Particulate matter (PM_{2.5}) exposure season-dependently induces neuronal apoptosis and synaptic injuries

Running title: PM_{2.5} causes neuronal apoptosis and synaptic injuries

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Abstract

Epidemiological studies have shown that particulate matter 2.5 (PM_{2.5}) not only increases the incidence of cardiopulmonary illnesses but also relates to the development of neurodegenerative diseases. Considering that PM_{2.5} is highly heterogeneous with regional disparity and seasonal variation, we investigated whether PM_{2.5} exposure induced neuronal apoptosis and synaptic injuries in a season-dependent manner. The results indicated that PM_{2.5} altered the expression of apoptosis-related proteins (mainly bax and bcl-2), activated caspase-3 and caused neuronal apoptosis. Additionally, PM_{2.5} decreased the levels of synaptic structural protein postsynaptic density (PSD-95) and synaptic functional protein N-methyl-D-aspartate (NMDA) receptor subunits (NR2B) expression. These effects occurred in a season-dependent manner, and PM_{2.5} collected from the winter showed the strongest changes. Furthermore, the effect was coupled with the inhibition of phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phosphorylated cAMP-response element binding protein (p-CREB). Based on the findings, we analyzed the correlations between the chemical composition of PM_{2.5} samples and the biological effects, and confirmed that winter PM_{2.5} played a major role in causing neuronal apoptosis and synaptic injuries among different season samples.

Keywords: Particulate matter (PM_{2.5}); Season-dependent effect; Neuronal apoptosis; Synaptic injury

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Introduction

Coal combustion constitutes the main energy structure of China, accounting for more than 70% of total energy demands (Chen et al., 2014). In the cities of northern China, dispersive use of coal combustion in rural areas and the extensive consumption of coal in residential stoves for cooking and heating has been the main source of PM_{2.5} in winter (Shen et al., 1994). A measurement at 74 major cities showed that the daily average concentrations of PM_{2.5} exceeded the Chinese pollution standard of 75 µg/m³ (about twice that of the US EPA standard of 35 µg/m³) for 69% of days in January, with a record-breaking daily concentration of 772 µg/m³. PM_{2.5} is a complex mixture of extremely small particles and liquid droplets that include various components including acids, organic chemicals (such as polycyclic aromatic hydrocarbons (PAHs), nitro- and oxygenated-PAHs), heavy metals, and soil or dust particles (Yue et al., 2015), which can directly affect the acidity of precipitation, degrade atmospheric visibility, and make the numerous harmful effects on individuals, even increased mortality or morbidity (Zhang et al., 2012, 2016; Eze et al., 2014). Epidemiological studies have indicated that PM_{2.5} is not only linked to an increased risk of hospitalization and mortality from cardiopulmonary diseases (Lo et al., 2016; Gillespie et al., 2013), but also associated with the development and progression of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Genc et al., 2012).

Neurodegenerative disorders originate from a progressive loss of neuronal function and structure in the nervous system, characterized by neuronal apoptosis (Okouchi et al., 2007) and synaptic plasticity changes (Lussier et al., 2016; Coleman and Yao, 2003). Previous studies have documented a phenomenon by which apoptosis accurately mediates the programmed natural death of neurons, suggesting that it is a vital process implicated in neurogenesis during maturation of the central nervous system (Okouchi et al., 2007). However, abnormal apoptosis, potentially triggered by dysfunction of the automatic nerves, promotes the pathogenesis of neurodegeneration (Li and Hu, 2015). Synaptic plasticity is essential for the development of the nervous system, especially regarding spatial

learning-related synaptic plasticity in telencephalic structures, which can regulate the ability of learning and remembering new information accordingly to adjust human functional behavior (Pooters et al., 2015). Synaptic plasticity is also crucial for neurological disorders, such as epilepsy, memory impairment and neurodegeneration (Yun et al., 2013). Protein phosphorylation is also involved in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease (AD). Phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP response element binding protein (CREB) are associated with long-term potentiation (LTP) (Yi et al., 2015), which is involved in synaptic plasticity and enhanced learning and memory function (Hölscher, 1999).

Considering that PM_{2.5} is highly heterogeneous with regional disparity and seasonal variation, here we aimed to investigate whether PM_{2.5} exposure induced neuronal apoptosis and synaptic injuries in a season-dependent manner, and to clarify the possible mechanism underlying the effects.

