the lowest standard concentrations, whereas the method quantitation limit (MQL) of each compound was calculated as the lowest concentration in the $PM_{2.5}$ samples. Owing to the uncertainty in the environmental sample concentration, eight to ten winter $PM_{2.5}$ samples from each region were prepared for quantitative detection to ensure the day-to-day reproducibility of the method.

For quality control, program blank samples and solvent blank samples were used to evaluate potential background interferences, and equal amounts of instrument internal standards (hexamethyl benzene, HMB) were added to each sample before analysis to eliminate instrument errors. In addition, all glassware was baked at 450 °C for 5 h prior to the analysis and the sample processing process was performed in a fume hood to avoid sample contamination.

2.5. Calculations for exposure assessment

It is well known that exposure of pollutants to different populations causes unequal health risks. In this investigation, preliminary assessments of human health risks were performed for adults and toddlers in an outdoor environment. The concentration of BTHs at each sampling point was used to calculate the estimated daily intake $EDI_{\alpha p}$ (ng kg-BW^{-1} day^{-1}) of various human exposures. Detailed calculation formulas and parameter settings can be obtained in the "Supplementary Materials" and Table S4.

2.6. Toxicity test in vitro

Mouse embryonic stem cells (mESCs) were used as a model for toxicity experiments. The cytotoxicity of six different BTHs was screened using an MTT assay kit (Beyotime Institute of Biotechnology, China) according to our previous publications (Qi et al., 2019; Qi et al., 2016). Briefly, six different BTHs were added to the wells of a microwell plate at final concentrations of 80, 250, 500, 1000, 2000 or 4000 μM and the cells were exposed for 48 h. Based on the measured dose-response curve, the half-inhibitory concentration (IC_{50}) of each substance for mESCs was calculated and used to assess the toxicity of the different BTHs.

From comparison of IC_{50} combined with the estimated results of daily average intake, 2-hydroxybenzothiazole (OTH) was selected as the analyte with the greatest environmental risk for subsequent toxicity examination. After mESC cells were treated with OTH at IC_{50} concentration, ROS detection, an apoptosis analysis, DNA damage and Western blot analysis were performed. A full description of the methods is available in the "Supplementary Materials and Methods" online.

2.7. Toxicity test in vivo

Zebrafish embryos were used as a biological model to explore the toxicity of OTH in vivo. The feeding and matching methods used for the zebrafish are shown in the "Supplementary Materials". For toxicity characterization assessment, synchronized 0 hpf zebrafish embryos (50 embryos/5 mL medium) were exposed to OTH at a concentration of 1, 100, 200, 300, 400, 500 or 750 μM with final concentration of dimethyl sulfoxide (vehicle) up to 0.1% (v/v) in E3 medium (344 mg NaCl, 15.2 mg KCl, 58 mg $CaCl_2 \cdot 2H_2O$, 98 mg $MgSO_4 \cdot 7H_2O$ and 60 μL 0.1% methylene blue/methylene blue were added to 20 mL of distilled water to obtain 60× E3 medium and diluted to 1× concentration to use). The exposure was followed up to 96 hr post fertilization (hpf). The mortality, deformity and heart rate of embryos were recorded by a stereo microscope (SZ780, OPTEC), and EC_{50} corresponding to the malformation rate and LC_{50} were calculated.

2.8. Metabolomics analysis

2.8.1. Metabolite extraction from zebrafish embryos

After exposure of zebrafish embryos at LC_{50} of OTH and 0.1% DMSO for 96 h, samples were collected from the treatment group and compared with those from a control group. Ten samples were collected for both groups, each sample containing 20 hatched larvae. Each sample was mixed with zirconia beads and 500 μL methanol-water (4:1, v/v) in a centrifuge tube, which was then placed in an automatic grinder operating at a constant frequency of 60 Hz for 20 s. Afterwards, the mixed samples were centrifuged at 13,000*g*, collecting 400 μL supernatant. After blowing dry with a gentle stream of nitrogen, 120 μL of methanol-water (1:1, v/v) was used to reconstitute the sample and the solution was transferred to a 250 μL inner lining pipe.

2.8.2. Non-targeted metabolomics analysis

In order to obtain high quality metabolic data, an UltiMate 3000 ultrahigh-performance liquid chromatography system coupled with a Q-Exactive focus Orbitrap mass spectrometer (Thermo Scientific, USA) was applied to take the non-targeted metabolomics, operating in both negative and positive ionization modes and equipped with an electrospray ionization (ESI) source and A Waters HSS T3 column (2.1 mm × 100 mm, 1.8 μm). The detailed chromatographic and mass spectrometric conditions for metabolomics can be found in the "Supplementary Materials".

2.9. Data analysis

For $PM_{2.5}$ composition data, the concentration below the MQL was set to 1/2 of the MQL before statistical analysis. One-way analysis of variance (one-way ANOVA) and principal component analysis (PCA) were performed to explore the contamination characteristics of BTHs in urban $PM_{2.5}$. $p < 0.05$ was statistically significant.

For the raw metabolomics data, the software MS-DIAL (ver. 3.98) was first used for deconvolution processing to obtain a data matrix of fragment ions (Arita, 2015). Subsequently, fragmented ions with missing value >20% were extracted, and after sum normalization, ions with relative standard deviation (RSD) > 20% were filtered. After completing the above data preprocessing, classical statistics (*t*-test and fold change) and multivariate statistics (PLS-DA and OPLS-DA), differential markers were screened for conditions of $p < 0.05$ and variable importance in projection (VIP) > 1. Finally, the R language-based software metID (Shen et al., 2019), MyCompoundID (Lin, 2013) and the HMDB database were used to perform secondary mass spectrometry identification of the markers. The data analyses were performed by MS-DIAL (ver. 3.98), R (ver. 3.5.2), Graphpad Prism 7, SPSS 22.0, Origin 8.1 and SIMCA 14.1 for Windows.

