Original Article

Aluminum and benzo[a]pyrene co-operate to induce neuronal apoptosis *in vitro*

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(Received December 7, 2014; Accepted March 14, 2015)

ABSTRACT — Toxic and harmful factors co-exist in the environment; these factors often interact to induce combined toxicity, which is the main focus of toxicological research. Furthermore, a large number of studies have shown that aluminum (Al) and benzo[a]pyrene (BaP) are neurotoxic and target the central nervous system to cause neuronal apoptosis. Because we are exposed to both Al and BaP in the air, water, food, and even medicine, the combined effects of these agents in humans must be examined. The present study examines the ability of Al and BaP co-exposure to intensify neuronal apoptosis. The primary neurons of newborn rats were cultured for 5 days, and cells from the same batch that were growing well were selected and assigned to the blank control group, the solvent control group (DMSO+S9+maltol), BaP groups (10, 40 $\mu\text{mol/L}$), Al (mal)₃ groups (50, 100, 400 $\mu\text{mol/L}$) and co-exposure groups with different combinations of BaP and Al (mal)₃. The cell viabilities indicated that 10 μM BaP or 50 μM Al (mal)₃ was mildly toxic, and we selected 10 μM BaP+50 μM Al (mal)₃ for subsequent co-exposure experiments. The morphological characteristics of cell apoptosis were much more obvious in the co-exposure group than in the Al-exposed cells or the BaP-exposed cells, as observed with a transmission electron microscope and a fluorescence inverted microscope. The apoptotic rates and caspase-3 activity quantitatively significantly differed between the co-exposure and Al-exposure groups, while the BaP-exposure group did not significantly differ from the control group. These results indicate that Al and BaP co-exposure exert synergistic effects on neuronal cell apoptosis.

Key words: Aluminum, Benzo[a]pyrene, Co-exposure, Neuron, Apoptosis

INTRODUCTION

Aluminum (Al) is the most abundant metallic element in the earth's crust and is widely used in industrial production and in daily life. It can enter the body via dust, drinking water, foods, cooking utensils, medicine and other avenues. Infant formulae are especially rich in aluminum (Burrell and Exley, 2010). Al concentrations in juice can be as high as 1.8 mM when acidic fruit is boiled in aluminum cookware (Fimreite *et al.*, 1997). Research shows that the exposure level of the general population to Al is 9-12 mg/d in China, which is 5 to 9 times higher than that in developed countries (Zhang and Gao, 2003). The permissible concentration-time weighted average (PC-TWA) exposure to Al is 3 mg/m³ in the workplace in China (GBZ2.1-2007, China). The neurotoxicity of Al has been repeatedly studied and confirmed by many authors

in different countries. At the same time, the heavily energy-consuming industrial structure and rapidly increasing number of cars running on the street are emitting high amounts of polycyclic aromatic hydrocarbons (PAHs), which pollute the air and water. Polluted drinking water and foods and polluted air ultimately enter humans via ingestion or inhalation. Benzo[a]pyrene (BaP), a representative substance of PAHs, has already garnered great concern and become an important public health problem in China (Cheng *et al.*, 2014). BaP was identified in fume samples from soybean and peanut oil in China at concentrations of 19.6 and 18.3 $\mu\text{g/m}^3$ (Chiang *et al.*, 1997). And the higher BaP level is 0.0303 $\mu\text{g/m}^3$, which is three times higher than the national standard in Taiyuan, Shanxi Province. The PAHs deposition flux in rural villages (3.91 $\mu\text{g/m}^2/\text{day}$) and urban areas (8.28 $\mu\text{g/m}^2/\text{day}$) was 3.8 and 9.1 times higher than that in background areas

