

# Adiponectin ameliorates the apoptotic effects of paraquat on alveolar type II cells via improvements in mitochondrial function

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**Abstract.** Previous studies have demonstrated that excessive reactive oxygen/nitrogen species (ROS/RNS)-induced apoptosis is an important feature of the injury to the lung epithelium in paraquat (PQ) poisoning. However the precise mechanisms of PQ-induced dysfunction of the mitochondria, where ROS/RNS are predominantly produced, remain to be fully elucidated. Whether globular adiponectin (gAd), a potent molecule protective to mitochondria, regulates the mitochondrial function of alveolar type II cells to reduce PQ-induced ROS/RNS production remains to be investigated. The current study aimed to investigate the precise mechanisms of PQ poisoning in the mitochondria of alveolar type II cells, and to elucidate the role of gAd in protecting against PQ-induced lung epithelium injury. Therefore, lung epithelial injury was induced by PQ co-culture of alveolar type II A549 cells for 24 h. gAd was administrated to and removed from the injured cells in after 24 h. PQ was observed to reduce cell viability and increase apoptosis by ~1.5 fold in A549 cells. The oxidative/nitrative stress, resulting from ROS/RNS and disordered mitochondrial function were evidenced by increased O<sub>2</sub><sup>•-</sup>, NO production and reduced mitochondrial membrane

potential ( $\Delta\Psi$ ), adenosine 5'-triphosphate (ATP) content in PQ-poisoned A549 cells. gAd treatment significantly reversed the PQ-induced cell injury and mitochondrial dysfunction in A549 cells. The protective effects of gAd were partly abrogated by an adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor, compound C. The results suggest that reduced  $\Delta\Psi$  and ATP content may result in PQ-induced mitochondrial dysfunction of the lung epithelium, which constitutes a novel mechanism for gAd exerting pulmonary protection against PQ poisoning via AMPK activation.

## Introduction

Previous studies have demonstrated that apoptosis is an important process in injured to the lung epithelium in paraquat (PQ) poisoning (1,2). The mechanisms of apoptosis are highly complex and involve oxidative/nitrative stress impairment to the function of mitochondria with the reduction of the mitochondrial membrane potential ( $\Delta\Psi$ ), resulting in an increase of superoxide formation and reduced production of adenosine 5'-triphosphate (ATP) (3,4). Previous studies suggest that adenosine 5'-monophosphate-activated protein kinase (AMPK) is one of the most important molecules in the stimulation of ATP production and protection against oxidative/nitrative stress in mitochondria (5,6). However, whether dysfunction of the mitochondria and reduction of ATP via inactivation of AMPK serve crucial roles in the PQ-induced apoptosis of alveolar epithelial type II cells remains to be fully elucidated.

Adiponectin (APN), an adipocytokine predominantly secreted by adipocytes and found at high levels in the plasma, has been reported to exhibit anti-insulin resistance and anti-inflammatory and anti-apoptotic properties (7-9). Circulating APN is present as trimeric, hexameric or oligomeric complexes of monomers, and cleavage to produce the C-terminal globular domain, gAd, has been proposed as an important regulatory step in the action of APN. This is due to the fact that this C-terminal fragment has been reported to mediate potent physiological effects (10,11). Previous studies have suggested that the potential role of gAd against apoptosis

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**Abbreviation:** ROS/RNS, reactive oxygen/nitrogen species; PQ, paraquat; gAd, globular adiponectin; ATP, adenosine 5'-triphosphate; AMPK, adenosine 5'-monophosphate-activated protein kinase

**Key words:** paraquat, gAd, reactive oxygen/nitrogen species, apoptosis, AMPK

is mediated via the activation of AMPK (12-14). APN-knockout mice exhibit inhibition of AMPK, a reduction in the levels of ATP, an increase of mitochondrial swelling and ROS/RNS production, resulting in augmented apoptosis, while administration of gAd reversed these effects. The protective effects of gAd have been reported to be reversed by administration of the AMPK inhibitor (compound C) (15). It remains unclear whether APN may protect alveolar type II cells from PQ poisoning via activation of AMPK and increasing the production of ATP in mitochondria.

