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Chlorogenic acid protects against aluminum toxicity via MAPK/Akt signaling pathway in murine RAW264.7 macrophages

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ABSTRACT

Aluminum (Al), which may bring about damage to the macrophages, has been implicated in the development of immunological diseases. It has been reported that chlorogenic acid (CGA, 5-caffeoylquinic acid, chemical formula: $C_{16}H_{18}O_9$) is a natural antioxidant and chelating agent with the capacity against Al (III)-induced biotoxicity. The present study was carried out to investigate whether CGA could reduce $AlCl_3$-induced cellular damage in RAW264.7 cells. After treatment with $AlCl_3$, the inhibition rate of viability and phagocytic activity of RAW264.7 cells was 54.5% and 27.6%, respectively. Administration of CGA significantly improved the integrity and phagocytic activity, and attenuated the accumulation of intracellular Al(III) level and oxidative stress in Al (III)-treated cells. Furthermore, CGA significantly inhibited Al(III)-induced increase of phospho-Jun N-terminal kinase (p-JNK), a pro-apoptotic Bcl-2 family protein (Bad), cytochrome *c* and decrease of extracellular regulated protein kinases (ERK1/2), protein kinase B (Akt) protein expressions. These results showed that CGA has a protective effect against Al(III)-induced cytotoxicity through mitogen-activated protein kinase (MAPK)/Akt-mediated caspase pathways in RAW264.7 cells.

1. Introduction

Aluminum (Al) is widely present in the environment and is known as a neurotoxin [1–4]. Aluminum age is a product of the developed world today, which means humans are experiencing chronic poisoning of Al(III) [5]. Al(III) migrates from the environment to water through the acidification of the soil and the use of Al(III)-containing water purifying agents [6]. The use of Al(III) compounds as additives in foods also increases its content in foods [7]. Moreover, the sources of Al(III) include drugs, cosmetics, tea, ware and containers [8], which have a serious impact on the health of humans and animals. More and more evidence showed that Al(III) has cumulative toxicity on experimental animals and humans [9,10]. Epidemiological and experimental studies have confirmed that excessive Al(III) exposure primarily causes neurological disorders such as Alzheimer [11,12], Parkinson and dialysis encephalopathy, accompanied by bone formation inhibition [13], liver dysfunction [14] and immunotoxicity in lymphocytes [15]. Most importantly, the immune system is the main target for Al(III) accumulation and exerts effect on Al(III) toxicity [16,17]. Studies have shown that Al(III) is an immune toxic by inhibiting immune function [18,19]. Although the use of Aluminum-based adjuvants in vaccines helps to stimulate the immune response of co-administered antigens [20]. However, the immune response induced by Al in the form of particulates in the vaccine is different from that of soluble Al(III). Importantly, Al adjuvants are toxic especially to the nervous system [21]. Therefore, it is urgent to study how to alleviate the immunotoxicity caused by Al (III).

Macrophages play a vital role in inflammation and host defense through both pro-inflammatory and anti-inflammatory mechanisms [22]. After 90 days of intragastric administration of $AlCl_3$ for female Wistar rats, the peritoneal inflammation increased and peritoneal macrophages were dysfunctional [23]. The study confirmed that Al(III) toxic effects on rat peritoneal macrophages, and at an exposure dose of 52 mg/kg Al(III) significantly inhibited the ability of phagocytic capacity of peritoneal macrophages [24]. RAW264.7 cells, mouse peritoneal macrophage cell lines, are one of the commonly used inflammatory cell models. The interest of present study has been focused on evaluation the Al(III)-induced damage to immune function in RAW264.7 cells.

Our laboratory is mainly devoted to studying the removal of metal toxicity by natural products. Our previous study has shown that

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chlorogenic acid (CGA) has the capacity of preventing on hepatotoxicity and hematotoxicity in acute Al(III) exposure mice [25]. Another study demonstrated that CGA attenuates Al(III)-induced cognitive dysfunction through chelation and antioxidation [26]. CGA (5-caffeoylquinic acid, chemical formula: $C_{16}H_{18}O_9$), caffeic and quinic acid condensed, is a phenolic compound widely which could be found in coffee, cocoa powder, fruits, vegetables, compositae and solanaceae and so on [27]. A large number of studies have shown that CGA has anti-inflammation [28], antioxidant activity [29], anti-virus [30]. Furthermore, studies have shown that CGA can exert anti-inflammatory effect by inhibiting prostaglandin E2 (PGE2) production in RAW264.7 cells [31].

Accordingly, the aim of this study was to investigate whether CGA has an effect on Al(III)-induced immunotoxicity of RAW264.7 cells. This was achieved by studying cell viability, cell morphology, antioxidant parameters, and the expression of apoptosis-related proteins.

2. Materials and methods

2.1. Materials and reagents

Chlorogenic acid (CGA) (CAS:327-97-9, purity ≥ 98%), aluminum chloride ($AlCl_3$, CAS:7446-70-0, purity ≥ 99%), CGA standard (CAS:327-97-9, purity ≥ 98%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red and 4′,6-diamidino-2-phenylindole (DAPI) were bought from Solarbio (Beijing, China). CGA and $AlCl_3$ were dissolved in water. Malondialdehyde (MDA) assay kit, superoxide dismutase (SOD) assay kit, catalase (CAT) assay kit, nitric oxide (NO) assay kit, glutathione (GSH) assay kit and acid phosphatase (ACP) assay kit were purchased from Comin Biotechnology (Suzhou, China). Antibodies for protein kinase B (Akt, AA326), extracellular signal-regulated protein kinase (ERK1/2, AF1051), phospho-Jun N-terminal kinase (p-JNK, AJ516), a pro-apoptotic Bcl-2 family protein (Bad, AB008), cytochrome *c* (AC909), β-actin (AF0003) and horseradish peroxidase (HRP)-conjugated secondary antibodies (A0216, A0208) were purchased from Beyotime Technology (Shanghai, China).

2.2. Cell culture and administration

RAW264.7 macrophage cells were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, PAN BIOTECH, Germany) at 37 °C in 5% CO_2 incubator (Thermo) [22]. Each group was treated as follows: Control (treated with RPMI 1640 for 24 h), Al (treated with 200 μg/mL $AlCl_3$ for 24 h), Al + CGA (L) (treated with a mixture of 200 μg/mL $AlCl_3$ and 37.5 μg/mL CGA for 24 h), Al + CGA (M) (treated with a mixture of 200 μg/mL $AlCl_3$ and 75 μg/mL CGA for 24 h), Al + CGA (H) (treated with a mixture of 200 μg/mL $AlCl_3$ and 150 μg/mL CGA for 24 h), CGA (treated with 150 μg/mL CGA for 24 h). The dose selection was based on our previous experiments, and the inhibition rate of $AlCl_3$ on cells was close to 50%, under this concentration. In our study, we selected the logarithmic cells for subsequent experiments. Each group set three parallels.

