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RESEARCH ARTICLE

Paraquat modulates immunological function in bone marrow-derived macrophages

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ABSTRACT

The herbicide paraquat (PQ) is known to affect the immune system. Many reports have indicated that PQ impacts on the viability and functions of the immune cells, however, the underlying mechanism in detail is still unknown. The aim of this study was to evaluate the effects of PQ on the free radical production, oxidative stress, cell death and pro-inflammatory gene expression of murine bone marrow-derived macrophages (BMDMs) from C57BL/6NJcl mice *in vitro*. BMDMs were incubated with PQ at 0, 200 and 400 µM concentrations for 24 h. Intracellular reactive oxygen species (ROS) production, apoptosis, cell viability, nitric oxide, inducible nitric oxide synthase (iNOS), and IL-6 expression levels were measured. The results revealed that PQ treatments led to a decrease in the cell viability and induced apoptotic cell death in a dose-dependent manner. Additionally, PQ also induced the generation of ROS. The mRNA level of the pro-inflammatory mediator genes iNOS and IL-6 were also elevated, while the level of lipid peroxide (malondialdehyde) production remained unaltered. Interestingly, the PQ treatment led to a decrease in the nitric oxide production. These results indicate that the increased cellular ROS production, due to the PQ treatment, induces apoptosis and the herbicide triggers production of iNOS and IL-6 in BMDMs.

KEYWORDS

paraquat, bone marrow-derived macrophages, reactive oxygen species, apoptosis, pro-inflammatory mediator

INTRODUCTION

Paraquat (PQ) is a herbicide that has been used in agricultural areas worldwide for several decades due to its excellent low cost and highly efficient broad spectrum herbicidal activity (Tsai, 2013). Because of the extensive use of this herbicide, its residual presence has been detected in all environmental compartments, including foodstuffs. Thus, agricultural workers and livestock animals are constantly exposed to this substance resulting from accidental oral ingestion, inhalation and dermal contact and this constitutes a health risk (Baharuddin et al., 2011).

PQ causes damage to multiple organs, including the lung, liver and kidney by disrupting various biological functions (Dinis-Oliveira et al., 2008; Wu et al., 2018). PQ toxication has a high mortality rate, nevertheless no specific medical therapy is available (Sun and Chen, 2016). The tissue injury is mainly due to the reactive oxygen species (ROS) that cause irreversible oxidative damage to the molecular structures and cell functions, as well as an exacerbated immune response (Wu et al., 2018). Recent studies have shown that PQ directly impacts on the immunity and the immune cell activities (Riahi et al., 2010; Hassuneh et al., 2012) with effects such as decreased interferon (IFN)-γ production and phagocytic activity of monocytes

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in mice (Riahi et al., 2011). PQ also inhibits the natural killer (NK) cell cytotoxic activity (Lim et al., 2015), macrophage and neutrophil infiltration in PQ-induced lung injury (Wu et al., 2020). Additionally, PQ behaves like a potent inhibitor of neutrophil apoptosis and stimulates production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α and induces ROS through the p38 MAPK/NF-kB pathways leading to tissue injury (Wang et al., 2014; Huang et al., 2019). Moreover, PQ impairs the phagocytic activity of monocytes and granulocytes (Jang et al., 2015), and induces apoptosis of the mature CD4 T cells and B cells during a primary immune response. The studies mentioned above have shown that the innate and adaptive immune cell functions were altered after PQ exposure (Shao et al., 2019). Likewise, PQ has also been found to influence the ROS production in human neural progenitor cells and to increase the malondialdehyde (MDA) level in the lungs (Chang et al., 2013; Toygar et al., 2015). These phenomena are in accordance with the study of Jang et al. (2015) who have found that PQ alters the mitochondrial activities leading to apoptosis.

Macrophages are a crucial component of the innate immune system that has important roles in homeostasis, tissue repair, host defense, phagocytosis and inflammation regulation (Wynn et al., 2013). During the response to infectious disease, macrophages produce various pro-inflammatory cytokines including IL-1, IL-6, IL-12 and TNF that increase the vascular permeability and the inflammatory cell migration, resulting in the inactivation or destruction of the invading microorganisms (Geissmann et al., 2010; Arango Duque and Descoteaux, 2014).

The immunotoxicity of PQ to macrophages is still poorly understood. As far as we know at present, there have not been any reports concerning the potential immunomodulatory effects of PQ to murine bone marrow-derived macrophages (BMDMs), which differ from the macrophage cell lines in the cytokine profiles, the phagocytic process and the ability to evoke oxidative burst (Trouplin et al., 2013). Therefore, the aim of this study was to evaluate the effects of PQ on the intracellular production of ROS, MDA, apoptosis, cell viability, nitric oxide (NO), and the expression levels of inducible nitric oxide synthase (iNOS) and IL-6 in BMDMs *in vitro*. Knowledge from this study is important to understand how PQ affects murine BMDMs viability and function that is linked to the possible risks to human and animal health. Also, it might be important for the future development of treatments for reducing paraquat toxicity.

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Animal Ethics Committee of the Mahasarakham University (IACUC-MSU 042/2019). This experiment adopted a completely randomized design. BMDM cells were incubated with PQ at concentrations of 0, 200 and 400 µM paraquat dichloride hydrate (Sigma-Aldrich) for 24 h. Each concentration of PQ was replicated in triplet and intracellular ROS production, MDA, cell viability, apoptotic cell death, NO, as well as the expression levels of iNOS and IL-6 in BMDMs were measured.