Therefore, the aims of the current study were: i) To determine whether mitochondrial function is impaired and ATP is reduced in PQ-poisoned alveolar type II cells via inactivation of AMPK; ii) to determine the effect of gAd against PQ-induced apoptosis and in alveolar type II cells; and if so, iii) whether gAd mediates protective effects via activation of AMPK and increasing production of ATP.

## Materials and methods

**Materials.** All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. RPMI 1640 medium was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Penicillin/streptomycin was obtained from Wisent, Inc. (Saint-Bruno, QC, Canada). PQ was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The globular domain of APN (gAd) was purchased from Beijing Adipobiotec, Inc. (Beijing, China). Compound C, an AMPK Inhibitor, was obtained from Merck Millipore (Darmstadt, Germany). The Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit I was from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). JC-1 and dihydroethidium (DHE) were purchased from Molecular Probes; Thermo Fisher Scientific, Inc. The Nitrate/Nitrite Colorimetric Assay kit was from Nanjing Jiancheng Biochemical Reagent Co. (Nanjing, China). The ATP Bioluminescent Somatic Cell assay kit was from Sigma-Aldrich.

**Cell cultures and treatments.** The human lung adenocarcinoma cell line A549 (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) was grown in a monolayer culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, 1% (v/v) streptomycin/penicillin. The culture medium was refreshed two to three times per week and was subcultured upon reaching 80% confluence. The cells were then seeded onto flat-bottom six-well or 96-well plates with growth medium at 37°C in a humidified atmosphere at 5% CO<sub>2</sub>, followed by administration of PQ at 300 µM for 24 h for the PQ, PA and PCA groups. Subsequently, gAd (2.5 µg/ml) was added and the cells were cultured for another 24 h, in order to investigate the role of gAd in PQ-induced cytotoxicity. PCA group cells were also treated with compound C (1 µM) for 30 min prior to the addition of PQ. The optimal doses and time points for gAd, PQ and compound C were determined through preliminary experiments (data not shown).

**Measurement of cell viability.** The 3-(4, 5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted as previously described (16). Briefly, following PQ

challenge, culture media was refreshed with media containing MTT reagent (5 mg/ml) and cells were incubated under standard conditions for an additional 4 h. The culture media was then carefully aspirated and 100 µl dimethylsulfoxide was added per well to solubilize the formazan crystals. Following agitation, absorbance was measured spectrophotometrically at a wavelength of 490 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Viabilities of the challenged cells were expressed relative to control cells.

**Measurement of necrosis and apoptosis.** Apoptotic and necrotic cell death were measured using flow cytometry with the Annexin V-FITC Apoptosis Detection kit I (17). At 24 h after administration of gAd, cells were trypsinized (Thermo Fisher Scientific, Inc.) without ethylenediaminetetraacetic acid and labelled with the fluorochromes in the absence of light for 10 min at room temperature. Fluorescence was measured by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) to monitor green fluorescence (525 nm band-pass filter) for Annexin V-fluorescein conjugate and red fluorescence (575 nm band-pass filter) for PI respectively. The Annexin V-FITC-/PI-cell population was regarded as normal, while Annexin V-FITC+/PI-cells were taken as an indicator of early apoptosis, Annexin V-FITC+/PI+ as late apoptosis and Annexin V-FITC-/PI+ as necrosis. The data were analyzed using CellQuest software (BD Biosciences). A minimum of 30,000 gated events were acquired per sample.

