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ALUMINUM IMPAIRS RAT NEURAL CELL MITOCHONDRIA *IN VITRO*

P.Y. NIU[1,2], Q. NIU[2], Q.L. ZHANG[2], L.P. WANG[2], S.C. HE[3], T.C. WU[1], P. CONTI[4], M. DI GIOACCHINO[4] and P. BOSCOLO[4]

[1] *Institute of Occupational Medicine, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P. R.China;* [2]*Department of Occupational Health, School of Public Health, Shanxi Medical University, Taiyuan, P. R. China;* [3]*Department of Psychology, Peking University, Beijing, P. R.China,* [4]*Faculty of Medicine and Surgery, G. D'Annunzio University, Chieti, Italy.*

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Exposure to aluminum has been reported to lead to neurotoxicity. Mitochondria are important organelles involved in maintaining cell function. This study investigates the effect of aluminum on mitochondria in rat neural cells. The ultrastructure of mitochondria was observed, and the cell death rate (CDR), reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and 3-[4,5demethyl-2-thiazalyl]-2,-5diphenyl-2H-tetrazolium bromide (MTT) were measured to investigate the effect of aluminum on the mitochondrial structure and its function in neural cells. Results observed from the mitochondrial ultrastructure show that aluminum may impair the mitochondrial membrane and cristae. Increased CDR, enhanced ROS, decreased MMP, and decreased enzyme activity in mitochondria were observed in the Al-exposed neurons (100 – 500 μM). The present study demonstrates that alteration in the mitochondrial structure and function plays an important role in neurotoxic mechanisms induced by aluminum.

Aluminum (Al) exposure is known to be neurotoxic (1-2). Al is considered to be an etiological factor in the development of several neurodegenerative disorders, including Alzheimer's disease (3-4), dialysis syndrome (5), Parkinson's disease (6-7) and amyotrophic lateral sclerosis (8). The molecular mechanism of Al-induced neurotoxicity has not yet been clarified, but it has been suggested that Al interferes with mitochondrial function (9). Evidence implicating energy defects in mithocondrial energy methabolism in neurodegenerative diseases comes from similarities to known mitochondrial disorders (10).

In this study, we investigated the effect of aluminum on mitochondria ultrastructure in neural cells and measured mitochondrial membrane potential (MMP) and enzyme activity in the mitochondria of cerebral neurocytes by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The relative content of reactive oxygen species (ROS) and cell death rate (CDR) were further assessed to determine the association between Al toxicity and mitochondria.

MATERIALS AND METHODS

Chemicals

Aluminum chloride ($AlCl_3$, AR) was purchased from Shanghai Chemical Co. (China) and dissolved in tris-distilled water in an Al^{3+} 100 mM stock solution.

Key words: Aluminum, rat neural cell, mitochondria, neurotoxixity, in vitro testing

Mailing address: Prof. Qiao Niu, MD, MPH, PhD
Department of Occupational Health
Shanxi Medical University
Taiyuan, 030001, P. R. China
Tel: 86-351-4135029,
Fax: 86-351-2027943
E-mail: SXYDWSXY@public.ty.sx.cn.

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Dulbecco's Modified Eagle's Medium (DMEM, high glucose) was obtained from Hyclone Co. (USA). FBS (fetal bovine serum), HS (horse serum), trypsin, poly-L-Lysine, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), rhodamine123 (Rh123), Propiodium Iodide (PI) were bought from Sigma Chemical Co. (USA), 2',7'-dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes Chemical Co. (USA), and Arabinosyl cytosine (Ara-C) was purchased from Shanghai Hualian Drug Making Company Limited (China).

Instruments

Transmission electron microscope (JEM-100_{CX}, JEOL Co., Japan); CO_2 Incubator (Heraeus and Lishen Co., Germany): Flow Cytometer (FACScan, Becton Dickinson Co., USA); Microplate Reader (Bio-Tek, EL_X800, USA).