Animal, bone marrow-derived macrophage isolation, culture and identification

Female 9-week-old C57BL/6NJcl mice were purchased from the Nomura Siam International Co., Ltd., Bangkok, Thailand. The mice were euthanized with carbon dioxide and cervical dislocation. Bone marrow-derived macrophages were generated from the precursors in the presence of macrophage colony stimulating factor (M-CSF) as previously described (Davis, 2013). Briefly, bone marrow cells were isolated from the tibias and femurs. The legs were sterilized by using 70% ethanol, then the skin and muscles were removed from the bones. The tibias and femurs were placed into 70% ethanol, then transferred to a tissue culture hood. The bones were washed twice with phosphate buffered saline (PBS), then cut at both ends and the bone marrow cavity was flushed out with a 25-gauge needle and syringe filled with RPMI 1640 medium (Gibco BRL, Life Technologies) until the bone cavity appeared white. The bone marrow cells were centrifuged at 300g, at 4 °C for 5 min. The supernatant was discarded and the cells were added to red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO) for removing red blood cells. The bone marrow cells were incubated at room temperature for 5 min, then centrifuged again at 300g at 4 °C for 5 min. The cell pellet was resuspended in the BMDMs growth medium. The cells were cultured in RPMI 1640 medium, containing 10% FBS (Invitrogen), 1% penicillin-streptomycin (Gibco BRL, Life Technologies) and 20 ng mL^{-1} recombinant mouse M-CSF (BioLegend, San Diego, USA) in tissue culture plate at 37 °C and 5% CO_2. The culture media was exchanged every 48 h and thus the non-adhered cells were removed. After 7 days, the adherent cells were harvested for the experiment.

The phenotypes of the BMDMs were identified by cell morphology and cell-specific surface markers. During cell culturing, the murine bone marrow cells were stimulated by M-CSF, then on days 2, 4 and 7 of incubation, the bone marrow-derived cell populations were observed by using an inverted microscope (Fig. 1a). Furthermore, cell surface markers (expression of $F4/80^+$ and $CD11b^+$) of the BMDMs were examined by using flow cytometry. Briefly, the BMDMs were incubated with anti-CD16/32 Fc blocker at 4 °C for 15 min, then stained with APC-conjugate anti-F4/80, and PE-conjugated anti-CD11b at 4 °C for 30 min. All antibodies were purchased from BioLegend, San Diego, CA. Flow cytometry was performed using a FACSCelesta (BD Biosciences, Franklin Lakes, NJ) instrument and the data were analyzed using the FlowJo Software (Davis, 2013). It was found that the proportion of $CD11b^+F4/80^+$ double-positive BMDM cells was greater than 98% (Fig. 1b).