1 Materials and methods

1.1 PM_{2.5} sampling, chemical analysis, and suspension preparation

PM_{2.5} samples were acquired with middle volume air samplers (TH-150CIII, Wuhan, China) in Taiyuan (112°21'E-112°34'E longitude, 37°47'N-37°48'N latitude), Shanxi Province, China. The flow rate of the sampler was 100 L/min, and the sampling time was 22 hr per day over a one-year period from March 2012 to March 2013: in spring, March 1, 2012 to April 30, 2012; in summer, May 1, 2012 to July 31, 2012; in autumn, August 1, 2012 to October 31, 2012; and in winter, November 1, 2012 to February 28, 2013 (considering the annual heating period in Northern China). Then, effective samples were 36, 54, 52 and 69 samples in spring, summer, autumn and winter, respectively, except for during the rainfall period, equipment failure, power outages and student holidays. The middle volume air samplers were set on the top of the living area building far from obstacles to obtain sufficient free-moving air. The PM samples were collected onto quartz filter membranes, and the quartz filter membrane weight was recorded before and after sampling.

Following this action, we cut 1/2-volume filters for the physical and chemical measurements and the other 1/2 for the biological experiments. PM_{2.5} mass concentrations in

ambient air were determined by the gravimetric method (Ambient air quality standards, 2012). With ion chromatography, inductively-coupled plasma-mass spectrometry, thermal/optical relection assay and gas chromatograph-mass spectrometer, the contents of the inorganic ions, elements, carbons, and PAHs were analyzed. For *in vitro* exposure, PM_{2.5} samples were extracted from the filters with ultrapure water ultrasonically. The suspensions were diluted to 10 mg/mL with 100 U/mL penicillin and 100 mg/mL streptomycin sulfate added. The samples were stored at -80°C prior to use. It should be noted that possible side effects to the PM size and composition from the procedure could not be entirely avoided.

1.2 Primary cortical neuron culture and treatment

Kunming mouse pups (postnatal day 0, P0) were purchased from the Animal Center of Hebei Medical University. The suckling mice were treated with 75% alcohol for disinfection and were then sacrificed by cervical dislocation, with immediate dissection of cortical tissue into a pre-chilled DMEM (high glucose, Hyclone Laboratories, China) nutrient solution. The cortical tissues were cut and incubated in oxygenated trypsin for 10 min at 37°C. The minced tissues were moved to DMEM medium containing 10% fetal calf serum (10099141, Gibco, USA) to terminate digestion, and the cells were separated by mechanical trituration. Then, the neurons were centrifuged at 1000 r/min for 10 min, resuspended in Neurobasal/B27 medium (Gibco® | Life Technologies, USA), and supplemented with 1.25 µmol/L L-glutamine, 25 µmol/L glutamate and penicillin/streptomycin.

The cells (5×10^5) were plated into poly-L-lysine-coated 96-well plates or 35-mm culture dishes for viability and western blot analysis, and the cells (5×10^4) were plated on poly-L-lysine-coated cover slips for TUNEL immunofluorescence analysis. The cultures and slips were used between 13 and 15 days *in vitro* (DIV) and were randomly divided into 5 groups of three samples each. The control group was incubated in Neurobasal/B27 medium with saline, and the different treatment groups were treated with the PM_{2.5} samples at different concentration. After treatment with PM_{2.5} for 24 hr, the cells were collected for further analysis.

1.3 Neuronal viability determination

Cortical neurons were plated on 96-well plates at a concentration of 2×10^5 cells/well, and the cultures were treated with spring, summer, autumn and winter PM_{2.5} samples at 12.5, 25, 50, 100 and 200 µg/mL at DIV14. After the treatments, neuronal viability was determined by MTT assay.