2.3. Assessment of cell viability and phagocytic activity

We used MTT assay to determine the effects of Al(III) and CGA on cell viability [32]. RAW264.7 cells were seeded equably in a 96-well plate at 5×10^4 cells/well and then administered in groups for 24 h. 20 μL of 5 mg/mL MTT (dissolving in PBS) was added to each well and incubated at 37 °C for 4 h. The medium was discarded and DMSO was added to dissolve the precipitate. After 10 min of shaking, the absorbance was measured at 570 nm using a microplate ELISA reader (Thermo Fisher, USA).

In addition, the phagocytosis activity assay was performed using neutral red [33]. After 24 h of administration, the medium was discarded. 100 μL of 0.1% neutral red (dissolving in 0.9% NaCl) was added to each well and incubated for 4 h. After the neutral red was discarded, the plate was washed three times with PBS. 200 μL of cell lysate (anhydrous ethanol: glacial acetic acid = 1: 1 (v:v)) was added to each well and the absorbance was measured at 540 nm by a microplate ELISA reader.

2.4. DAPI staining microscopy

RAW264.7 cells were seeded equably in 6-well plates at 1×10^5 cells/well and adhered for 4 h and then administered in groups for 24 h. Then the plate was washed three times with PBS, and fixed with 4% paraformaldehyde for 15 min. After discarding the solution, the 6-well plate was washed three times with PBS and stained with 100 ng/mL DAPI in the dark for 15 min. Then the plate was washed three times with PBS and observed by fluorescence microscopy (OLYMPUS, FSX100, Japan) [34].

2.5. Scanning electron microscope analysis

2 mL cell suspensions (5×10^4 cells/mL) were added to each well of a 6-well plate with a creep plate. After discarding the medium, the cells were administered in groups of 24 h. The groups of cell culture medium were then discarded and cells were fixed with 2.5% glutaraldehyde for 2 h. The water was gradually removed with 10%, 30%, 50%, 70%, 90%, 90%, 100% ethanol, and then examined for changes in cell state under scanning electron microscopy (Hitachi, SU1510, Japan) [34].

2.6. Determination of intracellular Al(III) level

The cells treated for 24 h were collected and washed three times with PBS (1000 rpm, 5 min) to remove the interference of extracellular Al(III). Then 0.1 g cells were added to a microwave digestion tube preplaced with 5 mL of HNO_3 (superior grade pure) and allowed to stand overnight. Before digestion, 2 mL of 30% H_2O_2 (superior grade pure) were added to per tube. After 2 h of digestion, the diluted digestion solution was purified by the membrane (0.22 μm, Filter Membranes-aquo System). Then the sample solution was detected by inductively coupled plasma mass spectrometry (ICP-MS) (CX7000, Aglient Corporation, USA), and the level of Al(III) in RAW264.7 cells was obtained [25,35].

2.7. Determination of CGA utilization

Cells were incubated in T25 flasks and administered for 24 h. 100 μL of cell medium supernatant and 400 μL of methanol were mixed with vortex for 2 min. After centrifugation (9000 rpm, 10 min), 400 μL of the supernatant was collected and dried through nitrogen. And then vortexed again with 100 μL of methanol for 2 min to obtain a crude sample. Finally, the coarse sample was passed through a 0.22 μm filter membrane to obtain a pure sample. High performance liquid chromatography (HPLC) (LC-20AT and SPD-20A, SHIMADZU, Japan) is used to detect CGA in samples. Analytical column C18 (Venusil MP 4.6 × 250 mm ID, 5 μm, Agela Technologies, Suzhou, China) was used and maintained at 35 °C. The gradient elution of mobile phase A (1% formic acid in water) and B (methanol) was as follows: 100% A (5 min), 90% A (10 min), 80% A (15 min), 60% A (5 min), 50% A (10 min), 20% A (10 min), 90% A (5 min). The flow rate was 0.8 mL/min and the injection volume was 10 μL. The detection wavelength of UV detector was set to 326 nm. The level of CGA in the sample was calculated by comparison with the retention time and peak area of the CGA standard [36].

2.8. Western blotting analysis

Cells treated for 24 h were collected and cleaved by cell lysates (Radio Immunoprecipitation Assay (RIPA): phenylmethanesulfonyl

fluoride (PMSF) = 100:1 (v:v)) to prepare different groups of protein samples. Followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% separation gel and 5% stacking gel) and transferred to nitrocellulose membrane (NC membrane, BIORAD, USA). 5% skimmed milk prevented non-specific sites. The primary antibody and HRP-conjugated secondary antibody (both 1:1000) were incubated on the NC membrane respectively. After the addition of the fluorescent luminescent solution, the expression of the protein was observed by developing [37].

2.9. Statistical analysis

Data were analyzed using SPSS 19.0 and expressed as mean ± standard deviation (SD). The differences between the treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's contrast. When the P value is < 0.05 is considered statistically significant.

3. Results

3.1. Effect of CGA on viability and phagocytosis of RAW264.7 cells induced by $AlCl_3$

We used MTT assay to detect the effect of chlorogenic acid (CGA) on the viability of RAW264.7 cells induced by $AlCl_3$. As shown in Fig. 1A, after 24 h treatment with 200 μg/mL $AlCl_3$, the viability of the cells was significantly inhibited and the inhibition rate was 44.6% as compared with the control group. The administration of CGA (37.5, 75, 150 μg/mL) remarkably reduced the rate of inhibition compared with the Al treated group. With the increase of CGA concentration, the cell viability was also increasing, which was positively correlated with the concentration of CGA.

As shown in Fig. 1B, RAW264.7 cells in the control group had relatively high phagocytic activity. After 24 h treatment with $AlCl_3$, the absorbance was significantly lower than that of the control group. The phagocytic activity of the cell which was exposed to 200 μg/mL $AlCl_3$ for 24 h was decreased to about 27.7% of the control. The phagocytic activity of the cell supplied with CGA (37.5, 75 and 150 μg/mL) increased, compared with $AlCl_3$ treatment alone. These results showed that CGA and $AlCl_3$ co-treatment can effectively reduce $AlCl_3$ inhibition of viability and phagocytosis of cells, and this protective effect has showed dose-dependence.

3.2. Effect of CGA on DAPI staining of RAW264.7 cells induced by $AlCl_3$

In order to investigate the apoptosis of RAW264.7 cells induced by $AlCl_3$ and the protective effect of CGA, we used DAPI, a fluorescent dye that binds to double-stranded DNA in the nucleus for staining. As shown in Fig. 2, the administration of CGA could markedly increase the number of cells, and inhibit Al(III)-induced nuclear fragmentation. With the additive concentration of CGA increased, the protective effect of CGA is more obvious.

3.3. Effect of CGA on morphology of RAW264.7 cells induced by $AlCl_3$

Morphological changes are one of the important signs of cell apoptosis. As shown in Fig. 3, RAW264.7 cells exposed to $AlCl_3$ were smaller in volume and exhibited apoptotic morphology. Compared with $AlCl_3$ treatment alone, cells supplied with CGA can obviously prevent apoptosis. Our results show that CGA can reduce the $AlCl_3$ damage to RAW264.7 cells by inhibiting apoptosis.