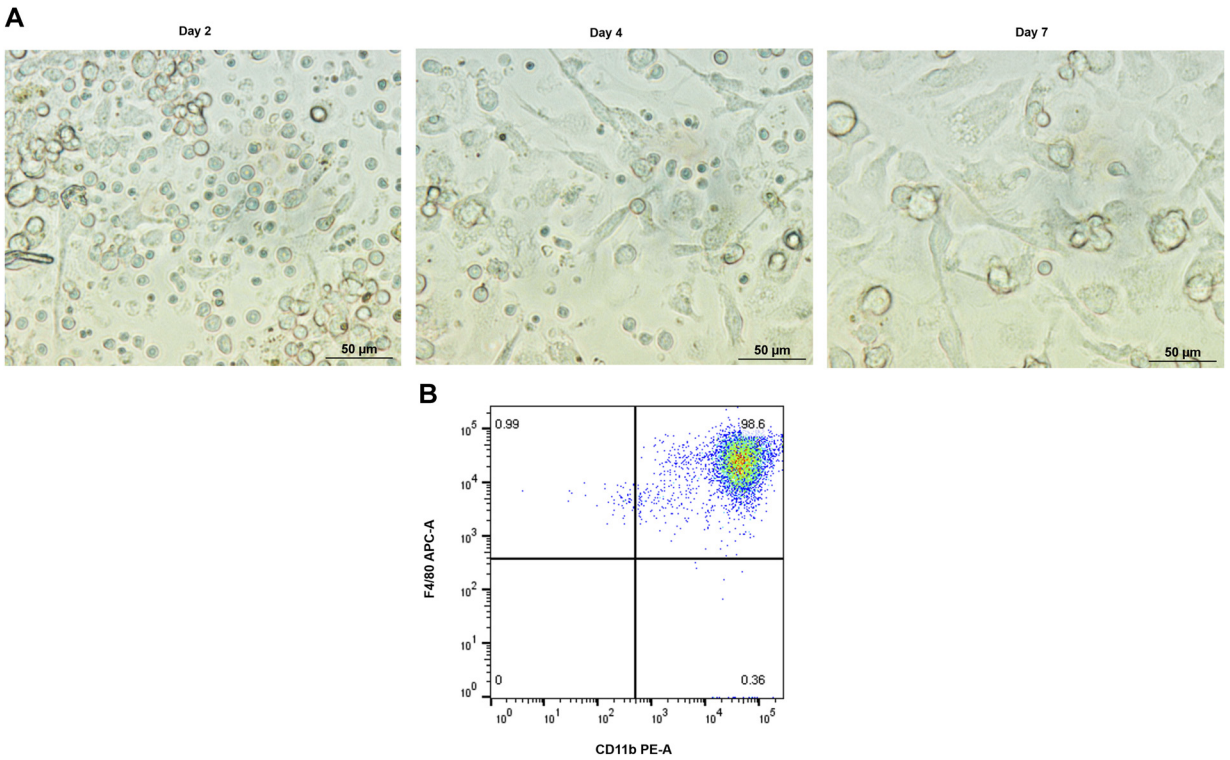

Fig. 1. Morphology of the BMDMs and expression of F4/80$^+$ and CD11b$^+$ on the cell surface of BMDMs. (1A.) Morphology of BMDMs on days 2, 4, and 7 of the culture period; (1B.) Expression of F4/80$^+$ and CD11b$^+$ on the BMDM cells' surface on day 7 of culture. The proportion of CD11b$^+$F4/80$^+$ double-positive cells was greater than 98%

Laboratory investigation

Cell viability. Cellular viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT solution was prepared by dissolving 50 mg of MTT powder (ab146345, Abcam, USA) in 10 mL PBS. The cells were seeded in 96-well plates (1×10^4 cells/well) and cultured for 24 h before adding PQ at 0, 200 and 400 µM concentrations. Following another 24-h incubation, the supernatant was removed and 100 µL of FBS-free culture medium was added to each well. Subsequently, the cells were incubated with MTT (5 mg mL^{-1}) at 37 °C and 5% CO_2 for 4 h. The supernatant was then removed completely, and the formazan crystals produced by the cells were dissolved by adding 200 µL of dimethyl sulfoxide (DMSO, AppliChem, Germany) to each well. The absorbance was measured at 450 nm with a Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland).

Apoptosis. The apoptosis of BMDMs was evaluated by using the FITC Annexin V Apoptosis Detection Kit (Biolengend, San Diego, CA) and analyzed by flow cytometry (FACSCelesta, BD Bioscience) and FlowJo software. Briefly, after culturing with PQ for 24 h, the BMDM cells were collected, washed with ice-cold PBS and resuspended in Annexin V binding buffer (containing Annexin V-FITC and propidium iodide (PI)). The cells were incubated in the dark at room temperature for 15 min and then analyzed by flow cytometry within 1 h following the manufacturer's protocol. The percentage of cells were distinguishable as follows: necrotic cells (Annexin V$^+$/PI$^+$); apoptotic cells (Annexin V$^+$/PI$^-$) and live cells (Annexin V$^-$/ PI$^-$).

Intracellular ROS production. Intracellular ROS was measured using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay kit (ab113851, Abcam, USA) according to the manufacturer's instruction. Briefly, the cells were seeded in 12-well plates at a density of 2×10^5 cells/well for 24 h, and then treated with PQ (0, 200 and 400 µM) for 24 h. At 24 h after PQ treatment, 20 mM DCFH-DA was added into the cells and incubated at 37 °C for 30 min. The cells were washed again with PBS. The fluorescence intensity was measured using a instrument FACSCelesta and analyzed using the FlowJo Software.

Nitric oxide. The NO concentration was measured using the Griess reagent (Csonka et al., 2015). Briefly, the BMDMs were cultured in RPMI 1640 culture medium at 37 °C for 24 h, and then the different doses of PQ were added. An equal volume of the supernatant and Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) were mixed and incubated at room temperature for 15 min. The absorbance was measured at 540 nm in a microplate reader (Tecan Trading AG, Männedorf, Switzerland), and the concentration of nitrite in the sample was calculated using sodium nitrite as a standard curve (Csonka et al., 2015).

Malondialdehyde. The level of MDA was measured by using the thiobarbituric acid reactive substance (TBARS) assay (Potter et al., 2011). Briefly, after incubating the BMDMs with PQ at the different concentrations for 24 h, the supernatants were collected. 100 µL of supernatant was added to the test tubes containing 450 µL 0.9% NaCl, 200 µL 0.12M thiobarbituric acid and 1,000 µL 10% (w/v) trichloroacetic acid and boiled for 30 min. The test tube was then cooled using running tap water and the mixture was centrifuged at 1,100 g for 10 min. Subsequently, 200 µL of the reaction mixture was transferred to the well of a 96-well plate and the absorbance of the mixture was measured at 532 nm in a microplate reader (Tecan Trading AG, Männedorf, Switzerland). The amount of TBARS was estimated using MDA as the standard (Potter et al., 2011).

iNOS and IL-6 expression. Total RNA from the cultured BMDM cells was isolated using a Nucleospin RNA kit (Macherey-Nagel Duren, Germany) according to the manufacturer's instructions, and the concentration of the RNA was determined using a Nanodrop 2,000 spectrophotometer (Thermo Fisher Scientific, USA). The absorbance ratios at 260/280 nm between 1.8 and 2.0 were used to assess the purity of the RNA for the subsequent analyses. Complementary DNA (cDNA) was synthesized using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) and Thunderbird SYBR qPCR Master Mix (TOYOBO, Osaka, Japan). The primer sequences are shown in Table 1. The gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The PCR was initiated at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C 15 s, and extension at 72 °C for 20 s. The melt-curve analysis was performed to confirm the specificity of the product. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The data were analyzed using the one-way analysis of variance. Means were separated by Duncan's multiple range tests. The level of significance was determined at $P < 0.05$.