3. Results and discussion

3.1. Method validation

Owing to the water solubility, weak polarity and relatively low volatility of BTHs, combined liquid chromatography and mass spectrometry or tandem mass spectrometry is one of the commonly used analytical methods for characterizing them (Hidalgo-Serrano et al., 2019; Wang et al., 2013a). However, when using electrospray ionization, problems may occur of co-elution and strong ion enhancement or suppression. Since the detection of BTHs in air samples is a trace analysis, matrix effects can also seriously affect the sensitivity and accuracy of detection. GC-EI-MS/MS is a useful alternative method. The chromatographic column used in this experiment was a Thermo TG-5MS column (30 m × 0.25 mm i.d., 0.25 mm film thickness), which is suitable for the separation of low polar compounds. The long column length enabled better separation of co-eluting compounds. The peak shape and height of the target ions were improved via the SRM mode of triple quadrupole mass spectrometry. The determination coefficients (R^2) of

the calibration curves of all the tested compounds were higher than 0.99 (Fig. S1), indicating a good linear relationship. The IDLs and MQLs of the analytes were placed in the range 0.002–0.40 ng mL^{-1} and 0.04–3.04 pg m^{-3}, respectively (Table S3). Compared with previous studies using PLE in combination with GC–MS (Maceira et al., 2018), the optimized method reduced MQL by more than 5 times, enabling high sensitivity for the detection of BTHs in our samples.

The optimized method was applied to solvent blanks ($n = 6$), program blank samples ($n = 6$) and QMF samples ($n = 6$) after spiking (1 μg mL^{-1} mixed standard) to evaluate the background interference of samples and the recovery of target substances in actual air samples. However, the six target BTHs were not detected in the solvent blanks and program blank samples. The recoveries for most analytes were between 60% and 105%, with the exception that the average recovery for CTH was only 55.4% (Fig. 1b). The total average recovery was 77.8% and the RSD value was below 10%, which proved this method to be with acceptable extraction efficiency following the European directive 96/23/EC. The peaks of the analytes in the $PM_{2.5}$ samples are shown in Fig. 1c.

3.2. Concentration and pollution characteristics of BTHs in $PM_{2.5}$

Serval publications have shown that BTHs can be detected in various gas samples (indoor air (Wan et al., 2016; Wang et al., 2013a), outdoor air (Simcox et al., 2011) and particulates (Maceira et al., 2018; Nunez et al., 2020)), indicating their widespread occurrence in the atmosphere. However, there have been only few reports about their detection in $PM_{2.5}$. Considering the current $PM_{2.5}$ pollution issue in China (Gao et al., 2016), our study focused on the pollution profiles and characteristics of BTHs in urban $PM_{2.5}$ samples. As we all know, as the weather changes, more fuel is burned, so $PM_{2.5}$ pollution in winter will be more serious than in the other three seasons. Eight to ten samples were collected at each sampling location (Guangzhou, Shanghai, and Taiyuan) during the winter from November 2018 to January 2019. Table 1 shows the results obtained, including the arithmetic mean, median value, concentration range and sample detection rate of the detected substances.

As shown in Table 1, the average $PM_{2.5}$ concentrations in winter in Guangzhou, Shanghai and Taiyuan were 88.0, 35.6 and 102 μg m^{-3}, respectively. The low concentration of $PM_{2.5}$ in Shanghai might be due to its relatively close geographical distance from the sea, whereas the high concentration in Taiyuan might be attributed to local coal combustion activities. Among the $PM_{2.5}$ samples at the three locations, most of analytes were found in all $PM_{2.5}$ samples with 100% detection rates, except for CTH in the Shanghai samples, for which the detection rate was only 87.5%. Such high detection rates indicate that BTHs were ubiquitous in the atmosphere.

In the three sampled cities, there were significant differences in the environmental concentrations of BTH, NTH, OTH, CTH and ΣBTHs (Fig. 2a), with OTH having the highest concentration. Although Taiyuan was the city with the most serious $PM_{2.5}$ pollution among the three locations, its ΣBTHs concentration was the lowest, with an average concentration of only 308 pg m^{-3}. Guangzhou had the highest concentration of BTHs, followed closely by Shanghai, with average concentrations of 525 and 369 pg m^{-3}, respectively, of which the OTH content accounted for more than 70% of the total BTHs (Fig. 2b). PCA was conducted on the target concentration data. Before performing PCA analysis, the concentration data was processed by Pareto scaling to make it normally distributed. After dimensionality reduction, two principal components (PC1 and PC2) were extracted, with proportions in the total variance of 77.6% and 12.2%, respectively. Fig. 2c presents a two-dimensional score chart of PCA. It can be seen that the data points for the three locations were well separated, indicating that the target component concentrations in $PM_{2.5}$ showed obvious regional differences. The corresponding load chart (Fig. 2d) showed that OTH had a higher load on PC1, indicating that OTH was the main contributor to the regional distribution of BTHs in atmospheric $PM_{2.5}$.

BTHs are a class of high yield chemicals that have been applied in lots of industrial and household products. The amount used as a vulcanization accelerator in rubber production is the largest, and the addition amount exceeds 1% (w/w) (Ni et al., 2008). Among the three sampled cities, the industry of Taiyuan is mainly dominated by heavy industries, such as coal and heavy metals production, whereas Guangzhou and Shanghai have mainly manufacturing industries, such as the manufacturing of automobiles, fine chemicals and electronic products. We had sorted out the previous researches on detection and source analysis of BTHs in various environmental matrices. It can be seen from Table S5 that the release of BTHs to the environment is mainly related to the wear of automobile tires and the use of rubber products. Although BTHs are a type of traffic pollutants, their source has little to do with vehicle exhaust emissions, which may be the reason for the non-correspondence between $PM_{2.5}$ and BTHs in atmospheric concentration.