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(0.82 $\mu\text{g}/\text{m}^2/\text{day}$) in the Beijing-Tianjin region of North China (Wang *et al.*, 2011). We are currently exposed to both Al and BaP in our living environment. Joint exposure to these two substances is a more serious problem for some occupations, such as aluminum electrolysis workers, who are simultaneously exposed to Al and BaP, and their exposure level to these pollutants is much higher than that of the general population (Tjoe Ny *et al.*, 1993; Boffetta *et al.*, 1997). Friesen *et al.* indicated that aluminum smelter workers are exposed to PAHs in many workplaces; even a modest association between aluminum and PAHs produces a high burden (Friesen *et al.*, 2010). Aluminum workers are exposed to coal tar fumes and dusts (contain BaP) during the manufacturing of anodes, which are made from a mixture of coke and coal tar pitch (van Schooten *et al.*, 1995). The involvement of Al in the etiology of Alzheimer's disease is a hot topic and has been debated for many years (Crapper *et al.*, 1973) because Al-induced neurotoxicity causes neuronal loss, A β deposition and neurofibrillary tangle-like fibrous structures (Mehta, 2007; Chen *et al.*, 2010). In addition to its carcinogenicity, BaP's toxic effect on the nervous system has aroused concern (He *et al.*, 2012). Scientists noticed that both Al and BaP can induce neural cell apoptosis (Zhao *et al.*, 2011; Oshima *et al.*, 2013), which is the basic characteristic of neurodegeneration.

We speculate that Al and BaP exert a combined effect on the neural cells to induce apoptosis. The present study mainly observed the aluminum-maltolate and BaP induced apoptosis in primary neurons of rats, and explored the possible mechanism of the combined effect of Al and BaP.

MATERIALS AND METHODS

Preparation of primary cultured cortical neurons (Gartlon *et al.*, 2006)

The primary neurons of newborn rats were obtained from the cerebral cortices of 0-3-d-old newborn Sprague Dawley rats (provided by the Laboratory of Physiology, Shanxi Medical University) using a method previously described by Gartlon *et al.* (2006). All animal experiments were approved the institutional Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Briefly, the newborn rats were placed in a 75% freezing ethanol solution to be anaesthetized. The cerebral cortices were then isolated, and the meninges and white matter were removed. The remaining tissue was dissociated with 0.25% trypsin (AMRESCO) for 15 min at 37°C. Subsequently, the dissociated cortical cells were filtered

through a 220-mesh nylon grid, and trypsinization was stopped by adding complete cell medium, which consisted of a DMEM (GIBCO, Grand Island, NY, USA) suspension, 10% fetal calf serum, and 10% horse serum (GIBCO) supplemented with 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. The density of cells was adjusted to approximately 1×10^6 cells/mL. The cells were plated in 35-mm tissue culture dishes (GIBCO) coated with poly-D-lysine (Solarbio, Beijing, China) and incubated in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C for 4 hr. The cells were then transferred to serum-free culture medium (Neurobasal-A+B27+Glutamine). The serum-free cell culture medium was replaced once every two days.

Preparation of liver post-mitochondrial supernatant (S9) and S9 mixture

The liver S9 fraction was a gift from the Shanxi Provincial Center for Disease Control and Prevention. The S9 mixture contained 5 mM glucose-6-phosphate, 4 mM NADP, 8 mM MgCl₂, 33 mM KCl, 100 mM sodium phosphate buffer (pH = 7.4), and 50 μL of the S9 fraction in a total volume of 0.5 mL. This mixture was prepared immediately before each treatment and maintained at 4°C.

Treatment of cells

Prior to each experiment, cortical neurons were cultured in triplicate at a density of 1×10^6 cells per dish in 35-mm tissue culture dishes. The cells were assigned to the following groups: blank control, solvent control (DMSO+S9+maltol), mono-exposure, i.e., BaP exposure (10, 40 $\mu\text{mol}/\text{L}$), Al (mal)₃ exposure (50, 100, 400 $\mu\text{mol}/\text{L}$), and co-exposure (10 μM BaP+50 μM Al (mal)₃, 10 μM BaP+100 μM Al (mal)₃, 10 μM BaP+400 μM Al (mal)₃, 40 μM BaP+50 μM Al (mal)₃, 40 μM BaP+100 μM Al (mal)₃, 40 μM BaP+400 μM Al (mal)₃). The cells were exposed to the assigned substances for 72 hr.

A stock solution of BaP was dissolved in dimethyl sulfoxide (DMSO) and was maintained at -20°C in the dark; a 3% S9 mixture was added to the culture medium to obtain the concentrations designated for each treatment. The Al solution was freshly prepared by diluting a 50 mM aluminum-maltolate stock solution in 3-hydroxy-2-methyl-4-pyrone (Maltol). The BaP exposure groups were treated with BaP at a final concentration of 10 or 40 $\mu\text{mol}/\text{L}$ in culture medium, and the Al exposure groups were treated with aluminum-maltolate at a final concentration of 50, 100 or 400 $\mu\text{mol}/\text{L}$ in culture medium. The co-exposure groups were treated with aluminum-maltolate at a final concentration of 50, 100 or 400 $\mu\text{mol}/\text{L}$ and BaP at a final concentration of 10 or 40 $\mu\text{mol}/\text{L}$ in

culture medium.