3.4. Effect of CGA on various enzyme activities induced by $AlCl_3$ in RAW264.7 cells

To explore the effect of $AlCl_3$ and CGA on macrophages sterilization, we measured intracellular acid phosphatase (ACP) activity. Compared with the control group, intracellular ACP activity decreased 51.0% (Fig. 4A). The administration of CGA (37.5, 75 and 150 μg/mL) enhanced the activity of ACP by 14.1%, 41.1% and 52.5% in cells, and it was positively correlated with the concentration of CGA added.

The level of MDA (the end product of lipid peroxidation) indirectly reflects the extent of oxidative damage to cells. As shown in Fig. 4B, the MDA level in RAW264.7 cells showed a significant increase responding to $AlCl_3$ exposure. The administration of CGA (150 μg/mL) significantly decreased the level of MDA by 32.4% in cells compared with $AlCl_3$ treatment alone.

Meanwhile, oxidative stress was assessed by measuring the level of GSH and the activities of SOD and CAT in cells. As shown in Fig. 4C–E, the exposure to $AlCl_3$ resulted in an obvious decrease of the activities of SOD and CAT and the level of GSH in cells. The administration of CGA (150 μg/mL) enhanced the activities of SOD, CAT and the level of GSH by 68.9%, 45.3% and 39.7%, respectively. It is suggested that CGA attenuates the immunotoxicity of $AlCl_3$ to cells by increasing the antioxidant capacity of cells.

As shown in Fig. 4F, the NO production increased significantly in the RAW264.7 cells exposed to $AlCl_3$, indicating that the cells were treated with $AlCl_3$ to produce oxidative stress. The administration of CGA (37.5, 75 and 150 μg/mL) resulted in significant decreases in NO production in cells, under that of $AlCl_3$.

3.5. Effect of CGA on Al(III) level and CGA utilization in RAW264.7 cells induced by $AlCl_3$

After microwave digestion of the cells, Al(III) level was determined by ICP-MS. As shown in Fig. 5A, the Al(III) level in cells with $AlCl_3$ exposure increased 170.3% over that of control, indicating that Al(III) was accumulated in the cells. The Al(III) levels in cells with the administration of CGA at doses of 1, 5 and 10 μM decreased by 5.81%, 15.7% and 31.4%, respectively, compared to that of $AlCl_3$ treatment

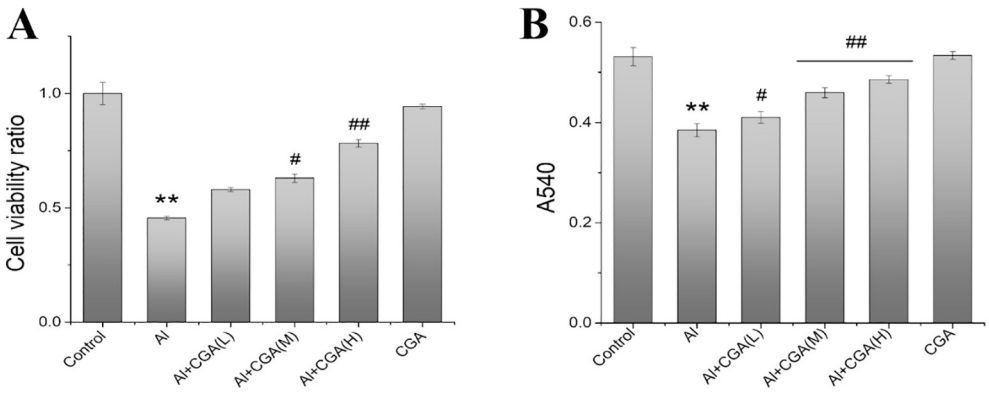

Fig. 1. Effect of chlorogenic acid (CGA) on viability (A) and phagocytic activity (B) of RAW264.7 cells induced by $AlCl_3$ for 24 h. Control, Al (treated with 200 μg/mL $AlCl_3$), Al + CGA (L) (treated with 200 μg/mL $AlCl_3$ + 37.5 μg/mL CGA), Al + CGA (M) (treated with 200 μg/mL $AlCl_3$ + 75 μg/mL CGA), Al + CGA (H) (treated with 200 μg/mL $AlCl_3$ + 150 μg/mL CGA), CGA (treated with 150 μg/mL CGA).Values are presented as means ± SD ($n = 6$), * ($p < 0.05$) and ** ($p < 0.01$) are compared with control, # ($p < 0.05$) and ## ($p < 0.01$) are compared with Al.