1.4 Western blot analysis

The medium was removed from the 35 mm culture dishes, which were washed with a pre-chilled PBS two times. Then, 40 µL of lysis buffer (1% Nonidet P40, 1 mmol/L EDTA, 8 mmol/L EGTA, 8 mmol/L PMSF, 2.5 µg/mL aprotinin, 5 µg/mL pepstatin, 50 µg/mL leupeptin, 25 µmol/L PMSF and 25 µg/mL trypsin inhibitor) were added to the culture dishes, and the cells were scraped with a cell scraper and collected to determine the protein concentration. The protein standard curve was prepared according to the Bradford method using bovine serum albumin (BSA) as the standard protein (Bradford, 1976). Each protein was quantified to 30-50 µg and the protein samples were loaded on a premade 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad). The blocking solution with PBS containing 3% bovine albumin (Solarbio, China) was added to incubate the membranes for 1 hr. After blocking, the membranes were incubated in rabbit polyclonal antibodies PSD95, NMDAR2B, bax, bcl-2, ERK1/2, phospho-ERK1/2, CERB, phospho-CREB (1:200, Biosynthesis, China), rabbit monoclonal antibodies for caspase-3, cleaved caspase-3 or β-actin (1:1000, Cell Signaling Technology, USA) overnight at 4°C. The membranes were then washed with PBS three times for 10 min and were exposed to fluorescent labeled secondary antibody (1:5000, LICOR Biosciences Corporation, USA) at room temperature for 1 hr. The membranes were washed again with PBS and were analyzed with the LI-COR Odyssey infrared scanning fluorescence detection system.

1.5 TUNEL staining

A KeyGen Biotech TUNEL Apoptosis Detection Kit (FITC labeled) was used to detect primary cultured cortical neuron apoptosis *in vitro* (KGI biological, China). FITC-labeled immunofluorescence indicated nuclear DNA fragmentation. The cells (5×10^4) were plated on

poly-L-lysine-coated cover slips and were cultured 13 days after PM_{2.5} exposure. The supernatant was removed after 24 hr, and the cover slips were fixed with 4% paraformaldehyde for 15-30 min, washed with PBS buffer three times for 5 min, permeabilized with 1% Triton X-100 for 3-5 min, and washed again three times for 5 min. After naturally drying, TUNEL reagent streptavidin-FITC was added to the coverslips at 37°C for 60 min in a humidified and dark chamber. After reaction with streptavidin, the cover slips were washed and dried, and the nuclei were stained with 4',6-amidino-2-phenylindole (DAPI) at room temperature for 10 min in the dark. The FITC-labeled DNA fragmentation images were then visualized and analyzed under a fluorescent microscope (Olympus, Japan) with Image-Pro Express software, version 6.0 (Media Cybernetics, USA), at 200× magnification.

1.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the mean differences among the treatment groups relative to the control group. All the variates were normally distributed after checking normality (Kolmogorov–Smirnov) using SPSS 17.0 statistical package (Wang et al., 2002). The interrelationships between physicochemical and molecular biological variables were analyzed using Pearson's correlation. The results are presented as the means ± SE. Values with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered statistically significant.

2 Results

2.1 Season-dependent effects of PM_{2.5} on neuronal viability

To evaluate the potential neurotoxicity of PM_{2.5} collected from different seasons, we first determined the neuronal viability. After a 24-hr treatment, all the PM_{2.5} samples decreased cell survival ratios in a concentration-dependent manner (**Fig. 1**). For the samples collected from spring and autumn, the significant differences were observed at 100 µg/mL. For the samples collected from summer and winter, the significant decreases occurred at 200 and 50 µg/mL, respectively.

2.2 Season-dependent effects of PM_{2.5} on neuronal apoptosis

Apoptosis is divided into two categories containing pro-apoptotic proteins and anti-apoptotic

protein (Qin et al., 2015). As shown in **Fig. 2a-d**, the protein expression of the pro-apoptotic gene (bax) increased to 1.56-, 1.11-, 1.37- and 2.19-fold of the control following the treatment of PM_{2.5} collected from spring, summer, autumn and winter; and the protein level of the anti-apoptotic gene bcl-2 showed the opposite result, at 0.82-, 0.90-, 0.85- and 0.78-fold of the control, respectively. Subsequently, we calculated the ratios of bax and bcl-2, and found that the bax/bcl-2 ratio displayed a distinct season-dependent change and that the most significant difference occurred after winter sample exposure (2.97-fold of the control).

To further verify the occurrence of apoptosis, we examined caspase-3 activation. As presented in **Fig. 2e**, cleaved caspase-3 expression showed an upward trend, and it significantly increased after spring and winter sample treatment (for spring, 1.21-fold of the control; and for winter, 1.34-fold of the control). Furthermore, we also determined the DNA damage (FITC, green fluorescence) by TUNEL staining to confirm the results. Consistent with caspase-3 activation, TUNEL staining-positive neuron numbers increased in a season-dependent manner, and the most severe apoptotic effect occurred after winter sample treatment (**Fig. 3**).