According to Fig. 2b, OTH was the most abundant BTHs in all three cities. It has been experimentally proven that OTH is an oxidation product of BTH (Haroune et al., 2002). Tests by Wang et al. have also shown that high temperature causes BTH to be converted to OTH in rubber particles of automobile tires (Wang et al., 2013b). Guangzhou and Shanghai are two developed cities with a large number of cars, and because of their geographical location, the winter temperature in both places is much higher than in Taiyuan (Table S2). We further compared the OTH concentration in $PM_{2.5}$ samples collected from these three places during summer and winter (basic information of sampling sites in summer was shown in Table S2) and found that the concentration in summer was significantly higher than that in winter (Fig. S2a). Moreover, there was a significant positive correlation between temperature and OTH concentration (Fig. S2b). Therefore, the BTH in high-speed friction car tires is more likely to be heated and oxidized into OTH in the air in Guangzhou and Shanghai. Based on the experimental results, the levels of OTH pollution in Guangzhou and Shanghai were indeed much higher than in Taiyuan. Therefore, it can be speculated that traffic pollution and air temperature played a key role in determining the concentrations of OTH in the atmospheric fine particles.

3.3. Human exposure assessment and toxicity screening

As the above results show, BTHs were widely present in the urban $PM_{2.5}$ samples. Due to the small particle size of $PM_{2.5}$, they can be easily deposited deeply in the lungs and pass through the lung epithelium and

Table 1
Concentrations of $PM_{2.5}$ (μg m^{-3}) and BTHs (pg m^{-3}) in outdoor air samples collected in Guangzhou, Shanghai and Taiyuan from November 2018 to January 2019.

Compounds	Guangzhou (n=8)				Shanghai (n=8)				Taiyuan (n=10)			
	Range	Mean	Median	DR (%)	Range	Mean	Median	DR (%)	Range	Mean	Median	DR (%)
$PM_{2.5}$	61.1–197	88.0	64.6	–	12.0–70.0	35.6	26.5	–	40.0–223	102	82.8	–
BTH	18.4–47.1	32.6	31.9	100	23.1–78.4	46.8	41.5	100	36.6–78.4	55.9	51.9	100
NTH	22.8–45.9	31.1	29.7	100	20.1–29.2	24.1	24.6	100	24.2–44.2	32.6	31.2	100
OTH	206–557	416	455	100	132–477	263	228	100	66.8–430	164	152	100
MSTH	10.8–29.1	19.8	18.9	100	9.38–28.2	18.1	17.5	100	14.0–39.5	25.4	21.6	100
CTH	4.91–16.9	8.99	6.21	100	n.d.–6.41	3.45	3.45	87.5	5.45–19.3	11.0	8.91	100
XTH	5.84–42.7	15.9	11.3	100	6.59–20.5	12.9	12.4	100	8.52–34.2	19.3	18.3	100
∑BTHs	274–695	525	564	–	209–619	369	325	–	165–626	308	305	–

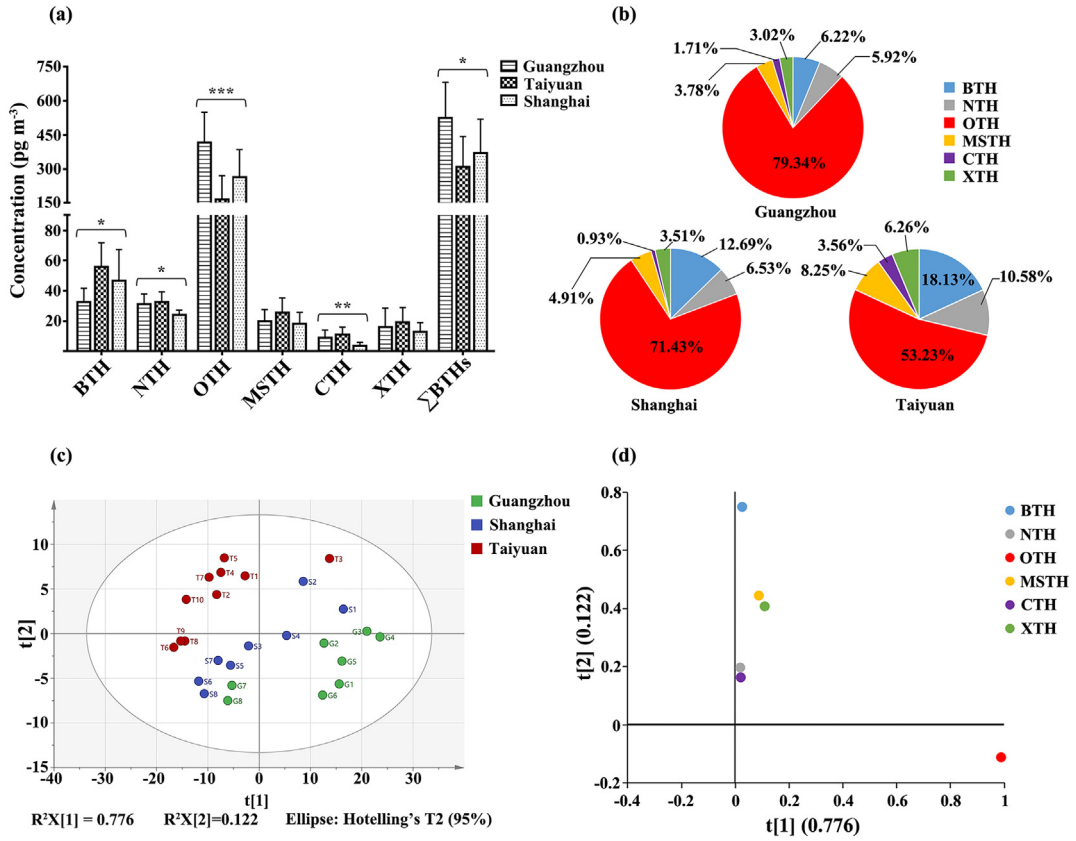

Fig. 2. Contamination profiles of BTHs in urban $PM_{2.5}$. (a) Concentrations of BTHs in $PM_{2.5}$ of Guangzhou ($n = 8$), Shanghai ($n = 8$), Taiyuan ($n = 10$) in winter. (b) Proportion of various BTHs in urban $PM_{2.5}$. (c) Two-dimensional score chart of PCA analysis based on analyte concentration data. (d) Load chart of PCA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ vs. control group.