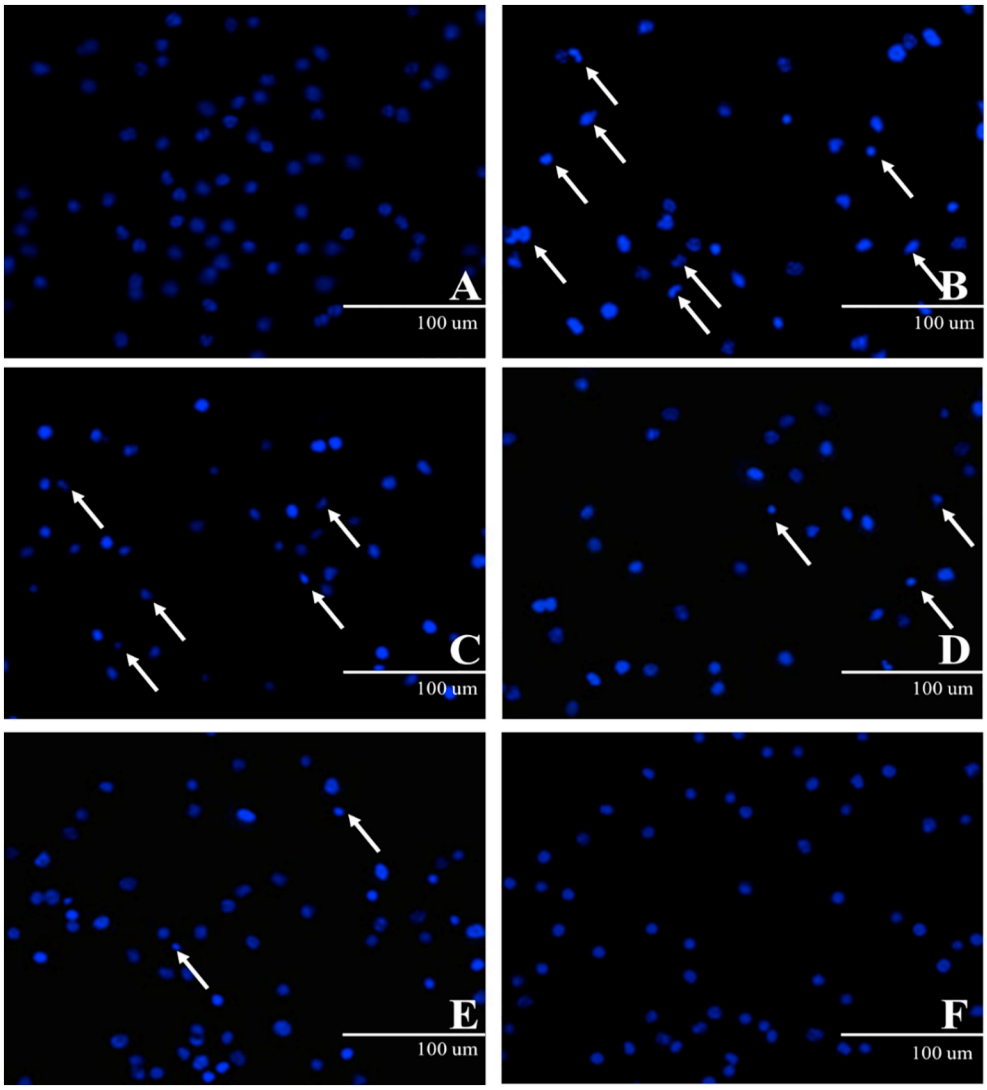

Fig. 2. Effect of chlorogenic acid (CGA) on DAPI staining of RAW264.7 cells induced by $AlCl_3$ for 24 h. A:the control group showed cells were full, the nuclei were intact and the chromatin was homogeneous; B: the Al group (treated with 200 μg/mL $AlCl_3$) showed the intracellular chromatin was condensed into granular substance, the final nucleus ruptured into pieces and the nucleus disintegrated; C–E: the Al + CGA (L, M, H) group (treated with 200 μg/mL $AlCl_3$ + 37.5, 75,150 μg/mL CGA) showed a decreased shrinkage of intracellular chromatin compared with Al-treated group; F: the CGA group (treated with 150 μg/mL CGA) was not significant compared to the control group.

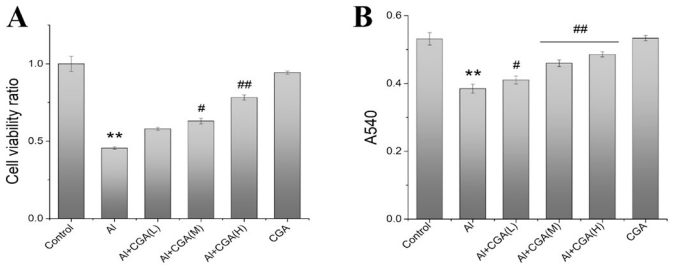

Fig. 3. Effect of chlorogenic acid (CGA) on morphology of RAW264.7 cells induced by $AlCl_3$ for 24 h. A: the control group showed cells were intact, and the surface has a large number of microvilli and clustering, along with regular arrangement; B: the Al group (treated with 200 μg/mL $AlCl_3$) showed the cell volume was reduced, and cell surface was deformed and disintegrated; C: the Al + CGA (H) group (treated with 200 μg/mL $AlCl_3$ + 150 μg/mL CGA) showed a significantly increase on cell volume, and small part of collapse appeared on cells; D: the CGA group (treated with 150 μg/mL CGA) showed normal cell volume and regular shape, and smaller protuberances and partial pseudopodia were observed on the cell surface.

alone. This shows that CGA can effectively reduce the accumulation of Al(III) in cells. The level of CGA in the culture supernatant was analyzed by HPLC. As shown in Fig. 5A, no CGA was detected in the control group and the Al group. The administration of CGA (37.5, 75 and 150 μg/mL) remarkably enhanced the CGA utilization rate to 61.1%, 70.3% and 81.8%, respectively. As shown in Fig. 5B, the CGA concentration in each group in the cell-free supernatant medium was higher than that in the cell medium (Supplementary material Fig. S1). In Al + CGA groups, the utilization of CGA in cells was positively correlated with the added concentration of CGA and correlated with the level of endogenous antioxidation.

3.6. Effect of CGA on protein expressions of RAW264.7 cells induced by $AlCl_3$

The expressions of p-JNK, Akt, ERK1/2, Bad, and cytochrome *c* (cyt-c) were measured in order to detect the effect of Al(III)-induced RAW264.7 cells apoptosis and CGA intervention on these proteins. The expression of p-JNK was up-regulated in cells exposed to $AlCl_3$. This indicated that the $AlCl_3$ stimulated cells produce a response that increased p-JNK expression. The administration of CGA (37.5, 75 and 150 μg/mL) significantly decreased the expression of p-JNK by 13.9%, 23.2% and 31.3%, respectively (Fig. 6B).

As shown in Fig. 6C, D, the expressions of Akt and ERK decreased 35.0% and 30.4% after $AlCl_3$ treatment in cells. Al + CGA groups could significantly inhibit Al(III)-induced Akt and ERK expression levels decreased, while the expression levels were positively correlated with the concentration of CGA added.

The Bcl-2 protein family is a special family that contains 25 kinds of homologous proteins, and some of its members promote apoptosis such

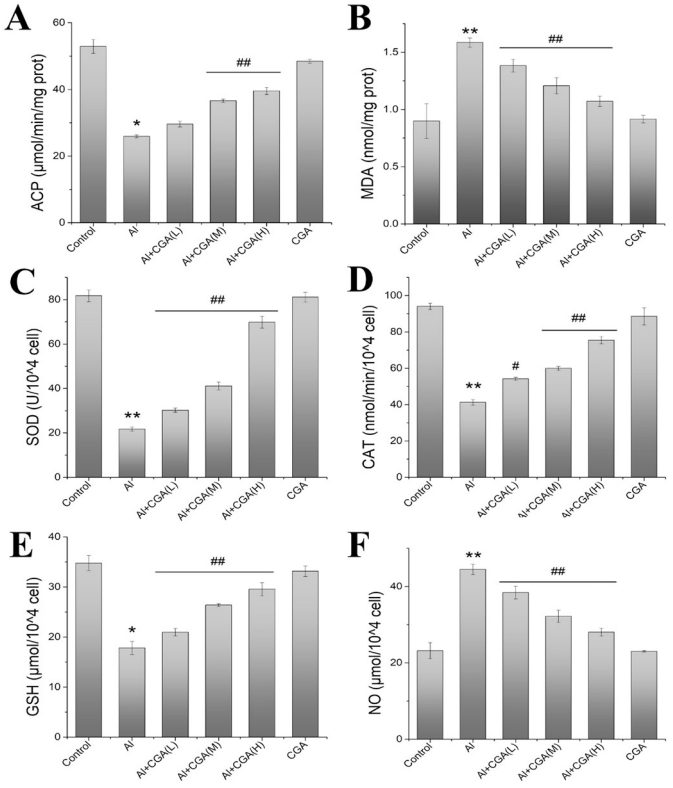

Fig. 4. Effect of chlorogenic acid (CGA) on enzyme activities (ACP(A), MDA(B), SOD(C), CAT(D), GSH(E), NO(F)) in $AlCl_3$-induced RAW264.7 cells for 24 h. Control, Al (treated with 200 μg/mL $AlCl_3$), Al + CGA (L) (treated with 200 μg/mL $AlCl_3$ + 37.5 μg/mL CGA), Al + CGA (M) (treated with 200 μg/mL $AlCl_3$ + 75 μg/mL CGA), Al + CGA (H) (treated with 200 μg/mL $AlCl_3$ + 150 μg/mL CGA), CGA (treated with 150 μg/mL CGA). Values are presented as means ± SD ($n = 6$), * ($p < 0.05$) and ** ($p < 0.01$) are compared with control, # ($p < 0.05$) and ## ($p < 0.01$) are compared with Al.

as Bad, Bid, Bax. As shown in the Fig. 6E, the Al group significantly up-regulated the expression of Bad protein compared with the control group, indicating that $AlCl_3$ enhanced the expression of pro-apoptotic proteins. The administration of CGA (150 μg/mL) significantly decreased the expression of Bad by 54.3%.

