Original Article

Edaravone attenuates paraquat-induced lung injury by inhibiting oxidative stress in human type II alveolar epithelial cells

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BACKGROUND: Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free-radical scavenger and has the antioxidant ability to inhibit lipid peroxidation. The study aimed to examine the effect of edaravone on protecting the acute injury of human type II alveolar epithelial cells (A549 cells) induced by paraquat (PQ) and the change of production of reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD).

METHODS: A549 cells were cultured and divided into PQ group (group P), edaravone-treated group (group E) and normal control group (group C). The cells in group P were exposed to paraquat (600 $\mu\text{mol/L}$), and the cells in group E were treated with edaravone (100 $\mu\text{mol/L}$) additionally, and no drug intervention was given to the cells in group C. Real-time monitoring by LSCM was used to detect the cell response and the intracellular dynamic change of ROS level in A549 cells after administration of PQ and edaravone. And the levels of SOD and MDA were detected respectively by biochemistry colorimetry. Data were expressed as mean $\pm$ standard error of the mean. Statistical analysis was carried out with the soft SPSS 16.0.

RESULTS: The concentration of intracellular ROS significantly increased when PQ was given to A549 cells. But after administration of edaravone, the concentration of intracellular ROS was decreased. Compared to the PQ group, the levels of SOD in the edaravone group were significantly increased while the levels of MDA were markedly decreased.

CONCLUSIONS: Paraquat can increase the oxidative stress, and induce the lipid peroxidation of A549 cells. Edaravone has the effect to scavenge reactive oxygen species, and to protect against the PQ-induced lung toxicity.

KEY WORDS: Paraquat; Intracellular reactive oxygen species; Edaravone; A549 cells; Poisoning

World J Emerg Med 2012;3(1):55-59
DOI: 10.5847/wjem.j.1920-8642.2012.01.010

INTRODUCTION

Paraquat (N,N-dimethyl-4-4'-bipyridinium, PQ) is a widely used herbicide. The chemical composition of paraquat is $\text{C}_{12}\text{H}_{14}\text{N}_2$ and its molecular weight is 186.3. The lipid hydroperoxide induced by paraquat destroys cell membranes and kills the tissue of green plants via an oxidative process, which is initiated by the formation

of superoxide as a result of the suppressed reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the course of photosynthesis when exposed to this herbicide. In humans, intentional or accidental ingestion of PQ is frequently fatal, resulting from significant lung injury.^[1,2] The most common cause of death from PQ poisoning is respiratory due to an oxidative

insult to the pulmonary parenchyma with subsequent pulmonary fibrosis.^[3] Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) as a potent free-radical scavenger has the antioxidant ability to inhibit lipid peroxidation.^[4] The purpose of this study was to investigate the effect of edaravone against reactive oxygen species (ROS) in A549 cells induced by paraquat.

METHODS

Drug and reagents

A549 cells were granted by the Cancer Center of Union Hospital, Wuhan. RPMI 1640 medium was purchased from Gibco Company. Paraquat (99% purity), trypsinase, and dimethyl sulfoxide were purchased from Sigma Company. Fetal bovine was purchased from Hangzhou Sijiqing Bioengineering Company. Superoxide dismutase (SOD) and malonyldialdehyde (MDA) kits were purchased from the Nanjing Jiancheng Bioengineering Research Institution. Apoptotic detection kit was purchased from Antgene Biotechnology Company. Reactive Oxygen Species Assay Kit was purchased from the Beyotime Institute of Biotechnology. Edaravone injection was from the Nanjing Xian Sheng Dongyuan Pharmaceutical Co., Ltd.

Cells culture and groups

Human type II alveolar epithelial cells (A549 cells) were maintained in endothelial cell growth medium (RPMI 1640) supplemented with 10% heat inactivated fetal bovine serum at 37 °C in a humid atmosphere of 5% CO₂. The medium was changed every other day. While the cells grow in 80% of the bottom area and 0.25% in trypsin digests and 1: 2-3 subcultures, the number of growing cells was used in the following experiments. A549 cells were collected and divided into PQ group (group P), edaravone-treated group (group E), and normal control group (group C). The cells in group P were exposed to paraquat (600 µmol/L), and the cells in group E were treated with edaravone (100 µmol/L) additionally, and no drug intervention was given to the cells in group C.

Reactive oxygen species (ROS) detected by LSCM

The activated A549 cells were plated on small glass cover slips in 6 wells culture dishes at a density of 5×10⁶/mL, and incubated in RPMI-1640 medium for 24 hours. After A549 cells were loaded with 5 µl of DCFH-DA (1: 1000 dilution) for 30 minutes at 37 °C under a mixed gas containing 95% air and 5% carbon dioxide, intracellular ROS was measured using an MRC-1024 type laser scanning

confocal microscope (LSCM, USA). The dynamic changes in individual A549 cell intracellular ROS after stimulation with 600 µmol/L paraquat and 100 µmol/L edaravone were recorded using LSCM and a computer. The concentration of A549 cell intracellular ROS was measured in terms of intracellular fluorescent intensity (FI). The change of ROS in A549 cells was examined with a LSCM using a 20 × objective lens and linear scan mean.