Cell Culture and Treatments

4 Sprague-Dawley rats (postnatal day 0-3) were killed by cervical dislocation. All efforts were made to minimize the number of animals killed and their suffering. The cerebrum discarded and the white matter was digested with 0.5% trypsin dissolved with D-Hank's solution for 10 minutes and was triturated with a fire-polished glass pipette. Neurons were plated on previously prepared poly-lysine sheet. The cells were maintained at a concentration of $5x10^5$/ml, in a 50 ml flask with DMEM (Hyclone, USA) supplemented with heat-inactivated fetal bovine serum (10 %), heat-inactivated horse serum (10 %), penicillin G (100 μ/ml), streptomycin (100 μ/ml), 25 mM HEPES, and 20 mM sodium bicarbonate at 37°C in a humidified atmosphere containing 5 % of CO_2. After 24 hours of culture, Ara-C was added to the culture medium at a final concentration of 3 μg/ml (11). The culture medium was changed every 3 days. After 3 days of culture, the cells were harvested and re-suspended at $1x10^5$ cells/ml, and transferred to 24-well plates. Al^{3+} solution was added to culture medium on the 8th day at final concentrations of 50 μM, 100 μM and 500 μM for 1 hour. The same volume of normal saline was added to serve as a negative control.

Preparation for Transmission Electron Microscopy

Following the Al^{3+} treatment, the cultured neural cells were trypsinized and collected into Eppendorff tubes after washing. The cells were rapidly fixed in 2.5 % glutaraldehyde diluted in 0.1 M phosphate buffer (pH 7.4) overnight at 4° C and washed twice in 0.1 M phosphate buffer (pH 7.4), fixed for 1h in 0.1 M phosphate buffer (pH 7.4) 1% osmium tetroxide (at room temperature) and washed several times in the buffer. Dehydration was then performed in ascending series of ethyl alcohol. After embedding in Epon-812, the samples were cut into ultra-thin sections (70 nm). The ultra-thin sections stained with uranium acetate and plumbum citrate were examined with a JEM-100cx electron microscope.

Cell Death Rate Assay

The cultured nerve cells were treated and washed as described above, and re-suspended in PBS at a concentration of 10^5 cells/ml. Then propidium iodide (PI) was added at a final concentration of 10 ng/ml in a 5 ml culture tube. After incubation at 37 °C for 30 min, cells were washed twice with PBS and then analyzed using FACS Vantage flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 620 nm (12). Fluoresecent signal intensity was examined with Cellguest software. For each sample 10,000 events were collected and cell death rate (CDR) was assessed by the mean of fluorescence intensity (MFI).

Reactive Oxygen Species (ROS) Assay

Following resuspension in PBS as previously described, the cell solution was added with DCFH-DA (2',7'-dichlorofluorescein diacetate) at a final concentration of 5 μM. After incubation at 37 °C for 30 min, cells were washed twice with PBS and then analyzed using FACS Vantage flow cytometer with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Fluoresecent signal intensity was examined with Cellguest software. For each sample 10,000 events were collected and the content of ROS was assessed by the mean of fluorescence intensity (MFI).

Determination of Mitochondrial Membrane Potential (MMP)

Similarly, the cells re-suspended in PBS were added with rhodamine 123 at a final concentration of 1 μM. After incubation at 37 °C for 30 min, cells were washed twice with PBS and then analyzed using FACS Vantage flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 525 nm (13). Fluoresecent signal intensity was examined with Cellguest software (14). For each sample 10,000 events were collected and MMP was assessed by the mean of fluorescence intensity (MFI).

MTT Assay

After the cultured and Al^{3+} added neural cells had been washed twice in 0.1 M phosphate buffer (pH 7.4), 10 μl of stock MTT solution (5.0 mg/ml) were added, then the cells were further incubated at 37 °C for 4 hours. The culture medium was carefully removed and 100 μl of 0.1 ml acidulated isopropanol (0.04 M HCl in absolute isopropanol) was added to each well, then the wells were

shaken for 10 min. The absorbance of samples was measured at a wavelength of 570 nm with a Microplate Reader. The negative control was conducted using only cell-free culture medium and each assay was performed in triplicate. The enzymatic activity of mitochondria was expressed by the absorbance values.

Statistical analysis

The statistical estimation for differences was performed with the one-way analysis of variance (ANOVA) followed by the Fisher's protected least significant difference multiple comparison post hoc analysis. The data were expressed as mean ± standard deviation of the mean.

