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PM_{2.5}, SO₂ and NO₂ co-exposure impairs neurobehavior and induces mitochondrial injuries in the mouse brain

Tingting Ku, Xiaotong Ji, Yingying Zhang, Guangke Li, Nan Sang*

College of Environment and Resource, Research Center of Environment and Health, Shanxi University, Taiyuan, Shanxi 030006, PR China

***Correspondence to:** Nan Sang, College of Environmental Science and Resources, Shanxi University, Taiyuan, Shanxi 030006, China Tel/Fax: +86-351-7011932 *E-mail:* sangnan@sxu.edu.cn.

Abstract: Air pollution is a serious environmental health problem that has been previously associated with neuropathological disorders. However, current experimental evidence mainly focuses on the adverse effects of a single air pollutant, ignoring the biological responses to the co-existence of these pollutants. In the present study, we co-exposed C57BL/6J mice to PM_{2.5}, SO₂ and NO₂ and explored their neurobehavior, histopathologic abnormalities, apoptosis-related protein expression and mitochondrial dysfunction. The results indicate that co-exposure to PM_{2.5}, SO₂ and NO₂ impaired spatial learning and memory and caused abnormal expression of apoptosis-related genes (p53, bax and bcl-2). Additionally, these alterations were related to morphological changes in mitochondria, a reduction of ATP, the elevation of mitochondrial fission proteins and the downregulation of fusion proteins. These findings provide a basis for the understanding of mitochondrial abnormality-related neuropathological dysfunction in response to co-exposure to ambient air pollutants, which suggests an adaptive response to the frangibility of the central nerve system.

Key words: air pollutant; co-exposure; spatial learning and memory; mitochondrial abnormality; neuronal apoptosis

11. Introduction

Ambient air pollution is a complex mixture of pollutants that includes both particulate and gaseous components, and global concern over its public health impact has been increasing. Among these gradients, fine particulate matter (PM_{2.5}), which contains multiple chemical components that can vary dynamically with time and space, has been attracting more and more attention. PM_{2.5} can be generated directly by combustion or mechanical processes and indirectly by the condensation of aerosol precursor gases (Lee et al., 2015). Water-soluble ions generally contribute to a large fraction of aerosols, and a number of field studies have been carried out to characterize water-soluble ions in fine aerosols (i.e., PM_{2.5}) in the past decade (Ocskay et al., 2006; Gao et al., 2012). However, sulfur dioxide (SO₂) and nitrogen oxides (NO_x) are the precursors of the formation of sulfate and nitrate PM, including acid and non-acid aerosols (secondary water-soluble aerosols) in the atmosphere (EPA, 2000), and secondary water-soluble ions (i.e., SO₄²⁻, NO₃⁻ and NH₄⁺) constituted a major portion of PM_{2.5} (i.e., 40%–57%) in eastern China (Gao et al., 2012). Therefore, PM_{2.5} and the gaseous pollutants SO₂ and NO₂ coexist in the atmosphere and play important roles in the process of secondary aerosol pollution.

Increasing numbers of epidemiological studies have indicated that air pollutants not only cause increased morbidity of respiratory diseases but are also associated with neuropathological dysfunction (Block et al., 2009; Guxens et al., 2012; Ranft et al., 2009; Calderón-Garcidueñas et al., 2015). Specifically, prolonged exposure to particulate matter has the potential to change the brain's inflammatory phenotype and facilitate the development of early Alzheimer's disease-like pathology (Field et al., 2010; Smith et al., 2012). SO₂ exposure disturbed neurobehavior and repressed memory-related kinase activation and glutamate receptor gene expression via neuroinflammation in rats and tended to cause neurodegeneration (Hong et al., 2002; Szyszkowicz et al., 2009; Smith et al., 2012; Yao et al., 2015). Additionally, an increased risk of nervous diseases, including neurodegenerative disorders, vascular dementia, and autism spectrum disorder, was induced by another important air pollutant, NO₂ (Zhu et al., 2012; Li and

¹Abbreviations: PM_{2.5}: fine particulate matter; SO₂: sulfur dioxide; NO_x: nitrogen oxides; HE: hematoxylin-eosin; PBS: phosphate buffer saline; TEM: transmission electron microscopy; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NC: nitrocellulose; BSA: bovine serum albumin; Mfn: mitofusin; OPA1: optic atrophy 1; Fis1: mitochondrial fission protein 1; Drp1: dynamin-related protein 1.

Xin, 2013; Jung et al., 2013; Kim et al., 2014). These findings highlight the contribution of PM_{2.5}, SO₂ and NO₂ to air pollution-related neurological dysfunction.

Neuronal apoptosis commonly occurs in neurological diseases, and the process is hallmarked by p53, the Bcl-2 family, c-myc and other factors. Mitochondria exist as a dynamic network and play crucial roles in numerous biochemical functions, including energy production, apoptosis induction, calcium signaling and cell growth (Hoppins, 2014). The activation of the mitochondrial pathway is an important indicator of the occurrence of apoptosis (Sassone et al., 2013; Peng et al., 2015). In vivo, mitochondria form elongated tubules that constantly undergo fission and fusion to maintain an interconnecting network (Hoppins, 2014), which are essential for maintaining normal cell functions (Westrate et al., 2014). A tightly controlled balance between fusion and fission is of utmost significance in the high energy-demanding biological process, which has a strong association with brain development and neurodegenerative diseases (Rousset et al., 2012). Mitochondrial dysfunction has been observed following individual exposure to SO₂, NO₂, black carbon and PM_{2.5} (Qin et al., 2012; Meyer et al., 2013; Colicino et al., 2014; Yan et al., 2015). However, little is known about whether co-exposure to air pollutants could trigger mitochondrial dysfunction and neurobehavior impairment.