RESULTS

Effect of PQ on the viability of BMDMs

As demonstrated by Fig. 2, PQ treatment reduced the BMDMs cell viability in a dose-dependent manner. The viability of BMDMs, incubated with PQ at 200 and 400 µM was significantly lower than that of the control group ($P < 0.05$). The viability of the BMDM cells incubated with PQ at 400 µM was significantly lower than that of the cells, treated with 200 µM ($P < 0.05$).

Induction of apoptosis and generation of ROS and NO in BMDMs

To investigate whether the PQ-induced cytotoxicity involves apoptosis, we assessed flow cytometric analysis of PQ treated BMDMs by Annexin V-FITC/PI double staining compared with the PQ untreated BMDMs. PQ significantly increased the percentage of both early apoptosis and late apoptosis. The proportion (25.7% and 48%) of apoptotic BMDM cells after incubation with 200 and 400 µM PQ, respectively, was found to be dose-dependent (Fig. 3).

As shown in Fig. 4, the ROS production of BMDMs, incubated with PQ at 400 uM concentarion, was significantly higher than that of BMDMs, incubated with PQ at 200 µM ($P < 0.05$).

The amount of released NO, examined by Griess assay was significantly lower ($P < 0.05$) in the PQ-treated BMDMs than in the control group (Fig. 5).

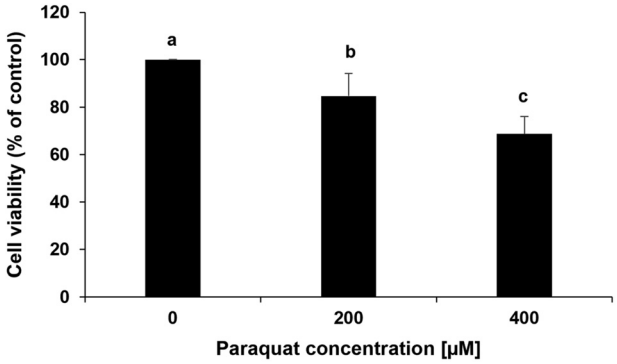

Fig. 2. Viability of BMDM cells, incubated with 0, 200 and 400 µM PQ for 24 h. *The values with different letters are significantly different ($P < 0.05$)*

Table 1. The sequence of the qPCR primers used in this study

Gene	Primer sequences (5′ to 3′)	Accession no.
IL-6	F: TTCCATCCAGTTGCCTTCTTG R: CATTTCCACGATTTCCCAGAG	NM_031168.2
iNOS	F: TTTGTGCGAAGTGTCAGTGG R: CCCTTTGTGCTGGGAGTCA	NM_010927.4
GAPDH	F: GGCATTGTGGAAGGGCTCAT R: GACACATTGGGGGTAGGAACAC	NM_001289726.1