**Measurement of superoxide production.** Production of superoxides was evaluated intracellularly using the superoxide-sensitive dye DHE (18). DHE is oxidized by superoxides to a novel product, which binds to DNA enhancing intracellular fluorescence. Culture medium was aspirated and cells were incubated with DHE (5 µM final concentration) for 10 min at 37°C in the dark. Following two rinses in phosphate-buffered saline, cells were photographed using an Axiostart 50 (Zeiss, Oberkochen, Germany) microscope equipped with a Canon PowerShot G5 epifluorescence attachment (Canon, Inc., Tokyo, Japan). Between five and six photographs were captured from each well. The fluorescence intensity values for each photo were determined using Adobe Photoshop software (version 4; Adobe Systems, Inc., San Jose, CA, USA).

**Measurement of nitrites.** Nitrites were measured in the culture supernatants by a colorimetric assay using a procedure based on the Greiss reaction with sodium nitrite as the standard (19). Culture medium was collected and stored at -80°C until analysis with the Nitrate/Nitrite Colorimetric Assay kit, according to the manufacturer's instructions.

**Measurement of  $\Delta\Psi$ .** The membrane potential assay was conducted as previously described (20). The relative  $\Delta\Psi$  was assessed using a laser scanning confocal microscope (SP8; Leica Microsystems GmbH, Wetzlar, Germany) analysis of cells stained with JC-1. Cells were incubated with 10 µg/ml JC-1 for 30 min at 37°C. Subsequent to applying the dye, the cells were scanned with the confocal microscope using a 10x objective lens. Fluorescence was excited by the 488-nm

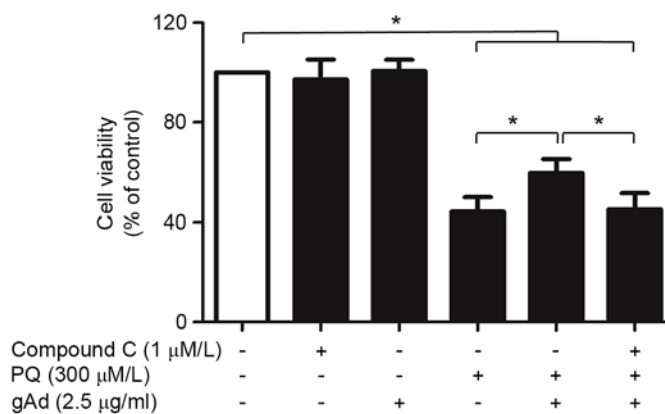


Figure 1. Cellular-protective effect of gAd on PQ-induced A549 cells. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C) pretreatment. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed at 24 h subsequent to the administration of gAd. The data are presented as the mean  $\pm$  standard deviation of five independent experiments, \* $P$ <0.05. gAd, globular adiponectin; PQ, paraquat.

line of an argon laser and the 543-nm line of a helium/neon laser. The red emission of the dye is due to a potential-dependent aggregation in the mitochondria, reflecting high  $\Delta\Psi$ . Green fluorescence indicates the monomeric form of JC-1, appearing in the cytosol following mitochondrial membrane depolarization, indicating low  $\Delta\Psi$ . The ratio of red/green indicated the mitochondrial membrane depolarization states of different cells.

**Measurement of ATP.** The amount of ATP was measured in the cultured cells using a luminometer (Bio Orbit 1251 Luminometer; Bio-Orbit, Turku, Finland) as previously described (21). Subsequent to exposure to different experimental conditions, cells were collected to react with different working solutions of from the ATP Bioluminescent Somatic Cell assay kit, according to the manufacturer's instructions. All reactions for a given sample were monitored simultaneously and calibrated with the addition of an ATP standard from the kit.

**Statistics.** Data are presented as the mean  $\pm$  standard error ( $n=5$ ) and were analyzed for statistical significance by one- or two-way analysis of variance with were least significant difference or Student-Newman-Keuls post hoc analyses. Statistical analysis and the significance of the data were determined using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA).  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**gAd reduced PQ-mediated cytotoxicity.** As presented in Fig. 1, PQ significantly reduced the cell viability of A549 cells, while gAd alleviated the PQ-induced cytotoxicity without any effects on the cells alone. However, pretreatment with compound C significantly reversed the protective effects of gAd on the viability reduced by PQ.

