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Research Paper

Astrocytes protect MN9D neuronal cells against rotenone-induced oxidative stress by a glutathione-dependent mechanism

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Abstract: Astrocytes maintain homeostasis of neuronal microenvironment, provide metabolic and trophic support to neurons and modulate neuronal responses to injury. Rotenone specifically inhibits mitochondrial complex I, and long exposure to rotenone may increase the risk for Parkinson's disease (PD) and cause Parkinsonism. However, little is known about the role of astrocytes in the process of rotenone-induced dopaminergic neuron injury. In order to investigate this issue, we used MN9D cells as a cell model of dopaminergic neurons and rotenone as a toxin to initiate mitochondrial deficiency. MN9D cells treated with the normal medium or astrocyte-conditioned medium (ACM) were exposed to different concentrations of rotenone for different time followed by cell viability measurement by MTT assay. Besides, various concentrations of ACM and temporally different treatments were devised to evaluate protective efficiency of ACM. Growth curve of cells in the normal medium or ACM was continuously assessed by cell counting for 8 d. The influence of rotenone and ACM on cellular oxidative stress was determined by DCFH-DA staining followed by flow cytometric analysis. Glutathione (GSH) content after treatment of ACM or rotenone was measured by GSH assay kit. Our results showed that rotenone decreased viability of MN9D cells in a dose-dependent manner and ACM treatment significantly attenuated rotenone toxicity at each concentration. No significant difference in growth rate was observed between the normal medium and ACM treatment. Four concentrations of ACM, namely 1/3ACM, 1/2ACM, 2/3ACM and pure ACM, all displayed protection, increasing cell viability to (124.15±0.79)%, (126.59±0.82)%, (125.84±0.61)% and (117.15±1.63)% of the cells exposed directly to rotenone, respectively. Treatment with ACM through the whole experiment except the initial 24 h, 24 h before or at the same time of rotenone addition all exerted protective effects, with cell viability being (110.11±2.52)%, (113.30±2.36)%, (114.42±2.00)% of the cells exposed directly to rotenone, respectively. Conversely, ACM treatment 12 h after rotenone addition had no protective effect, with cell viability being (102.54±1.36)% of the cells exposed directly to rotenone. Moreover, ACM treatment up-regulated GSH level in MN9D cells nearly twofold. Incubation with 100 nmol/L rotenone for 24 h depleted GSH level by nearly two thirds of the control, but ACM treatment mitigated the drop of GSH level, maintaining its content at (147.83±0.63)% of the control. Consistent with GSH change, rotenone administration resulted in a positive rate of 96.24% of DCF staining, implying a great extent of oxidative stress, whereas treatment with ACM reduced the extent of oxidative stress to a positive rate of 78.31%. Taken together, these findings suggest that astrocytes protect MN9D cells from oxidative stress caused by rotenone, and GSH partially accounts for the protection. Therefore, astrocytes may play a protective role in the process of PD.

Key words: astrocytes; oxidative stress; rotenone; glutathione

星形胶质细胞通过谷胱甘肽保护 MN9D 细胞抵抗鱼藤酮所致氧化应激

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摘 要：星形胶质细胞维持神经元微环境，给予营养和代谢支持，并调节其对损伤的反应。鱼藤酮特异阻断线粒体复合物

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I，长期暴露于鱼藤酮可能增加患帕金森病的几率，并引起帕金森综合征。然而，星形胶质细胞在鱼藤酮所致多巴胺能神经元损伤过程中的作用尚无报道。本研究采用多巴胺能神经元细胞系 MN9D 细胞模型，将经过或未经过星形胶质细胞条件培养基处理的 MN9D 细胞暴露于不同浓度的鱼藤酮中，用计数法测生长曲线，MTT 法测细胞活性，DCFH 染色流式细胞仪测氧化应激水平，比色法测还原型谷胱甘肽含量。结果显示，MN9D 细胞在条件和普通培养基培养条件下生长曲线无明显差别；鱼藤酮浓度依赖性地降低细胞活性；不同浓度鱼藤酮作用 24、48 h 后，经条件培养基处理的细胞其活性显著高于普通培养基培养的细胞；不同浓度的条件培养基都有保护作用，纯的条件培养基保护作用稍弱；预先 24 h 条件培养基处理或同时给予鱼藤酮和条件培养基处理都有保护作用，鱼藤酮作用 12 h 后再给予条件培养基则无保护作用；经条件培养基处理的细胞氧化应激水平降低；另外，条件培养基提高了细胞内还原型谷胱甘肽含量，缓解了鱼藤酮所致的谷胱甘肽耗竭。结果提示，星形胶质细胞可保护 MN9D 细胞抵抗鱼藤酮所致的氧化应激，还原型谷胱甘肽可能参与了该保护过程。