lung blood barrier (Rothen-Rutishauser et al., 2008). Once they enter the blood, the toxic substances in $PM_{2.5}$ can be rapidly distributed in various organs in the body, posing a serious risk to human health (Ali et al., 2019; Brook et al., 2002). Therefore, it is essential to conduct an intake assessment and toxicity screening for BTHs. Table 2 shows the calculated EDI of inhalation exposure in the three Chinese cities for two groups of people (toddlers and adults). The results for the average daily intake of ΣBTHs were roughly similar to those calculated by Alba et al. (Maceira et al., 2018). Furthermore, separate EDI calculations were performed for the six different BTHs. It was found that the highest intake was that of OTH and the outdoor exposure risk of toddlers was much higher than that of adults. Currently, there have been only a few studies on the toxicity of these six BTHs, making it difficult to conduct a more in-depth environmental risk assessment.

This study focused on the growth and developmental toxicity of BTHs. Mouse embryonic stem cells (mESCs) were exposed to the six different BTHs at various concentrations, and an initial screening of cytotoxicity was performed by MTT assay. The IC_{50} data of the BTHs are shown in Fig. S3 and Table 2. Among them, OTH was found to be the most cytotoxic, which is similar to data obtained by Ye et al. using a human carcinoma cell line (Ye et al., 2014). Combined with its higher concentration in urban $PM_{2.5}$ than other BTHs, OTH poses serious health risks for the growth and development of young children. Therefore, it is urgent to conduct in-depth toxicity research on OTH.

Table 2
Estimated daily intake (EDI, 10^{-3} ng kg-bw^{-1} day^{-1}) of BTHs through inhalation of outdoor air ($PM_{2.5}$) in three Chinese cities for various age groups and corresponding BTH *in vitro* toxicity data.

Compound	EDI(G)[a]				EDI(S)[b]				EDI(T)[c]				IC_{50} (μM)
	Toddlers		Adults		Toddlers		Adults		Toddlers		Adults		
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	
BTH	6.73–17.2	11.9	2.35–6.02	4.16	8.45–28.6	17.1	2.95–10.0	5.97	13.4–28.6	20.4	4.67–10.0	7.14	1254
NTH	8.32–16.8	11.3	2.91–5.86	3.96	7.34–10.7	8.79	2.57–3.72	3.07	8.83–16.1	11.9	3.09–5.64	4.16	812
OTH	75.4–203	152	26.3–71.1	53.1	48.4–174	96.2	16.9–60.9	33.6	24.4–157	60.0	8.52–54.9	21.0	659
MSTH	3.96–10.6	7.24	1.38–3.71	2.53	3.42–10.3	6.61	1.20–3.60	2.31	5.10–14.4	9.29	1.78–5.04	3.25	682
CTH	1.79–6.15	3.28	0.63–2.15	1.15	0.00–2.34	1.26	0.00–0.82	0.44	1.99–7.05	4.01	0.70–2.46	1.40	742
XTH	2.13–15.6	5.79	0.75–5.44	2.02	2.41–7.47	4.72	0.84–2.61	1.65	3.11–12.5	7.05	1.09–4.37	2.46	756
∑BTHs	100–254	192	35.0–88.7	66.9	76.4–226	135	26.7–78.9	47.1	60.3–228	113	21.1–79.8	39.4	–

[a] Estimated daily intake of BTHs in Guangzhou.
[b] Estimated daily intake of BTHs in Shanghai.
[c] Estimated daily intake of BTHs in Taiyuan.

3.4. In vitro toxicity study of OTH

In the light of the increased accumulation in $PM_{2.5}$ and higher health risk of OTH, we selected it as a representative chemical to investigate the cytotoxicity of BTHs. After treatment of mESCs with OTH (IC_{50} concentration, 659 μM) for 48 h (Table 2), DNA damage was examined by alkaline comet assays. In comparison with the control group, significant DNA damage was found in the OTH exposure group and the Olive tail moment (OTM) was 31.6 ± 7.3 (Fig. 3a), suggesting increased ROS in the cells (Fig. 3d). The genotoxicity and cytotoxicity of BTHs have been shown in bacteria and human cells (Hornung et al., 2015; Ye et al., 2014). However, the underlying mechanisms are still unclear. Once DNA damage occurs, ESCs may rapidly initiate a DNA damage response and DNA repair to ensure their genomic stability (Liu et al.,