**gAd reduced PQ-mediated apoptosis.** The results indicated that there was a low level of cell death in the control group (Fig. 2A). However, PQ was observed to induce apoptosis in ~37.5% cells (Fig. 2B). However, gAd significantly reversed the PQ-induced

cytotoxicity, reducing the percentage of apoptotic cells to 22.3% ( $P$ <0.01). However, the pretreatment with compound C significantly reversed gAd's protective effect (Fig. 2).

**gAd reduced PQ-mediated superoxide production.** As presented in Fig. 3, control cultures generally exhibited low fluorescence intensity while PQ-treated cultures exhibited bright red fluorescence. This indicated that PQ significantly increased  $O_2^{\cdot -}$  production in cells compared with that of the controls ( $P$ <0.001). gAd inhibited the  $O_2^{\cdot -}$  production induced by PQ significantly. However, pre-treatment with compound C partly reversed gAd's effects on  $O_2^{\cdot -}$  production.

**gAd reduced PQ-mediated nitrite production.** Addition of PQ to the cultures significantly increased nitrite production compared with the controls ( $P$ <0.01). Administration of gAd or compound C significantly reversed the increase, however pre-treatment with compound C did not reverse the effects of gAd on nitrite production (Fig. 4).

**gAd reduced PQ-mediated  $\Delta\Psi$  depolarization.** Incubation with PQ depolarized the  $\Delta\Psi$  significantly, resulting in a low ratio of red/green. Treatment with gAd repolarized the  $\Delta\Psi$  of the cells, resulting in a greater ratio of red/green, compared with PQ group ( $P$ <0.01). However, compound C reversed the repolarization of gAd, resulting in a lower ratio of red/green as compared with the gAd group ( $P$ <0.01; Fig. 5).

**gAd partly reversed the PQ-mediated reduction in ATP.** Addition of PQ significantly reduced the intracellular ATP concentration, as compared with the initial control levels: 21% after 24 h, irrespective of the presence of other medium supplements or stimulation ( $P$ <0.05). Conversely, treatment with gAd increased the ATP level to ~50% of that of the control group ( $P$ <0.05). However, compound C reversed the gAd-mediated increase ( $P$ <0.05; Fig. 6).

## Discussion

The most prevalent cause of morbidity and mortality in patients with PQ poisoning is organ injury, in particular, injury to the

