Aluminum Nanoparticles Induce ERK and p38MAPK Activation in Rat Brain

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Aluminum nanoparticles (Al-NPs) are one of the most widely used nanomaterial in cosmetics and medical materials. For this reason, Al-NP exposure is very likely to occur via inhalation in the environment and the workplace. Nevertheless, little is known about the mechanism of Al-NP neurotoxicity via inhalation exposure. In this study, we investigated the effect AL-NPs on the brain. Rats were exposed to Al-NPs by nasal instillation at 1 mg/kg body weight (low exposure group), 20 mg/kg body weight (moderate exposure group), and 40 mg/kg body weight (high exposure group), for a total of 3 times, with a 24-hr interval after each exposure. Inductively coupled plasma mass spectrometry (ICP-MS) analysis indicated that the presence of aluminum was increased in a dose-dependent manner in the olfactory bulb (OFB) and the brain. In microarray analysis, the regulation of mitogen-activated protein kinases (MAPK) activity (GO: 0043405), including Ptprc, P2rx7, Map2k4, Trib3, Trib1, and Fgd4 was significantly over-expressed in the treated mice than in the controls ($p = 0.0027$). Moreover, Al-NPs induced the activation of ERK1 and p38 MAPK protein expression in the brain, but did not alter the protein expression of JNK, when compared to the control. These data demonstrate that the nasal exposure of Al-NPs can permeate the brain via the olfactory bulb and modulate the gene and protein expression of MAPK and its activity.

Key words: Aluminum nanoparticles, Mitogen-activated protein kinases, Nasal instillation, Neurotoxicity, Olfactory bulb

INTRODUCTION

Due to the nature of lightweight and good strength, aluminum has been widely used in many industries such as transportation, packaging, construction and electronics industry to improve the affordability. However, these industries have process of melting operations, and nanoparticles can be scattered into the atmosphere of their workplace (1,2). This is because metals can be converted into nanomaterials by nucleation and growth processes from the surface at a melting point during heating (3,4). In general, aluminum has known to be of low acute toxicity when ingested or dermally exposed, but chronic exposure of aluminum may induce neurological disorders such as Alzheimer's disease (AD) (5-7).

Recently, numerous studies have been reported about the adverse effect of engineered nanoparticles (8,9). In particular, inhaled nanomaterials can invade alveolar region of the respiratory tract and other organs due to their size (10,11). Nevertheless, the use of nanomaterials has become widely available in various consumer products, since nanomaterials have unique physico-chemical properties such as surface reaction and strength compared with bulk materials (12,13). With increasing manufacture of products containing nanomaterials, workers have potential to be highly exposed to nanomaterials by inhalation in the workplace.

Our previous study showed that inhaled nanoparticles were able to penetrate blood-brain barrier (BBB) and blood-testis barrier (BTB) (10). In addition, nanoparticles by inhalation exposure could translocate to the brain from the nasal cavity via the olfactory nerve (11). Thus, inhalation of Al-NPs can be translated that it could affect the brains and may

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cause neurotoxicity. However, until now, the effect of inhaled Al-NPs in the brains is unclear. In this study, we assessed rapid toxicity screen of Al-NPs by microarray and Western blot analysis to present its toxic effects as well as gene and protein expression in the brains.