In this study, we exposed C57BL/6J mice to PM_{2.5}, SO₂ and NO₂ and determined spatial learning and memory, histopathologic abnormality, apoptosis-related protein expression and mitochondrial dysfunction (mainly including mitochondrial ultrastructure, ATP content and mitochondrial fission/fusion) in the brain. The study attempted to provide experimental evidence for the increased risk of neurological disorders in response to co-exposure to ambient air pollutants.

2. Materials and methods

2.1. PM_{2.5} sampling

PM_{2.5} were acquired with middle volume air samplers (TH-150CIII and TH-150C, Wuhan, China) from Shanxi University (112°57'E longitude, 37°73'N latitude), Taiyuan City, Shanxi Province of China. The middle volume air samplers were set on the top of the living area of a building far from obstacles to obtain sufficient free-moving air. The flow rate of the sampler was 100 L·min⁻¹, and the sampling time was 22 h each day during the period from November 2013 to February 2014. The PM samples were collected onto a quartz filter membrane (Φ90 mm, Munktel,

Sweden), and a PM_{2.5} suspension was prepared by soaking filters with PM_{2.5} in Milin-Q deionized water, followed by immersion and ultrasonic vibration more than three times. Then, the PM_{2.5} suspension was processed with a vacuum freeze-drying technique. The dried samples were diluted with sterilized 0.9% physiological saline and swirled for 10 min before using them for animal experiments, and an aqueous suspension was pooled and frozen at -20 °C.

2.2. Animal treatment

Healthy male C57BL/6 mice (8 weeks old) were obtained from Beijing HFK Bioscience Co., LTD (Beijing, China). The mice were housed under standard conditions (24 ± 2 °C, 50 ± 5% humidity, and 12:12 h light:dark cycle). After one week of adaptation, the mice were randomly divided into three groups. For the control group, mice were exposed to filtered air (6 h/d, 28 d) and received an oropharyngeal aspiration of saline every other day. For the lower concentration group, mice were treated with 0.5 mg·m⁻³ SO₂ and 0.2 mg·m⁻³ NO₂ via dynamic inhalation simultaneously (6 h/d, 28 d) followed by oropharyngeal aspiration of PM_{2.5} 1 mg·kg⁻¹ every other day. For the higher concentration group, mice were exposed to 3.5 mg·m⁻³ SO₂, 2 mg·m⁻³ NO₂ and 3 mg·kg⁻¹ PM_{2.5} using the same protocol. When not being treated, the animals had free access to water and standard feed. Mice were sacrificed 18 h after the final exposure. The brains were excised, quick frozen in liquid nitrogen, and stored at -80 °C. All of the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of the People's Republic of China. The protocol was approved by the Shanxi University's Institutional Animal Care and Use Committee (Approved Animal Use Protocol Number: HZ20140503).

For dynamic inhalation, the animals were placed in a dynamic inhalation chambers without anaesthetization. SO₂ and NO₂ gases were diluted with fresh air at the intake port of the chambers to yield the desired concentrations, and the diluted gases were evenly distributed across the whole chamber by two perforated gas radiant plates, with one located in the intake port and the other connected to a gas outlet matched with an aspirator pump. The concentrations of SO₂ and NO₂ within the chambers were measured using pararosaniline hydrochloride spectrophotometry at 577 nm (Goyal et al., 2001) and the Saltzman colorimetric method at 540 nm (Kumie et al., 2009), respectively. For PM_{2.5} administration, we used the oropharyngeal aspiration technique (Rao et al. 2003). Briefly, the mice were anesthetized with isoflurane (Yi Pin

Pharmaceutical Co., Ltd., He Bei, China) and placed on a slant board, and the tongue was gently held at full extension while a 20- μ L suspension of particles was pipetted onto the base of the tongue. For the control group, ambient air was cleaned with an air filter consisting of multilayer synthetic fibers to remove particles.

2.3. Behavioral tests

Morris water maze studies with mice have principally been performed to measure spatial-based learning and memory. A circular tank (diameter 100 and 75 cm in high) was filled with water rendered opaque by the addition of white, non-toxic paint. A circular platform with a diameter of 15 cm was placed 1 cm below the water surface in the center of a specific quadrant of the tank. The animals were given training sessions using visible and invisible platforms for a period of 8 consecutive days (8 sessions in total). In the non-spatial training, the mice received 3 days of training to find the same platform elevated above the water surface. Then, invisible platform training was performed continuously for 5 identical daily sessions with 4 trials per day. For each trial, the mouse was released from the wall of the tank and allowed to search, find, and stand on the platform for 10 s within the 60-s trial period. If an animal failed to reach the platform, it was gently guided to the location and was allowed to stay on it for 10 s. For each training session, the sequence designed for the animals' training was determined in a random manner that varied each day so that it was different between the separate sessions for each animal and was different for individual animals. Using an Etho Vision video tracking device (Noldus, Wageningen, Netherlands), we recorded the latency in reaching the target as the task performance. The average latencies on each hidden platform training day were recorded and compared. Memory tests were conducted in a probe trial performed 24 h after the last training trail. A probe trial was performed in which the platform was removed from the tank and the animals were allowed to swim for 60 s. The number of target annulus crossovers and the time spent in the target quadrant were measured.