RESULTS

Rat neuronal cells mitochondria ultrastructural analysis

Electron micrographs of rat neural cells treated with saline showed intact inner mitochondrial membrane and regular mitochondrial cristae. Instead, neural cells treated with aluminum showed unstructured inner mitochondrial membranes and irregular mitochondrial cristae. In the Al^{3+} 50 μM group, inner mitochondrial membrane and mitochondrial cristae were unclear. Mitochondrial swelling, local cavitation and reduced mitochondria occurred in the neural cells treated with 100 μM and 500 μM Al^{3+} (Fig. 1).

Determination of CDR, ROS, MPP and MTT

CDR increased significantly in neural cells treated with 500 μM Al^{3+} ($P<0.01$) (Fig. 2), DCF MFI and Rh123 MFI enhanced in 100 μM ($P<0.01$) and 500 μM Al^{3+} treated group ($P<0.01$), compared to saline treated cells (Table I, Fig. 2, 3, and 4).

As shown in Table II and Fig. 5, compared with normal saline group, the enzyme activity in mitochondria decreased significantly in the 500 μM Al^{3+} group ($P<0.01$). However, no significant difference was observed between the cells treated with 50 μM and 100 μM Al^{3+}.

DISCUSSION

It is very important to maintain normal mitochondrial function in neurons. Alterations in mitochondrial structure and function decrease the energy level in neurons and lead to a series of events in the cell such as nerve cell membrane depolarization, influx of calcium ions. As a result, neural cells are impaired and die, leading to neurodegeneration.

In this study, we investigated the effect of Al^{3+} on mitochondria ultrastructure and function in neural cells. Tang et al (16) showed that Al^{3+} (intraperitoneal injection to SD rats, 5 mg/kg per day for 8 weeks) can cause mitochondrial swelling and destruction of mitochondrial cristae in nerve cells. The ultrastructure of mitochondrial membranes and cristae were found changed in the cultured cells. In addition, Al^{3+} caused local cavitation and degradation of mitochondria (17-18). Therefore, mitochondria were considered to be an organelle sensitive to aluminum. However, it is unclear how aluminum caused the alteration of mitochondrial ultrastructures. In this study, we found

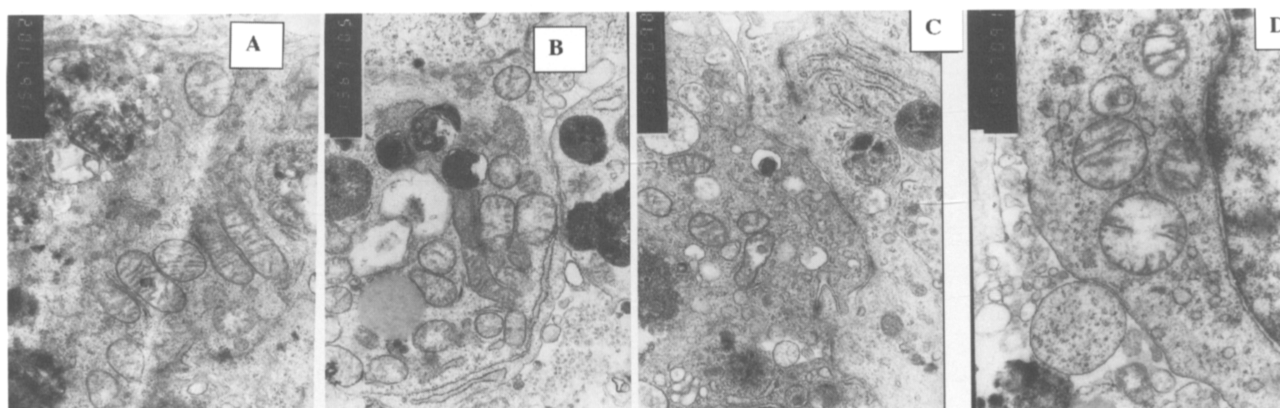

Fig. 1. *Ultrastructural analysis of rat neuronal cell mitochondria after treatment with Al^{3+}; magnification:15,000X.; A) negative control (normal saline): intact inner mitochondrial membrane and regular mitochondrial cristae; B) 50 μM: irregular mitochondrial cristae. C) 100 μM: irregular or unclear mitochondrial cristae and local cavitation; D) 500 μM: swelling and reduced mitochondria, and destructured mitochondrial cristae.*