MATERIALS AND METHODS

Experimental design. The current study is a continuation of our previous study for pulmonary toxicity of Al-NPs by intranasal instillation of Al-NPs (14), Our previous study was investigated pulmonary toxicity except for neurotoxicity effect. For this reason, we used the olfactory bulb (OFB) and brains samples of our previous study for analysis of microarray and proteins expression as investigation of neurotoxicity effect of Al-NPs. Briefly, specific pathogen free (SPF) male Sprague-Dawley (SD, aged 7 weeks) rats were purchased from Orient Bio Inc (Seongnam, Korea). Animals were acclimatized for at least 1 week prior to beginning the study. Rats were divided into four groups (n = 10): one control group and three exposure groups. Each group was studies as follows: investigation of gene expression in the brain (n = 5), analysis of aluminum burden in the brain and histopathological examination (n =5). Al-NPs used in this study were purchased from the Sigma-Aldrich (Sigma-Aldrich, MO, USA). Al-NPs were dispersed in sterile saline and prepared by a sonication 10 min before the beginning of the experiment. Animals were exposed to 1 mg/kg body weight (low exposure group), 20 mg/kg body weight (middle exposure group) and 40 mg/kg body weight (high exposure group) by intranasal instillation. In 1 week, rats were instilled totally 3 times with an interval of one day under anesthesia by intraperitoneal injection of 50 mg/kg tiletamine plus zolazepam (Zoletil; Virbac, Carros, France) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Leuverkeusen, Germany). Control group was exposed to saline as vehicle. Animals were sacrificed at 48 hrs after the last instillation. All animal experiments were performed according to the guideline for care and use of National Institute of Environmental Research.

Preparation of Al-NPs. Al-NPs used in this study were purchased from the Sigma-Aldrich (Sigma-Aldrich, MO, USA). Al-NPs were dispersed in sterile saline and prepared by a sonication 10 min before the beginning of the experiment. Particle size was measured by transmission electron microscopy (TEM). To investigate the state of dispersion of Al-NPs when placed into saline as vehicles, samples were analyzed by dynamic light scattering (DLS). The size of Al-NPs was measured after suspension in saline at concentrations of 10~100 ppm.

Aluminum burden. Brains and OFBs were collected under anesthesia and freezed at -20°C overnight. Then, the samples were weighed, and the aluminum burdens in the brains were quantified with an inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS, Australia) after microwave-assisted digestion of the samples with HNO$_3$ using a microwave digestion system.

Histopathological examination. For histopathological analysis, nasal cavity and brains were collected and the samples were fixed in 10% neutral buffered formalin. Nasal cavity was decalcified in formic acid and trimmed in four transverse nasal sections. After the routine processing, the tissues were embedded in paraffin, and the tissue sections, 3~5 μm in thickness, were stained with hematoxilin and eosin (H&E) for histopathological examination, which was later performed by two toxicologic pathologists. If there was discrepancy in diagnosis between the pathologists, the final consensus would be made by discussing the disagreements in diagnosis under the dual microscopes (Olympus Co., Tokyo, Japan).

Gene expression in the brain. For investigation of gene expression profiling, a microarray for changes in whole gene expression in the brains was performed by Geno-Check (GenoCheck Co. Ltd, Ansan, Korea) using an Agilent rat genome 8 × 60 K arrays. For hybirdizations, RNA was labeled with fluorescents and incubated at Agilent hybrization system (Agilent technology, CA, USA). After a series of washes, the hybridized array was scanned using a 2303C agilent scanner and analyzed by Feature Extraction software (v10.7.3.1, Agilent technolgy, CA, USA) for gene expression analysis. The quantified sample was examined for functional analysis through Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, GeneSpring and JAK software (GenoCheck Co. Ltd., Ansan, Korea).

Western blot analysis. The total protein of brains was extracted using protein extraction solution (PRO-PREPTM, iNtRON Biotechnology, Sungnam, Korea), and samples were quantified using a Bradford assay kit (Bio-Rad, Hercules, CA, USA). The concentration of protein related to mitogen-activated protein kinases (MAPK) pathway such as extracellular signal-regulated kinase (ERK1), and c-Jun amino-terminal kinase (JNK), p38 was analyzed by Western blot analysis. Antibodies were purchased from Santa Cruz. Biotechnology (Santa Cruz, CA, USA). Equal amounts of protein samples were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked for 1 hr in Tris-buffered saline with Tween (TBST) containing 5% skim milk. Immunoblotting was performed by incubating the membranes overnight with their corresponding primary antibodies at 4°C. After washed in TBST, the membranes were incubated with a horseradish peroxi-

dase (HRP)-labeled secondary antibody (Zymed, Carlsbad, CA, USA). The bands of interest were detected and results were quantified using a luminescent image analyzer system (Multi Gauge version 2.02 program of the LAS-3000).