Cell viability assay

The Cell Counting Kit-8 (CKK-8; Dojindo Laboratories, Kumamoto, Japan) produces a highly water-soluble tetrazolium salt, i.e., WST-8[2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. In the present study, the Cell Counting Kit-8 was used to assay cell cytotoxicity as an alternative to the MTT method because the former method is more sensitive.

Acridine orange (AO)-ethidium bromide (EB) double Staining

To observe the morphology of dead cells, we stained cells with AO-EB. After the treatment period, the cells were washed with PBS; the cells were incubated with AO and EB at a concentration of 1 µg/mL for 10 min at room temperature in the dark. The cells were then observed under an inverted research microscope (Olympus IX-71, Tokyo, Japan) using a filter for the 510 nm wavelength.

Transmission electron microscopy (TEM)

TEM was performed to determine the ultra-structure of the cells. First, the cells were collected by centrifugation (1,000 rpm, 10 min) to form grain-like cell clusters (1×10^6 cells). The cells were then fixed with 3% glutaraldehyde, followed by 1% osmium tetroxide. The cells were then suspended in 2% molten agar and subsequently passed through a series of ethanol dehydration steps. The dehydrated agar blocks were suspended in propylene oxide for 20 min at 4°C, followed by treatment with a 1:1 mixture of propylene oxide and araldite for 1 hr at 60°C. The resin blocks were carefully trimmed to expose the underlying agar blocks. Sections of various thicknesses (200 nm, 300 nm, and 500 nm) were cut using a Leica Ultracut UCT microtome and transferred to 300-mesh copper grids. A 10% alcohol solution of uranyl acetate was prepared and centrifuged to remove any precipitate. The sections on copper grids were stained for 1 hr with uranyl acetate in the dark at room temperature, washed thoroughly with distilled water, and observed with a Jeol (JEM 2000 FX, Tokyo, Japan) electron microscope at 80 kV.

Annexin V-FITC/PI assay

Apoptosis was detected using an Annexin V-FITC/PI detection kit (Bipece Biopharma Corp., Cambridge, MA, USA) according to the manufacturer's directions. Briefly, the cells were harvested and re-suspended in buffer (1×10^6 cells/mL). Aliquots of 10^5 cells were mixed with 5 µL of Annexin V-FITC and 5 µL of PI. After incubating the cells for 15 min at room temperature in the dark, the fluorescence was detected by flow cytometry (Millipore Corporation, Inc., Boston, MA, USA). The percentage of apoptosis was determined from the number of Annexin V(+)/PI(-) cells relative to the number of Annexin V(+)/PI(-) cells. The experiment was completed within 1 hr with flow cytometry.

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Measurement of activities of caspase-3

The activities of caspase-3 were measured using a caspase-3/PPP32 colorimetric assay kit (BioVision, Inc., Mountain View, CA, USA). Briefly, cultured cortical neurons were lysed using the cell lysis buffer provided in the kit. After determining the protein content, samples containing 50 µg of total cell lysate (caspase-3) were assembled in duplicate in 96-well plates with freshly prepared 2x reaction buffer (containing 10 mM DTT), and the 200 µM DEVD-pNA substrate was added to each well. The reaction mixture was incubated at 37°C for 2 hr. The pNA light emission from the substrate was then quantified using an enzyme-linked immunosorbent assay plate reader at 405 nm.

Statistical analysis

The data were analyzed using the SPSS11.5 (SPSS Inc., Chicago, IL60606-6412, USA) statistical package and expressed as the mean $\pm$ standard deviation ($\pm$ S.D.). In each phase, a single-factor ANOVA was used to analyze the samples, and a Fisher LSD post hoc test was used when warranted. Additionally, the interaction term of a 2×2 factorial design (0/10 µM BaP and 0/50 µM Al (mal)₃) was used to test for synergism in phase II (Jonker *et al.*, 2004; Elder and Athanasiou, 2008). Significance was defined as $p < 0.05$.