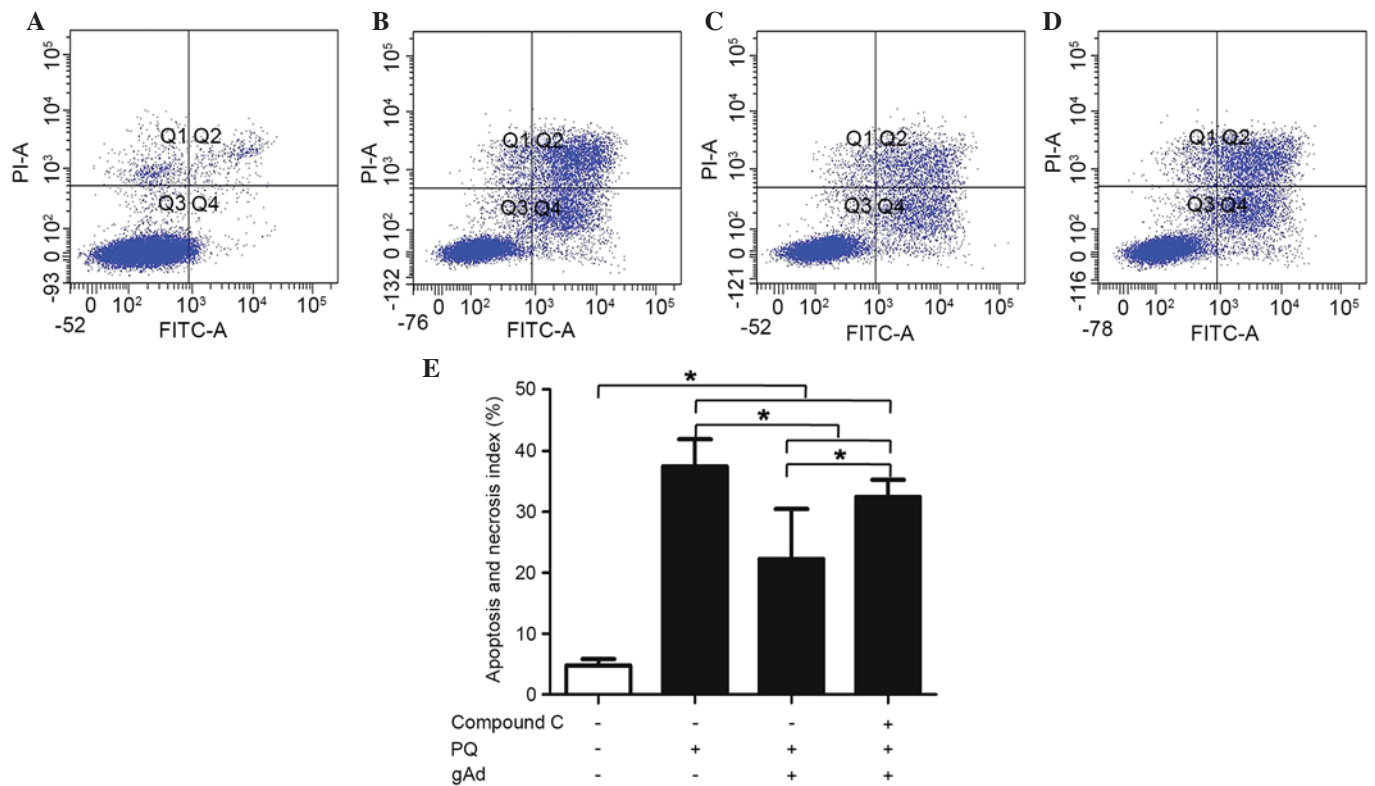


Figure 2. Anti-apoptotic effects of gAd on PQ-induced A549 cells. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C) pretreatment. The flow cytometry assay was conducted 24 h subsequent to administration of gAd. Flow cytometry analysis of the (A) control, (B) PQ, (C) PA and (D) PCA groups are presented. (E) Percentage of apoptosis and necrosis for the four groups are presented as the mean  $\pm$  standard deviation of five independent experiments, \*P<0.05. gAd, globular adiponectin; PQ, paraquat; PI, propidium iodide; FITC, fluorescein isothiocyanate.