Statistical analysis.   The analysis of variance (ANOVA) test and Student's *t*-test (Graphpad Software, CA, USA) were used to compare test groups with the unexposed control group. The level of significance was set at $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSION

Characterizations of Al-NPs.   The average primary sizes of Al-NPs were measured by TEM, demonstrating a variety of shapes of nanoparticles the size range of approximately 5~100 nm with aggregation (Fig. 1A). Our previous study, the size of Al-NPs in the water was approximately 100 nm. However, in present study showed that Al-NPs
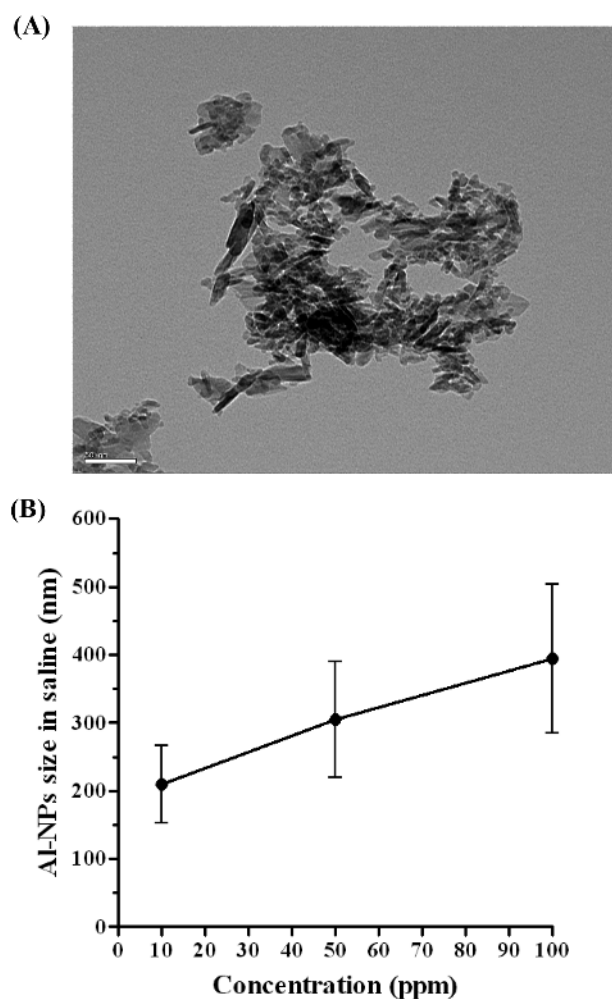
(A)

(B)

Fig. 1. Characterization of exposure conditions. (A) TEM image of aluminum nanoparticles. Scale bar: 50 nm. (B) DLS analysis of aluminum nanoparticles in saline. Mean ± SD.

were agglomerated and dispersion of Al-NPs was observed over the range of 200 to 400 nm in saline in a dose-dependent manner by DLS analysis (Fig. 1B). In the previous study, the increase in particles size and agglomeration of Al-NPs in cell media was significant when compared to primary particle size measured by TEM (15). In the present study, our results of DLS were consistent with the previous observation. Thus, this result suggests that Al-NPs were easily aggregated in high concentration and salt solution.

