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Paraquat Induces Apoptosis through a Mitochondria-Dependent Pathway in RAW264.7 Cells

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Abstract

Paraquat dichloride (N,N-dimethyl-4-4′-bipiridinium, PQ) is an extremely toxic chemical that is widely used in herbicides. PQ generates reactive oxygen species (ROS) and causes multiple organ failure. In particular, PQ has been reported to be an immunotoxic agrochemical compound. PQ was shown to decrease the number of macrophages in rats and suppress monocyte phagocytic activity in mice. However, the effect of PQ on macrophage cell viability remains unclear. In this study, we evaluated the cytotoxic effect of PQ on the mouse macrophage cell line, RAW264.7 and its possible mechanism of action. RAW264.7 cells were treated with PQ (0, 75, and 150 μM), and cellular apoptosis, mitochondrial membrane potential (MMP), and intracellular ROS levels were determined. Morphological changes to the cell nucleus and cellular apoptosis were also evaluated by DAPI and Annexin V staining, respectively. In this study, PQ induced apoptotic cell death by dose-dependently decreasing MMP. Additionally, PQ increased the cleaved form of caspase-3, an apoptotic marker. In conclusion, PQ induces apoptosis in RAW264.7 cells through a ROS-mediated mitochondrial pathway. Thus, our study improves our knowledge of PQ-induced toxicity, and may give us a greater understanding of how PQ affects the immune system.

Key Words: Paraquat, RAW264.7 cells, Apoptosis, Mitochondria, ROS

INTRODUCTION

The field of immunotoxicology has greatly progressed over the last several decades; however, the immunotoxic effects of agrochemical compounds, such as pesticides, remain unclear and require further study (Neishabouri *et al*., 2004; Riahi *et al*., 2010). This is especially important, because several widely used agricultural products, including organochlorine, organophosphate, and carbamate pesticides, are immunotoxic (Riahi *et al*., 2010).

Paraquat dichloride (N,N-dimethyl-4-4′-bipiridinium, PQ), a non-selective and non-systemic herbicide, has been used worldwide for several decades; however, its immunotoxic potential remains unclear (Schenker *et al*., 2004). Studies have shown that PQ exerts its immunotoxic effects by suppressing Concanavalin A (Con A)-induced rat splenocyte proliferation, decreasing thymic and splenic weight, and reducing the splenic cellular content (Caroleo *et al*., 1996; Schenker *et al*., 2004). PQ was also shown to inhibit lipopolysaccharide (LPS)-induced lymphocyte proliferation, interferon (IFN)-γ production, and monocyte phagocytosis (Styles, 1974; Riahi *et al*., 2010). Combined, these data support an immunotoxic role for PQ.

Macrophages are immune cells that play an essential role in antigen presentation and phagocytosis, thereby mediating immune responses. As a result, macrophages play a key role in the initiation of adaptive immunity, regulate many physiological and immunological functions, and are susceptible targets for chemical oxidants (Zirk *et al*., 1999; Deng *et al*., 2013). PQ dose- and time- dependently decreases the number of viable peritoneal and alveolar macrophages (Styles, 1974; Dusinska *et al*., 1998). Furthermore, PQ dysregulates macrophage activity and decreases phagocytosis(Dusinska *et al*., 1998). However, the effects of PQ-induced cytotoxicity on macrophage cell viability and its mechanism of action remains unclear.

In this study, we evaluated the underlying mechanism of PQ-induced cytotoxicity in macrophages. We hypothesized that PQ induces apoptosis via the intrinsic mitochondrial pathway. RAW264.7 cells were treated with PQ and assessed for changes in mitochondrial membrane potential (MMP), the ex-

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pression of cleaved caspase-3 and Bax/Bcl-2, and intracellular reactive oxygen species (ROS) levels. Our study established a novel role for PQ in RAW264.7 cell apoptosis, and elucidated the mechanism of PQ cytotoxicity.

MATERIALS AND METHODS

Materials

PQ was purchased from Sigma Chemical Co. (St Louis, MO, USA). PQ was dissolved in distilled water (100 mM) and was diluted to final concentrations of 75 and 150 μM. Phosphate buffered saline (PBS), fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco (Carlsbad, CA, USA). The Cell Counting Kit 8 was purchased from Dojindo Molecular Technologies (Dojindo Lab, Tokyo, Japan). SuperScript® III First-Strand Synthesis System and Trizol® reagent were purchased from Invitrogen (Carlsbad, CA, USA). iQ™ SYBR® Green Supermix was purchased from BIO-RAD (Hercules, CA, USA). The anti-β-actin, Bcl-2, Bax, and cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-caspase-3 antibody and all secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The Mitochondria Isolation Kit for Mammalian Cells was purchased from Thermo Scientific (Hudson, NH, USA). Carboxy-H_2DCF-DA and the MitoProbe JC-1 Assay Kit for flow cytometry were purchased from Molecular Probes (Eugene, OR, USA). The Annexin V/FITC-PI apoptosis detection kit for flow cytometry was purchased from BD Biosciences (San Jose, CA, USA).