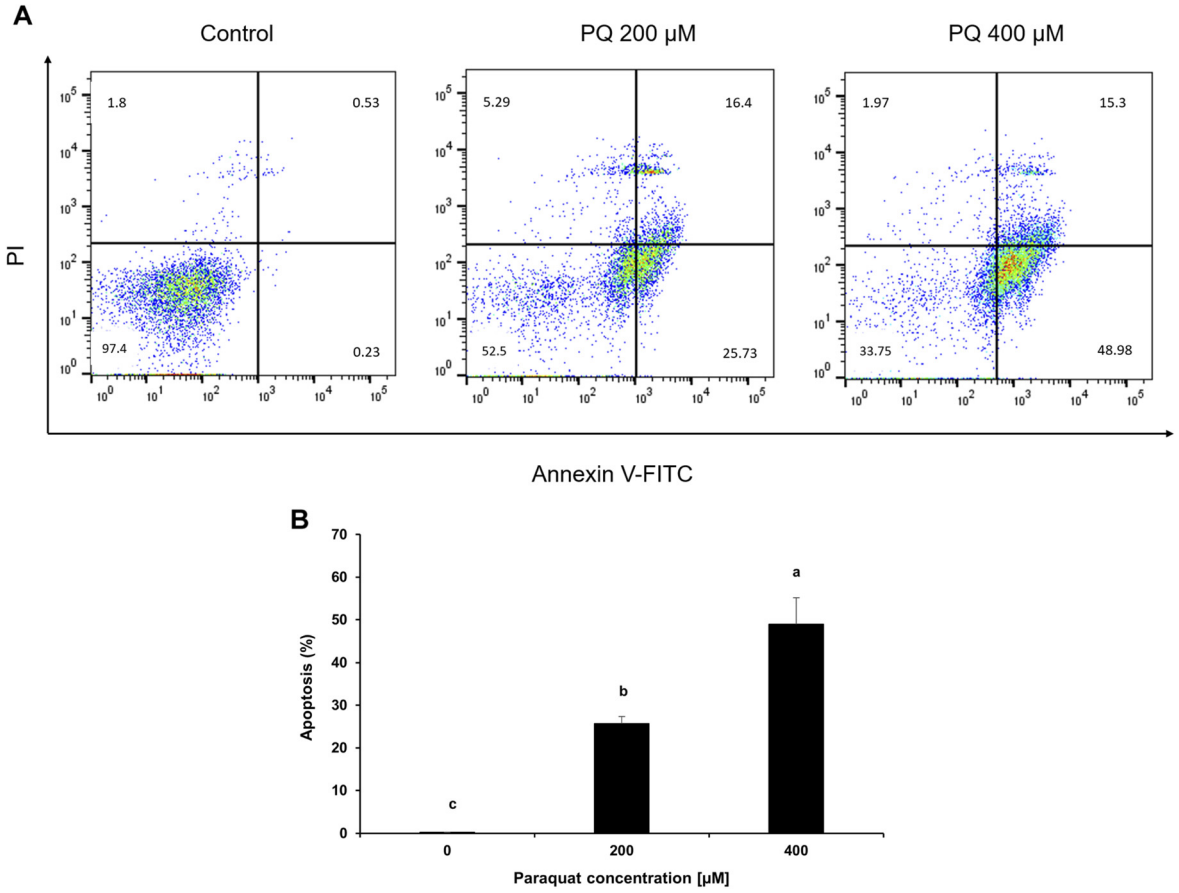

Fig. 3. Apoptotic BMDM cells in samples incubated with 0, 200 and 400 µM paraquat (PQ) for 24 h, (3A) Detection of apoptosis by staining with annexin V-FITC and PI. (3B) Percentage of apoptotic cells in the groups, incubated with 0, 200 and 400 µM PQ. *The values with different letters are significantly different* ($P < 0.05$)

Effect of PQ on the lipid peroxidation

As it can be seen in Fig. 6, the MDA level in the PQ-treated and control group were not significantly different ($P > 0.05$).

Effect of PQ on the expression of *iNOS* and *IL-6*

The amount of mRNA from the *iNOS* gene was significantly ($P < 0.05$) higher in the PQ treated groups than in the untreated control (Fig. 7A). The *IL-6* specific mRNA level was significantly ($P < 0.05$) higher in the group incubated with 400 µM PQ than in the control and the 200 µM PQ-treated BMDMs (Fig. 7B).

DISCUSSION

Generally, the ROS play a key role in the normal cellular functions and physiological processes (Milkovic et al., 2019). Excessive production of intracellular ROS might lead to oxidative damage to the DNA, including apoptosis (Finkel, 2003) and pathologic condition (Milkovic et al., 2019). This was in accordance with the report of Chang et al. (2013) who have found that exposure to increased amounts of ROS was possibly the cause of the apoptotic cell death. PQ is widely accepted to have the ability to induce cells producing superoxide free radicals (O_2-) via a redox cycle and to cause induction of intracellular ROS generation (Cristóvão et al., 2009). In the present study, we found that PQ at 400 µM concentration induced BMDMs to produce higher levels of ROS than the control group. In addition, there were more apoptotic BMDM cells after the incubation with PQ than in the control group. These findings were similar to the report of Doonan and Cotter (2008) who have found that the exposure to lower concentarions of PQ resulted in the induction of morphological apoptotic changes. Jang et al. (2015), when studying the effect of PQ on RAW264.7 cells have found that PQ increased the intracellular ROS level in a dose-dependent manner, which could result in apoptosis through a caspase-dependent mitochondrial pathway.

Cell viability is an important indicator of cellular activity. Chemical agents may cause toxicity in cells through different pathways. The cell viability *in vitro* can be measured by using the MTT colorimetric assay that indicates the cellular metabolic activity (Aslantürk, 2018). In the present study, we found that PQ at 200 and 400 µM concentrations decreased the BMDM cell viability. This finding is in accordance with the study of Yang and Tiffany-Castiglioni (2005) who have found that PQ caused reduced cell viability in the human SYS5 neuroblastoma cell line in a dose-dependent manner. Furthermore, the decrease in the cell