Measurement of superoxide dismutase content

The A549 cells were cultured in 6-well plates and divided into groups P, E, and C. After 12 hours, the cells were collected into the tubes. Cell lysis was performed by means of 3 cycles of freezing and thawing. SOD content was measured by the thiobarbituric acid assay according to the manual of the kit and was calculated according to the following formula:

$$\text{SOD (U/mL)} = \frac{\text{OD of NCG} - \text{OD of exp.}}{\text{dilution multiples} \times \text{sample dilution}} \times 1$$

Measurement of malonyldialdehyde content

The A549 cells were cultured in 6-well plates and divided into groups P, E, and C. After 24 hours, the cells were collected into the tube. Cell lysis was performed by means of 3 cycles of freezing and thawing. MDA content was measured by the thiobarbituric acid assay according to the manual of the kit and calculated according to the following formula:

$$\text{MDA (nmol/mL)} = \frac{\text{OD of exp.} - \text{OD of Bla.}}{\text{con. 10 nmol/mL} \times \text{sample dilution}} \times 2$$

Assay for apoptosis

Assay for apoptosis was performed as described by Monsigny. Apoptotic cells were quantified by annexin V-FITC and propidium iodide (PI) double staining, using a kit purchased from Antgene Biotechnology Company. The cells were harvested and resuspended in binding buffer at a cell density of 5×10⁶/L. Then they (100 µL) were transferred to the tubes added with 5 µL of annexin V-FITC and 10 µL of PI. After incubation for 15 minutes at room temperature in the dark, 400 µL of binding buffer was added into the tubes. The samples were analyzed to apoptosis rate (AR) by flow cytometry.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for windows. To compare three or more groups, one-way ANOVA was used. To compare two groups, matched *t*-test was used. *P*<0.05 was considered statistically significant.

RESULTS

Effects of edaravone on intracellular ROS in A549 cells

Intracellular ROS was increased by addition of paraquat (600 $\mu\text{mol/L}$), as compared to the stimulation-free state (FI: 564.8637 ± 89.50969 in stimulation-free state vs. 971.4825 ± 52.6687 after paraquat stimulation, $P < 0.005$). Experimental results showed that intracellular ROS of A549 cells decreased significantly after the administration of edaravone (100 $\mu\text{mol/L}$), as compared to the stimulation of paraquat (FI: 569.6635 ± 50.01203 after administration of edaravone vs. 971.4825 ± 52.6687 after paraquat stimulation, $P < 0.005$) (Figures 1 and 2). These data indicated that edaravone could inhibit oxidation stress.

Effect of EDA on release of SOD and contents of MDA in A549 cells

The SOD activity decreased in the extract of

A549 cells treated with PQ after 12 hours, indicating a damage to the cellular membrane. The SOD activity in the extract of A549 cells obviously increased after treatment with edaravone, showing that edaravone protected cellular membrane against damage induced by PQ (Table 1).

While A549 cells were exposed to PQ for 24 hours, the MDA level in A549 cells was higher than that of the control group. When the cells were treated with the indicated concentration of edaravone, a significant reduction of MDA was observed.

Table 1. Changes of every group on the aspect of oxidative damage (mean $\pm$ SD, $n=3$)

Groups	SOD (U/mL)	MDA (nmol/mL)
C	172.32 ± 5.485	7.60 ± 0.29
P	$88.78 \pm 3.382^*$	$18.32 \pm 0.17^*$
E	$142.55 \pm 2.58^{*#}$	$10.45 \pm 0.53^{*#}$

Compared with group C, $^*P < 0.05$; compared with group E, $^{\#}P < 0.05$.

Figure 1. The change of intracellular of product of ROS after stimulation with paraquat. With the addition of 600 $\mu\text{mol/L}$ paraquat at 600 seconds, the level of intracellular ROS in A549 cells increased gradually.

Figure 2. The change of intracellular of product of ROS after administration of edaravone. When paraquat was administered at a final concentration of 100 $\mu\text{mol/L}$, the concentration of ROS in A549 cells increased to a peak level, and then gradually reduced at 780 seconds after the administration of edaravone (100 $\mu\text{mol/L}$).

Figure 3. Apoptotic rates of cells in the three groups. The apoptosis rates of groups P, E and C were significantly different ($P < 0.05$).