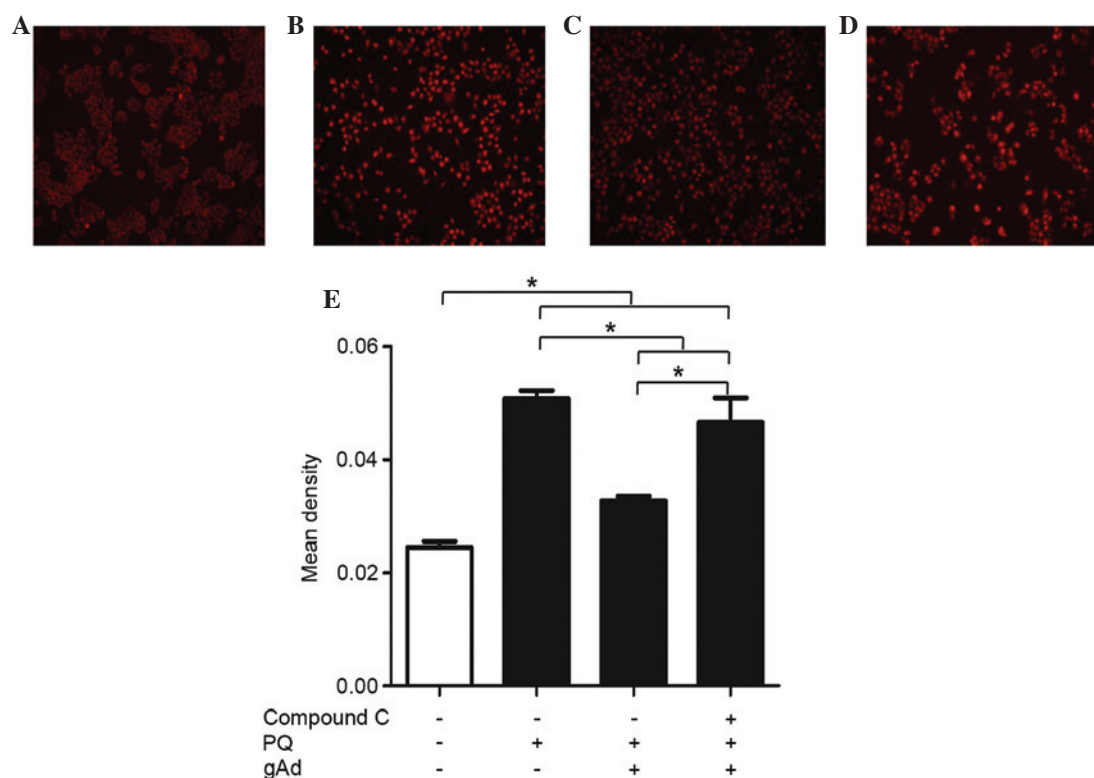


Figure 3. Anti-oxidative stress effects of gAd on PQ-induced A549 cells. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C) pretreatment. The fluorescence intensity of dihydroethidium was performed at 24 h after the administration of gAd. (A) Control group; (B) PQ group; (C) PA group; (D) PCA group; (E) Quantification of fluorescence data. The data are presented as the mean  $\pm$  standard deviation of five independent experiments, \*P<0.05. gAd, globular adiponectin; PQ, paraquat.



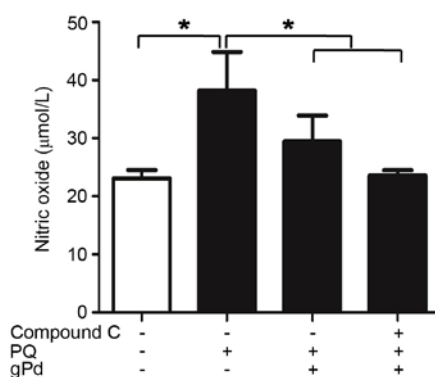


Figure 4. Anti-nitrative stress effects of gAd on PQ-induced A549 cells. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C). A colorimetric assay was performed 24 h subsequent to the administration of gAd. The data are presented as the mean  $\pm$  standard deviation of five independent experiments, \*P<0.05. gAd, globular adiponectin; PQ, paraquat.