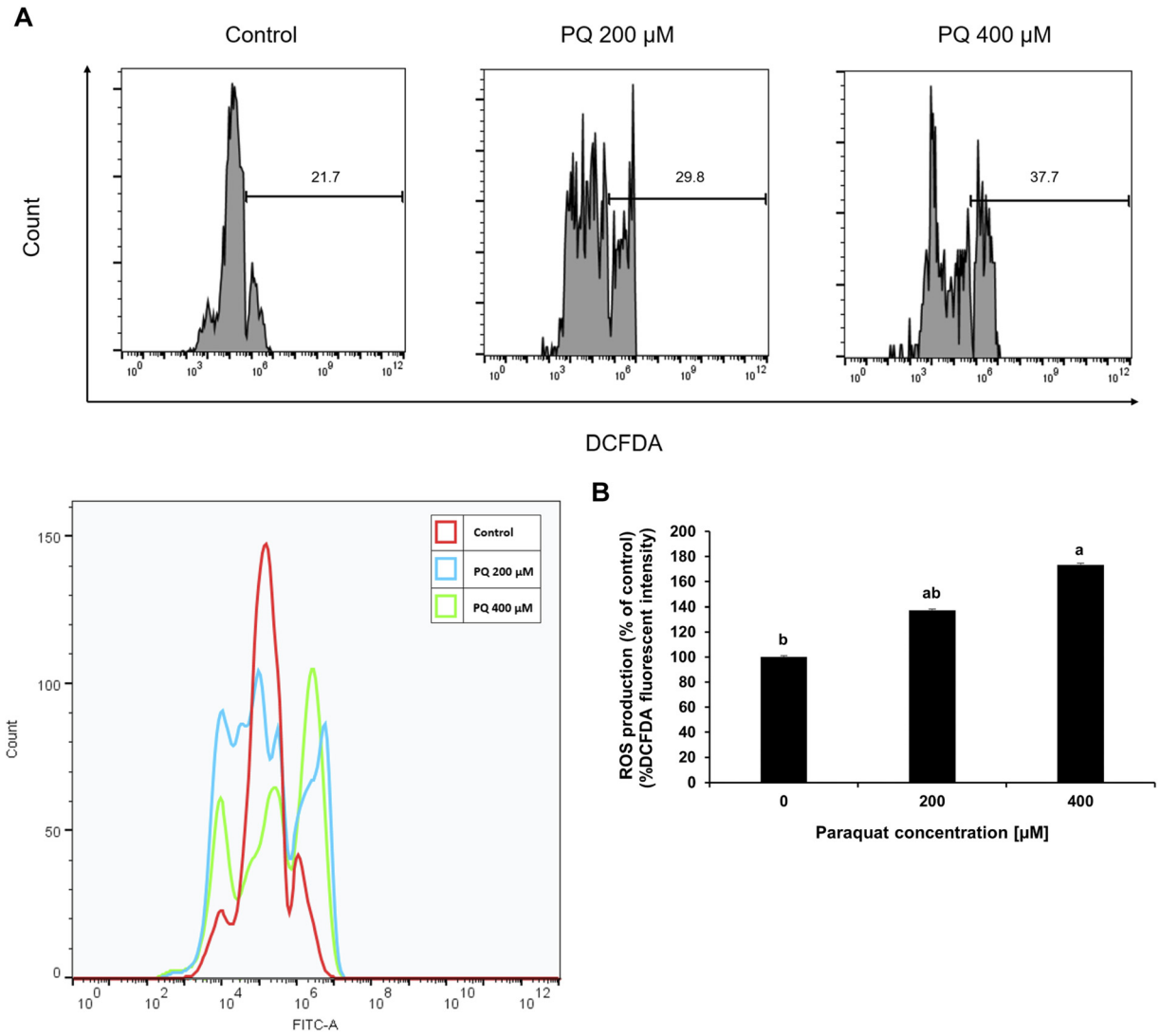

Fig. 4. The production of reactive oxygen species (ROS) by BMDM cells, incubated with paraquat (PQ) at 0, 200 and 400 µM for 24 h. (4A); Detection of ROS using carboxy-H2DCFDA dye. (4B). ROS production (fluorescence intensity, % of control) of BMDMs incubated with PQ at 0, 200 and 400 µM. *The values with different letters are significantly different* ($P < 0.05$)

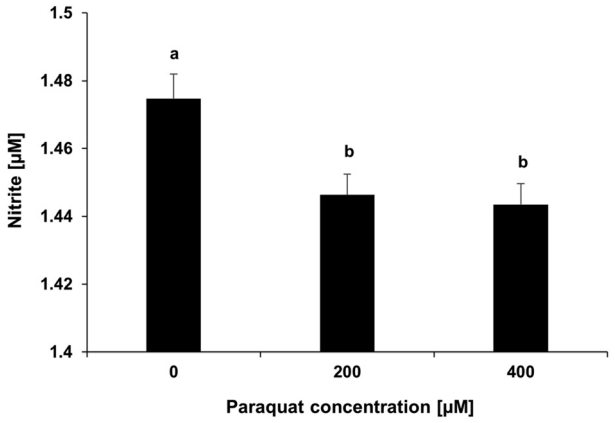

Fig. 5. The amount of nitric oxide in the supernatant of BMDMs, incubated with 0, 200 and 400 µM PQ for 24 h. *The values with different letters are significantly different* ($P < 0.05$)

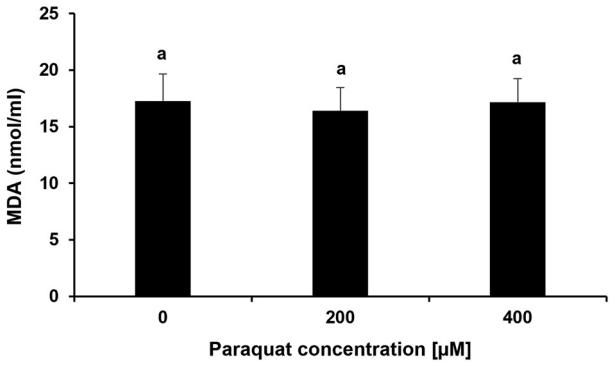

Fig. 6. The malondialdehyde level in the supernatant of BMDMs, incubated with 0, 200 and 400 µM PQ for 24 h. *The values with different letters are significantly different* ($P < 0.05$)