Cell culture and treatment

RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cells were cultured in a humidified CO_2 incubator at 37°C. When adherent cells became confluent, the cells were treated with 0, 75, or 150 μM PQ for 24 h.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit 8. RAW264.7 cells were plated in 96-well plates (5×10^3 cells/well) for 24 h, and then treated with PQ (0-200 μM) for 24 h. CCK-8 (10 μL) was added to the treated cells and incubated for 1 h at 37°C in dark. Absorbance was measured at 450 nm with FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices; Sunnyvale, CA, USA).

DAPI staining for apoptosis

RAW264.7 cells were plated in 4-well plates (5×10^4 cells/well) for 24 h, and then treated with PQ (0, 75, or 150 μM) for 24 h. Cells in each well were fixed in 4% formaldehyde for 15 min at 37°C. Triton X-100 (0.5%) was added, and cells were incubated for 15 min at 37°C. Finally, cells were incubated with 2 nM DAPI staining solution for 10 min in the dark. Apoptotic cells were observed through confocal microscopy (LSM 710 Meta Confocal Microscope, Zeiss, Welwyn Garden City, UK).

Annexin-V staining assay

RAW264.7 cells were plated in 6-well plates (5×10^5 cells/well) for 24 h, and then treated with PQ (0, 75, or 150 μM) for 24 h. The cells were then collected, washed with ice-cold PBS, and suspended in 1 mL Annexin V binding buffer containing 5 μL Annexin V-FITC and propidium iodide (PI). Cells were incubated for 15 min at room temperature in the dark. The fluorescence was measured using a FACS Calibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson Immunocytometry System; Franklin Lakes, NJ, USA), and fluorescence data was collected from 10,000 cells.

Measurement of ROS generation

Intracellular ROS levels were determined using a fluorometric assay (DCF-DA assay). RAW264.7 cells were plated in 6-well plates (5×10^5 cells/well) for 24 h, and then treated with PQ (0, 75 or 150 μM) for 24 h. Cells were then incubated with 10 μM carboxy-H2DCF-DA at 37°C for 60 min. The plates were washed three times with PBS, and the fluorescence was measured using flow cytometry, as described above. The fluorescence intensity is proportional to the amount of ROS produced by the cells.

MMP assay

MMP (ψ_m) was measured using the JC-1 Detection Kit according to the manufacturer's instructions. RAW264.7 cells were plated in 6-well plates (5×10^5 cells/well) for 24 h, and then treated with PQ (0, 75, or 150 μM) for 24 h. Cells were incubated with 2 μM JC-1 for 30 min at 37°C in the dark, and then analyzed by flow cytometry. The JC-1 fluorescence was also measured by confocal microscopy. Cells were plated in 24-well plates (5×10^4 cells/well) for 24 h, and then treated with PQ for 24 h. Cells were then incubated with 2 μM JC-1 for 30 min, as described above. JC-1 fluorescence was photographed with a confocal microscope. JC-1 is a fluorescent carbocyanine dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm; monomer) to red (~590 nm; dimer). Consequently, a decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. At a highly polarized MMP, JC-1 aggregates into dimers and emits red fluorescence; however, when MMPs are depolarized (such as during apoptosis), it forms monomers and emits green fluorescence. Thus, the cells with decreased MMP were indicated by a higher percentage of green fluorescence from JC-1 monomers.

RNA isolation, cDNA synthesis and quantitative real-time PCR amplification

Real-time PCR was performed to analyze gene mRNA expression in treated RAW264.7 cells. Cells were plated in 6-well plates (5×10^5 cells/well) for 24 h, and then incubated with PQ (0, 75, or 150 μM) for 24 h. RNA was extracted using Trizol reagent. For quantitative real-time PCR, 0.1 μg of the total RNA was reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR. Real-time PCR was performed in duplicate using the MyiQ single-Color Real-Time PCR Detection System and an iQ SYBR Green Supermix (Bio-Rad). Relative expression was normalized to GAPDH levels. The primer sequences used were: GAPDH, forward (5′-TTCACCACCATGGAGAAGGC-3′) and reverse (5′-GGCATGGACTGTGGTCATGA-3′); Bax, forward (5′-TGCAGAGGATGATTGCTGAC-3′) and reverse (5′-GATCAGCTCGGGCACTTTAG-3′); Bcl-2, forward (5′-CAGATGCCG GTTCAGGTACT-3′) and reverse (5′-AAGCTGTCACAGAGGGGC-

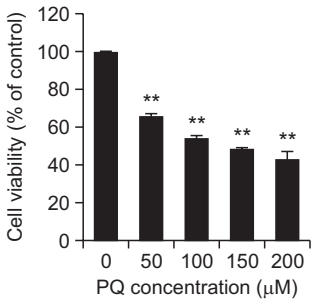

Fig. 1. Effect of PQ on RAW264.7 cell viability. Cell viability was assessed using the CCK method. Cells were treated with various concentrations of PQ for 24 h, followed by the addition of 10 μL CCK to each well. Following 1 h incubation at 37°C, absorbance was measured at 450 nm with microplate reader. The cell viability was expressed as the optical density percentage of the treatment versus the control. ***p*<0.01 indicates a significant difference as compared to the control group.

TA-3′). The thermal cycling conditions were pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 63.5°C for 15 sec, and extension at 72°C for 20 sec.