Histopathological findings.   No treatment-related lesions were observed in the nasal cavity, and brain of the rats exposed to AL-NPs by intranasal route at concentrations of 1, 20, and 40 mg/kg body weight (data not shown). A case of meningioma in the brain was observed in a rat of the middle dose group (20 mg/kg B.W.). However, the incidences of the lesions were ranged within the historical control range, which is considered to be incidental. In contrast, the previous study showed that intracerebroventricular injection of aluminum induced severe anterograde degeneration of cholinergic terminals in the brain of rats (16). These discrepancies between our results and those of the previous study may be due to differences in the study design and exposure methodology to the brain. In this study, our investigation focused on abnormal regions in the brain by H&E staining, but there is weakness of detecting toxicity indexes such as cholinergic markers. H&E stains are difficult to distinguish toxicity response by morphological changes, because it has limitations to confirming histopathological findings at molecular level (17). Therefore, further studies are needed for the approach of molecular pathology to detect brain damage by nasal instillation of Al-NPs.

Al-NPs deposition in brains.   To determine the translocation of Al-NPs to the brain from the nasal cavity, alumi-
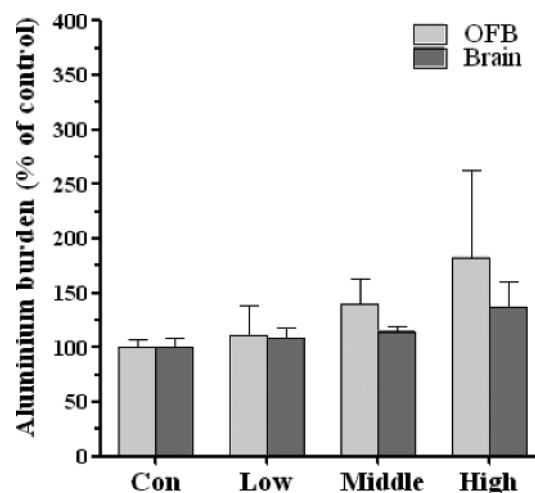
Fig. 2. Aluminum burdens in olfactory bulbs and brains of rats exposed to aluminum nanoparticles. Mean ± SEM.

num burden in OFBs and brains were measured by ICP-MS. Previous studies reported that inhaled silver nanoparticles were distributed in various organs or tissues including the brain and OFB (18,19). The results showed higher accumulation of silver in OFB than the brain. In the present study, as shown in Fig. 2, the content of aluminum in the samples was dose-dependently increased by nasal instillation of Al-NPs compared to the control. Specially, aluminum burden in OFB was higher than the brain sample, and the high dose group was clearly increased compared to other groups (Fig. 2). These results indicate that Al-NPs penetrated into the brains through the olfactory nerve and may affect the brain. Therefore, the high dose group of the brains was used in the following analysis for gene expression.

Gene expression in brain. The use of microarray analysis has been on the rise for rapid toxicity screening of various chemicals in the environmental or workplace (20,21). Gene expression patterns in target organs can play an important role in toxicity prediction. Table 1 showed that the count of differentially expressed gene in the brain exposed to Al-NPs was identified 449 genes, a significant increase by 1.5 fold in change of expression compared with the control ($p < 0.05$ by unpaired *t*-test). Results of functional analysis of genes revealed that the major categories in the biological process ontology were signal transduction, cell differentiation, transcription, transport, response to stress and apoptosis. In the present study, our results showed that the regulation of MAP kinase activity (GO: 0043405, p value 0.0027) such as Ptprc, P2rx7, Map2k4, Trib3, Trib1 and Fgd4 was significantly over-expressed. Thus, MAPK signal pathway was found to be mainly related to gene or protein expression in the brain exposed by nasal instillation of Al-NPs. Therefore, as the next step, we analyzed protein

expression related to MAPK signal pathway in the brain.