2.3 Season-dependent effects of PM_{2.5} on synaptic protein expression

As indicated in **Fig. 4**, PSD-95 expression decreased following PM_{2.5} sample treatment, and the significant differences occurred after spring and winter sample treatment (for spring, 0.83-fold of the control; and for winter, 0.75-fold of the control). Similarly, NR2B expression attenuated in a season-dependent manner, decreasing to 0.87-, 0.92-, 0.89- and 0.76-fold of the control for spring, summer, autumn, and winter, respectively; a significant difference was observed only after winter sample exposure.

2.4 Concentration-dependent effects of winter PM_{2.5} on neuronal apoptosis and synaptic protein expression

To confirm above mentioned neurotoxicity, we further determined the concentration-dependent effects on neuronal apoptosis and synaptic protein expression based on winter PM_{2.5} exposure. As presented in **Fig. 5**, winter PM_{2.5} concentration-dependently increased caspase-3 activation and attenuated the expression of PSD95 and NR2B, and

significant differences were observed at 50 µg/mL.

2.5 Season-dependent effects of PM_{2.5} on phosphorylated protein kinase expression

Protein phosphorylation plays a key role in neuronal survival and synaptic plasticity. Here, we showed that the total ERK1/2 and CREB proteins remained unchanged under different treatment conditions. The ratio of the phosphorylation of ERK1/2 and total ERK1/2 attenuated to 0.81-, 0.93-, 0.83-, and 0.76-fold of the control by PM_{2.5} samples collected from spring, summer, autumn, and winter, respectively, and significant differences were observed after spring, autumn and winter sample exposure (**Fig. 6a**). Similarly, the ratio of phosphorylated CREB to total CREB decreased to 0.83-, 0.97-, 0.88-, and 0.76-fold of the control for spring, summer, autumn, and winter, respectively, and significant differences occurred after spring and winter sample treatment (**Fig. 6b**).

2.6 Correlative analysis between PM_{2.5} physicochemical characteristics and neuronal injuries

Following the above season-dependent neuronal apoptosis and synaptic injuries of PM_{2.5} exposure, we further detected the chemical components of PM_{2.5} samples collected from different seasons. The results showed that significant seasonal differences in PM_{2.5} components occurred in the levels of PAHs and carbon load (Table S1–S4) and that the winter samples presented the highest PAHs load and carbon contents. Subsequently, we performed the correlation analysis between PM_{2.5} concentration and the PM_{2.5} composition (Table S5–S8) and the correlation analysis between PM_{2.5} composition and neuronal injuries (Tables 1–4). Following this, the maximum correlation coefficient was observed between PM_{2.5} concentration and PAHs and OC contents. Consistently, the maximum correlation occurred between PAHs and OC load and neuronal injuries. It implicates that PAHs played the important role in PM_{2.5} induced season-dependent neuronal apoptosis and synaptic injuries.

3 Discussion

Previous studies linked an association between the PM_{2.5} exposure and neurodegenerative diseases (Genc et al., 2012). Considering that PM_{2.5} has a significant seasonal differences in

size distribution and chemical composition (Huang and Wang, 2011), it is essential to explore the possibility of PM_{2.5} regulating gene expression of neuronal apoptosis and synaptic functions based on seasonal variations.

Currently, among the known apoptotic proteins, the bcl-2 family plays a critical role in apoptotic processes due to external stimuli. The main members of the bcl-2 proteins family are the anti-apoptotic protein bcl-2 and the pro-apoptotic protein bax. An increased bcl-2 expression indicates the suppression of apoptosis, while enhanced bax expression indicates increased apoptosis; and apoptosis is the result of an imbalance between these two mutually-antagonistic proteins (Qin et al., 2015). In the present study, the bax/bcl-2 ratio increased in season-dependent manner, and the most obvious enhancement was observed after winter PM_{2.5} sample treatment. It was reported that the ratio of bax /bcl-2 could determine the activation of caspase-3 protein kinase, generating cleaved caspase-3 and leading to apoptosis (Crocker et al., 2011). Following this, we found caspase-3 activation and increased TUNEL staining-positive neurons. Consistently, these results showed season-dependent properties, and the winter PM_{2.5} sample caused the most obvious changes. The synapse is the key structure in mediating information transmission between neurons and synaptic degeneration is an early event in neurodegenerative diseases (Taft et al., 2013). Knockdown of the PSD-95, one of the PSD-related proteins, caused synaptic changes and enhanced long-term potentiation (Park et al., 2015), thereby decreasing the abilities of learning and memory (Hölscher, 1999). And PSD-95 downregulation regulates postsynaptic glutamate receptor clustering (El-Husseini et al., 2000). Here, we showed that PM_{2.5} exposure season-dependently attenuated PSD-95 and glutamate receptor NR2B expression. Importantly, winter PM_{2.5} sample caused the most serious neuronal injuries, and the effects showed the concentration-dependent property.