关键词：星形胶质细胞；氧化应激；鱼藤酮；谷胱甘肽
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Parkinson's disease (PD) is a common motor disorder of the elderly. The pathological hallmark of PD is a preferential loss of dopaminergic neurons in the substantia nigra pars compacta and concomitant reduction of dopamine content in the striatum to which these neurons project[1]. The mechanism of PD, although poorly understood, is believed to involve an interaction between genetic and environmental factors[2]. Epidemiological studies suggest that exposure to environmental agents, such as pesticides, may increase the risk for PD[3].

As a common lipophilic pesticide, rotenone is also a well-characterized, high-affinity and specific inhibitor of mitochondrial complex I. Distinct from other mitochondrial inhibitors such as 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and paraquat, rotenone does not concentrate in dopamine neurons due to its low affinity for the dopamine transporter. Intravenous or subcutaneous administration of rotenone replicated parkinsonism in rats[4,5], and the latter even caused α-synuclein aggregation, a major pathological feature of PD[5].

Astrocytes play an important role in maintaining the homeostasis of neuronal microenvironment, such as ion homeostasis, uptake of neurotransmitters and the formation of blood-brain barrier[6,7]. Astrocytes are activated in PD characterized by increased expression of glial fibrillary acidic protein (GFAP), enlarged cell body and projections in the injured area[8]. Many lines of evidence suggest a neuroprotective role for activated astrocytes in PD and in experimental models of PD[9,10]. Infusion of IL-1β into the substantia nigra of rats led to increased activation of astrocytes which correlated with the protection of dopaminergic neurons from 6-hydroxydopamine (6-OHDA) toxicity[9]. It is also well recognized that astrocytes possess trophic properties. Relevant to this is the observation that oligodendrocyte-type 2 astrocyte-derived trophic factors increase survival of developing dopamine neurons through inhibition of apoptotic cell death[11]. Additionally, astrocytes are able to protect neurons from nitric oxide toxicity by a glutathione (GSH)-dependent mechanism[12].

However, despite the protective effects of astrocytes enumerated above, little is known about their role in environmental factors leading to PD. To address this question, in the present study MN9D cells were used as a cell model of dopaminergic neurons and rotenone as a toxin to initiate mitochondrial deficiency. In this way, we tested the hypothesis whether astrocyte-conditioned medium (ACM) could protect MN9D cells from rotenone-induced neuronal toxicity.

1 MATERIALS AND METHODS

1.1 *Primary astrocyte culture and conditioned medium collection*

Primary astrocytes were prepared from 1-day-old Sprague-Dawley rats provided by the Animal Center of Capital Medical University. Briefly, the cerebral cortex, striatum and ventral mesencephalon of each brain were isolated in ice-cold PBS. After blood vessels and meninges were removed, tissue was mechanically dissociated and resuspended in Dulbecco's modified Eagle medium/F12 (DMEM/F12) (Gibco) supplemented with 10% newborn calf serum (NCS) (Hyclone) and 1% penicillin/streptomycin. Cell suspensions were centrifuged at 1 000 r/min, resuspended, seeded at a density of 2×10^6 in flasks precoated with poly-*D*-lysine and maintained at 37 °C in 5% CO_2 for 9-14 d. The medium was changed every 3 d. When reaching confluence, mixed glial cultures were placed in a rotary shaker at 250 r/min. 15-18 h later, cells of the surface layer were dislodged and the underlying cell monolayer was predominantly astrocytes. The purity of astrocyte

cultures was determined by double immunofluorescent labeling using anti-GFAP antibody and 4'-6-diamidino-2-phenylindole (DAPI). Our result showed that more than 95% cells were GFAP-immunoreactive (data not shown).