Western blot analysis

The expression of Bax, Bcl-2, and caspase-3 was detected by Western blot. PQ-treated RAW264.7 cells were harvested and lysed in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitor cocktail tablets [Roche]) on ice. The protein concentrations of the cytosolic extracts were measured using the BCA protein assay with bovine serum albumin as standard. Equal amounts of protein extracts were separated on acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a PVDF membrane and incubated with specific primary antibodies (rabbit polyclonal anti-Bcl-2, rabbit polyclonal anti-Bax, rabbit polyclonal anti-Caspase-3) overnight at 4°C. Each membrane was washed three times, and then incubated with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies for 1 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody. Immunodetection was performed using Amersham™ ECL™ western blotting detection reagents (GE Healthcare, Chalfont St. Giles, UK). Expression levels were quantified by densitometry, followed by normalization to the β-actin control. The signals were analyzed and quantified by Image J software (NIH, Bethesda, MD, USA).

Analysis of cytochrome c release

Mitochondria were isolated with the Mitochondria Isolation Kit for Mammalian Cells according to the manufacturer's instructions. Protein concentrations were measured by the BCA protein assay, and 25 μg protein was loaded to an SDS-PAGE gel and transferred to PVDF, as described above. The primary antibody, mouse monoclonal anti-cytochrome C, was added overnight at 4°C, and HRP-labeled anti-mouse secondary antibody was incubated for 1 h at room temperature. Immunodetection was performed using Amersham™ ECL™ western blotting detection reagents.

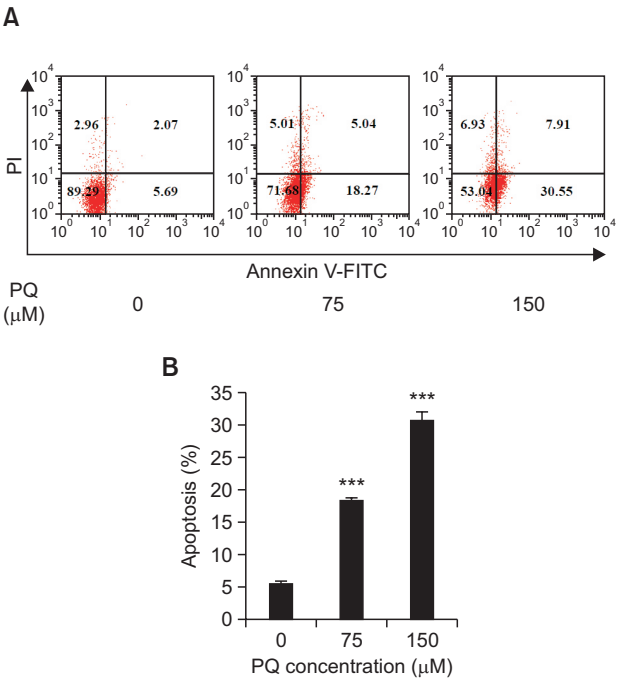

Fig. 2. Effect of PQ on the apoptosis of RAW264.7 cells. Cells were treated with various concentrations of PQ for 24 h, followed by Annexin V-FITC/PI double staining to evaluate apoptosis. The degree of apoptotic cell death was quantified by measuring fluorescence with a FACS Calibur™ flow cytometer. Data represent the mean ± SD of at least three independent experiments. ****p*<0.001 indicates a significant difference as compared to the control group.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using a one-way analysis of variance (ANOVA) and Tukey's test. Statistical significance was accepted when the *p*-value was less than 0.05.

RESULTS

PQ decreases the viability of RAW264.7 cells

To detect the effects of PQ on RAW264.7 cell viability, the cells were incubated with PQ (0-200 μM) for 24 h (Styles, 1974; Cappelletti *et al*., 1998; Mitsopoulos and Suntres, 2010, 2011; Cheng *et al*., 2012; Kang *et al*., 2013; Wang *et al*., 2014). After incubation, cell viability was evaluated using a CCK-8 assay. As shown in Fig. 1, PQ dose-dependently decreased cell viability. Treatment of RAW264.7 cells with 150 μM PQ for 24 h reduced cell viability approximately 50%.

PQ induces apoptosis in RAW264.7 cells

To quantify PQ-induced apoptosis, we performed a fluorometric analysis after annexin V-FITC/PI double staining. The rate of apoptosis in control cells was low (5.69%; Fig. 2). However, apoptotic cells, as indicated by Annexin V-FITC positive cells, were dose-dependently increased by PQ (18.27 and 30.55% compared to the control; Fig. 2). To investigate the effects of PQ on the nucleus, cells were stained with DAPI. PQ induced prominent nuclear changes in treated cells (Fig. 3).

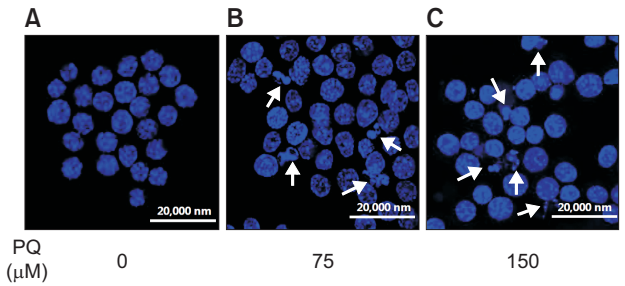

Fig. 3. Effect of PQ on nuclear alterations in RAW264.7 cells. PQ induces apoptosis in RAW264.7 cells. Cells were incubated with various concentration of PQ for 24 h. Nuclear alterations were examined using DAPI staining and photographed by confocal microscopy. RAW264.7 cells with 0 (A), 75 (B), 150 μM (C) PQ (magnification, 400 ×).