Activation of the ERK/CREB pathway played an important role in the process of forming and maintaining neuronal plasticity. CREB could promote synaptic formation and growth (Katz and Shatz, 1996) and that its transcriptional activity could be enhanced by phosphorylation. Thus, the phosphorylation of CREB (p-CREB) contributes to the transcription of target genes, including the Arc, c-Fos and glutamate receptor subunit NR2B (Abad et al., 2015). In the present study, p-CREB decreased with a season-dependent property, thus regulating the expression of the synapse-associated proteins, NR2B and PSD-95. At the

same time, due to the reportedly positive effects of CREB on neuroprotective function (Cutler et al., 2015), CREB is responsible for the regeneration, survival and repair of damaged neurons under oxidative stress. It also can regulate the expression of neurotrophins and anti-apoptotic gene levels (bcl-2) (Huang et al., 2015). Thus, decreased p-CREB also affects neuronal apoptosis-related proteins, such as bax, bcl-2 and cleaved caspase-3. The activation of ERK1/2 in the ERK/MAPK signaling pathway has an impact on the phosphorylation of CREB at serine 133; and the phosphorylation of ERK could enhance neurotransmitter release, promote cell proliferation and differentiation (Sun et al., 2015), and inhibit neuronal apoptosis (Li et al., 2002). Moreover, inhibited ERK1/2 increased the secretion rate of synaptic vesicles and suppressed the release of neurotransmitters (Subramanian and Morozov, 2011), and induced neuronal apoptosis. Here, we further found that ERK1/2 activation attenuated in a season-dependent manner. These data suggested that PM_{2.5} samples tended to cause neuronal apoptosis and synaptic injuries accompanied by the limiting of ERK and CREB activation in a network of cortical areas.

Because the neuronal apoptosis and synaptic injuries resulting from PM_{2.5} changed as a function of season and the concentration and chemical composition of PM_{2.5} exhibited seasonal differences, we analyzed the correlation between PM_{2.5} concentration and the PM_{2.5} composition and the correlation between PM_{2.5} composition and neuronal injuries. The result, the maximum correlation coefficient between PM_{2.5} concentration and PAHs and OC contents and the maximum correlation between PAHs and OC load and neuronal injuries, suggests that PAHs played the important role in PM_{2.5} induced season-dependent neuronal apoptosis and synaptic injuries,

4 Conclusions

In summary, PM_{2.5} caused neuronal apoptosis and synaptic injuries in a season-dependent manner, and winter sample played the most important role during the process. Furthermore, the action was coupled with the inhibition of phosphorylated ERK1/2 and phosphorylated CREB.

Acknowledgments

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Figure legends

Fig. 1 Season-dependent effects on neuronal viability of PM_{2.5} collected from spring (a), summer (b), autumn (c) and winter (d). The value of each treated group is expressed as the fold increase compared with the mean value in the control group, which was assigned an arbitrary value of 1. The data are expressed as the means $\pm$ SE ($n=3$); * $p<0.05$ and ** $p<0.01$, compared with the negative control.

Fig. 2 Season-dependent effects of PM_{2.5} on neuronal apoptosis. (a) The bands show representative Western blot results of Bax, Bcl-2, Cleaved caspase-3, Caspase-3 and β-actin; (b) Bax protein expression after treatment of PM_{2.5} collected from spring, summer, autumn and winter; (c) Bcl-2 protein expression after treatment of PM_{2.5} collected from different seasons; (d) Bax/Bcl-2 ratio after treatment of PM_{2.5} collected from different seasons; and (e) Caspase-3 activation after treatment of PM_{2.5} collected from different seasons. The value of each treated group is expressed as the fold increase compared with the mean value in the control group, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (*n*=3); **p*<0.05, ***p*<0.01 and ****p*<0.001, compared with the negative control.