Effect of EDA on PQ-induced apoptosis in A549 cells

Apoptotic cells were quantified by annexin V-FITC and PI double staining. Annexin V-FITC positive cells were dramatically increased after treatment with PQ for 12 hours. The apoptosis rates of groups C, P and E were $(3.56 \pm 0.34)\%$, $(34.98 \pm 0.85)\%$ and $(19.27 \pm 0.52)\%$, respectively ($P < 0.05$). The results showed that PQ induces apoptosis of A549 cells. Edaravone can inhibit apoptosis induced by PQ.

DISCUSSION

Paraquat, a well-known herbicide, causes severe injury to the lungs and other organs mediated by toxic reactive oxygen species^[5,6] as well as lipid peroxidation that may result in cell death.^[7,8] ROS as a natural byproduct of the normal metabolisms of oxygen may play important roles in cell signaling. However, during paraquat poisoning, ROS levels increase dramatically and result in a significant damage to cell structures. This cumulates into a situation known as oxidative stress. Paraquat is unusually toxic to the lung, presumably due to its selective uptake by lung cells and localized redox cycling in the alveolar epithelium.^[9,10] Damage caused by PQ to the alveolar epithelium results in alveolitis and further extensive lung fibrosis.^[11] In the current study, Nordberg et al^[12,13] found that the potential mechanism for PQ toxicity is the production of superoxide radicals. The study also indicated that exposure to PQ increased the intracellular production of ROS^[14] and was injurious to a variety of biomolecules such as DNA, lipids, and proteins. According to this point as well as the intracellular metabolic process, alveolar macrophages and other inflammatory cells are possible sources of ROS that may damage the lung epithelium.^[15] The chemical cascades leading to reduction of paraquat, the generation of free radicals, and lipid peroxidation are the main factors leading to lung tissue damage.

Redox cycling has been recognized as an important pathway by which cells can utilize oxygen to generate ROS.^[16] NADPH oxidase catalyzes the transfer of electrons from NADPH to oxygen, resulting in the formation of superoxide anion. Superoxide anion rapidly dismutates into hydrogen peroxide.^[17] In further studies, we characterized the paraquat-stimulated NADPH oxidase activity in lung cell lysates. The one-electron reduction of paraquat by this enzyme would be expected to generate paraquat radicals. Paraquat was also found to stimulate production of superoxide anion and hydroxyl

radicals. The accumulation of ROS was dependent on NADPH.^[18]

Therefore, living cells developed various mechanisms to protect cellular constituents against oxidative damage. Superoxide dismutases (SODs) are a ubiquitous family of enzymes efficiency catalyzing the dismutation of superoxide anions to molecular oxygen and hydrogen peroxide according to the following equation: $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$.^[19,20] The primary function of SODs is the detoxification of cell-damaging superoxide anions, but they also play important roles in phytopathogenesis since oxidative stress is an important component of the organism defense response against microbial invasion.^[21]

More importantly, ROS may attack mitochondria to induce the creation of apoptosome, which cleaves procaspase-3 to form caspase-3 and activates the apoptosis cascade.^[22] Caspase-3 has been shown to be the end product for both external and internal apoptotic cascades.^[23] The caspase-3 protein level reflects the extent of apoptosis. The PQ-induced increase in cleaved caspase-3 protein indicates that the triggering of apoptosis cascades leading to cell death is likely one of the most important mechanisms of PQ cytotoxicity.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) as a potent free-radical scavenger has the antioxidant ability to inhibit lipid peroxidation.^[4] To study the antioxidant effect of edaravone, the levels of ROS, SOD and MDA were measured in the study as indicators of oxidative stress. The increased ROS level after stimulation with paraquat was detected by LSCM. The endogenous protective antioxidant SOD was consumed by the burst of ROS, thus leading to a significant reduction of SOD activity. The increased levels of A549 cells MDA showed that ROS caused cell injury by compromising the integrity of cell membrane via the oxidation of membrane phospholipids (lipid peroxidation). However, treatment with edaravone significantly decreased the levels of ROS and MDA, while the level of endogenous SOD of the cells was significantly increased. Accordingly, in the present study, a paraquat-induced lung injury model was used with A549 cells to investigate the ability of edaravone to inhibit pulmonary injury and attenuate ROS. Lifshitz et al^[24,25] found that edaravone acts as a radical scavenger, a stimulator of prostaglandin (PG) production, an inhibitor of lipoxigenase and a protector against cell membrane damage.

In conclusion, edaravone protection of A549 cells injuries by paraquat induces a significant reduction in free radical precursors and their metabolites compared

with untreated controls. Furthermore, this study could be helpful in basic research and clinical treatment of paraquat poisoning.

Funding: This work was supported by a grant from the Natural Science Foundation of China (Grant No.C030105).

Ethical approval: Not needed.

Conflicts of interest: The authors have no financial or other conflicts of interest regarding this article.

Contributors: Cheng ZQ proposed and drafted the manuscript. All authors read and approved the final manuscript.

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Received September 19, 2011

Accepted after revision February 10, 2012