A factorial design analysis of variance was used to analyze the data when a combined effect was studied. If an interaction effect was identified, the type of combined effect was assessed by calculating the separate effect and estimating the marginal means of the data. The marginal means were estimated as follows: if the curves are parallel to each other, it means that there is no interaction. With an increase in the dose, if the curves are far apart, the combined effect can be determined as a synergistic interaction. If the curves are close to each other with the increased dose, the effect is judged to be antagonism.

RESULTS

Culture conditions of rat neuronal cell

On the fifth day of cell culture, the cultured cells were observed under an optical microscope (Fig. 1). As the

Fig. 1. Primary cultured neurons under an optical microscope (magnification 200 ×). Primary cultured neurons showed membrane integrity, a plump cytoplasm, and many cell junctions, and the synapses were embedded within an elaborate plexus of axons and dendrites.

Fig. 2. Primary cultured neurons were immunostained with neuron-specific class iii β -tubulin (magnification 200 ×). The cultured cells were immunostained with tubulin-3, which is a specific antigen for neurons.

incubation time was extended, the trunk and branches of the neurites were significantly longer and thicker, the neurite network became denser, and the neuronal cell bodies gradually increased. Neuron-specific class iii β -tubulin, the protein specifically expressed in neurons, was immunohistochemically stained to ensure that the cultured cells are neurons (Fig. 2).

Cell viability assay

The cell viabilities of mono-exposure groups (40 μ M BaP group; 100 μ M Al (mal)₃ group, 400 μ M Al (mal)₃ group) and co-exposure groups (10 μ M BaP+50 μ M Al (mal)₃ group, 10 μ M BaP+100 μ M Al (mal)₃ group, 10 μ M BaP+400 μ M Al (mal)₃ group, 40 μ M BaP+50 μ M Al (mal)₃, 40 μ M BaP+100 μ M Al (mal)₃ group, 40 μ M BaP+400 μ M Al (mal)₃) decreased significantly ($P < 0.05$) compared with the control groups. However, the viabilities of the 10 μ M BaP group and 50 μ M Al (mal)₃ group only slightly decreased ($P > 0.05$), indicating that 10 μ M BaP or 50 μ M Al (mal)₃ are only weakly toxic (Table 1). Thus, we selected 10 μ M BaP and 50 μ M Al (mal)₃ as “starting doses” to induce apoptosis. We then assessed whether joint exposure to 10 μ M BaP and 50 μ M Al (mal)₃ exacerbated this effect.

The cell viability of the 10 μ M BaP+50 μ M Al (mal)₃ group was significantly lower ($P < 0.05$) than those of the control groups and mono-exposure groups (10 μ M BaP, 40 μ M BaP; 50 μ M Al (mal)₃, 100 μ M Al (mal)₃), suggesting that the combined toxicity of 10 μ M BaP+50 μ M Al (mal)₃ is much greater than mono-exposure to BaP or

Al (mal)₃. Furthermore, increasing the co-exposure dose (10 μ M BaP+400 μ M Al (mal)₃ group, 40 μ M BaP+50 μ M Al (mal)₃ group, 40 μ M BaP+100 μ M Al (mal)₃ group, 40 μ M BaP+400 μ M Al (mal)₃) significantly decreased the cell viability ($P < 0.05$), which demonstrates that BaP and Al co-exposure exerted a significant toxic effect. Overall, 10 μ M BaP+50 μ M Al (mal)₃ co-exposure was significantly more toxic than mono-exposure. Second, the concentrations of aluminum and BaP are lower in real environments. Thus, the use of 10 μ M BaP+50 μ M Al (mal)₃ as a co-exposure dose for further studies is realistic and reasonable to mimic the “real exposure” principle of modern toxicological research.

The factorial design analysis of variance shows an interaction between BaP and Al (mal)₃ ($F = 14.12$, $P < 0.01$). The type of combined effect was assessed by estimating the marginal means of the data. The curves diverged as the doses increased, which indicated that these compounds exerted a synergistic effect (Rothman, 1976; Lee *et al.*, 2011) (Fig. 3, Table 1).