2.4. Hematoxylin-eosin (HE) staining

The tissues were quickly harvested, washed with 0.1 M phosphate buffered saline (PBS, pH 7.4) and fixed in 10% formalin at room temperature for 24 h. The fixed tissues were then dehydrated by graded ethanol and embedded in paraffin. Finally, 5-6- μ m-thick sections were deparaffinized with xylene, stained with hematoxylin and eosin, and then observed by microscopy

(Olympus ix71, Japan).

2.5. Transmission electron microscope (TEM) observation

Approximately 1-mm³ cortical pieces were fixed, en bloc stained, dehydrated and embedded in beam capsules. Then, 70-80-nm-thick sections of embedded tissue were collected onto grids, stained with uranyl acetate and lead citrate. The transmission electron microscope (JEOL, JEM-1011, Japan) was used for observation.

2.6. Assay of ATP

According to the protocol of the ATP detection kit (Beyotime, China), the determination of ATP was performed by the luciferinluciferase method. Approximately 50 mg of cortical tissues was homogenized with 500 μ L of lysis buffer from the ATP detection kit. After being centrifuged at 12,000 g for 10 min at 4 °C, the supernatant was transferred to a new tube for ATP testing. The luminescence from a 100- μ L sample was assayed in a Varioskan Flash Multimode Reader (Thermo Scientific, Vantaa, Finland) together with 100 μ L of ATP detection buffer from the ATP detection kit. The standard curve of ATP concentration was prepared from a known quantity (1 nM–1 μ M).

2.7. Immunoblot analysis

Proteins were extracted from cortical tissues and quantified according to our previously described method (Yan et al., 2016). Then, 50 μ g of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane. The membranes were blocked with 3% bovine serum albumin (BSA) and probed with the indicated antibodies specific for mice rabbit β -actin (1:1000, Cell Signaling Technology, USA), p53 (1:1000, Affinity, USA), bax, bcl-2, mitofusin (Mfn)1, Mfn2, optic atrophy 1 (OPA1), mitochondrial fission protein 1 (Fis1), and dynamin-related protein 1 (Drp1) (1:200, Bioss, China) at 4 °C overnight. The membranes were washed with PBS, exposed to IR Dye 800CW-conjugated secondary antibody (1:5000, LiCor Biosciences, USA), and then scanned with a LI-COR Odyssey Infrared Fluorescent Instrument. The density of each band was quantified using Image-Pro Express software, version 6.0 (Media Cybernetics, USA).

2.8. Statistical analysis

The results are expressed as the means $\pm$ SE. The data were analyzed by one-way ANOVA

to detect the significant differences among different exposure groups and the control group. The results with $p < 0.05$ were considered significant.

3. Results

3.1. Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on body weight, brain weight and brain-to-body weight ratio

Mouse body weights were obtained after treatment for 7 and 28 d. At the beginning of the experiment, the mean body weight gained was not different in mice co-exposed to PM_{2.5}, SO₂, and NO₂ compared to the control group. However, the weights of the high concentration group significantly dropped after co-exposure for 28 d, even though the weights of all groups were significantly increased compared to the initial values (Fig. 1A). After the final exposure, the brains were excised and weighed. The brain weight and ratio of the brain to body weight of the mice declined in a dose-dependent manner and were significantly reduced to 0.86 ± 0.05 - and 0.94 ± 0.02 -fold of the control in response to the higher concentration group (Fig. 1B, C). These observations implicate that co-exposure might have an adverse effect on mouse brain.

3.2. Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on spatial learning and memory

To determine whether the behavioral performance of mice was impaired following PM_{2.5}, SO₂ and NO₂ co-exposure, we used the Morris water maze test. The results indicate that co-exposure impaired the spatial learning and memory of mice. During the training trials, the mice showed a longer time to find the hidden platform in response to PM_{2.5}, SO₂ and NO₂ co-exposure at higher doses (Fig. 2A). In the probe trial, the number crossing the target zone (Fig. 2B) and the length of time the animals stayed in the target quadrant (Fig. 2C) significantly decreased in the higher concentration group.

3.3. Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on cortical damage

The histopathology of the cortex was detected by performing HE staining. As presented in Fig. 3A, the neurons in control groups exhibited a normal morphology and were neatly arranged. With increased concentration, neurons appeared slightly swollen or shrunken with the unclear edge of cells (Fig. 3B). Apparently, the histopathologic changes of neurons in the higher concentration group were severe and were characterized by a massive infiltration of inflammatory cells, necrotic neurons and pycnotic or condensed nuclei in the apoptotic bodies (Fig. 3C).