Cyt-c is an important protein in the mitochondrial electronic respiratory chain. Compared with the control group, the up-regulated cyt-c in the Al group showed that $AlCl_3$ induced apoptosis and cyt-c was released from the mitochondria into the cytoplasm. Al + CGA groups can effectively inhibit the up-regulation of Al-induced cyt-c expression. And with the increase of CGA concentration, cyt-c release gradually decreased, indicating that the protective effect gradually increased (Fig. 6F).

4. Discussion

It is inevitable that aluminum (Al) enters the human body in a variety of ways, accumulates and even produces toxicity. To date, more and more researchers pay attention to the damage of Al(III) to the nervous system and how to prevent and alleviate it. However, Al(III) is also toxic to macrophages that play an important role in immune organs [38]. Hence, it is extremely urgent to select a substance that is safe, low-dose effective against Al(III)-induced macrophage toxicity. Chlorogenic acid (CGA) is a kind of phenolic acid which has a wide range of sources, easy to extract and high content, with a variety of physiological activity. Our previous study confirmed that CGA has anti-inflammatory effects and was resistant to Al(III)-induced inflammation of the liver and blood. This study provides a strong evidence that CGA protects RAW264.7 cells from $AlCl_3$-induced cytotoxicity.

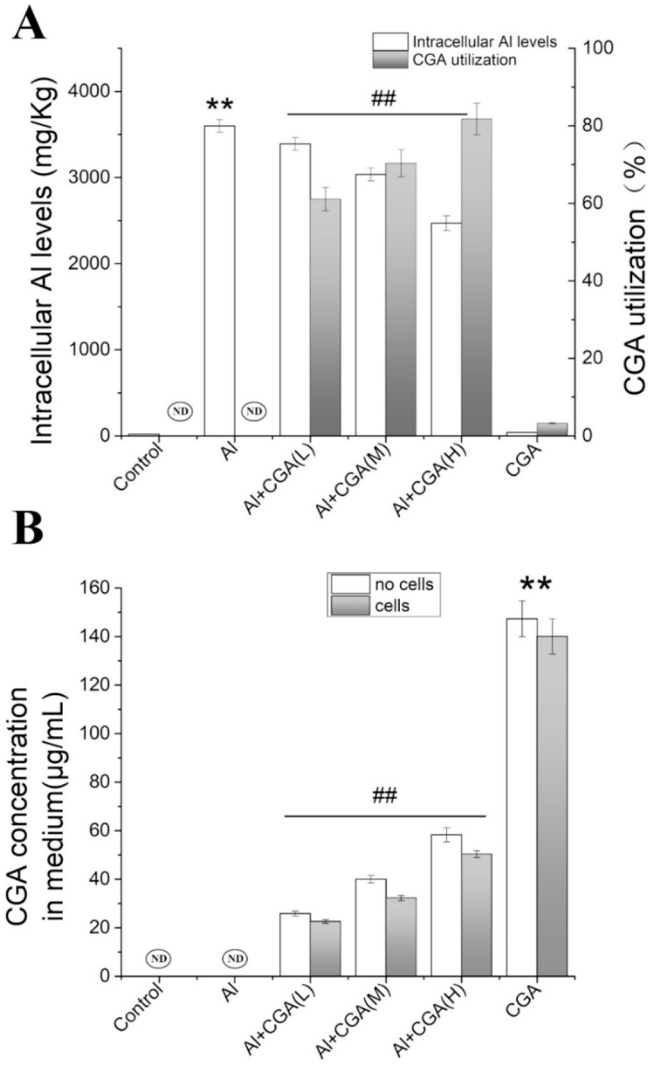

Fig. 5. Effect of chlorogenic acid (CGA) on $AlCl_3$ level (A) in Al-treated RAW264.7 cells for 24 h. CGA concentration in medium with or without Al-treated RAW264.7 cells for 24 h (B). Control, Al (treated with 200 μg/mL $AlCl_3$), Al + CGA (L) (treated with 200 μg/mL $AlCl_3$ + 37.5 μg/mL CGA), Al + CGA (M) (treated with 200 μg/mL $AlCl_3$ + 75 μg/mL CGA), Al + CGA (H) (treated with 200 μg/mL $AlCl_3$ + 150 μg/mL CGA), CGA (treated with 150 μg/mL CGA). ND: not detected. Values are presented as means ± SD ($n = 6$), * ($p < 0.05$) and ** ($p < 0.01$) are compared with control, # ($p < 0.05$) and ## ($p < 0.01$) are compared with Al.

We determined the cytotoxicity induced by $AlCl_3$ and evaluated it by MTT. In rat peritoneal macrophages, Al(III) decreased chemotaxis, adhesion and phagocytosis at a dose of 0.97 mM [39]. After oral administration of 0, 13, 26 and 52 mg/kg Al(III) in drinking water for 120 days, Al(III) had significant toxicity to rat peritoneal macrophages [40]. In vitro studies have confirmed at least three different metal-specific toxic modes that affect the vitality and phagocytosis of earthworm coelomocytes. Therefore, our findings in RAW264.7 cells are consistent with those derived from other macrophages. Now, the viability and phagocytosis of RAW264.7 cells treated with $AlCl_3$ and CGA has improved. These results indicate that CGA plays a role in the alleviation of Al(III)-induced cytotoxicity.