Early and late apoptosis detected by AO/EB double staining

The fluorescence microscopy images obtained from AO/EB double staining are shown in Fig. 4. Uniformly green cells with a normal morphology were observed in the control groups, whereas green-stained early apoptotic cells with nuclear margination and chromatin condensation were evident in the BaP-exposure and Al-exposure groups. However, many orange-stained late-stage apop-

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Fig. 3. Mean interaction diagram of cell viability. The dose-effect curves of BaP-exposure and Al-exposure cells diverged as the dose increased, and the cell viability decreased, which indicated that BaP and Al co-exposure exerted a significant synergistic toxic effect. (Each inflection point represents a different experimental group).

otic cells with fragmented chromatin and apoptotic bodies were observed after the co-exposure to 10 μM BaP+50 μM Al (mal)₃. The results suggest that Al and BaP can induce apoptosis in primary cultured neurons and that this effect is exacerbated by the combination of BaP and Al (Fig. 4).

Apoptosis morphology observed with transmission electron microscopy

The electron microscope images showed regular nuclei and evenly distributed chromatin in the control group (A), but those of the BaP-exposure group (B) and Al-exposure group (C) showed nuclear membrane shrinkage, chromatin condensation (e.g., half-moon or strips attached to the nuclear membrane), and slightly swollen mitochondria. The co-exposure group (D) showed an obviously pyknotic nuclear membrane, nuclear fragmentation, mitochondrial swelling and apoptotic bodies (Fig. 5).

Apoptosis rate with Annexin-V/PI double staining

Flow cytometry was used as described above to quantitatively measure the neuronal apoptosis rate. Compared with the control group, the increase in the apoptosis rate of the BaP-exposure group was not statistically significant.

Table 1. The cell viability by BaP and aluminum ($\bar{X} \pm \text{S.D.}$, $n = 3$).

Group	Dosage units (μM) BaP Al (mal) ₃		Cell viability (OD value)
1 (blank control)	0	0	100 [#]
2 (solvent control)	0	0	98.84 $\pm$ 0.54 [#]
3	10	0	97.26 $\pm$ 0.56 [#]
4	40	0	89.19 $\pm$ 1.83 ^{*#}
5	0	50	96.64 $\pm$ 0.90 [#]
6	0	100	89.82 $\pm$ 3.43 ^{*#}
7	0	400	81.25 $\pm$ 2.42 [*]
8	10	50	77.70 $\pm$ 2.53 [*]
9	10	100	74.01 $\pm$ 1.09 [*]
10	10	400	66.56 $\pm$ 1.57 ^{*#}
11	40	50	70.62 $\pm$ 1.57 ^{*#}
12	40	100	56.23 $\pm$ 2.96 ^{*#}
13	40	400	48.75 $\pm$ 3.12 ^{*#}
F value	14.12		P < 0.01
F(Al) value	225.40		P < 0.01
F(BaP) value	333.01		P < 0.01

Compared with the blank control group, ^{*}P < 0.05; compared with the group 8, [#]P < 0.05

Fig. 4. Primary cultured neurons were stained with AO/EB and observed under a fluorescence microscope (magnification 200 $\times$ /400 $\times$). Green-stained live cells showed a normal morphology in the control; green-stained early apoptotic cells showed nuclear margination and chromatin condensation; yellowish orange-stained later apoptotic cells showed fragmented chromatin and apoptotic bodies. All experiments were repeated three times. [Note] A: blank control group; B: 10 μM BaP groups; C: 50 μM Al (mal)₃ group; D: 10 μM BaP+50 μM Al (mal)₃ group.

cant ($P > 0.05$). However, the increase observed in the Al-exposure group was significant ($P < 0.05$), and that of the co-exposure group was much more significant ($P < 0.01$) (Fig. 6). The factorial design analysis of variance indicated an interaction effect ($F = 878.65$, $P < 0.01$) (Table 2). The type of combined effect was assessed by estimating the marginal means of the data. The curves diverged as the dose increased, which indicated a synergistic interaction.