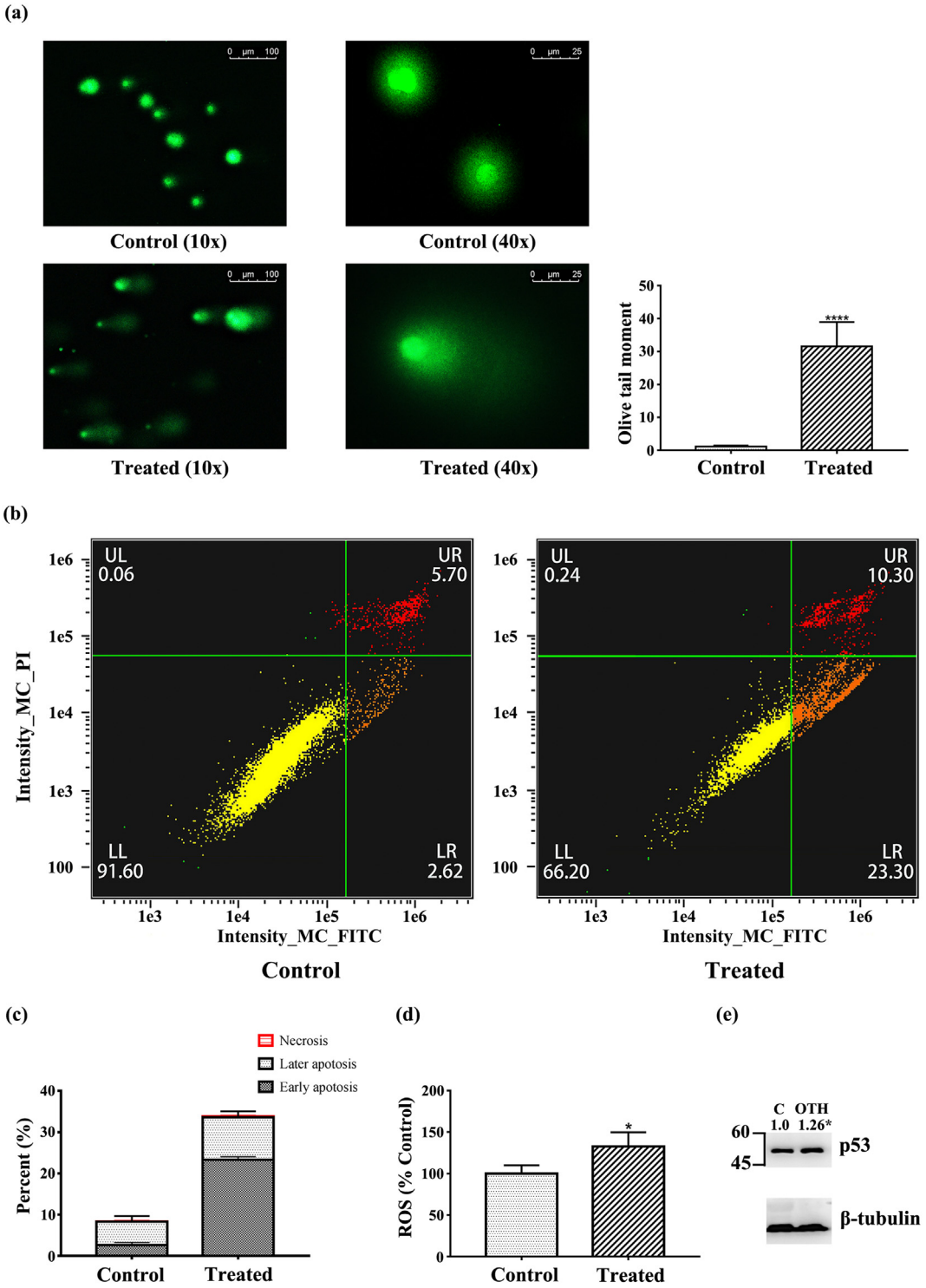

Fig. 3. The cytotoxicity of OTH in mESCs. (a) The representative images of comet assay after exposure mESCs to OTH. (b) High levels of apoptosis in ESCs were detected by flow cytometry in OTH treated group. (c) Percentages of early apoptotic and late apoptotic/necrotic cells. (d) Oxidative stress induced by OTH on mESCs. The intracellular ROS generation caused by OTH in mESCs was measured by DCF fluorescence assay. (e) The expression of p53 significantly increased in the OTH exposure group. Fold change was normalized against β-tubulin and compared with the control. * $p < 0.05$, **** $p < 0.001$ versus the control group.

2014). High levels of apoptosis in ESCs is one of the most important pathways to remove impaired cells from the stem cell pool (Lai et al., 2011; Hong and Stambrook, 2004; Qin et al., 2007). Fig. 3b-c show results obtained from the flow cytometry assay. It can be seen that OTH exposure significantly increased the apoptotic populations compared with those in the untreated control (OTH, 33.6 ± 2.2% vs. control, 8.32 ± 2.03%; $p < 0.01$). At the same time, increased expression of p53 was detected in the OTH exposure group (Fig. 3e). Taken together, these results suggest that exposure of mESCs to OTH (659 μM) for 48 h significantly increased the intracellular ROS and induced DNA damage and apoptosis via functionally activating p53 expression.

Zeng et al. investigated the potential cytotoxicity of 12 BTHs in two rainbow trout epithelial cell lines (RTgill-W_1 and RTL-W_1) and concluded that OTH could induce transitory ROS levels and DNA damage, consistent with our findings in mESCs (Zeng et al., 2016). However, neither necroptosis nor apoptosis was detected in their BTH treatment group. The reason for this difference in inducing apoptosis may be the different exposure dose between our experiments and Zeng et al. Moreover, one essential factor that cannot be omitted is that ESCs are sensitive to DNA damage, resulting in severe apoptosis (Nagaria et al., 2013; Qin et al., 2007).

3.5. Toxicity characterization of zebrafish embryo after exposure to OTH

In this study, zebrafish embryo was employed to verify the developmental toxicity of OTH. After 96 h exposure of zebrafish embryos to a gradient concentration of OTH, the numbers of deaths and deformities were counted and used to calculate the LC_{50} of 358 μM and EC_{50} of 287 μM (Fig. 4a and b). At the same time, the heart rate of the zebrafish larvae was recorded after 96 h exposure in each group. It was found that with increasing exposure concentration, the heart rate decreased significantly (Fig. 4c) accompanied by malformations, such as notochord deformity, pericardial edema and yolk sac edema (Fig. 4d). In summary, OTH showed obvious growth and developmental toxicity. mESCs and zebrafish are the most acceptable test models to explore the effects of toxic chemicals on development and reproduction at the gene expression and metabolic levels. In our study, using both in vivo and in vitro experiments to investigate the toxicities and potential mechanisms of BTHs can discover more valuable and reliable results than operating separated in vivo or in vitro experiments.