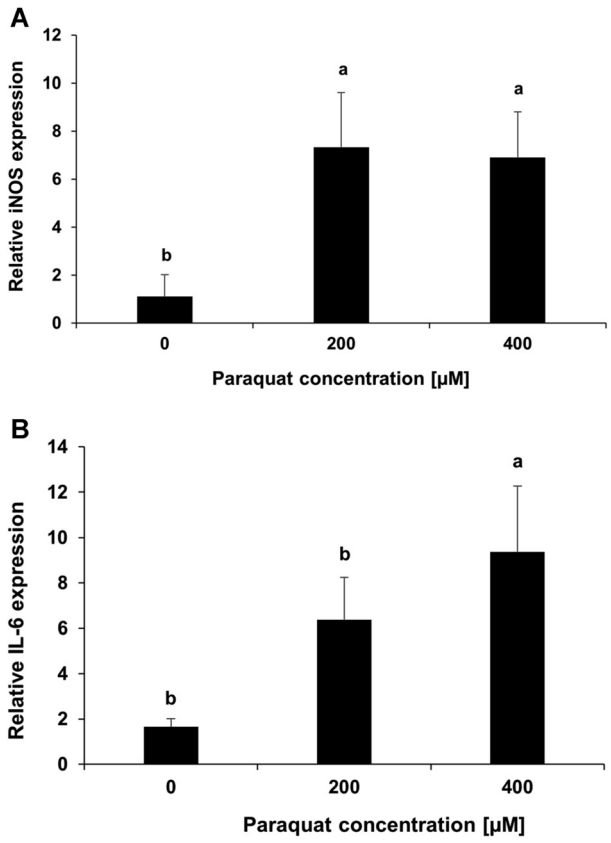

Fig. 7. The mRNA transcription of iNOS (7A) and IL-6 (7B) in BMDMs, incubated with 0, 200 and 400 μM for 24 h. *The values with different letters are significantly different* ($P < 0.05$)

viability, after incubation with PQ in the present study, was also in harmony with the report of González-Polo et al. (2004) who have found that incubation with PQ for 24 h markedly decreased the viability of cerebellar granule cells. When considering the relationship among the generation of ROS, the rates of cell viability and the induction of apoptosis, our results and previous studies indicated that PQ inhibited the viability of BMDMs partially by induction of apoptosis through enhancing the ROS production.

Excess NO generation has been implicated in cytotoxic mediation in pathophysiological processes (Lu et al., 2003). The NO promotes an increased rate of apoptosis in many cell types including macrophages (Gotoh et al., 2002). Berisha et al. (1994) have reported that PQ-induced lung tissue damage depended on the amount of NO-related activity. In our study, we found that the expression of iNOS was higher in the BMDMs incubated with PQ at 200 and 400 μM concentrations than in the control group. At the same time, the amount of NO in the BMDMs incubated with PQ (at 200 and 400 μM) was lower than in the control group. The reduction of NO is similar to the report of Fukushima et al. (2002) who have found that PQ effectively inhibited NO synthesis by using the NO synthase transferred electrons to synthesize superoxide. Moreover, NO has been found to be depleted through a redox-cycling metabolism, by interacting with superoxide induced by PQ to form the harmful anion peroxynitrite ($ONOO^-$) (Day et al., 1999; Djukic et al., 2007).

Our present study provided other evidence that the content of MDA, a lipid peroxidation biomarker (Ogata and Manabe, 1990) of BMDMs incubated with PQ (200 and 400 μM) was not different from that of the control group. This finding is similar to those reported by Ogata and Manabe (1990) who have found that PQ-mediated toxicity in pulmonary macrophages did not result from lipid peroxidation. It is probable that the damage to BMDMs during incubation with PQ may not occur through lipid peroxidation, which is in accordance with the reduction of NO (Fukushima et al., 2002). This is because, under the increase of oxidative burst conditions after exposure to PQ, excess superoxide anion radical may reduce via peroxy-nitrite formation resulting in decreased MDA. Conversely, Liu et al. (2018) have reported that the MDA level was increased in the lung and spleen on day 3 after PQ exposure. In addition, the increase of MDA also confirmes the ability of PQ to induce damage to SH-SY5Y human neuroblastoma cells from oxidative stress (Yang and Tiffany-Castiglioni, 2005).

The ROS release by the macrophages could induce signaling and transducing mediators that play an important role during inflammation (Virág et al., 2019). The upregulation of the genes of proinflammatory molecules such as IL-6, TNF-α, ROS and NO may trigger different molecular pathways during progression of the inflammatory process (Geissmann et al., 2010; Naik and Dixit, 2011; Wynn et al., 2013;

Arango Duque and Descoteaux, 2014). In addition, Naik and Dixit (2011) have reported that mitochondrial ROS influences the balance of regulators, which cause an increase in the expression of proinflammatory cytokines such as IL-6. IL-6 is a multifunctional cytokine, and is produced by activating immune cells including macrophages. It accelerates inflammatory injury and tissue damage (Hu et al., 2016). In the present study, the transcription level of IL-6 in BMDMs, incubated with PQ at 400 µM concentration, was higher than in the 200 µM PQ and the control group. The increase of IL-6 expression in BMDMs after incubating with PQ has been reported by Hu et al. (2016) who has also found that PQ induced alveolar macrophages to produce IL-6, and caused pulmonary fibrosis.

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REFERENCES

Arango Duque, G. and Descoteaux, A. (2014): Macrophage cytokines: involvement in immunity and infectious diseases. Front. Immunol. **5**, 491.

Aslantürk, Ö. S. (2018): In vitro cytotoxicity and cell viability assays: principles, advantages, and disadvantages. In Genotoxicity - A Predictable Risk to Our Actual World.