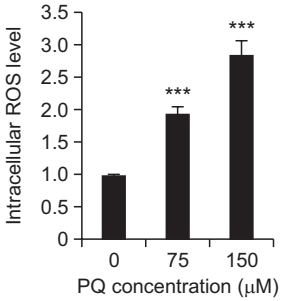

Fig. 4. PQ induced ROS generation in RAW264.7 cells. Cells were treated with various concentration of PQ for 24 h. After treatment, the cells were incubated with 10 μM carboxy-H_2DCF-DA for 60 min, and the intracellular ROS levels were measured by flow cytometry. The fluorescence intensity is proportional to the amount of ROS produced by the cells. Data represent the mean ± SD of at least three independent experiments. ***p<0.001 indicates a significant difference as compared to the control group.

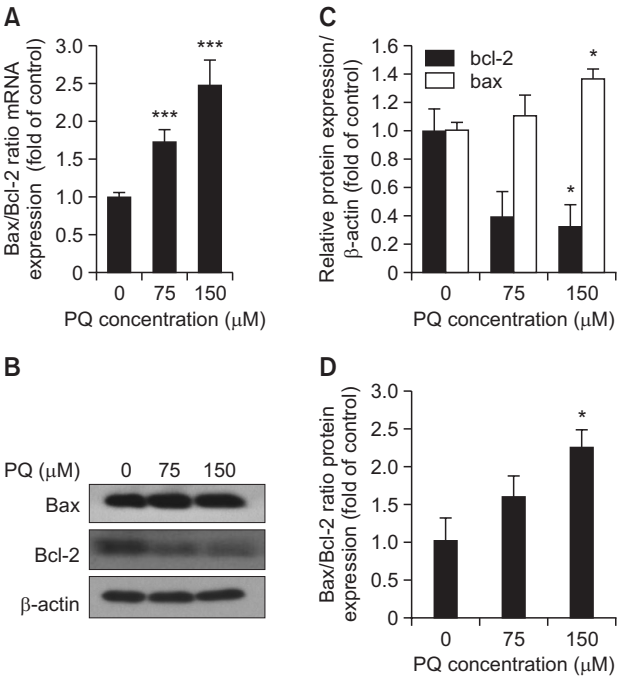

Fig. 5. Effects of PQ exposure on mRNA and protein expression levels of Bax/Bcl-2 in RAW264.7 cells. Cells were treated with various concentrations of PQ for 24 h. Quantitative Bax/Bcl-2 mRNA levels were normalized to GAPDH. Protein levels of Bax and Bcl-2 were evaluated by immunoblot, followed by densitometry analysis relative to β-actin expression. The data revealed an increase in Bax and decrease in Bcl-2 protein expression. Data are expressed as a percent of the control, and are the mean ± SD; n=3. *p<0.05, ***p<0.001 indicate a significant difference as compared to the control group.

The nuclei were round, intact, and uniformly stained in control cells; however, PQ exposure induced nuclear condensation, resulting in smaller nuclei that displayed membrane blebbing and fragmentation as the cells died. At 75 and 150 μM PQ, a number of cells exhibited nuclear condensation, membrane blebbing, and apoptotic bodies. Importantly, these aberrant nuclear alterations were not observed in control cells. Thus, PQ induced apoptosis in RAW264.7 cells.

PQ increases intracellular ROS levels in RAW264.7 cells

To investigate whether ROS mediate PQ-induced apoptosis, we measured the intracellular ROS levels after PQ treatment. Because PQ is known to induce ROS formation through the activation of enzymes initiating redox cycling of the herbicide, it would not be unexpected that ROS generation mediates apoptosis (Ali *et al*., 1996; Castello *et al*., 2007; Mussi and Calcaterra, 2010). We used carboxy-H_2DCF-DA, a ROS-sensitive fluorometric probe, to measure ROS production by flow cytometry. As shown in Fig. 4, PQ treatment significantly enhanced ROS generation in RAW264.7 cells.

PQ induces apoptosis by decreasing the Bax/Bcl-2 ratio

The Bcl-2 family, including Bcl-2, Bcl-X_L, Bax, and Bad, regulates various steps in apoptosis. Bcl-2 and Bcl-X_L inhibit cell death, whereas Bax and Bad promote programmed cell death (Shi *et al*., 2010). To further investigate whether PQ modulates the expression of pro- and anti-apoptotic genes, we measured the mRNA and protein expression levels of Bax and Bcl-2 in RAW264.7 cells after PQ treatment. After normalization to GAPDH, the expression level of Bcl-2 mRNA was dramatically decreased. Furthermore, compared to the control group, the Bax/Bcl-2 ratio was significantly and dose-dependently increased (Fig. 5A). Bcl-2 and Bax protein levels were also measured by western blot (Fig. 5B). Our results showed that the band corresponding to Bcl-2 (26 kDa) decreased in a dose-dependent manner. However, the pro-apoptotic protein, Bax (23 kDa), was increased (Fig. 5C).