The medium was changed to DMEM/F12 supplemented with 2% NCS, collected 48 h later as ACM, centrifuged at 3 000 r/min for 15 min to remove cellular debris and then stored at -80 °C. ACM was mixed with the normal medium (1:1 in volume) and NCS concentration was adjusted to 10% before use.

1.2 *MN9D cell culture and treatment*

MN9D neuronal cells were a generous gift from Dr. Bastian Hengerer (Novartis Company). MN9D, capable of expressing tyrosine hydroxylase, synthesizing, storing and releasing dopamine, is a hybridoma cell line established by fusing embryonic primary cells from mouse ventral midbrain with cells from mouse neuroblastoma cell line N18TG2. Cells were divided into 4 groups. The first was a control, in which MN9D cells grew for 72 h in the normal medium (DMEM/F12); in the second group, culture medium was replaced by ACM 24 h after initial plating and were cultured in ACM for the next 48 h; in the third group, 48 h after initial plating cells were exposed to rotenone (Sigma) for 24 h; in the fourth group, cells were treated with ACM through the whole experiment except the initial 24 h, 24 h before, at the same time of, or 12 h after rotenone addition, and incubated with rotenone for 24 h totally. Lastly, cells of 4 groups were tested for cell viability, 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining by flow cytometry and GSH content.

1.3 *Immunocytochemistry*

Cells were fixed in 4% paraformaldehyde for 20 min. The fixed cells were washed by PBS three times, immersed in 0.5% Triton X-100 in PBS (PBST) for 10 min, blocked in 5% nonspecific goat serum diluted by PBS, and then incubated with a mouse anti-GFAP monoclonal antibody (Chemicon) overnight at 4 °C. After washed in PBS, cells were incubated with FITC-anti-mouse IgG (Beijing Zhongshan Biotechnology Company) for 30 min at 37 °C, washed with PBS and stained with DAPI. Observations were done under a fluorescent microscope.

1.4 *Cell growth curve assay*

MN9D cells were plated on 24-well culture plates at a density of 5×10^3 cells in 1 mL of complete medium per well. One day later, half of medium in the wells was replaced with ACM, and then cells in the normal medium and ACM were counted respectively. Cell counting was conducted as before for the next 7 d with 3 wells per group.

1.5 *Cell viability assay*

MN9D cells were plated on 96-well culture plates at a density of 5×10^3 cells in 100 μL complete medium per well. After the treatment described in section 1.2, cell viability was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 20 μL of 5 mg/mL MTT solution was added to each well and incubated at 37 °C in 5% CO_2 for 4 h. Then the supernatant was discarded and 100 μL DMSO was used to resolve formazan crystals followed by measurement of absorbance at 570 nm.

1.6 *Measurement of oxidative stress*

MN9D cells were washed with PBS, trypsinized and harvested into DMEM/F12 medium supplemented with 10% NCS. The cell suspension was centrifuged at 1 000 r/min for 10 min before the supernatant was discarded. Then the cell pellets was resuspended in NCS-free medium and centrifuged at 1 000 r/min for 10 min to abandon the supernatant. This process was repeated once, and then cell pellets was resuspended in 1 mL NCS-free medium and 5 μmol/L DCFH-DA was loaded. After incubation at 37 °C for 15 min, cells were rinsed with NCS-free medium three times and analyzed by flow cytometry.

1.7 *Measurement of GSH content*

GSH levels were determined by GSH assay kit (Calbiochem). Cells were harvested into 500 μL ice-cold metaphosphoric acid, homogenized and centrifuged at 3 000 *g*, 4 °C for 10 min. 150 μL aliquots of supernatant were mixed with solution 3 to final volume of 900 μL. The mixture was added 50 μL solution R1 and 50 μL solution R2 successively. After incubation at 25 °C for 10 min in the dark, absorbance at 400 nm was measured.