3.4. Effects of PM_{2.5}, SO₂ and NO₂ on apoptosis-related protein expression in the cortex

Numerous studies have revealed that apoptosis modulates the strict and procedural inherent death of neurons, and during maturation of the central nervous system, apoptosis is a physiologically crucial process in neurogenesis (Li and Hu, 2015). In this study, the apoptosis-related expression of p53, bax and bcl-2 were examined following co-exposure for 28 d. The significantly increased protein levels of p53, bax and the bax/bcl-2 ratio were observed in the higher concentration group and, respectively, increased by 1.46-, 1.31- and 1.28-fold compared to the normal counterpart. However, in all of the treatment groups, the anti-apoptotic protein bcl-2 showed no significant differences compared to the control (Fig. 4).

3.5. Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on mitochondrial morphological changes

Mitochondria are essential organelles in cells and play important roles in triggering apoptosis (Sassone et al., 2013; Peng et al., 2015). To identify potential mitochondrial damage, we first discovered whether the mitochondrial ultrastructure changed following PM_{2.5}, SO₂ and NO₂ co-exposure. As shown in Fig. 5, pathological alterations obviously appeared in exposure groups with breached mitochondrial membranes and cristae disorders, including fragmentation and loss. More serious injuries were observed with collapsed or even dissolved mitochondria in response to higher concentration treatment.

3.6. Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on ATP content

Cellular mitochondria generate abundant ATP to maintain endergonic processes, which play crucial roles in apoptosis, cell death and cell signaling (Mattson, 2003). By detecting the ATP content in the mouse cortex, we evaluated the effect of air pollutant exposure on mitochondrial function. The results in Fig. 6 suggested that co-exposure to three air pollutants reduced ATP levels in a dose-dependent manner, and we found considerable ATP deficits in the brain cortex after treatment with higher concentrations.

3.7. Effects of PM_{2.5}, SO₂ and NO₂ on the expression of mitochondrial fusion/fission genes in the mouse cortex

Mitochondrial fusion and fission are evolutionarily conserved membrane remodeling processes that respond to a number of physiologic cues or external stimuli (Westrate et al., 2014; Li et al., 2015). Western blotting was used to detect the protein levels of fusion/fission-related genes in the cortex of mice at different concentrations following PM_{2.5}, SO₂ and NO₂ co-exposure for 28 d. As shown in Fig. 7, Mfn1, Mfn2 and OPA1 expression in the higher concentration group

was significantly decreased and was attenuated, respectively, to 0.69-, 0.66-, and 0.77-fold compared to the control groups ($p<0.05$ or $p<0.01$), while Fis1 and Drp1 expression were significantly increased in a dose-dependent manner ($p<0.05$) to 1.41- and 1.46-fold compared to controls, respectively. No significant translational differences in five mitochondrial fusion/fission genes were observed in mice after co-exposure to lower doses.

4. Discussion

Many studies have documented a positive association between exposure to air pollution containing NO₂, SO₂, O₃, PM₁₀ and PM_{2.5} and an increased risk of central nervous system diseases (Szyszkowicz et al., 2009; Kim et al., 2014; Calderón-Garcidueñas et al., 2015). However, as major environmental pollutants, most experimental studies on ambient PM_{2.5}, SO₂ and NO₂ mainly focus on the adverse health effects of single air pollutants, ignoring biological responses to the co-existence of these pollutants. In the present study, we first report that PM_{2.5}, SO₂ and NO₂ co-exposure decreased the brain-to-body mass ratio and impaired the spatial learning and memory of mice, and this action was coupled with inflammatory cell infiltration and neuronal cell loss in the cortex.

Programmed cell death, or apoptosis, is an evolutionarily conserved process triggered by a variety of stimuli (Qin et al., 2015). Apoptosis is controlled by the complex interaction of numerous pro-survival and pro-death signals, including the bcl-2 family of proteins, which can be anti-apoptotic (bcl-2 and bcl-xL) or pro-apoptotic (bax, bak and bid). These two types of proteins function in concordance with the regulation of the apoptosis mechanism and are important in determining cell fate (Yun et al., 2010). Previous studies have shown that particulate matter aroused neuronal apoptosis by remarkably elevating the bax/bcl-2 ratio in the rat brain (Guo et al, 2012). Additionally, SO₂ inhalation caused obvious alterations on the transcription and translation of several apoptosis-related genes (p53, bax, bcl-2, c-fos, and c-jun) in the rat hippocampus according to real-time RT-PCR analysis and western blotting, respectively (Yun et al., 2010). In accordance with the apoptotic signal described above, destruction of the concordance occurred following co-exposure due to the rising ratio of the pro-apoptotic bax protein to anti-apoptotic bcl-2. P53, a key factor closely related to apoptosis (Marchenko et al., 2000), was also activated in response to PM_{2.5}, SO₂ and NO₂ co-exposure.