3.6. Metabolomics analysis

In order to explore the toxic mechanism of OTH on growth and development, an overall metabolomics study was conducted on the exposed zebrafish larvae. It can be seen in the PLS-DA score chart (Fig. S4a, S4b) that the data points of the QC group were densely clustered, indicating that the instrument method was reproducible. Moreover, the data points of the three groups (treated, control and QC) were clearly separated from each other, showing that OTH had a serious effect on the metabolism of zebrafish larvae. Further, OPLS-DA analysis was performed on the data of the treated and control group to calculate the VIP value corresponding to each fragment ion. Using VIP > 1 and p-value <0.05 as screening conditions (Fig. S4c, S4d), the screened ions were identified by secondary mass spectrometry. The verification of the PLS/OPLS-DA model is available in "Supplementary Material" and Fig. S5. A total of 41 qualifying metabolites were identified in the positive and negative modes (Table S6), and metabolic pathway analysis was performed.

Among the possible metabolic pathways (Fig. S6), phenylalanine, tyrosine and tryptophan biosynthesis pathways were the most severely affected (Fig. 5). After exposure to OTH, the concentration of phenylalanine, phenylpyruvic acid, tyrosine and acetylphenylalanine all increased significantly. Phenylpyruvic acid and acetylphenylalanine are the intermediate products or catabolism byproducts of phenylalanine metabolism, and acetylphenylalanine is a harmful amphiphilic compound

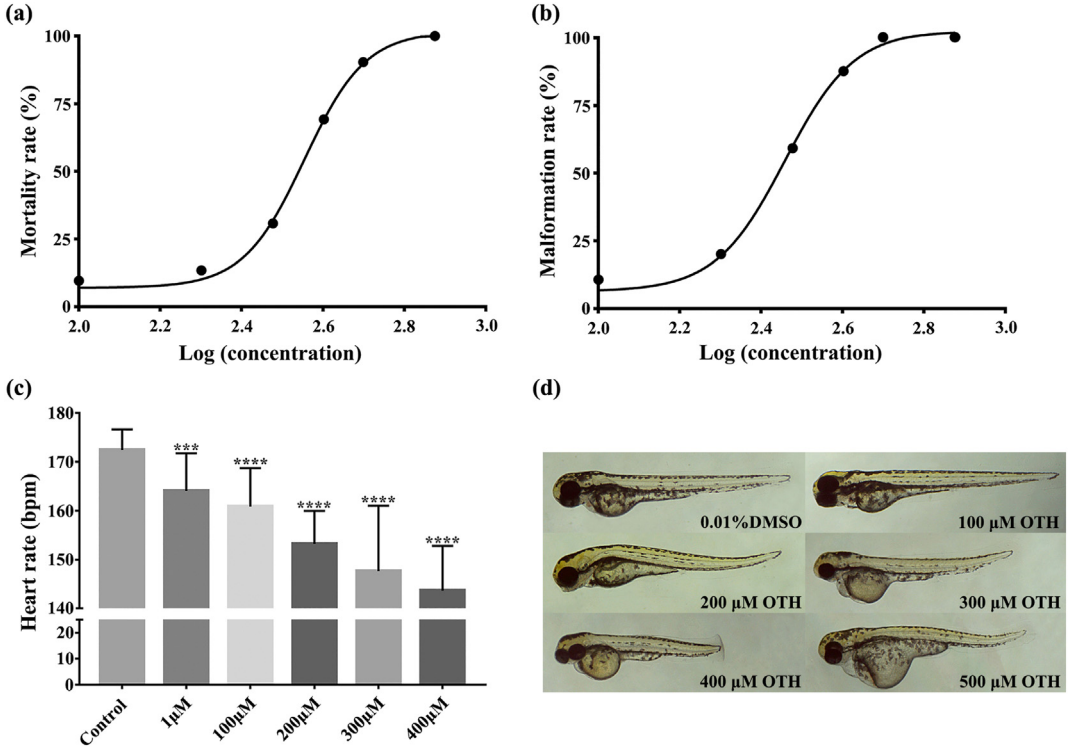

Fig. 4. Effects of OTH on zebrafish embryonic development. (a-b) Logarithmic-concentration vs. response graph. The zebrafish embryos were cultured with various concentrations of OTH (1, 100, 200, 300, 400, 500 and 750 μM) for 96 hpf. The LC_{50} concentration was 358 μM and the EC_{50} concentration of malformation was 287 μM. (c-d) Changes in the heart rate and morphology of zebrafish larvae after OTH exposure for 96 hpf. ***$p < 0.005$, ****$p < 0.001$ vs. control group.