Fig. 3 Season-dependent effects of PM_{2.5} on TUNEL staining-positive neurons. Red arrows show the apoptotic cells. Magnification, 400×; Scale bar, 50 μ m.

Fig. 4 Season-dependent effects of PM_{2.5} on PSD95 and NR2B expression. (a) PSD-95 expression after exposure of PM_{2.5} collected from spring, summer, autumn and winter; (b) NR2B expression after exposure of PM_{2.5} collected from different seasons. The value of each treated group is expressed as the fold increase compared with the mean value in the control group, which was assigned an arbitrary value of 1. The data are expressed as the means $\pm$ SE ($n=3$); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared with the negative control.

Fig. 5 Concentration-dependent effects of winter PM_{2.5} on the cleaved caspase-3/caspase-3 ratio and PSD95 and NR2B expression. (a) Cleaved caspase-3/caspase-3 ratio after winter PM_{2.5} treatment; (b) PSD-95 and NR2B protein expression after winter PM_{2.5} exposure. The value of each treated group is expressed as the fold increase compared with the mean value in the control group, which was assigned an arbitrary value of 1. The data are expressed as the means $\pm$ SE ($n=3$); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared with the negative control.

Fig. 6 Season-dependent effects of PM_{2.5} on phosphorylated protein kinases. (a) Total and p-ERK1/2 expressions after treatment of PM_{2.5} collected from spring, summer, autumn and winter; (b) Total and p-CREB expression after treatment of PM_{2.5} collected from different seasons. The value of each treated group is expressed as the fold increase compared with the mean value in the control group, which was assigned an arbitrary value of 1. The data are expressed as the means $\pm$ SE ($n=3$); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared with the

negative control.

Table 1 Pearson's correlation between inorganic ion level and biological responses.

Inorganic ion	Bax/Bcl-2	Cleaved caspase-3	PSD-95	NR2B	p-ERK1/2	p-CREB
F ⁻	0.729	0.669	-0.795	-0.697	-0.482	-0.625
Cl ⁻	0.974*	0.917	-0.936	-0.983*	-0.753	-0.852
NO ₃ ⁻	-0.928	-0.895	0.844	0.956*	0.792	0.839
SO ₄ ²⁻	-0.707	-0.793	0.816	0.639	0.855	0.856
Na ⁺	0.015	0.125	-0.197	0.078	-0.259	-0.232
NH ₄ ⁺	0.245	0.117	-0.070	-0.334	0.072	0.006
K ⁺	0.925	0.847	-0.855	-0.952*	-0.659	-0.763
Mg ²⁺	0.200	0.023	-0.099	-0.262	0.275	0.118
Ca ²⁺	-0.714	-0.820	0.720	0.690	0.950*	0.877

* $p < 0.05$, represent the significant correlation between inorganic ion level and biological responses.

Table 2 Pearson's Correlation between Elements Level and Biological Responses.

Element	Bax/Bcl-2	Cleaved caspase-3	PSD-95	NR2B	p-ERK1/2	p-CREB
Zr	-0.512	-0.657	0.578	0.460	0.853	0.755
Al	-0.732	-0.820	0.833	0.668	0.883	0.880
Sr	-0.277	-0.279	0.119	0.343	0.316	0.247
Mg	-0.307	-0.397	0.477	0.217	0.476	0.481
Ti	-0.551	-0.633	0.478	0.567	0.760	0.663
Ca	-0.269	-0.337	0.157	0.307	0.476	0.357
Fe	-0.697	-0.805	0.701	0.674	0.941	0.863
Ba	-0.187	-0.103	0.001	0.276	0.001	0.009
Li	-0.435	-0.486	0.593	0.354	0.488	0.538
Be	-0.262	-0.342	0.437	0.173	0.403	0.420
Na	0.107	0.220	-0.286	-0.014	-0.352	-0.325