Changes in mitochondrial dynamics, such as structural and functional aberrations in

mitochondria, have been implicated in several neurological dysfunctions (Mattiasson et al., 2003). They perform many critical cellular functions that are involved in the generation of cellular ATP that is required for endergonic processes (Duchen et al., 1999), in the compartmentalization of metabolic pathways (Benard et al., 2008) and in the generation of reactive oxygen species, and they also play an important role in apoptosis (Mattson et al., 2003), cell death and cell signaling (Mironov et al., 2009). Alterations of the mitochondrial energy metabolism were possibly generated by massive ATP depletion in a dose-dependent manner following PM_{2.5}, SO₂ and NO₂ co-exposure. The consumption of ATP by endergonic processes is matched by the production of ATP, which results in the maintenance of a good energy level in the form of ATP for normal biological reactions, and ATP deficits may have an adverse effect on cell function. The efficient production of intracellular ATP mainly depends on the structural and functional integrity of the mitochondrion (Cheng et al., 2012). To determine whether biochemical alterations were also reflected in the morphology of mitochondria in response to co-exposure, the mitochondrial ultrastructure was observed using TEM. It exhibited membrane breaches and cristae fragmentation or loss and even collapsed and dissolved after exposure at higher concentrations. These characteristics indicated that mitochondrial function were negatively influenced following co-exposure to ambient air pollutants.

Mitochondrial networks are dynamic systems in which mitochondria grow, divide, fuse and move along cytoskeletal filaments in an active fashion for the preservation of normal mitochondrial function. Meanwhile, mitochondria are among the most important organelles in apoptosis, and their fusion and fission may release large amounts of apoptosis-promoting factors. Our findings previously indicated that co-exposure to ambient pollutants caused apoptosis and mitochondrial dysfunction. Thus, we further investigated the factors related to mitochondrial fusion and fission to comprehensively reveal the relationship between them. Mitochondrial fusion in mammals is modulated by three proteins: mitofusins (Mfn1 and Mfn2) in the outer membrane and OPA1 in inner membrane (Youle and van der Bliek, 2012). Previous studies proposed that Mfn1/2 and OPA1 overexpression facilitated mitochondrial fusion and networking (Griparic et al., 2004), whereas Fis1 participated in mitochondrial fission by translocating the cytoplasmic Drp1 onto the surface of mitochondria. The inhibition of Drp1 via a dominant negative mutant or siRNA can prevent mitochondrial fragmentation (Cho et al., 2010).

The activation of the mitochondrial fusion proteins Mfn1/2 or OPA1 may inhibit mitochondrial fragmentation, as well as anti-apoptotic progression, and the pharmacologic inhibitor of Drp1 prevented tBid-dependent cytochrome c release from isolated mitochondria (Lackner et al., 2010). The overexpression of rat Fzo1 (a homolog of human Mfn1) suppresses cytochrome c release, Bax translocation, and Bax oligomerization caused by apoptotic stimuli (Sugioka et al., 2004), and the inhibition of Mfn1 was able to elicit the permeabilization of mitochondrial membranes, thereby causing the release of apoptotic factors from mitochondria into the cytosol (Li et al., 2014). Additionally, inhibiting OPA1 cleavage within mitochondria could accelerate cell proliferation and resistance to apoptosis and maintain the normal morphogenesis of mitochondrial cristae (Merkwirth et al., 2008). Conversely, mitochondrial fission is more amenable to bax/bak channel formation, with Drp1 promoting bax oligomerization (Montessuit et al., 2010). Similar results have been demonstrated by Fis1 overexpression, which induces apoptosis in some cell types and suggests that mitochondrial fission may be a driver of apoptosis (Alirol et al., 2006). Thus, both mitochondrial fusion and fission proteins appear to modulate apoptosis by participating in the activities of members of the Bcl-2 activities, but are different from their characteristics in mitochondrial dynamics. Our results showed that the fusion protein decreased, but the fission protein significantly increased, when apoptosis occurred following co-exposure. Our findings and previous evidence suggest that mitochondrial fusion and fission play a causative role in the process of pro-apoptotic functions.

5. Conclusions

In conclusion, PM_{2.5}, SO₂ and NO₂ co-exposure repressed the ratio of brain to body weight and deteriorated spatial learning and memory. Abnormal neurobehavior was characterized by fusion- and fission-related mitochondrial dysfunction and following neuronal apoptosis in the mouse cortex. This study provides the basis for understanding mitochondrial abnormality-related neuropathological dysfunction in response to co-exposure to ambient air pollutants and the fragility of the central nervous system.