Measurement of activities of caspase-3

The caspase-3 activity was determined in primary cul-

Fig. 5. Apoptosis of primary cultured neurons under a transmission electron microscope (magnification 15,000 $\times$). Electron microscopy the confirmed apoptotic characteristics, such as chromatin condensation, mitochondrial swelling, organelle disruption and apoptotic bodies, in Al- and BaP-exposure neurons. The arrows indicate the different morphological patterns. Three samples were mixed for each experiment. All experiments were repeated three times. [Note]: (A) blank control group; (B) 10 μ M BaP groups: arrows show slightly swollen mitochondria; (C) 50 μ M Al (mal)₃ group: arrows shows lightly swollen mitochondria; (D) 10 μ M BaP + 50 μ M Al (mal)₃ group: arrows show the formation of apoptotic bodies.

tured neurons treated with 10 μ M BaP alone, 50 μ M Al (mal)₃ alone and 10 μ M BaP+50 μ M Al (mal)₃ cultured for 72 hr. Compared with the control group, the increase in the caspase-3 activity in the 10 μ M BaP-exposure group was not statistically significant ($P < 0.05$), but the increase in the 50 μ M Al-exposure group was significant ($P < 0.05$), and that in the co-exposure group was even more significant ($P < 0.01$). (Fig. 7)

DISCUSSION

Humans are often exposed to a combination of BaP and Al in their living and occupational environments via various exposure pathways. Neural cells may be highly susceptible to BaP-induced damage (He *et al.*, 2012), and Al has been considered a neurotoxin that may cause cognitive deficiencies and dementia when it enters the brain (Kawahara, 2005; Kawahara and Kato-Negishi, 2011; Shaw *et al.*, 2014). In addition, the Al concentration is reportedly increased in the brains of AD patients (Andrási *et al.*, 2005; Walton, 2009; Rusina *et al.*, 2011; Bhattacharjee *et al.*, 2013). BaP was reported to induce neurobehavioral deficits in both coking plant workers (Niu *et al.*, 2010) and rats (Nie *et al.*, 2013), and BaP-induced neurotoxicity is involved in the mechanism of neuron apoptosis (Nie *et al.*, 2014). Because both BaP and Al are neurotoxic and humans are often co-exposed to both chemicals, these chemicals are likely to synergistically damage the central nervous system to exacerbate human health problems.

Cytotoxic chemicals often induce apoptotic cell death. Apoptosis is one of the main types of programmed cell death and involves a series of biochemical events that lead to specific cell morphology characteristics and ultimately the death of cells. Apoptosis is triggered by multi-signal pathways and regulated by complex multi-extrinsic and intrinsic ligands. Mitochondrion-dependent signaling pathways and death receptor-dependent signaling pathways are two major pathways related to apoptosis (Zádor *et al.*, 2003; Liu *et al.*, 2013). In the mitochondrion-de-

Table 2. Neurons apoptosis rate in primary cultured neurons of rat ($\bar{X} \pm S.D.$, $n = 3$).

Al-exposure (μ M)	BaP-exposure (μ M)		F (BaP) value	P (BaP) value
	0	10		
0	1.96 $\pm$ 0.07	2.51 $\pm$ 0.15	1085.00	< 0.001
50	2.66 $\pm$ 0.34*	13.05 $\pm$ 0.43**		
F (Al) value	1146.52		F value	878.65
P (Al) value	< 0.001		P value	< 0.001

Compared with the blank control group, * $P < 0.05$ and ** $P < 0.01$

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Fig. 6. Neurons apoptosis rate in primary cultured rat neurons. (a) Apoptosis measurement chart of primary cultured rat neurons. Apoptosis was analyzed by AnnexinV-FITC/PI staining 72 hr after treatment. The figures depict representative data. [Note] A: blank control group; B: 10 μ M BaP groups; C: 50 μ M Al (mal)₃ group; D: 10 μ M BaP + 50 μ M Al (mal)₃ group. (b) Mean interaction diagram of apoptosis rates. The dose-effect curves of BaP-exposure and Al-exposure cells diverged as the dose increased, which indicated that BaP and Al (mal)₃ acted synergistically to induce apoptosis. The impact of both BaP and Aluminum on neural cell apoptosis is synergistic.