Activation of MAPK signaling. To confirm the activation of MAPK signaling by exposure of Al-NPs, we investigated the expression of three subfamilies of MAPK including ERK, p38 MAPK and JNK proteins in the brains. The MAPK pathways in the signaling transduction play an important role in the regulation of cell proliferation and apoptosis (22). Previous studies showed that aluminum induced oxidative stress as neurotoxicity and osteomalacia with chronic renal failure and microcytic anemia without iron deficiency in cell lines and animals (23). Also, it may be related to Alzheimer disease (AD) and Parkinson disease (PD) (24). MAPK signaling in neurodegenerative disorders was activated by various mechanism including oxidative stress and apoptosis. Specially, JNK and p38 MAPK pathways were activated in response to oxidative stress (25). In our study, the brains of rats after nasal instillation of Al-NPs induced activation of p38 and ERK, whereas JNK showed no change of protein expression differently from control (Fig. 3). These results are thought to be caused by a rapid
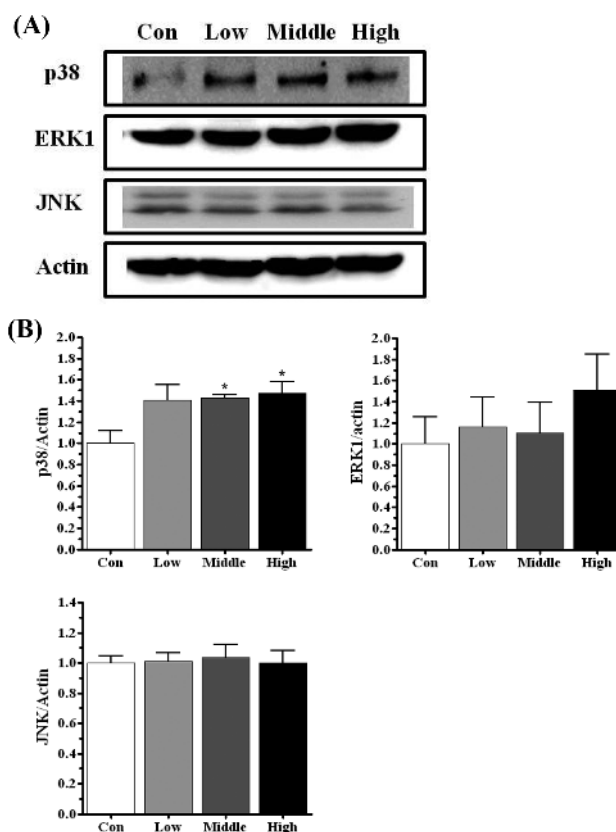

Fig. 3. Effect on MAPK signaling pathway in the brains by nasal instillation of aluminum nanoparticles. (A) Lysates from the brains were analyzed by Western blot for p38, ERK1 and JNK protein level. (B) Densitometric analysis. Data were normalized to actin. Mean ± SEM * $p < 0.05$ *vs.* control.

Table 1. Gene expression analysis in brains of rats after nasal instillation of aluminim nanoparticles

Count of differentially expressed gene

1.5 fold over (significant, $p < 0.05$)		1.5 fold down (significant, $p < 0.05$)	
2156 (230)		2260 (219)	

Biological functional classification

Term	Count	Term	Count
Signal transduction	67	Cell differentiation	35
Transcription	34	Transport	34
Response to stress	28	Apoptosis	22
Cell cycle	14	Cell proliferation	14
Immune response	12	Lipid metabolism	8
Inflamamatory response	5	Protein biosynthesis	3
Cell growth	3		

The genes presented in table are the ones with fold changes more than 1.5 and *P* value less than 0.05. Data represents mean of three animals. Data are mean ± SEM for n = 3.

increase of ERK and p38 activation, and a delayed activation of JNK in the brain. Judging from the above results, nasal exposure of aluminum activates MAPK signaling pathway directly; such direct p38 and ERK activation in the brains. However, our results need to be further confirmed by the evidence that long term exposure of AL-NPs induces activation of JNK in the brains.

In the present study, we investigated the gene and protein expression in the brain of rats exposed by nasal instillation. Our study showed that Al-NPs were found to accumulate in OFB and brains via nasal exposure, inducing enrichments of gene expression of cell communication and activation of ERK and p38MAPK proteins. Therefore, inhalation of Al-NPs might induce neurotoxicity by MAPK signal pathway.

ACKNOWLEDGEMENTS

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