P	-0.526	-0.605	0.674	0.445	0.655	0.673
K	0.042	0.157	-0.223	0.051	-0.296	-0.265
Sc	-0.605	-0.644	0.741	0.533	0.618	0.681
V	-0.529	-0.595	0.678	0.449	0.618	0.653
Cr	-0.894	-0.832	0.796	0.932	0.685	0.755
Mn	-0.938	-0.873	0.863	0.965*	0.709	0.796
Co	-0.522	-0.474	0.359	0.592	0.403	0.402
Ni	-0.550	-0.524	0.397	0.613	0.487	0.468
Cu	-0.883	-0.898	0.810	0.902	0.88	0.876
Zn	0.973*	0.989*	-0.949	-0.969*	-0.950*	-0.975*
Rb	-0.928	-0.932	0.863	0.944	0.888	0.904
Y	-0.696	-0.734	0.817	0.630	0.705	0.766
Mo	-0.294	-0.200	0.378	0.262	-0.018	0.146
Cd	0.525	0.531	-0.385	-0.580	-0.548	-0.499
Sn	0.410	0.500	-0.571	-0.324	-0.574	-0.580
Sb	-0.062	-0.139	0.246	-0.029	0.209	0.222
Cs	-0.857	-0.871	0.936	0.809	0.803	0.877
La	-0.452	-0.503	0.609	0.372	0.503	0.555
Ce	0.525	0.445	-0.357	-0.600	-0.318	-0.350
Sm	-0.587	-0.649	0.727	0.510	0.665	0.703
W	0.054	0.017	0.125	-0.136	-0.007	0.041
Tl	0.901	0.884	-0.955*	-0.867	-0.763	-0.862
Pb	0.988*	0.988*	-0.999**	-0.969*	-0.906	-0.969*
Bi	-0.019	0.071	0.116	-0.015	-0.265	-0.114
Th	-0.277	-0.347	0.450	0.189	0.392	0.419
U	-0.407	-0.464	0.569	0.324	0.478	0.522

* $p < 0.05$ and ** $p < 0.01$, represent the significant correlation between elements level and biological responses.

Table 3 Pearson's correlation between PAHs level and biological responses.

PAHs	Bax/Bcl-2	Cleaved caspase-3	PSD-95	NR2B	p-ERK1/2	p-CREB
Naphthalene (NA)	0.934	0.855	-0.888	-0.949	-0.656	-0.774
Acenaphthylene (ACL)	0.560	0.417	-0.429	-0.627	-0.161	-0.285
Acenaphthene (AC)	0.467	0.434	-0.589	-0.410	-0.297	-0.424
Fluorene (FLU)	0.965*	0.906	-0.912	-0.982*	-0.742	-0.836
benzo[g,h,i]pyrene (BPE)	0.983*	0.937	-0.960*	-0.985*	-0.784	-0.880
indeno[1,2,3-cd]pyrene (IPY)	0.980*	0.929	-0.946	-0.987*	-0.772	-0.868
dibenzo[a,h]anthracene (DBA)	0.986*	0.941	-0.962*	-0.988*	-0.792	-0.886
benzo[b]fluoranthene (BbF)	0.981*	0.930	-0.945	-0.988*	-0.774	-0.869
coronene (COR)	0.988*	0.968*	-0.994**	-0.974*	-0.853	-0.935
Phenanthrene (PHE)	0.936	0.870	-0.862	-0.964*	-0.704	-0.793
Anthracene (ANT)	0.935	0.863	-0.866	-0.961*	-0.685	-0.783
fluoranthene (FA)	0.941	0.870	-0.875	-0.965*	-0.693	-0.791
benzo[a]anthracene (BaA)	0.963*	0.900	-0.913	-0.978*	-0.730	-0.829
Chrysene (CHR)	0.975*	0.921	-0.931	-0.987*	-0.764	-0.857
Pyrene (PYR)	0.940	0.869	-0.874	-0.964*	-0.691	-0.790
benzo[a]pyrene (BaP)	0.966*	0.904	-0.921	-0.979*	-0.735	-0.835
benzo[e]pyrene (BeP)	0.978*	0.926	-0.945	-0.986*	-0.767	-0.864
benzo[k]fluoranthene (BkF)	0.967*	0.906	-0.924	-0.979*	-0.735	-0.837

* $p < 0.05$ and ** $p < 0.01$, represent the significant correlation between PAHs level and biological responses.

Table 4 Pearson's correlation between carbon level and biological responses.

Carbon	bax/bcl-2	cleaved caspase-3	PSD-95	NR2B	p-ERK1/2	p-CREB
OC	0.964*	0.962*	-0.912	-0.974*	-0.900	-0.937
EC	0.890	0.922	-0.836	-0.898	-0.927	-0.915
TC	0.952*	0.958*	-0.900	-0.962*	-0.910	-0.932

* $p < 0.05$, represent the significant correlation between carbon level and biological responses.