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References

- Alirol, E., James, D., Huber, D., Marchetto, A., Vergani, L., Martinou, J.C., Scorrano, L., 2006. The mitochondrial fission protein hFis1 requires the endoplasmic reticulum gateway to induce apoptosis. *Mol. Biol. Cell* 17, 4593–4605.
- Benard, G., Faustin, B., Galinier, A., Rocher, C., Bellance, N., Smolkova, K., Casteilla, L., Rossignol, R., Letellier, T., 2008. Functional dynamic compartmentalization of respiratory chain intermediate substrates: implications for the control of energy production and mitochondrial diseases. *Int. J. Biochem. Cell Biol.* 40, 1543–1554.
- Block, M.L., Calderón-Garcidueñas, L., 2009. Air pollution: mechanisms of neuroinflammation and CNS disease. *Trends Neurosci.* 32, 506–516.
- Calderón-Garcidueñas, L., Kulesza, R.J., Doty, R.L., D'Angiulli, A., Torres-Jardón, R., 2015. Megacities air pollution problems: Mexico city metropolitan area critical issues on the central nervous system pediatric impact. *Environ. Res.* 137, 157–169.
- Cheng, G., Kong, R., Zhang, L., Zhang, J., 2012. Mitochondria in traumatic brain injury and mitochondrial-targeted multipotential therapeutic strategies. *Br. J. Pharmacol.* 167, 699–719.
- Cho, S.G., Du, Q., Huang, S., Dong, Z., 2010. Drp1 dephosphorylation in ATP depletion-induced mitochondrial injury and tubular cell apoptosis. *Am. J. Physiol. Renal Physiol.* 299, F199–F206.
- Colicino, E., Power, M.C., Cox, D.G., Weisskopf, M.G., Hou, L., Alexeeff, S.E., Sanchez-Guerra, M., Vokonas, P., Spiro, A., Schwartz, J., Baccarelli, A.A., 2014. Mitochondrial haplo groups modify the effect of black carbon on age-related cognitive impairment. *Environ. Health* 13, 42.
- Duchen, M.R., 1999. Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J. Physiol.* 516, 1–17.
- EPA. 2000. National Air Pollutant Emission Trends 1900–1998. US Environmental Protection Agency.
- Field, R., Champion, S., Warren, C., Murray, C., Cunningham, C., 2010. Systemic challenge with the TLR3 agonist poly I:C induces amplified IFN α / β and IL-1 β responses in the diseased

brain and exacerbates chronic neurodegeneration. *Brain. Behav. Immun.* 24, 996–1007.

Gao, X., Xue, L., Wang, X., Wang, T., Yuan, C., Gao, R., Zhou, Y., Nie, W., Zhang, Q., Wang, W., 2012. Aerosol ionic components at Mt. Heng in central southern China: Abundances, size distribution, and impacts of long-range transport. *Sci. Total. Environ.* 433, 498–506.

Goyal, S.K., 2001. Use of rosaniline hydrochloride dye for atmospheric SO₂ determination and method sensitivity analysis. *J. Environ. Monit.* 3, 666–670.

Griparic, L., van der Wel, N.N., Orozco, I.J., Peters, P.J., van der Bliek, A.M., 2004. Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. *J. Biol. Chem.* 279, 18792–18798.

Guo, L., Zhu, N., Guo, Z., Li, G., Chen, C., Sang, N., Yao, Q.C., 2012. Particulate matter (PM₁₀) exposure induces endothelial dysfunction and inflammation in rat brain. *J. Hazard. Mater.* 213–214, 28–37.

Guxens, M., Sunyer, J., 2012. A review of epidemiological studies on neuropsychological effects of air pollution. *Swiss Med. Wkly.* 141, w13322.

Hong, Y.C., Lee, J.T., Kim, H., Ha, E.H., Schwartz, J., Christiani, D.C., 2002. Effects of air pollutants on acute stroke mortality. *Environ. Health Perspect.* 110, 187–191.

Hoppins, S., 2014. The regulation of mitochondrial dynamics. *Curr. Opin. Cell Biol.* 29C, 46–52.

Jung, C.R., Lin, Y.T., Hwang, B.F., 2013. Air pollution and newly diagnostic autism spectrum disorders: a population-based cohort study in Taiwan. *PloS One* 8, e75510.

Kim, E., Park, H., Hong, Y.C., Ha, M., Kim, Y., Kim, B.N., Kim, Y., Roh, Y.M., Lee, B.E., Ryu J.M., Kim, B.M., Ha, E.H., 2014. Prenatal exposure to PM₁₀ and NO₂ and children's neuro development from birth to 24 months of age: mothers and Children's Environmental Health (MOCEH) study. *Sci. Total Environ.* 481, 439–445.

Kumie, A., Emmelin, A., Wahlberg, S., Berhane, Y., Ali, A., Mekonen, E., Worku, A., Brandstrom, D., 2009. Sources of variation for indoor nitrogen dioxide in rural residences of Ethiopia. *Environ. Health.* 8, 130–142.

Lackner, L.L., Nunnari, J., 2010. Small molecule inhibitors of mitochondrial division: tools that translate basic biological research into medicine. *Chem. Biol.* 17, 578–583.

Lee, C.J., Martin, R.V., Henze, D.K., Brauer, M., Cohen, A., Donkelaar, Av., 2015. Response of global particulate-matter-related mortality to changes in local precursor emissions. *Environ.*

Sci. Technol. 49, 4335–4344.

Li, H., Xin, X., 2013. Nitrogen dioxide (NO₂) pollution as a potential risk factor for developing vascular dementia and its synaptic mechanisms. *Chemosphere* 92, 52–58.

Li, J., Li, Y., Jiao, J., Wang, J., Li, Y., Qin, D., Li, P., 2014. Mitofusin 1 is negatively regulated by microRNA 140 in cardiomyocyte apoptosis. *Mol. Cell. Biol.* 34, 1788–1799.

Li, L., Hu, G.K., 2015. Pink1 protects cortical neurons from thapsigargin-induced oxidative stress and neuronal apoptosis. *Biosci. Rep.* 35, e00174.

Li, R., Kou, X., Geng, H., Xie, J., Tian, J., Cai, Z., Dong, C., 2015. Mitochondrial damage: an important mechanism of ambient PM_{2.5} exposure-induced acute heart injury in rats. *J. Hazard. Mater.* 287, 392–401.