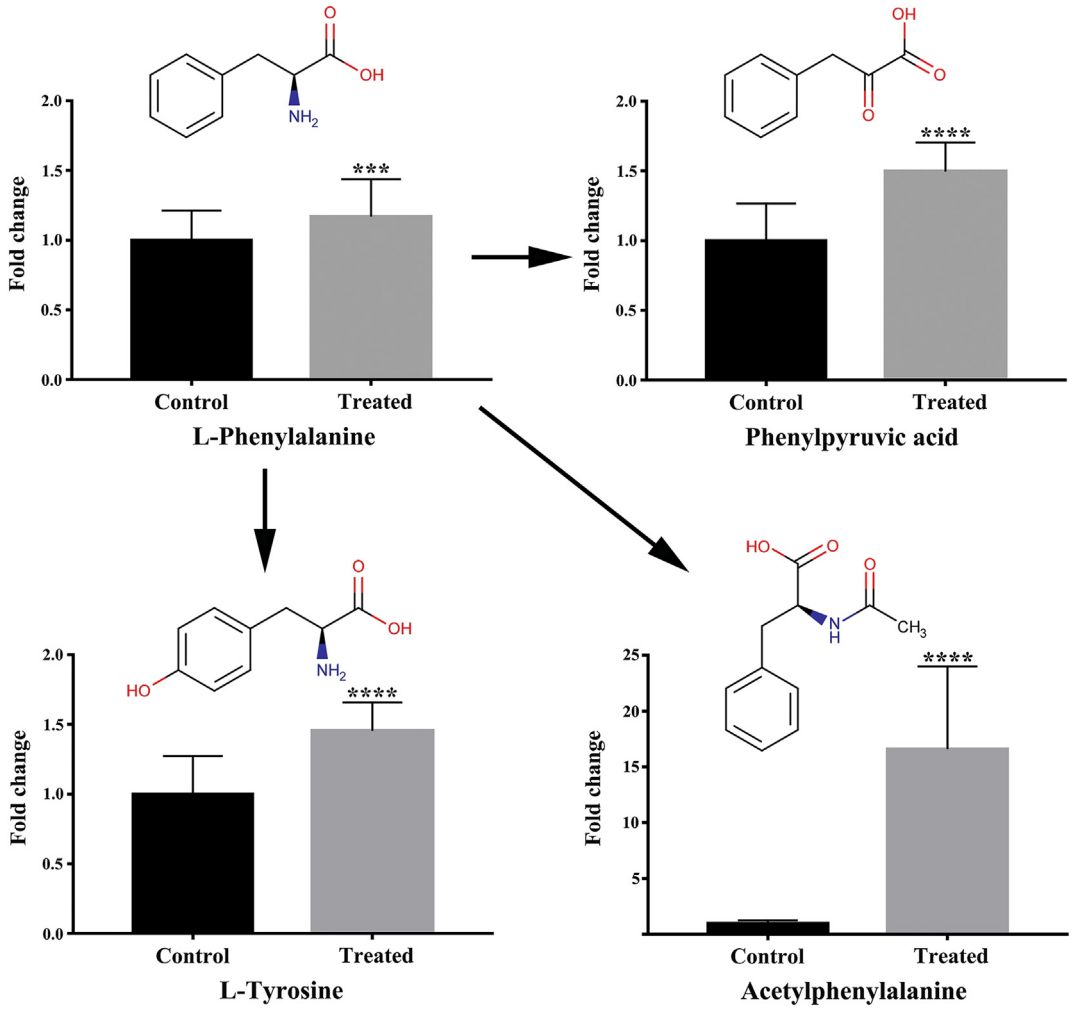

Fig. 5. Changes of metabolites in phenylalanine pathways in zebrafish larvae. $^{***}p < 0.005$, $^{****}p < 0.001$ vs. control group.

(Michals and Matalon, 1985; Nierop Groot and de Bont, 1998; Okajima et al., 1985). High levels of phenylpyruvic acid have been found in the urine of phenylketonuria (PKU) patients, and PKU can cause symptoms such as mental retardation and microcephaly in toddlers (Langenbeck et al., 1981; Monch et al., 1990). PKU is caused by a lack of phenylalanine hydroxylase (PAH). Tetrahydrobiopterin is a coenzyme of PAH which acts as an electron carrier in the enzymatic reaction and also as a reducing agent (Blau and Trefz, 2002; Ye et al., 2009). Isoflavopterin is a metabolite of tetrahydrobiopterin and has been used as a possible indicator of levels of tetrahydrobiopterin (Blau et al., 1996). This study found that after exposure to OTH, the concentration of isoxanthine decreased significantly, which may indicate that the hydroxylation reaction of phenylalanine was blocked, resulting in the conversion of a large amount of phenylalanine to phenylpyruvic acid.

In addition to the metabolism of phenylalanine, OTH also affected the metabolic pathways of glutamic acid (Fig. 6). As shown in Table S6, the concentrations of pyroglutamic acid, glutamine and cysteineglutathione disulfide in zebrafish larvae increased markedly after OTH exposure, whereas the levels of glutamic acid and glutathione (reduced) decreased significantly. Glutathione is a common antioxidant in the body and is composed of glutamic acid, cysteine and glycine. It has two forms: reduced (G-SH) and oxidized (G-S-S-G). Under physiological conditions, reduced glutathione is predominant. Betaine, which acts as an antioxidant in vivo and plays an important role in embryo development (Amiraslani et al., 2012), also showed significantly decreased concentrations after OTH treatment. Pyroglutamic acid is a cyclic derivative of L-glutamic acid and is an unusual amino acid derivative. When the concentration of pyroglutamic acid in the body is in sufficiently high levels, it can be used as an acidogen and metabotoxin, which can have a variety of adverse effects on many organ systems. In infants with acidosis, adverse symptoms such as malnutrition, vomiting, loss of appetite, muscle weakness, and lack of energy can occur. These can progress to heart, liver, and kidney abnormalities, seizures, coma and possibly death (Creer et al., 1989). After OTH exposure, oxidative stress was found to occur in zebrafish larvae, Significantly increased concentrations of aminoadipic acid, a small molecular marker of oxidative stress (Yuan et al., 2011), may indicate that G-SH was converted into cysteineglutathione disulfide (Cys-S-S-G) under stress(Oz et al., 2007), increasing the concentration of Cys-S-S-G in vivo. However, glutamate, a precursor of glutathione, may instead be converted to toxic pyroglutamic acid under stress or glutamine through the addition of an amine functional group to enhance the immune system function (Wada et al., 2005). These conversions reduce the concentration of G-SH, causing a serious loss of the body's antioxidant capacity.

In summary, acute exposure to OTH caused the phenylalanine hydroxylation reaction in zebrafish larvae to be blocked, the accumulation of toxic phenylpyruvate and acetylphenylalanine triggered PKU, which seriously affected zebrafish larvae's growth and development. In addition, the larvae's organs and tissues were further damaged through oxidative stress.