1.8 *Statistical analysis*

Data were presented as means±SEM of independent experiments. Statistical analyses were performed by SPSS software using one-way analysis of variance (ANOVA) followed by multiple comparison test if appropriate. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Effects of ACM on MN9D cell growth rate

The number of MN9D cells gradually increased over time both in the normal medium and ACM, but no significant differences were found after cell counting for continuous 8 d (Fig.1).

2.2 Effects of ACM on MN9D cell viability

To test the hypothesis whether astrocytes protect MN9D

cells from mitochondrial inhibition and subsequent oxidative stress induced by rotenone, MN9D cells were treated with rotenone in the presence or absence of ACM. While exposure to rotenone for 24 h decreased cell viability in a dose-dependent manner, treatment with ACM greatly attenuated such rapid viability decline, with higher cell viability than that in the corresponding normal medium at each rotenone concentration. Similar protective trend was also shown when MN9D cells were incubated with various concentrations of rotenone for 48 h (Fig.2).

To assess protective effects of ACM at different concentrations, pure ACM was mixed with DMEM/F12 by different volume ratios, namely 1:2, 1:1 and 2:1, respectively. ACM at 3 different concentrations as well as the pure one were used to determine whether the protective effects of ACM are concentration-dependent. Our data indicated that viability of MN9D cells treated with 4 kinds of ACM were (124.15±0.79)%, (126.59±0.82)%, (125.84±0.61)% and (117.15±1.63)% of the cells exposed directly to 100 nmol/L rotenone for 24 h, suggesting all the concentrations of ACM protected against rotenone toxicity, but the pure one was less effective than the other three (Fig.3).

While exposure to 100 nmol/L rotenone for 24 h decreased cell viability to about (71.14±2.21)% of the control, treatment with ACM through the whole experiment except the initial 24 h, 24 h before or at the same time of rotenone administration all significantly alleviated the decrease in cell viability without statistical difference among these 3 groups. However, addition of ACM 12 h after rotenone administration did not show any protective effect. Besides, the protective effects of ACM in the above three methods did not significantly differ from each other (data not shown). Accordingly, we added ACM from cortex 24 h before and at the same time of rotenone addition in the following experiments as a protective model (Fig.4).

2.3 Effects of rotenone and ACM on cellular oxidative stress in MN9D cells

DCFH-DA is a specific probe for reactive oxygen species (ROS). The principle of this assay is that DCFH-DA diffuses through cell membrane and is enzymatically hydrolyzed by esterase to become non-fluorescent DCFH, which reacts with ROS to form a fluorescent product, DCF. In the present study, incubation with 100 nmol/L rotenone for 24 h resulted in a positive rate of 96.24% of DCF staining, implying a great extent of oxidative stress, whereas treatment with ACM reduced the extent of oxidative stress to 78.31% (Fig.5).

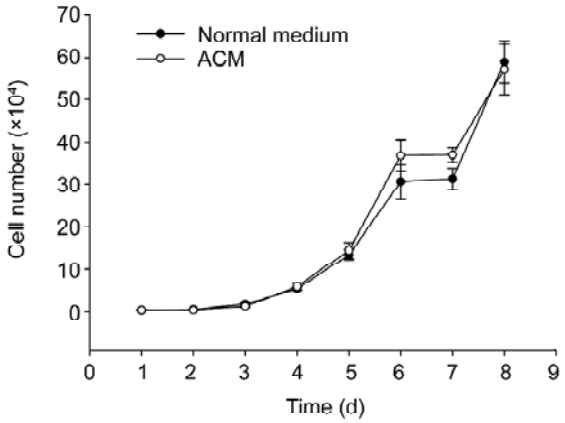

Fig. 1. Astrocyte-conditioned medium (ACM) did not affect growth rate of MN9D cells. MN9D cells were cultured in the normal medium and ACM, respectively. There was no significant difference in growth rate after cell counting for continuous 8 d (P>0.05). Data were means±SEM of three independent experiments.