PQ decreases mitochondrial membrane potential ($\Delta\psi_m$) in RAW264.7 cells

To investigate whether changes in MMP regulate PQ-induced apoptosis, we evaluated the effect of PQ on the MMP. After PQ treatment, the fluorescence of JC-1 was measured by flow cytometry or confocal microscopy. As shown in Fig. 6, the control cells exhibited strong red fluorescence with a high MMP, whereas the PQ treated cells showed green fluorescence, due to the presence of monomeric JC-1. Thus, our data indicate that PQ decreases the MMP, leading to mitochondrial depolarization.

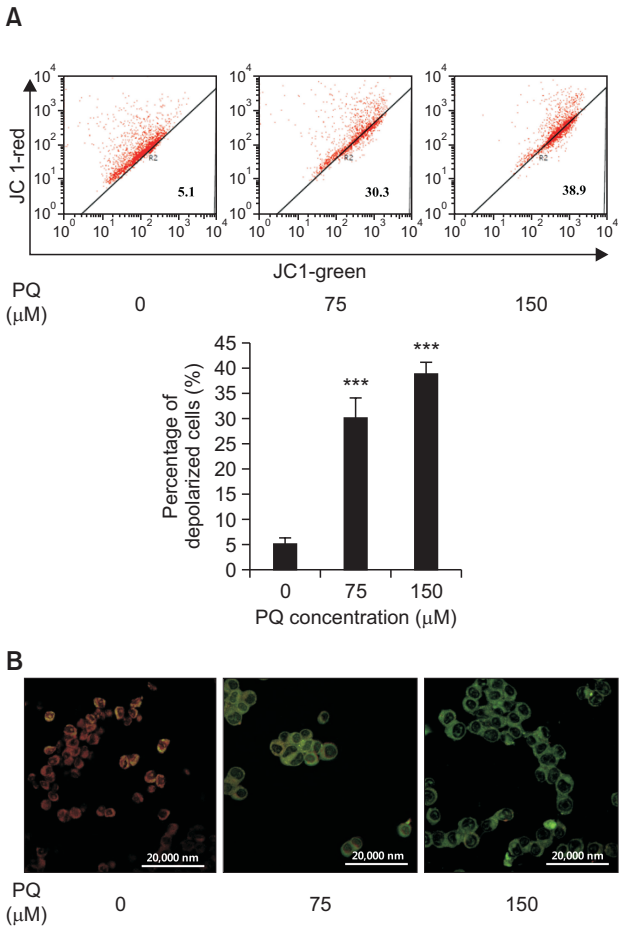

Fig. 6. Effects of PQ on mitochondrial membrane potential (MMP) depolarization in RAW264.7 cells. Following PQ treatment, cells were incubated with JC-1 (2 μM) for 30 min. Labeled cells were analyzed for changes in MMP by flow cytometry, with an excitation at 514 nm and emission at 529 nm (green) or 590 nm (red) (A) and confocal microscopy (B). Data represent the mean ± SD of at least three independent experiments. ***p<0.001 indicates a significant difference as compared to the control group.

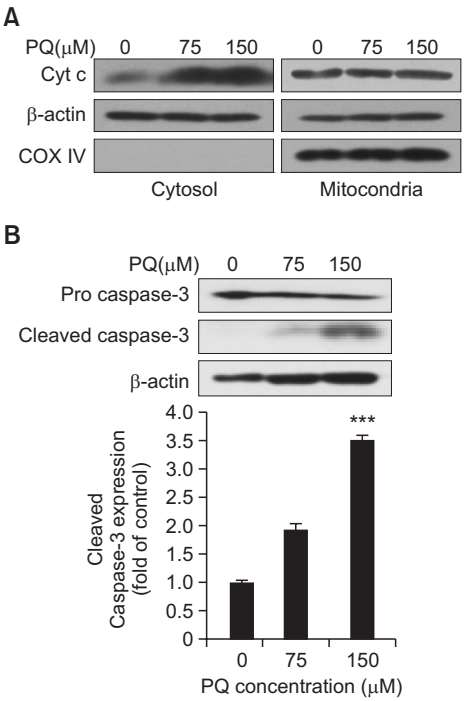

Fig. 7. Effects of PQ on apoptosis-associated proteins in RAW 264.7 cells. Cells were treated with PQ for 24 h, and then harvested for western blot to examine the protein levels of cytochrome c (Fig. 7A) and caspase-3 (Fig. 7B). Densitometry was performed following normalization to β-actin. Data represent the mean ± SD of at least three independent experiments. ***p<0.001 indicates a significant difference as compared to the control group.

PQ precedes the release of cytochrome c from mitochondria into cytosol and activation of caspase-3 in RAW264.7 cells

To determine if loss of MMP results in the release of cytochrome c, we evaluated cytochrome c content. After PQ treatment, mitochondria were separated from the cytosol, and cytochrome c was analyzed by western blot. As shown in Fig. 7A, cytochrome c appeared in the cytosol after PQ treatment. However, cytochrome c content in the mitochondria was not altered. Depolarization of MMP triggers the release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c oxidase activity was identical in the mitochondrial extracts, and was absent in the cytosolic extracts. Caspase proteins play a pivotal role downstream of the Bcl-2 family by initiating cellular breakdown during apoptosis. In the intrinsic cell death pathway, which is mediated by mitochondria, cytochrome c activates caspase-9, which then activates caspase-3. To determine whether caspase-3 is activated after PQ treatment, the cells were harvested for western blot after exposure to PQ. As shown in Fig 7B, PQ increased the protein levels of cleaved caspase-3, indicating that PQ promotes the release of cytochrome c from the mitochondria, thereby activating caspase-3.