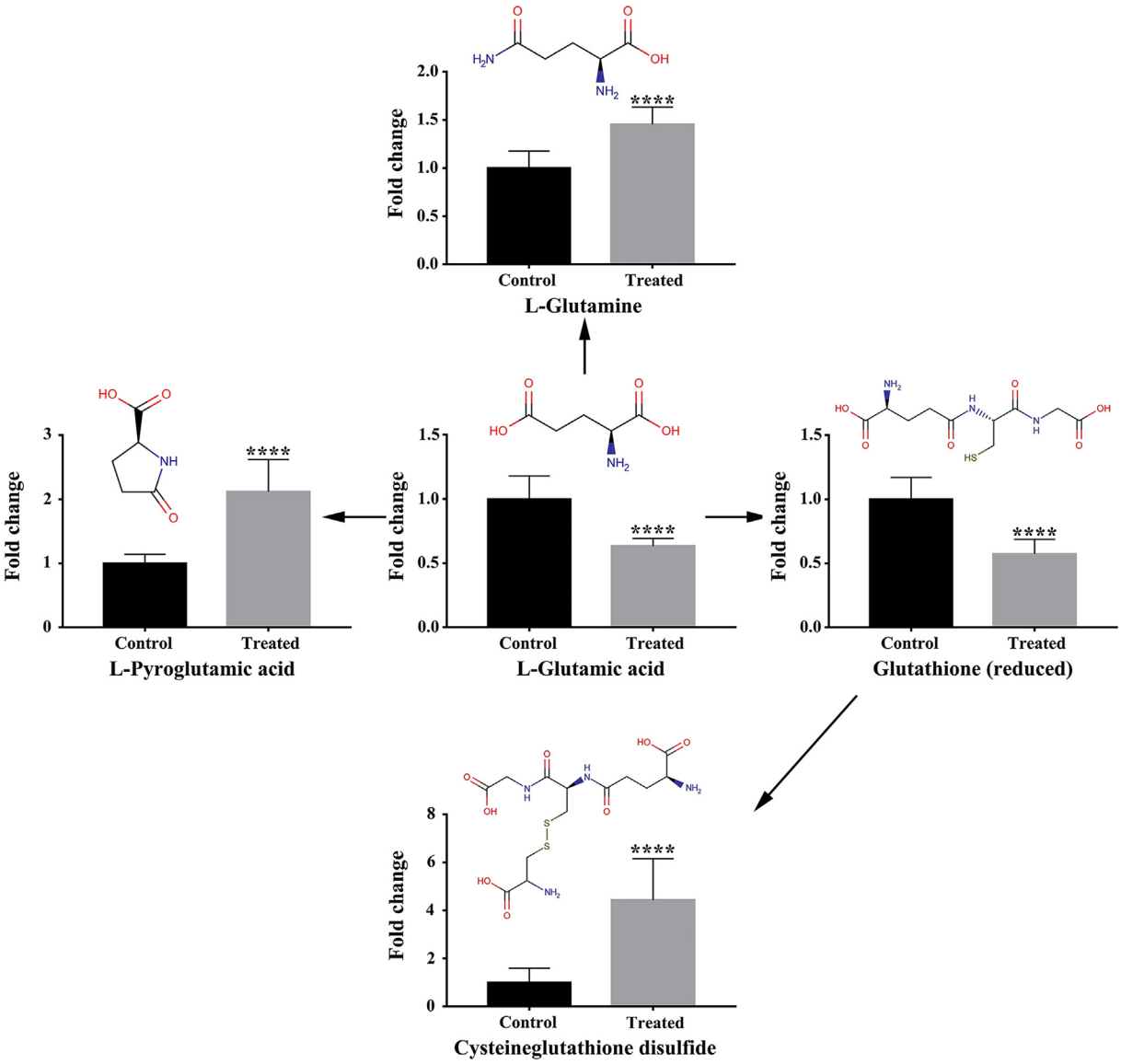

Fig. 6. Changes of metabolites in glutamic acid pathways in zebrafish larvae. ****$p < 0.001$ vs. control group.

4. Conclusions

The present study was designed to determine the contamination profiles and health risks of $PM_{2.5}$-bound BTHs from three cities of China (Guangzhou, Shanghai and Taiyuan) during the winter of 2018. The highest concentration of BTHs was detected in $PM_{2.5}$ from Guangzhou, followed by Shanghai and Taiyuan, which indicated that the concentration profiles of BTHs in $PM_{2.5}$ samples were independent on the $PM_{2.5}$ mass concentration in these three cities. In addition, OTH was the most abundant BTHs in all $PM_{2.5}$ samples and it has the greatest health risk than the other five BTHs, especially for young children. The toxicology studies in vivo and in vitro also revealed that OTH could negatively affect the developmental and reproduction for zebrafish embryos and mESCs. The present investigation has therefore revealed the contamination profile and health risks of BTHs combined with $PM_{2.5}$ and emphasized the potential growth and development toxicity of OTH, laying a theoretical foundation for future related research.

CRediT authorship contribution statement

Xiaoliang Liao: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Ting Zou:** Conceptualization, Investigation, Data curation, Supervision. **Min Chen:** Data curation, Supervision. **Yuanyuan Song:** Conceptualization, Supervision. **Chun Yang:** Data curation. **Bojun Qiu:** Data curation. **Zhi-Feng Chen:** Conceptualization, Data curation, Supervision. **Suk Ying Tsang:** Writing - review & editing, Supervision. **Zenghua Qi:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Zongwei Cai:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision.

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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Appendix A. Supplementary data

Figs. S1–S6, Tables S1–S6 and supporting methods are available in the supplementary data, which is free of charge via the Internet. Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2020.142617.

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