Marchenko, N.D., Zaika, A., Moll, U.M., 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J. Biol. Chem.* 275, 16202–16212.

Mattiasson, G., Friberg, H., Hansson, M., Elmér, E., Wieloch, T., 2003. Flow cytometric analysis of mitochondria from CA1 and CA3 regions of rat hippocampus reveals differences in permeability transition pore activation. *Neurochem.* 87, 532–544.

Mattson, M.P., 2003. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med.* 3, 65–94.

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Löwer, B., Wunderlich, F.T., von Kleist-Retzow, J.C., Waisman, A., Westermann, B., Langer, T., 2008. Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. *Genes Dev.* 22, 476–488.

Meyer, J.N., Leung, M.C., Rooney, J.P., Sandoel, A., Hengartner, M.O., Kisby, G.E., Bess, A.S., 2013. Mitochondria as a target of environmental toxicants. *Toxicol. Sci.* 134, 1–17.

Mironov, S.L., 2009. Complexity of mitochondrial dynamics in neurons and its control by ADP produced during synaptic activity. *Int. J. Biochem. Cell Biol.* 41, 2005–2014.

Montessuit, S., Somasekharan, S.P., Terrones, O., Lucken-Ardjomande, S., Herzig, S., Schwarzenbacher, R., Manstein, D.J., Bossy-Wetzel, E., Basañez, G., Meda, P., Martinou, J.C., 2010. Membrane remodeling induced by the dynamin-related protein Drp1 stimulates Bax oligomerization. *Cell* 142, 889–901.

Ocskay, R., Salma, I., Wang, W., Maenhaut, W., 2016. Characterization and diurnal variation of

size-resolved inorganic water-soluble ions at a rural background site. *J. Environ. Monit.* 8, 300–306.

Peng, C., Rao, W., Zhang, L., Wang, K., Hui, H., Wang, L., Su, N., Luo, P., Hao, Y.L., Tu, Y., Zhang, S., Fei, Z., 2015. Mitofusin 2 ameliorates hypoxia-induced apoptosis via mitochondrial function and signaling pathways. *Int. J. Biochem. Cell Biol.* 69, 29–40.

Qin, G., Wang, J., Huo, Y., Yan, H., Jiang, C., Zhou, J., Wang, X., Sang N., 2012. Sulfur dioxide inhalation stimulated mitochondrial biogenesis in rat brains. *Toxicology* 300, 67–74.

Qin, G., Wu, M., Sang, N., 2015. Sulfur dioxide and benzo(a)pyrene trigger apoptotic and anti-apoptotic signals at different post-exposure times in mouse liver. *Chemosphere* 139, 318–325.

Ranft, U., Schikowski, T., Sugiri, D., Krutmann, J., Krämer, U., 2009. Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. *Environ. Res.* 109, 1004–1011.

Rao, G.V., Tinkle, S., Weissman, D.N., Antonini, J.M., Kashon, M.L., Salmen, R, Battelli, L.A., Willard, P.A., Hoover, M.D., Hubbs, A.F., 2003. Efficacy of a technique for exposing the mouse lung to particles aspirated from the pharynx. *J Toxicol. Environ. Health. A.* 66,1441-1452.

Rousset, C.I., Baburamani, A.A., Thornton, C., Hagberg, H., 2012. Mitochondria and perinatal brain injury. *J. Matern. Fetal Neonatal Med. Suppl* 1,35–38.

Sassone, J., Maraschi, A., Sassone, F., Silani, V., Ciammola, A., 2013. Defining the role of the Bcl-2 family proteins in Huntington's disease. *Cell Death Dis.* 4, e772.

Smith, J.A., Das, A., Ray, S.K., Banik, N.L., 2012. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain. Res. Bull.* 87, 10–20

Sugioka, R., Shimizu, S., Tsujimoto, Y., 2004. Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. *J. Biol. Chem.* 279, 52726–52734.

Szyszkowicz, M., Rowe, B.H., Kaplan, G.G., 2009. Ambient sulphur dioxide exposure and emergency department visits for migraine in Vancouver, Canada. *Int. J. Occup. Med. Environ. Health.* 22, 7–12.

Westrate, L.M., Drocco, J.A., Martin, K.R., Hlavacek, W.S., Mackeigan, J.P., 2014. Mitochondrial morphological features are associated with fission and fusion events. *PLoS One* 9, e95265.