DISCUSSION

Although PQ is a widely used herbicide in agriculture, there is little information regarding its influence on the immune system. Some studies have shown that PQ has an immunosuppressive effect (Caroleo *et al*., 1996; Riahi *et al*., 2010; Riahi *et al*., 2011). Furthermore, the presence of PQ in the immune and hematopoietic systems, including the bone marrow, spleen, and thymus, has been reported (Riahi *et al*., 2011). PQ was shown to significantly decrease the viability of rat peritoneal and alveolar macrophages (Styles, 1974). More recently, a study demonstrated that PQ suppresses monocyte and granulocyte phagocytosis. Thus, it is clear that PQ can enter the host immune system and suppress macrophage activity. However, the specific mechanisms of PQ action have not been elucidated.

Although it is widely accepted that PQ generates ROS, such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), its detailed toxic actions at the single cell level have not yet been fully described (Ali *et al*., 1996; Castello *et al*., 2007; Mussi and Calcaterra, 2010). In the present study, we provide *in vitro* evidence indicating that oxidative stress promotes PQ-induced macrophage death

via mitochondrial damage and caspase-3 activation, thereby leading to apoptosis. We used RAW264.7 cells isolated from the ascites of BALB/c mice to investigate the relationship between PQ and cell apoptosis (Deng *et al*., 2013). Our results revealed that PQ induced the intrinsic apoptotic pathways in RAW264.7 cells.

This study showed, for the first time, that the PQ-mediated cytotoxic effects in RAW264.7 cells were associated with increased intracellular ROS levels (Fig. 4), MMP depolarization (Fig. 6), release of cytochrome c (Fig. 7), and the activation of caspase-3 (Fig. 7). As shown in the present study, PQ increased intracellular ROS levels and decreased MMP (Fig. 4 and 6). Immune cells, especially phagocytic cells, such as macrophages and neutrophils, use ROS to support their functions. Thus, low levels of ROS are essential for daily survival (Boxer *et al*., 1979; Riahi *et al*., 2011; Victor *et al*., 2004). Nevertheless, ROS overproduction can induce cellular damage (Victor *et al*., 2004; Rio and Velez-Pardo, 2008). Phagocytic cells are particularly sensitive to oxidative stress because of the high proportion of polyunsaturated fatty acids in their plasma membranes and their high ROS production, which contribute to damage. Oxidative injury to the mitochondrial membrane can also occur, resulting in membrane depolarization and, ultimately, mitochondrial damage, release of cytochrome c, activation of caspases and resultant apoptosis (Hughes, 1999; Victor *et al*., 2003, 2004). Thus, oxidative stress induced by PQ is likely an important mechanism of PQ-induced cytotoxicity and immunotoxicity.

The CCK-8 assay indicates that PQ dose-dependently induces cytotoxicity in RAW264.7 cells (Fig. 1). This observation is consistent with previous studies that showed PQ-induced cytotoxicity in human lymphocytes, A549 cells, U2 cells, and THP-1 cells (Lee *et al*., 1993; Don Porto Carero *et al*., 2001; Rio and Velez-Pardo, 2008). In previous studies, the PQ concentration varied from 1 μM to 10 mM (Styles, 1974; Cappelletti *et al*., 1998; Mitsopoulos and Suntres, 2010, 2011; Cheng *et al*., 2012; Kang *et al*., 2013; Wang *et al*., 2014). In particular, in some studies on apoptosis, high concentrations of PQ, ranging from 100 μM to 800 μM, were used for 24 h (Cappelletti *et al*., 1998; Cheng *et al*., 2012; Kang *et al*., 2013). In this study, we used a lower concentration range for PQ treatments (0, 75, 150 μM). From the CCK-8 assay in our study, the highest concentration of PQ (150 μM) caused a 50% decrease in cell viability compared to the control group. Additionally, the induction of apoptosis was examined through flow cytometry after Annexin V/PI staining. PQ dose-dependently increased the percentage of apoptotic cells (Fig. 2).