At present, microscopic observation, flow cytometry and enzyme activity detection methods are used for apoptosis detection. Nevertheless, the morphological changes of apoptotic cells are generally recognized as the basis for evaluating apoptosis. Therefore, in order to study the protective effect of CGA on Al (III)-induced apoptosis of RAW264.7 cells, we observed the nuclear morphology by

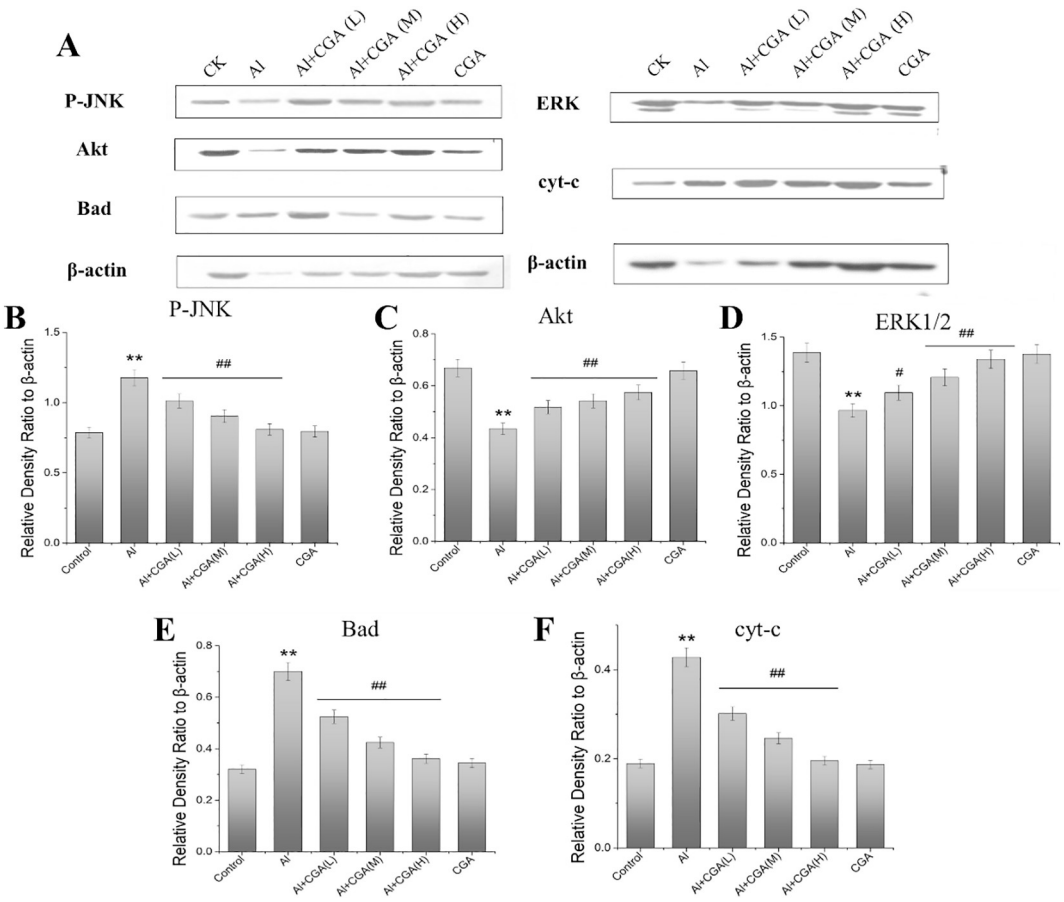

Fig. 6. Effect of CGA on protein expressions in RAW264.7 cells induced by $AlCl_3$ for 24 h. Control, Al (treated with 200 μg/mL $AlCl_3$), Al + CGA (L) (treated with 200 μg/mL $AlCl_3$ + 37.5 μg/mL CGA), Al + CGA (M) (treated with 200 μg/mL $AlCl_3$ + 75 μg/mL CGA), Al + CGA (H) (treated with 200 μg/mL $AlCl_3$ + 150 μg/mL CGA), CGA (treated with 150 μg/mL CGA). Values are presented as means ± SD ($n = 6$), * ($p < 0.05$) and ** ($p < 0.01$) are compared with control, # ($p < 0.05$) and ## ($p < 0.01$) are compared with Al.

fluorescence microscopy and the morphology of cells by scanning electron microscopy (SEM). In our study, most cells of Al + CGA groups were observed with clear margins and fine dendritic networks. DAPI staining showed that the cells co-treated with $AlCl_3$ and CGA were round, the chromatin was evenly distributed without dark spots, and no obvious morphological changes were observed. Similarly, typical apoptotic condensation and fragmentation of chromatin were observed in astrocytes and cortical neurons treated with Al(III) [40,41]. Nuclear chromatin condensation occurred in the root tip cells of peanut exposed to $AlCl_3$, and nuclear damage increased with increasing $AlCl_3$ treatment time [42].

Acid Phosphatase (ACP) is the material basis for phagocytic cell sterilization. It can destroy and eliminate foreign bodies invading the body, and play a role in defending the body. Yousef et al. found that ACP activity in sperm of male New Zealand white rabbits decreased significantly after 4 h of treatment with 1–20 mM $AlCl_3$ [43]. In addition, the activity of ACP in liver and testis decreased significantly after oral administration of $AlCl_3$ (34 mg/kg BW/day) for 16 weeks [44]. At present, we found that $AlCl_3$ inhibited the ACP activity of RAW264.7 cells, whereas CGA was effective against Al(III)-induced inhibition. Therefore, it can be concluded that $AlCl_3$ weakened the bactericidal ability of RAW264.7 cells and inhibited their activation, and CGA played a significant role in reducing $AlCl_3$ toxicity.

Oxidative stress is one of the most common forms of metal toxicity. The definition of oxidative stress is the imbalance between reactive oxygen species (ROS) and antioxidant defense systems (GSH, SOD, CAT, etc.). MDA is a decomposition product of lipid peroxides induced by ROS, and its content is an indicator of lipid peroxidation and cell damage. It has been determined that the level of MDA is increased, the activities of SOD and CAT and the level of GSH are reduced in cells treated with Al(III), which resulted in a decrease in RAW264.7 cells viability and phagocytic activity. Studies have shown that treatment of primary hippocampal neurons with Al (III) for 24 h resulted in significant changes in MDA levels, SOD and CAT activities [45]. In our previous studies, Al (III) increased MDA accumulation by inhibiting liver SOD and CAT activities [25]. Similarly, Wang et al. suggested that pretreatment of primary hippocampal neuronal cells with CGA before exposure to $AlCl_3$ significantly restored the activities of superoxide dismutase, catalase, glutathione peroxidase (GPx), and glutathione S-transferase and attenuated Al(III)-induced MDA levels in cells [35]. Moreover, MDA, GSH and GPx in Al(III)-treated kidney proximal tubular cells were significantly higher than those in the control group [46]. The results of Fig. 4B–E showed that CGA could effectively inhibit the decrease of the antioxidant capacity of the Al (III)-induced cells.

Nitric oxide (NO), second messenger molecule, regulates various cell functions, and widely involved in the body's physiological and pathological processes [47]. Satoh et al. found that 150 μM Al(III) was used to treat PC12 cells for 48 h, resulting in NO production in cells [48]. Several studies have shown that damage caused by Pb^{2+} is accomplished by the production of ROS and NO [49]. In this study, intracellular levels of NO in cells treated with CGA and $AlCl_3$ for 24 h were significantly lower than those treated with $AlCl_3$ alone, indicating that CGA had a certain anti-inflammatory effect and showed a dose-dependent inhibitory effect. All of these results reveal that CGA exerts its protective effect in relation to its antioxidant activity.