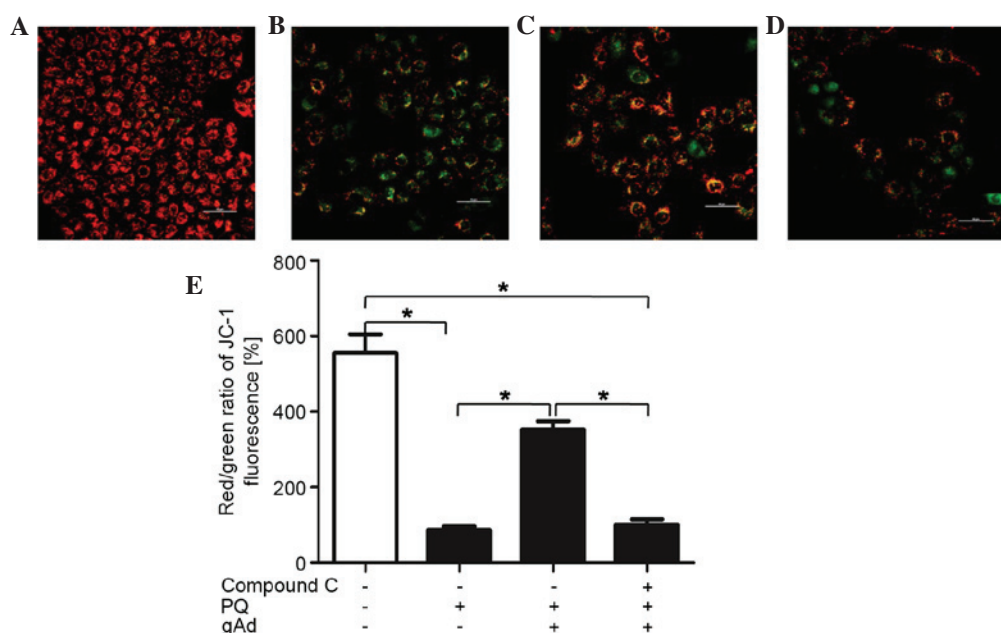


Figure 5. Effect of gAd on PQ-mediated  $\Delta\Psi$  depolarization. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C). Confocal microscopy analysis was performed 24 h subsequent to the administration of gAd on the (A) control, (B) PQ group, (C) PA group and (D) PCA group. The red emission of the dye is due to a potential-dependent aggregation in the mitochondria, indicating high  $\Delta\Psi$ . Green fluorescence indicates the monomeric form of JC-1, appearing in the cytosol subsequent to mitochondrial membrane depolarization, indicating low  $\Delta\Psi$ . The ratio of red/green indicated the mitochondrial membrane depolarization states of different cells. (E) Quantification of the red/green ratio of the four groups is presented as the mean  $\pm$  standard deviation of five independent experiments, \*P<0.05. gAd, globular adiponectin; PQ, paraquat;  $\Delta\Psi$ , mitochondrial membrane potential.

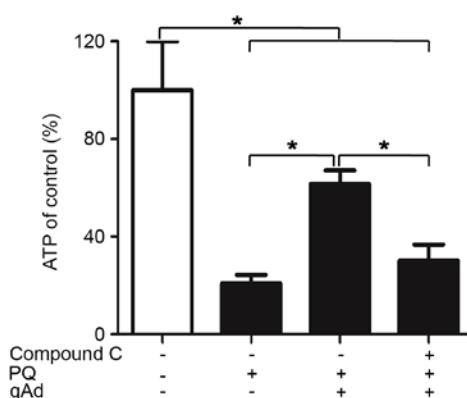


Figure 6. Effect of gAd on PQ-mediated reduction in ATP. Cells were treated with 300  $\mu$ M PQ followed by 2.5  $\mu$ g/ml gAd with or without adenosine 5'-monophosphate-activated protein kinase inhibitor (compound C). The luminometer assay was performed 24 h subsequent to gAd administration. The data are presented as the mean  $\pm$  standard deviation of five independent experiments, \*P<0.05. gAd, globular adiponectin; PQ, paraquat; ATP, adenosine 5'-triphosphate.