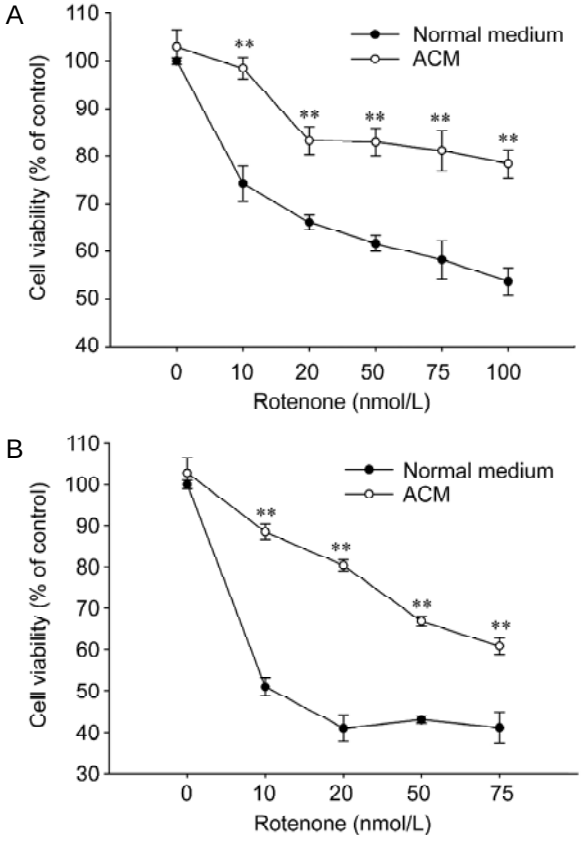

Fig.2. Astrocyte-conditioned medium (ACM) protected MN9D cells against rotenone toxicity. Exposure to rotenone for 24 h or 48 h decreased cell viability of MN9D cells in a dose-dependent manner, but treatment with ACM greatly attenuated such rapid viability decline, with higher cell viability than that in the corresponding control at each rotenone concentration. *A*: 24 h. *B*: 48 h. Data were means±SEM of three independent experiments expressed as percentage of the control. $^{**}P$<0.01 *vs* normal medium.

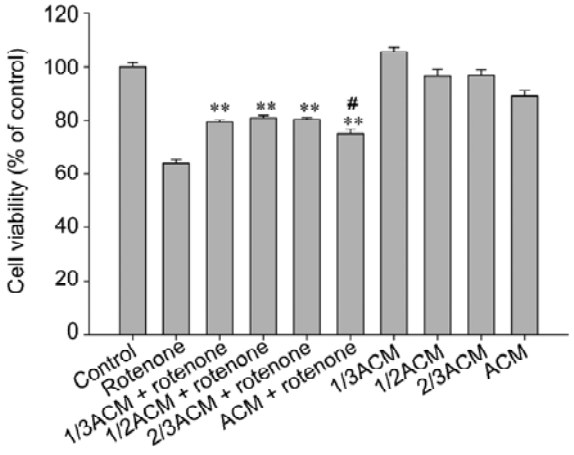

Fig. 3. Comparison of protective effects of astrocyte-conditioned medium (ACM) at different concentrations. 1/3ACM, 1/2ACM, 2/3ACM and pure ACM all attenuated decrease in cell viability caused by 24-hour incubation with 100 nmol/L rotenone, but the pure one was less effective than the other three. Data were means±SEM of three independent experiments expressed as percentage of the control. $^{**}P<0.01$ *vs* rotenone; $^{\#}P<0.05$ *vs* 1/3ACM + rotenone, 1/2ACM + rotenone and 2/3ACM + rotenone.