Cell apoptosis is a highly regulated process of programmed cell death that is important for modulating cell numbers (Fiers *et al*., 1999; Fadeel and Orrenius, 2005). Many intrinsic and extrinsic factors, especially caspases (Fuchs and Steller, 2011; Mao *et al*., 2013) and Bcl-2 superfamily proteins (Martinou and Youle, 2011) regulate apoptosis (Fuchs and Steller, 2011). PQ has been reported to induce apoptosis in some mammalian cells (Cappelletti *et al*., 1998; Li and Sun, 1999; Rio and Velez-Pardo, 2008). In the present study, a significant decrease in Bcl-2 was observed after exposure to PQ for 24 h (Fig. 5). The Bax/Bcl-2 ratio was increased, leading to apoptosis. PQ also dose-dependently increased the cleavage of caspase-3 (Fig. 7). The mitochondria, a critical intracellular organelle, plays an essential role in the regulation of apoptosis (Green and Kroemer, 2004; Orrenius, 2004; Mao *et al*., 2013). ROS-induced MMP depolarization is involved in apoptosis related to mitochondrial dysfunction (Chipuk *et al*., 2006). MMP depolarization induces a permeability transition, allowing for the release of pro-apoptotic factors, including cytochrome c, from mitochondria into the cytosol, thereby triggering caspase-dependent or caspase-independent cytosolic signaling events (Green and Kroemer, 2004; Orrenius, 2004; Mao *et al*., 2013). In this study, exposure of RAW264.7 cells to PQ induced MMP depolarization. The present study suggests that mitochondrial injury may be associated with PQ-mediated RAW264.7 cells apoptosis. ROS are known to induce the intrinsic apoptotic cascade through interactions with proteins of the mitochondrial permeability transition complex (Tsujimoto and Shimizu, 2007; Mao *et al*., 2013), and have been suggested to play an important role as a mediator in apoptosis (Circu and Aw, 2010). PQ has been reported to produce oxidative stress in the rat midbrain and various cell lines, such as human lung carcinoma cells, hepatocytes, and retinal pigmented epithelial cells (Castello *et al*., 2007; Haley, 1979; Lu *et al*., 2006; Kim *et al*., 2011). In this study, ROS generation was observed in RAW264.7 cells after PQ exposure (Fig. 4). Combined, these data demonstrate that PQ can cause apoptosis through a ROS-mediated mitochondrion-dependent pathway.

In summary, the present study revealed that PQ dose-dependently induces apoptosis in RAW264.7 cells, via a caspase-dependent mitochondrial pathway. Additionally, exposure of RAW264.7 cells to PQ increased intracellular ROS levels and induced MMP depolarization. Our study highlights PQ-induced cytotoxicity in macrophages, and may be useful for further investigations of PQ-mediated immunotoxicity.

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REFERENCES

Ali, S., Jain, S. K., Abdulla, M. and Athar, M. (1996) Paraquat induced DNA damage by reactive oxygen species. *Biochem. Mol. Biol. Int.* **39**, 63-67.

Boxer, L. A., Harris, R. E. and Baehner, R. L. (1979) Regulation of membrane peroxidation in health and disease. *Pediatrics* **64**, 713-718.

Cappelletti, G., Maggioni, M. G. and Maci, R. (1998) Apoptosis in human lung epithelial cells: triggering by paraquat and modulation by antioxidants. *Cell Biol. Int.* **22**, 671-678.

Caroleo, M. C., Rispoli, V., Arbitrio, M., Strongoli, C., Rainaldi, G., Rotiroti, D., Nistico, G. (1996) Chronic administration of paraquat produces immunosuppression of T-lymphocytes and astrocytes in rats. *Toxic Subst. Mech.* **15**, 183-194.

Castello, P. R., Drechsel, D. A. and Patel, M. (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J. Biol. Chem.* **282**, 14186-14193.

Cheng, Z. Q., Han, J. Y., Sun, P., Weng, Y. Y., Chen, J., Wu, G. Y. and Ma, H. X. (2012) Edaravone attenuates paraquat-induced lung injury by inhibiting oxidative stress in human type II alveolar epithelial cells. *World J. Emerg. Med.* **3**, 55-59.

Chipuk, J. E., Bouchier-Hayes, L. and Green, D. R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ.* **13**, 1396-1402.

Circu, M. L. and Aw, T. Y. (2010) Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **48**, 749-762.

Deng, S., Yuan, H., Yi, J., Lu, Y., Wei, Q., Guo, C., Wu, J., Yuan, L. and He, Z. (2013) Gossypol acetic acid induces apoptosis in RAW264.7 cells via a caspase-dependent mitochondrial signaling pathway. *J. Vet. Sci.* **14**, 281-289.

Don Porto Carero, A., Hoet, P. H., Verschaeve, L., Schoeters, G. and Nemery, B. (2001) Genotoxic effects of carbon black particles, diesel exhaust particles, and urban air particulates and their extracts on a human alveolar epithelial cell line (A549) and a human monocytic cell line (THP-1). *Environ. Mol. Mutagen.* **37**, 155-163.

Dusinska, M., Kovacikova, Z., Vallova, B. and Collins, A. (1998) Responses of alveolar macrophages and epithelial type II cells to oxidative DNA damage caused by paraquat. *Carcinogenesis* **19**, 809-812.

Fadeel, B. and Orrenius, S. (2005) Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J. Intern. Med.* **258**, 479-517.

Fiers, W., Beyaert, R., Declercq, W. and Vandenabeele, P. (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene* **18**, 7719-7730.

Fuchs, Y. and Steller, H. (2011) Programmed cell death in animal development and disease. *Cell* **147**, 742-758.

Green, D. R. and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science* **305**, 626-629.

Haley, T. J. (1979) Review of the toxicology of paraquat (1,1'-dimethyl-4,4'-bipyridinium chloride). *Clin. Toxicol.* **14**, 1-46.

Hughes, D. A. (1999) Effects of dietary antioxidants on the immune function of middle-aged adults. *Proc. Nutr. Soc.* **58**, 79-84.