Aluminum is a well-known toxic reagent and accumulates in cells and animals. In our previous study, mice injected with $AlCl_3$ intraperitoneally had significantly increased levels of Al(III) in the liver and blood [25]. Guo et al. found that the Al(III) concentration in rat plasma and erythrocytes was significantly increased after intraperitoneal injection of $AlCl_3$ at a dose of 27 mg/Kg body weight for 3 weeks [50]. In our results, it was found that the co-treatment with CGA and $AlCl_3$ effectively reduced the intracellular Al(III) level, which was lower than that of Al group. Moreover, we analyzed the CGA concentration in each group. It has been demonstrated that the accumulation of CGA in CGA-treated primary hippocampal neuronal cell, and the level of CGA was positively correlated with the CGA added concentration [45], consistent with our results. According to the liquid chromatogram that was supplemented (Supplementary material Fig. S1), the retention time of CGA in the cell-free supernatant medium was almost the same as that of the cell-containing supernatant medium, indicating that CGA can still be detected after chelating with $AlCl_3$. Furthermore, the detected concentration of CGA in the cell-free supernatant medium was higher than that of the cell-containing supernatant medium. The degree of oxidative damage of cells was negatively correlated with the utilization of CGA. Thus, these results show that decreased CGA in cell-containing supernatant medium is utilized by cells compared to cell-free supernatant medium. These reduced CGA have a contribution to the clearance of $AlCl_3$ toxicity and may function as an antioxidant component.

Our previous studies suggest the CGA to be effective not only in reducing the biochemical variables indicative of oxidative stress but also in preventing the accumulation of Al(III) in the liver and blood in the Al(III)-treated (intraperitoneally) mice [25]. Although we find that CGA-mediated protection against Al (III)-cytotoxicity is connected with CGA antioxidation, we should not exclude the contribution of CGA chelation properties. We hypothesize that the cytoprotection role of CGA against Al(III)-toxicity involve chelation and antioxidant effects. These two protective mechanisms' respective contribution to CGA mediated protection against Al(III)-toxicity need further study.

To investigate the effects of $AlCl_3$ and CGA at the molecular level on RAW264.7 cells, we selected five crucial proteins for western blotting. Mitogen-activated protein kinases (MAPKs) mainly include extracellular regulatory protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. ERKs play an important role in cell viability and differentiation, while JNK and p38MAPKs are responsible for regulating cellular stress response. Fu et al. found that the level of JNK phosphorylation in cortical neurons exposed to $AlCl_3$ decreased with the prolongation of time, but was still higher than those in the control group [41]. Studies have shown that compared with the control group, the expression of ERK in hippocampus of Wistar rats was decreased [51].

Akt protein kinase regulates cell viability and growth, involved in cell processes including apoptosis and glucose metabolism. Akt plays a role in the anti-apoptotic effect of phosphorylation of target proteins through downstream routes. In Al(III)-induced long-term impairment in rats, it was confirmed that the relative intensity of active caspase-3 increased, and the relative intensity of Akt and phosphorylation glycogen synthase kinase (p-GSK-3β) decreased with the increase of Al(III) concentration in a dose-dependent manner [52].

It is well-known that apoptosis-related proteins in the Bcl-2 family are key regulators of apoptosis. In the Bcl-2 family, anti-apoptotic protein Bcl-2 inhibits apoptosis, and pro-apoptotic proteins Bad inhibit or enhance the release of cyt-c, respectively [53]. One of the important indicators of cell apoptosis is the release of cyt-c from mitochondria to cytoplasm. Our results showed that CGA down-regulated the expressions of Bad and cytochrome *c* induced by $AlCl_3$ and showed a dose-response relationship. Ghribi et al. found direct injection of aluminum complex into the rabbit brain induced cyt-c release from mitochondria and reduced Bcl-2 protein in mitochondria and endoplasmic reticulum [54]. Moreover, treating rabbits with malto-aluminum resulted in the translocation of cyt-c, down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3. It has been observed that cyt-c is released from mitochondria into the cytoplasm of RAW264.7 cells treated with Al(III), which has the same effect on rat hippocampus [55] and human teratocarcinoma (NT2) precursor cells [56] as reported in other reports.

5. Conclusion

In summary, chlorogenic acid (CGA) has cytoprotective effect against Al(III)-induced functional and biochemical changes, which might be through PI3K/Akt and MAPK signaling pathways. The accumulation of oxidative stress induced by Al (III) initiated the activation of MAPKs (up-regulation of p-JNK) and blocked the Akt. As a result, the expression of cytochrome *c* was up-regulated and the cell apoptosis was induced. We hypothesize that the cytoprotective role of CGA against Al (III)-toxicity involves antioxidant and chelation effects, while these two mechanisms' respective contribution to CGA-mediated protection is unclear. Therefore, for humans, exposure to Al(III) should be monitored and limited, CGA supplementation may be favorable to alleviate the immunotoxicity caused by Al(III).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2018.11.001.

Conflict of interest

No conflict of interest exists in the submission of this manuscript.

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References

[1] E. Andrási, N. Páli, Z. Molnár, S. Kösel, J. Alzheimers Dis. 7 (2005) 273–284.
[2] A. Mahboob, S.M. Farhat, G. Iqbal, M.M. Babar, N.S. Zaidi, S.M. Nabavi, T. Ahmed, Brain Res. Bull. 122 (2016) 19–28.
[3] A.N. Hashmi, A. Yaqinuddin, T. Ahmed, Int. J. Neurosci. 125 (2015) 277–287.
[4] N. Xu, V. Majidi, W.R. Markesbery, W.D. Ehmann, Neurotoxicology 13 (1992) 735–743.
[5] C. Exley, Morphologie 100 (2016) 51–55.
[6] D. Cheng, J. Tang, X. Wang, X. Zhang, S. Wang, Ecotoxicol. Environ. Saf. 157 (2018) 201–206.
[7] G.F. Deng, K. Li, J. Ma, F. Liu, J.J. Dai, H.B. Li, Food Addit. Contam., Part B 4 (2011) 248–253.
[8] A.-S.A. Newairy, A.F. Salama, H.M. Hussien, M.I. Yousef, Food Chem. Toxicol. 47 (2009) 1093–1098.
[9] D. Krewski, R.A. Yokel, E. Nieboer, D. Borchelt, J. Cohen, J. Harry, S. Kacew, J. Lindsay, A.M. Mahfouz, V. Rondeau, J. Toxicol. Environ. Health Part B 10 (2007) 1–269.
[10] T. Singh, R.K. Goel, Neurotoxicology 49 (2015) 1–7.
[11] J.R. Walton, J. Alzheimers Dis. 35 (2013) 7–43.
[12] S.K. Ravi, B.N. Ramesh, R. Mundugaru, B. Vincent, Environ. Toxicol. Pharmacol. 58 (2018) 202–211.
[13] X. Sun, H. Wang, W. Huang, H. Yu, T. Shen, M. Song, Y. Han, Y. Li, Y. Zhu, Chemosphere 176 (2017) 1–7.
[14] F. Xu, Y. Liu, H. Zhao, K. Yu, M. Song, Y. Zhu, Y. Li, J. Biol. Inorg. Chem. 174 (2017) 55–62.
[15] J.H. Zhang, C.W. Hu, Y.Z. Zhu, S.M. Liu, C.S. Bai, Y.F. Han, S.L. Xia, Y.F. Li, Biol. Trace Elem. Res. 154 (2013) 275–280.
[16] B.I. Synzynys, A.N. Sharetskiĭ, O.V. Kharlamova, Gig. Sanit. 4 (2004) 70–72.
[17] Nesse Lauricella, Ther. Drug Monit. 15 (1993) 137.
[18] C.C. Willhite, G.L. Ball, C.J. Mclellan, Crit. Rev. Toxicol. 42 (2012) 358–442.
[19] Y. Zhu, Y. Li, L. Miao, Y. Wang, Y. Liu, X. Yan, X. Cui, H. Li, Chemosphere 104 (2014) 1–6.
[20] P. He, Y. Zou, Z. Hu, Immunotherapy 11 (2015) 477–488.
[21] C.A. Shaw, D. Li, L. Tomljenovic, Immunotherapy 6 (2014) 1055–1071.