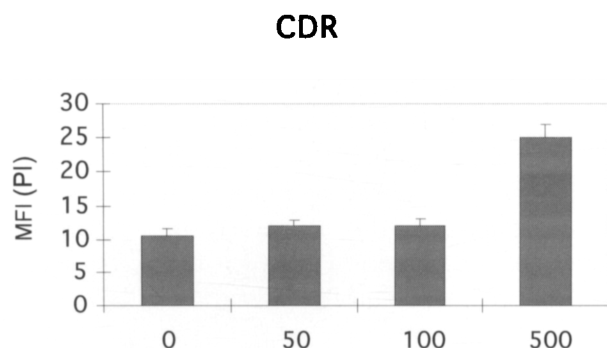

Fig. 2. *Reactive oxygen species (ROS) in rat neuronal cells treated with increasing concentrations of Al^{3+}. ROS intracellular content was measured using the oxidation-sensitive fluorescent probe DCFH-DA and cytofluorimetric analysis. Data are shown as Mean Fluorencence Intensity (MFI)± SD of triplicates.*

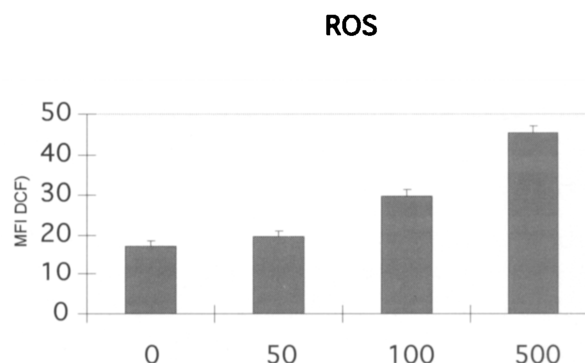

Fig. 3. *Cell death rate (CDR) in rat neuronal cells treated with increasing concentrations of Al^{3+} measured by propidium iodide (PI) uptake and cytofluorimetric analysis. Data are shown as Mean Fluorencence Intensity (MFI)± SD of triplicates*

that the destruction of mitochondria was aggravated along with the increasing Al^{3+} concentration. To explore the alterations of ultrastructure in nerve cells treated by aluminum, the damage of mitochondrial function was further studied.

Reactive oxygen species (ROS) are constantly formed in the human body and removed by antioxidant defenses. ROS include superoxide anion, hydroxy, hydrogen peroxide, etc. (19). The mitochondrion is the major site of ROS production. At the same time, mitochondria are also the organelle mostly impaired by ROS. 2',7'-dichlorofluorescin diacetate (DCFH-DA) has been used as a substrate for measuring intracellular oxidant production. DCFH-DA is hydrolyzed by esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. DCFH-DA cannot be appreciably oxidized to a fluorescent state without prior hydrolysis. Fluorescence intensity (FI) is directly related to the production of ROS in cells (13-14). In this study, we compared the FI of cells treated with normal saline to that of cells treated with Al^{3+} (100-500 μM), the latter was much higher than the former. The cell death rate (CDR) was significantly higher in Al^{3+} (500 μM) treated neural cells than that of normal saline treated group. The results showed that more ROS can be induced by aluminum and increased ROS lead to neural cell injury and necrosis (Fig. 5)

Rhodamine123 (Rh123), a lipophilic cationic fluorochrome, may pass through phospholipid biolayer of cell membranes. The fluorescent signal from Rh123 is concentrated in the mitochondria. The change in FI of Rh123 reflects the change in mitochondrial membrane potential (MMP) (20-21).