- 466 Yan, W., Ji, X., Shi, J., Li, G., Sang, N., 2015. Acute nitrogen dioxide inhalation induces
467 mitochondrial dysfunction in rat brain. *Environ. Res.* 138, 416–24.
- 468 Yan, W., Yun, Y., Ku, T., Li, G., Sang, N., 2016. NO₂ inhalation promotes Alzheimer's disease-like
469 progression: cyclooxygenase-2-derived prostaglandin E2 modulation and monoacylglycerol
470 lipase inhibition-targeted medication. *Sci. Rep.* 6, 22429.
- 471 Yao, G., Yue, H., Yun, Y., Sang, N., 2015. Chronic SO₂ inhalation above environmental standard
472 impairs neuronal behavior and represses glutamate receptor gene expression and
473 memory-related kinase activation via neuroinflammation in rats. *Environ. Res.* 137, 85–93.
- 474 Youle, R.J., van der Bliek, A.M., 2012. Mitochondrial fission, fusion, and stress, *Science* 337,
475 1062–1065.
- 476 Yun, Y., Li, H., Li, G., Sang, N., 2010. SO(2) inhalation modulates the expression of
477 apoptosis-related genes in rat hippocampus via its derivatives in vivo. *Inhal. Toxicol.* 22,
478 919–929.
- 479 Zhu, N., Li, H., Han, M., Guo, L., Chen, L.Q., Yun, Y., Guo, Z., Li, G., Sang, N., 2012.
480 Environmental nitrogen dioxide (NO₂) exposure influences development and progression of
481 ischemic stroke. *Toxicol. Lett.* 214, 120–130.

Figure Captions

Fig. 1 Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on body weight (A), brain weight (B) and the brain-to-body weight ratio (C). C57BL/6 mice were exposed to PM_{2.5}+SO₂+NO₂ (L) (0.5 mg·m⁻³ SO₂ and 0.2 mg·m⁻³ NO₂ (6 h/d, 28 d) followed by PM_{2.5} 1 mg·kg⁻¹ every other day) or PM_{2.5}+SO₂+NO₂ (H) (3.5 mg·m⁻³ SO₂, 2 mg·m⁻³ NO₂ (6 h/d, 28 d) followed by PM_{2.5} 3 mg·kg⁻¹ every other day). The control group was exposed to filtered air and saline using the same schedule. Data were expressed as the means ± SE (n=6 mice/group), **P*<0.05 vs. control.

Fig. 2 Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on spatial learning and memory in C57BL/6J mice. (A) Learning curve for 5 days of invisible training to locate the hidden platform in the Morris water maze. Retention memory was determined using a probe trial test conducted 24 h after 5 days of training. During the probe test, the platform was removed from the pool. (B) Number of times crossing the target zone. (C) Percentage of time remaining in the target quadrant. Data were expressed as the means ± SE (n=14 mice/group), **P*<0.05, ***P*<0.01 vs. control. C = control; L = low concentration air pollutants PM_{2.5}+SO₂+NO₂ (L); H = high concentration air pollutants PM_{2.5}+SO₂+NO₂ (H).

Fig. 3 Representative histopathological images of HE staining in the cortex after PM_{2.5}, SO₂ and NO₂ co-exposure. (A) Control group; (B) Low concentration air pollutants group PM_{2.5}+SO₂+NO₂ (L); (C) High concentration air pollutants group PM_{2.5}+SO₂+NO₂ (H) (n=2 mice/group). Black arrows show the necrotic neurons; Red arrows show the apoptotic bodies; Green arrows show the infiltration of inflammatory cells. Scale bars = 25 μm.

Fig. 4 Effects of PM_{2.5}, SO₂ and NO₂ co-exposure on apoptosis-related gene protein expression in the mouse cortex. (A) Protein bands; (B) Protein expression of p53; (C) Protein expression of bax; (D) Protein expression of bcl-2; (E) Ratio of bax to bcl-2. Data were expressed as the means ± SE (n=6 mice/group), **P*<0.05 vs. control. C = control; L = low concentration air pollutants PM_{2.5}+SO₂+NO₂ (L); H = high concentration air pollutants PM_{2.5}+SO₂+NO₂ (H).

Fig. 5 Electron micrographs of neuronal cell mitochondria (40,000×) from the cortex of the mouse brain following PM_{2.5}, SO₂ and NO₂ co-exposure. (A) Control group; (B) Low

concentration group $\text{PM}_{2.5}+\text{SO}_2+\text{NO}_2$ (L); (C) High concentration group $\text{PM}_{2.5}+\text{SO}_2+\text{NO}_2$ (H) (n=2 mice/group). After co-exposure, the cristae within mitochondria exhibited fragmentation and loss, and the membrane became ruptured. The highest concentration of co-exposure showed serious injuries, with the mitochondria appearing collapsed or even dissolved. M — mitochondrion; MM — mitochondrial membrane; C — cristae. Scale bars =1 μm .

Fig. 6 Effects of $\text{PM}_{2.5}$, SO_2 and NO_2 co-exposure on ATP content in the mouse cortex. Data were expressed as the means $\pm$ SE (n=6 mice/group), * $P<0.05$ vs. control. C = control; L = low concentration air pollutants $\text{PM}_{2.5}+\text{SO}_2+\text{NO}_2$ (L); H = high concentration air pollutants $\text{PM}_{2.5}+\text{SO}_2+\text{NO}_2$ (H).

Fig. 7 Effects of $\text{PM}_{2.5}$, SO_2 and NO_2 co-exposure on the mitochondrial fusion/fission gene expression in the mouse cortex. (A) Protein bands; (B) Expression of the Mfn1, Mfn2, OPA1, Fis1 and Drp1 proteins in mitochondria of the cortex of mice treated with different co-exposure concentrations. The control group was exposed to filtered air and saline using the same schedule. Data were expressed as the means $\pm$ SE (n=6 mice/group), * $P<0.05$, ** $P<0.01$ vs. control.

Figure 1

Figure 2

Figure 3

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