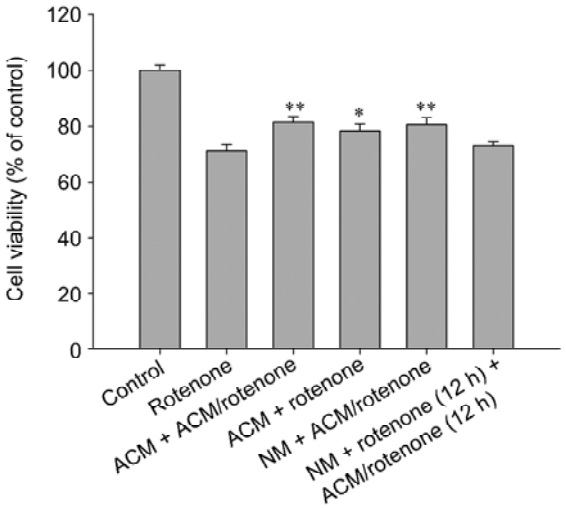

Fig.4. Effects of temporally different astrocyte-conditioned medium (ACM) treatments on cell viability. MN9D cells were treated with ACM through the whole experiment except the initial 24 h (ACM + ACM/rotenone), 24 h before rotenone addition (ACM + rotenone), at the same time of rotenone addition (NM + ACM/rotenone) or 12 h after rotenone addition [NM + rotenone (12 h) + ACM/rotenone (12 h)]. After incubation with 100 nmol/L rotenone for 24 h, cell viability was estimated using MTT assay. Data were means±SEM of three independent experiments expressed as percentage of the control. $^{*}P<0.05$, $^{**}P<0.01$ *vs* rotenone. NM, the normal medium.

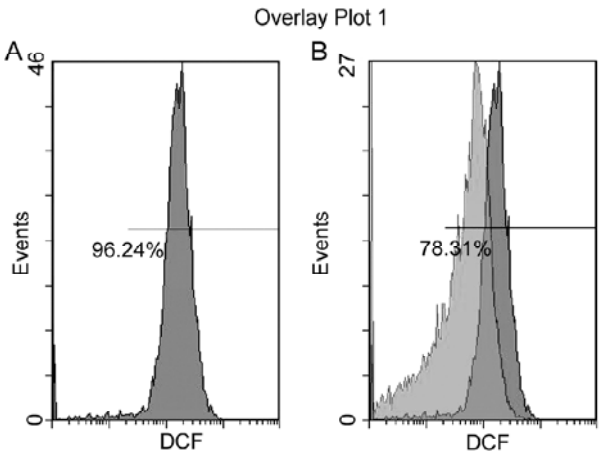

Fig.5. DCFH-DA assay by flow cytometry. MN9D cells treated with or without astrocyte-conditioned medium (ACM) were incubated with 100 nmol/L rotenone for 24 h before stained by DCFH-DA. *A*: Without ACM treatment; *B*: With ACM treatment.

2.4 Effects of rotenone and ACM on cellular GSH content in MN9D cells

GSH is one of important components of antioxidant machinery in the central nervous system. In our experiments, cellular GSH content was increased nearly twofold when cells were treated with ACM alone. However, the addition of 100 nmol/L rotenone to MN9D cells decreased GSH level to 33% of the control. By contrast, ACM treatment attenuated rotenone-induced GSH depletion, with its content being about 150% of the control (Fig.6).

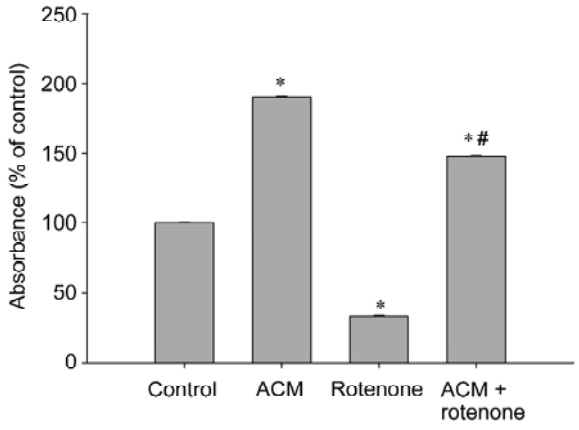

Fig.6. Astrocyte-conditioned medium (ACM) treatment increased GSH levels in MN9D cells and alleviated subsequent rotenone-induced GSH depletion. GSH levels were determined by a GSH assay kit. Data were means±SEM of three independent experiments expressed as percentage of the control. $^{*}P<0.01$ *vs* control. $^{\#}P<0.01$ *vs* rotenone.