Kang, H. J., Hong, Y. B., Yi, Y. W., Cho, C. H., Wang, A. and Bae, I. (2013) Correlations between BRCA1 defect and environmental factors in the risk of breast cancer. *J. Toxicol. Sci.* **38**, 355-361.

Kim, H., Lee, S. W., Baek, K. M., Park, J. S. and Min, J. H. (2011) Continuous hypoxia attenuates paraquat-induced cytotoxicity in the human A549 lung carcinoma cell line. *Exp. Mol. Med.* **43**, 494-500.

Lee, T. C., Lai, G. J., Kao, S. L., Ho, I. C. and Wu, C. W. (1993) Protection of a rat tracheal epithelial cell line from paraquat toxicity by inhibition of glucose-6-phosphate dehydrogenase. *Biochem. Pharmacol.* **45**, 1143-1147.

Li, X. and Sun, A. Y. (1999) Paraquat induced activation of transcription factor AP-1 and apoptosis in PC12 cells. *J. Neural Transm.* **106**, 1-21.

Lu, L., Hackett, S. F., Mincey, A., Lai, H. and Campochiaro, P. A. (2006) Effects of different types of oxidative stress in RPE cells. *J. Cell. Physiol.* **206**, 119-125.

Mao, Z., Xia, W., Wang, J., Chen, T., Zeng, Q., Xu, B., Li, W., Chen, X. and Xu, S. (2013) Perfluorooctane sulfonate induces apoptosis in lung cancer A549 cells through reactive oxygen species-mediated mitochondrion-dependent pathway. *J. Appl. Toxicol.* **33**, 1268-1276.

Martinou, J. C. and Youle, R. J. (2011) Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev. Cell* **21**, 92-101.

Mitsopoulos, P. and Suntres, Z. E. (2010) Cytotoxicity and gene array analysis of alveolar epithelial A549 cells exposed to paraquat. *Chem. Biol. Interact.* **188**, 427-436.

Mitsopoulos, P. and Suntres, Z. E. (2011) Protective effects of liposomal N-acetylcysteine against paraquat-induced cytotoxicity and gene expression. *J. Toxicol.* **2011**, 808967.

Mussi, M. A. and Calcaterra, N. B. (2010) Paraquat-induced oxidative stress response during amphibian early embryonic development. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* **151**, 240-247.

Neishabouri, E. Z., Hassan, Z. M., Azizi, E. and Ostad, S. N. (2004) Evaluation of immunotoxicity induced by diazinon in C57bl/6 mice. *Toxicology* **196**, 173-179.

Orrenius, S. (2004) Mitochondrial regulation of apoptotic cell death. *Toxicol. Lett.* **149**, 19-23.

Riahi, B., Rafatpanah, H., Mahmoudi, M., Memar, B., Brook, A., Tabasi, N. and Karimi, G. (2010) Immunotoxicity of paraquat after subacute exposure to mice. *Food Chem. Toxicol.* **48**, 1627-1631.

Riahi, B., Rafatpanah, H., Mahmoudi, M., Memar, B., Fakhr, A., Tabasi, N. and Karimi, G. (2011) Evaluation of suppressive effects of paraquat on innate immunity in Balb/c mice. *J. Immunotoxicol.* **8**, 39-45.

Rio, M. J. and Velez-Pardo, C. (2008) Paraquat induces apoptosis in human lymphocytes: protective and rescue effects of glucose, cannabinoids and insulin-like growth factor-1. *Growth Factors* **26**, 49-60.

Schenker, M. B., Stoecklin, M., Lee, K., Lupercio, R., Zeballos, R. J., Enright, P., Hennessy, T. and Beckett, L. A. (2004) Pulmonary function and exercise-associated changes with chronic low-level paraquat exposure. *Am. J. Respir. Crit. Care Med.* **170**, 773-779.

Shi, L., Chen, J., Yang, J., Pan, T., Zhang, S. and Wang, Z. (2010) MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. *Brain Res.* **1352**, 255-264.

Styles, J. A. (1974) Studies on the effects of paraquat and diquat on cells in culture. Viability of macrophages and fibroblasts incubated with paraquat and diquat. *Br. J. Exp. Pathol.* **55**, 71-77.

Tsujimoto, Y. and Shimizu, S. (2007) Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* **12**, 835-840.

Victor, V. M., Rocha, M. and De la Fuente, M. (2003) Regulation of macrophage function by the antioxidant N-acetylcysteine in mouse-oxidative stress by endotoxin. *Int. Immunopharmacol.* **3**, 97-106.

Victor, V. M., Rocha, M. and De la Fuente, M. (2004) Immune cells: free radicals and antioxidants in sepsis. *Int. Immunopharmacol.* **4**, 327-347.

Wang, X., Luo, F. and Zhao, H. (2014) Paraquat-induced reactive oxygen species inhibit neutrophil apoptosis via a p38 MAPK/NF-kappaB-IL-6/TNF-alpha positive-feedback circuit. *PLoS One* **9**, e93837.

Zirk, N. M., Hashmi, S. F. and Ziegler, H. K. (1999) The polysaccharide portion of lipopolysaccharide regulates antigen-specific T-cell activation via effects on macrophage-mediated antigen processing. *Infect. Immun.* **67**, 319-326.