lungs (22). It has been demonstrated that excessive ROS/RNS stress resulting from impaired mitochondria, is an important feature of the injured lung epithelium in PQ poisoning, though the underlying mechanism responsible for the mitochondrial dysfunction remains unclear (23). Therefore, studies have focussed upon clarifying the underlying mechanisms for PQ-associated mitochondrial injury of alveolar type II cells (24,25) and developing novel strategies for the treatment of PQ-poisoned patients (26). In the present study, an established PQ poisoning model with A549 cells cultured with PQ for 24 h was used, and it was confirmed that apoptosis and cell death were exacerbated in the presence of PQ. Furthermore, it was indicated that PQ markedly disturbed mitochondrial function, depolarizing  $\Delta\Psi$  and reducing ATP production, resulting in progressive increases of  $O_2^-$  and NO. This may be explained by the fact that  $PQ^{2+}$  induced the release of  $Ca^{2+}$  from cytoplasm (27), which may induce the depolarization of mitochondria and reduce  $\Delta\Psi$  (28). This results in reductions of ATP synthesis to less than 50% of the normal level, thus activating pro-apoptotic and pro-necrotic proteins, including Bax and caspase 3/7/8/9 (29,30). As a result, the injured mitochondria produce excessive  $O_2^-$  and NO, which attached to the mitochondrial membrane in turn, and resulted in increases in ROS/RNS production and ultimately inducing apoptosis (31).

Injury resulting from PQ poisoning was however, reversed by treatment with gAd in the current study. Although the associated mechanisms remain to be fully elucidated, a previous study demonstrated the potential cellular protective effects of gAd against apoptosis via inhibiting mitochondrial dysfunction and ROS/RNS attachment (32). Therefore, the current study suggested that gAd may protect the alveolar type II cells against PQ-induced cell injury via inhibiting mitochondrial dysfunction and reducing oxidative/nitrative injury.

In order to further elucidate the underlying mechanisms for the cellular-protective effects of gAd in PQ poisoning against mitochondrial dysfunction and oxidative/nitrative injury, the signaling of AMPK, a protective kinase in oxidative/nitrative injury and a downstream mediator of gAd's mitochondrial protective actions, was investigated (33). It was identified that the protective effects of gAd could be reversed by an AMPK inhibitor, compound C, which has been previously identified to block the cardiac protective benefits of gAd treatment against cardiac myocardial hypoxia/reoxygenation injury (15). This result clarified the critical role of AMPK in the pneumonocyte-protective effects of gAd, indicating it may be used in the therapy for PQ poisoning. This observation is consistent with a previous study by Yan *et al* (15), which demonstrated that gAd was able to activate AMPK-peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$ , resulting in increased ATP production, improving  $\Delta\Psi$ , thus leading to return to normal function. These results indicate that AMPK may be one of the key molecules in gAd's inhibition of mitochondrial dysfunction induced by PQ. However, interspecies differences (34,35), animal model establishment (36,37) and the injury procedure (38) may affect whether AMPK signaling serves a beneficial or detrimental role in the mitochondria. Experiments using animal models should be conducted in order to further clarify the mechanisms.

There were limitations in the present study. The inactivation of AMPK was not introduced when PQ was added to the cells,

due to the high rate of mortality, thus the ability to directly demonstrate the role of AMPK in PQ-poisoned mitochondria following oxidative/nitrative injury was compromised. In addition, gAd treatment in PQ poisoning may modulate numerous other key molecules in addition to AMPK-ATP (39-41). Thus, investigation of these molecules in oxidative/nitrative injury requires additional investigation.

In conclusion, it was demonstrated that the  $\Delta\Psi$  exhibited progressive reductions with increased oxidative/nitrative injury in PQ-induced apoptosis. In addition, it was identified that in PQ poisoning,  $\Delta\Psi$  is increased in response to gAd treatment, which significantly attenuated mitochondrial dysfunction and oxidative/nitrative injury in PQ-poisoned alveolar type II cells. The present study provided indirect evidence for the possible role of the AMPK-ATP signaling pathway in gAd against oxidative/nitrative injury originating from the mitochondria following PQ poisoning. To the best of our knowledge, this is the first study to demonstrate the ability of gAd in attenuating oxidative/nitrative injury in PQ poisoning of lung cells beyond its metabolic actions. These observations add to the current understanding of the pathophysiology of PQ poisoning and the mechanisms of the widely used gAd therapy. This may aid in the development of future therapeutic strategies capable of assisting gAd in protecting against PQ-induced lung injury.

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