Fig. 7. The activities of caspase-3 in primary cultured rat neurons. [Note]: * $P < 0.05$ versus blank control, # $P < 0.05$ versus 10 μ M BaP+50 μ M Al (mal)₃ group.

pendent signaling pathway, members of the Bcl-2 family may adjust the release of cytochrome C and the activation of specific proteases (caspase, a proteolytic enzyme that plays a key role in the nuclear fracture and apoptosis), resulting in the inhibition or promotion of apoptosis. This process is known as the “intrinsic pathway” (Stefanis, 2005). The death receptor Fas can specifically bind to Fas ligand (FasL) to induce apoptosis, which is known as the “extrinsic pathway”. Both signaling pathways converge at caspase-3, which appears to be the major effector of neuronal apoptosis in response to a variety of stimuli (Sarlak *et al.*, 2013). The activation of caspase-3 then induces the activation of caspase-activated deoxyribonuclease and finally results in cell apoptosis. Neuronal apoptosis is well known to play a key role in the neurodegeneration (Naoi *et al.*, 2012; Jain *et al.*, 2013; Sarlak *et al.*, 2013).

The present experiment focused on Al (mal)₃ as the Al treatment agent. Unlike the vast majority of Al salts, Al (mal)₃ can form a metastable solution under physiological conditions and release a large number of Al³⁺ ions. Thus, it offers physiologically realistic aluminum doses in an experimental setting that are useful for Al-related neu-

rotoxicity research (Johnson *et al.*, 2005). Al (mal)₃ can cause primary cultured rat hippocampal neurons apoptosis in neuron aging and in a concentration-dependent manner (Johnson *et al.*, 2005). Neurons are sensitive to low concentrations of Al; 0-2 mmol/L Al can induce neuron apoptosis (Niu *et al.*, 2007). Studies have shown that Al can cause neural cell apoptosis via the mitochondrial pathway and a caspase cascade reaction (Zhang *et al.*, 2005; Kaneko *et al.*, 2007; Niu *et al.*, 2007). BaP is strongly fat-soluble and easily crosses the blood-brain barrier. One of its neurotoxic mechanisms is the induction of neuronal progenitor cell apoptosis via a caspase cascade (Zhao *et al.*, 2011). Kim showed that BaP-induced apoptosis in RL95-2 cells is accompanied by the activation of caspase (Kim *et al.*, 2007). Nie provided direct evidence that BaP induces apoptotic death of cerebral neurons (Nie *et al.*, 2014).

In the present study, the characteristic morphology of apoptosis, such as a pyknotic nuclear membrane and chromatin margination, were observed under an electron microscope. The cells treated with a combination of BaP and Al showed nuclear corpuscles, cracking and even apoptotic bodies, which indicated that the joint effect of the two substances is far greater than their individual effects. Moreover, the AO-EB fluorescence dye staining revealed uniformly green cells and intact connections among the cells in the control group, which indicated live cells. The BaP-exposure group showed a large number of green live cells and a few early apoptotic cells, which contained green or yellow-green bead bodies (apoptotic bodies formed by the fracture of chromatin), and a number of yellow-colored late apoptotic cells appeared in the Al-exposure cells. However, apoptosis was more pronounced in the co-exposure group, as evidenced by orange fluorescence dyed bead apoptotic bodies in the plasma, accompanied by nuclear shrinkage and cleavage, or even some necrotic cells, which fluoresced bright red. Moreover, the apoptosis rate significantly increased in the co-exposure group compared with the control group, and a factorial design analysis of variance showed a significant interaction between BaP and Al (mal)₃, which indicated a synergistic effect. Because the activation of caspase-3 induces DNA fragmentation, nuclear chromatin condensation, and cell apoptosis (Li *et al.*, 2008), we assessed the caspase-3 activation and found that it significantly increased in the co-exposure group compared with the Al (mal)₃ alone or BaP alone groups, which indicated a synergistic effect. Tu Baijie's study found that BaP and lead exert synergistic effects; BaP can enhance the neurotoxicity of lead (Tu *et al.*, 2004). Both aluminum and lead are neurotoxic metals and are thought to be related to neurodegenerative dis-

eases (Weidenhamer *et al.*, 2014); their toxicity mechanisms are similar (Tüzmen *et al.*, 2014). According to the present study, BaP may enhance the neurotoxicity of aluminum, which warrants further study.

In summary, Al and BaP exert synergistic effects on neuronal cell apoptosis. However, the specific mechanism of this synergy has not been clarified and requires further research. We will examine these issues in future studies.

ACKNOWLEDGMENTS

This research work was supported by the National Natural Science Foundation of China (81372698, 81430078).

Conflict of interest---- The authors declare that there is no conflict of interest.

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