Table I. *Alteration of CDR, ROS and MPP induced by aluminum in cultured rat neural cells. (Mean±SD of MFI).*

Al^{3+} (μM)	Repeated tubes	CDR (PI MFI)	ROS (DCF MFI)	MPP (Rh123 MFI)
0 (normal saline)	3	10.53±1.07	17.12±1.49	8.03±0.88
50	3	11.99±0.94	19.71±1.27	8.02±0.97
100	3	12.03±0.96	29.67±1.63**	4.69±0.66**
500	3	25.00±1.87**	45.4±1.61**	3.01±0.35**

*Note: **P<0.01 vs normal saline group*

Table II. *Alteration of enzyme activity in mitochondria in cultured rat neural cells treated with aluminum. (Mean±SD).*

Al^{3+} (μM)	Repeated tubes	Enzyme activity in mitochondria (A_{570nm})
0 (normal saline)	3	0.56±0.08
50	3	0.47±0.10
100	3	0.42±0.08
500	3	0.32±0.04**

*Note: **P<0.01 vs normal saline group*

MMP will decrease when a change in mitochondrial function occurs. Thus it is vital for living cells to maintain MMP (22). Decreased MMP inhibits the enzyme activity in the electron transport chain, decreasing ATP and increasing electron leakage. This situation leads to the overproduction of endogenous superoxide anions. Cell apoptosis and necrosis are induced by ROS (23). In this study, we investigated the alteration of MMP and ROS synchronization. ROS first attacks the mitochondria as the target organelle and alters the structure and function, finally the neural cells die.

Then, we further observed the mitochondrial function by detecting the enzyme activity in mitochondria with MTT assay. Orange MTT can be deoxidised to purple crystals by succinic dehydrogenase. The production of purple crystals is directly related to the activity of enzymes in the mitochondria, thus $OD_{570 nm}$ (optical density) obtained by colorimetry may reflect the enzyme activity in mitochondria (24). Al^{3+} significantly decreased the enzyme activity in mitochondria. The ultrastructure of mitochondria, the level of ROS in neural cells, cell death rate and MTT were investigated in this study. We explored the possible cyto-neurotoxic mechanisms induced by aluminum from an oxidation and injury to mitchondria point of view.

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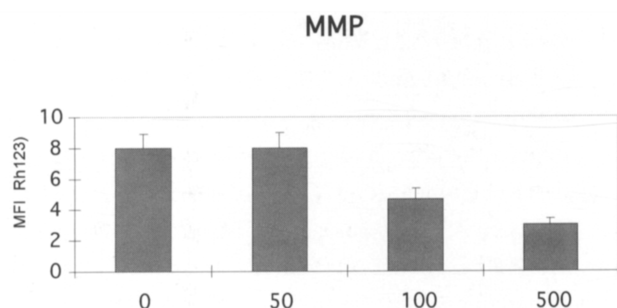

Fig. 4. *Mitochondrial Membrane Potential (MMP) in rat neuronal cells treated with increasing concentrations of Al^{3+}. MMP was measured by cytofluorimetric analysis using rhodamine123 (Rh123) as a fluorescent probe. Data are shown as Mean Fluorencence Intensity (MFI)± SD of triplicates.*

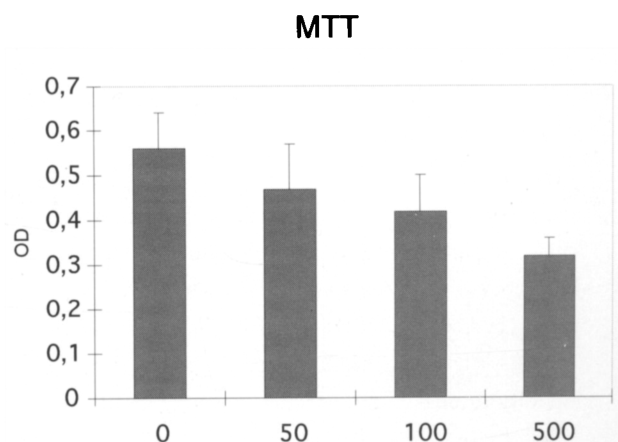

Fig. 5. *Enzymatic mithocondrial activity in rat neuronal cells treated with increasing concentrations of Al^{3+} measured by MTT assay using DCFH-DA as a chromogenic substrate. Data are shown as Optical Density (OD_{570nm})± SD of triplicates.*

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