3 DISCUSSION

Our results showed that viability of MN9D cells treated with ACM is higher than that of the cells directly exposed to rotenone at different concentrations and for different incubation time. To exclude the possibility that the increased cell viability is due to distinct cell growth rate, we compared cell growth curve both in the normal medium and ACM and did not find significant difference between them. Therefore, it can be concluded that ACM did not increase cell viability by accelerating cell growth. In addition, 1/3ACM, 1/2ACM and 2/3ACM mitigated rotenone-induced cell viability decline more effectively than pure ACM, suggesting that some active agents in ACM may have their own optimal concentration range, beyond which they could not exert protective effects thoroughly. Moreover, four temporally different treatments were used to compare the protective effects of ACM. The first was to pretreat MN9D cells with ACM for 24 h and then change to fresh medium immediately before rotenone exposure. The second was to change the medium to ACM at the same time of rotenone administration. The third was to treat cells with ACM through the whole experiment except the initial 24 h. The fourth was to treat cells with ACM 12 h after rotenone exposure and incubate for another 12 h at the same concentration of rotenone. Our data indicated that the former three methods of ACM treatment all displayed protection against rotenone toxicity without significant difference, but the last one did not function at all. Perhaps 12 h of incubation with rotenone was long enough to bring about some irreversible deleterious events in MN9D cells, so ACM treatment could not reverse or mitigate such damage. It seemed that ACM exerted its protection only before toxin exposure or at the early phase of cell injury. Our work was similar to that of Amano *et al*.[13], but the latter showed ACM protected N18-RE105 hybrid cells against glutamic acid only when ACM was given before, not at the same time of glutamic acid addition. This variance was probably ascribed to different cell model, toxin or mechanism of protection.

Inhibition of mitochrondrial complex I by rotenone leads to ROS production, because a site of electron leak exists upstream of the rotenone-binding site in mitochrondrial complex I[14]. Many lines of evidence suggested that oxidative stress, compared with bioenergetic defects, was a primary mechanism of rotenone toxicity, as only rotenone was toxic when rotenone and deoxyglucose caused similar extent of ATP loss[15]. Moreover, antioxidants such as α-tocopheral and coenzyme Q_{10} blocked rotenone toxicity *in vitro* and were potential therapies for PD[16]. In the present study, MN9D cells treated with ACM produced less ROS than the cells without ACM treatment when exposed to rotenone according to DCFH-DA staining by flow cytometric assay, which meant ACM could attenuate rotenone-induced oxidative stress.

There are several mechanisms explaining the neuroprotective effects of ACM against oxidative stress caused by rotenone. A metabolic link about GSH exists between neurons and astrocytes[17]. Astrocytes can release GSH by multidrug resistance associated protein (MRP), and then GSH is digested by γ-glutamyltranspeptidase (γ-GT), present on membrane of astrocytes, to form dipeptide cysteinylglycine (CysGly), on which neurons depend for *de novo* GSH synthesis. As a result, small antioxidants such as GSH or its precursor in ACM could be utilized by MN9D cells to scavenge reactive oxygen/nitrogen species induced by rotenone. Our results also showed that ACM exposure alone produced a significant increase in GSH content and partially attenuated subsequent rotenone-induced GSH depletion. Further evidence of the role of GSH in protection is that GSH-deficient astrocytes fail to enhance neuronal survival and protect against extra toxin[12,18,19].

Not only can ACM enhance level of small antioxidant molecules like GSH, but also it may strengthen activity of antioxidant enzyme. Iwata-Ichikawa *et al*. found that unidentified diffusible factors in ACM enhance neuronal resistance to 6-OHDA and H_2O_2 by increasing transcription of *L*-glutamate: *L*-cysteine γ-ligase (GCL), a rate limiting enzyme of GSH synthesis[20]. This process is mediated by activation of antioxidant response element (ARE), a *cis*-acting regulatory element located in the 5' flanking region of *GCL* genes. Since ARE regulates many protective genes other than *GCL*, such as NAD(P)H:quinone oxidoreductase (*NQO1*), and glutathione *S*-transferase (*GST*), the possibility can not be excluded that ACM could upregulate these genes to combat rotenone-induced oxidative stress[21].

In conclusion, the current study reported that astrocytes protect against rotenone-induced oxidative stress. Because both oxidative stress and astrocytes play important roles in the pathogenesis of PD, a better understanding of the downstream signaling mechanism of astrocyte protection may uncover new therapies for PD.

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