[22] Q. Xu, M. Liu, Q. Liu, W. Wang, Y. Du, H. Yin, Carbohydr. Polym. 174 (2017) 1138–1143.
[23] N.N. Ayuob, Immunology 284 (2013) 104–110.
[24] C. Hu, J. Li, Y. Zhu, H. Sun, H. Zhao, B. Shao, Y. Li, Biol. Trace Elem. Res. 144 (2011) 1032–1038.
[25] D. Cheng, X. Zhang, L. Xu, X. Li, L. Hou, C. Wang, Chem. Biol. Interact. 273 (2017) 125–132.
[26] X. Wang, Y. Xi, X. Zeng, H. Zhao, J. Cao, W. Jiang, J. Funct. Foods 40 (2018) 365–376.
[27] M.N. Clifford, J. Sci. Food Agric. 79 (1999) 362–372.
[28] H.S. Shin, H. Satsu, M.J. Bae, Z. Zhao, H. Ogiwara, M. Totsuka, M. Shimizu, Food Chem. 168 (2015) 167–175.
[29] L. Rui, M. Xie, B. Hu, L. Zhou, M. Saeeduddin, X. Zeng, Carbohydr. Polym. 170 (2017) 206–216.
[30] H. Shi, L. Dong, X. Dang, Y. Liu, J. Jiang, Y. Wang, X. Lu, X. Guo, Inflamm. Res. 62 (2013) 581–587.
[31] J. Shan, J.Z. Fu, Int. Immunopharmacol. 9 (2009) 1042–1048.
[32] P. Wei, P. Ma, Q.S. Xu, Q.H. Bai, J.G. Gu, H. Xi, Y.G. Du, Glycoconj. J. 29 (2012) 285–295.
[33] M. Ishida, K. Nishi, N. Kunihiro, H. Onda, S. Nishimoto, T. Sugahara, J. Sci. Food Agric. 97 (2017) 4727–4736.
[34] D. Cheng, X. Zhang, M. Meng, L. Han, C. Li, L. Hou, W. Qi, C. Wang, Int. J. Biol. Macromol. 92 (2016) 88–95.
[35] X. Wang, X. Fan, S. Yuan, W. Jiao, B. Liu, J. Cao, W. Jiang, Food Funct. 8 (2017) 2924–2934.
[36] D. Cheng, Y. Xi, J. Cao, D. Cao, Y. Ma, W. Jiang, Neurotoxicology 45 (2014) 111–120.
[37] P. Ma, H.T. Liu, P. Wei, Q.S. Xu, X.F. Bai, Y.G. Du, C. Yu, Carbohydr. Polym. 84 (2011) 1391–1398.
[38] S. Guis, J.F. Pellissier, F. Nicoli, D. Reviron, J.P. Mattei, R.K. Gherardi, J. Pelletier, G. Kaplanski, D. Figarella-Branger, J. Roudier, Arthritis Rheum. 46 (2002) 2535–2537.
[39] M. Djouina, N. Esquerre, P. Desreumaux, C. Vignal, M. Body-Malapel, Food Chem. Toxicol. 91 (2016) 108–116.
[40] Y. Zhao, S. Bhattacharjee, B.M. Jones, P. Dua, P.N. Alexandrov, J.M. Hill, W.J. Lukiw, Neuroreport 24 (2013) 318–323.
[41] H.J. Fu, Q.S. Hu, Z.N. Lin, T.L. Ren, H. Song, C.K. Cai, S.Z. Dong, Brain Res. 980 (2003) 11–23.
[42] H. He, W. Huang, T.L. Oo, M. Gu, L.F. He, J. Hazard. Mater. 333 (2017) 285–292.
[43] M. Yousef, K.G. Kamel, Mi, F. El-Demerdash, Toxicology 239 (2007) 213–223.
[44] M.I. Yousef, Toxicology 199 (2004) 47–57.
[45] X. Wang, X. Fan, S. Yuan, W. Jiao, B. Liu, J. Cao, W. Jiang, Food Funct. 8 (2017) 2924–2934.
[46] M. Sargazi, A. Shenkin, N.B. Roberts, J. Trace Elem. Med. Biol. 19 (2006) 267–273.
[47] B. Mayer, B. Hemmens, Trends Biochem. Sci. 22 (1997) 477–481.
[48] E. Satoh, I. Yasuda, T. Yamada, Y. Suzuki, T. Ohyashiki, Biol. Pharm. Bull. 30 (2007) 1390–1394.
[49] V. Matović, A. Buha, D. Đukić-Ćosić, Z. Bulat, Food Chem. Toxicol. 78 (2015) 130–140.
[50] C.H. Guo, G.S. Hsu, L.Y. Lin, Y.H. Wang, C.Y. Lin, M.S. Yeh, Biol. Trace Elem. Res. 101 (2004) 61–71.
[51] X. Cui, B. Wang, Z. Zong, S. Liu, W. Xing, Food Chem. Toxicol. 50 (2012) 315–319.
[52] H. Zhang, X. Yang, X. Qin, N. Qiao, Neurotox. Res. 29 (2016) 484–494.
[53] S.H. Graham, J. Chen, R.S. Clark, J. Neurotrauma 17 (2000) 831–841.
[54] O. Ghribi, M.M. Herman, M.S. Forbes, D.A. Dewitt, J. Savory, Neurobiol. Dis. 8 (2001) 764–773.
[55] D.R. Sharma, W.Y. Wani, A. Sunkaria, R.J. Kandimalla, R.K. Sharma, D. Verma, A. Bal, K.D. Gill, Neuroscience 324 (2016) 163–176.
[56] K.J.S. Griffioen, O. Ghribi, N. Fox, J. Savory, D.A. Dewitt, Neurotoxicology 25 (2004) 859–867.