Baharuddin, M. R., Sahid, I. B., Noor, M. A., Sulaiman, N. and Othman, F. (2011): Pesticide risk assessment: a study on inhalation and dermal exposure to 2,4-D and paraquat among Malaysian paddy farmers. J Environ Sci Health B **46**, 600–607.

Berisha, H. I., Pakbaz, H., Absood, A. and Said, S. I. (1994): Nitric oxide as a mediator of oxidant lung injury due to paraquat. Proc. Natl. Acad. Sci. USA. **91**, 7445–7449.

Chang, X., Lu, W., Dou, T., Wang, X., Lou, D., Sun, X. and Zhou, Z. (2013): Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells. Chem. Biol. Interact. **206**, 248–255.

Cristóvão, A. C., Choi, D. H., Baltazar, G., Beal, M. F. and Kim, Y. S. (2009): The role of NADPH oxidase 1-derived reactive oxygen species in paraquat-mediated dopaminergic cell death. Antioxid. Redox Sign. **11**, 2105–2118.

Csonka, C., Páli, T., Bencsik, P., Görbe, A., Ferdinandy, P. and Csont, T. (2015): Measurement of NO in biological samples. Br. J. Pharmacol. **172**, 1620–1632.

Davis, B. K. (2013): Isolation, culture, and functional evaluation of bone marrow-derived macrophages. Methods Mol. Biol. **1031**, 27–35.

Day, B. J., Patel, M., Calavetta, L., Chang, L. Y. and Stamler, J. S. (1999): A mechanism of paraquat toxicity involving nitric oxide synthase. Proc. Natl. Acad. Sci. U. S. A. **96**, 12760–12765.

Dinis-Oliveira, R. J., Duarte, J. A., Sánchez-Navarro, A., Remião, F., Bastos, M. L. and Carvalho, F. (2008): Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit. Rev. Toxicol. **38**, 13–71.

Djukic, M., Jovanovic, M. C., Ninkovic, M., Vasiljevic, I. and Jovanovic, M. (2007): The role of nitric oxide in paraquat-induced oxidative stress in rat striatum. Ann. Agric. Environ. Med. **14**, 247–252.

Doonan, F. and Cotter, T. G. (2008): Morphological assessment of apoptosis. Methods **44**, 200–204.

Finkel, T. (2003): Oxidant signals and oxidative stress. Curr. Opin. Cell Biol. **15**, 247–254.

Fukushima, T., Tanaka, K., Lim, H. and Moriyama, M. (2002): Mechanism of cytotoxicity of paraquat. Environ. Health Prev. Med. **7**, 89–94.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M. and Ley, K. (2010): Development of monocytes, macrophages, and dendritic cells. Science **327**, 656–661.

González-Polo, R. A., Rodríguez-Martín, A., Morán, J. M., Niso, M., Soler, G. and Fuentes, J. M. (2004). Paraquat-induced apoptotic cell death in cerebellar granule cells. Brain Res. **1011**, 170–176.

Gotoh, T., Oyadomari, S., Mori, K. and Mori, M. (2002): Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. J. Biol. Chem. **277**, 12343–12350.

Hassuneh, M. R., Albini, M. A. and Talib, W. H. (2012): Immunotoxicity induced by acute subtoxic doses of paraquat herbicide: implication of shifting cytokine gene expression toward T-helper (T(H))-17 phenotype. Chem. Res. Toxicol. **25**, 2112–2116.

Hu, L., Yu, Y., Huang, H., Fan, H., Hu, L., Yin, C., Li, K., Fulton, D. J., and Chen, F. (2016): Epigenetic regulation of interleukin 6 by histone acetylation in macrophages and its role in paraquat-induced pulmonary fibrosis. Front. Immunol. **7**, 696.

Huang, J., Ning, N. and Zhang, W. (2019): Effects of paraquat on IL-6 and TNF-α in macrophages. Exp. Ther. Med. **17**, 1783–1789.

Jang, Y. J., Won, J. H., Back, M. J., Fu, Z., Jang, J. M., Ha, H. C., Hong, S., Chang, M. and Kim, D. K. (2015): Paraquat induces apoptosis through a mitochondria-dependent pathway in RAW264.7 cells. Biomol. Ther. **23**, 407–413.

Lim, J. H., Won, J. H., Ahn, K. H., Back, M. J., Fu, Z., Jang, J. M., Ha, H. C., Jang, Y. J. and Kim, D. K. (2015): Paraquat reduces natural killer cell activity via metallothionein induction. J. Immunot. **12**, 342–349.

Liu, Y., Li, Z., Xue, X., Wang, Y., Zhang, Y. and Wang, J. (2018): Apigenin reverses lung injury and immunotoxicity in paraquat-treated mice. Int Immunopharmacol **65**, 531–538.

Livak, K. J. and Schmittgen, T. D. (2001): Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, **25**, 402–408.

Lu, J., Moochhala, S., Shirhan, M., Ng, K. C., Tan, M. H., Teo, A. L. and Ling, E. A. (2003): Nitric oxide induces macrophage apoptosis following traumatic brain injury in rats. Neurosci. Lett. **339**, 147–150.

Milkovic, L., Cipak Gasparovic, A., Cindric, M., Mouthuy, P. A. and Zarkovic, N. (2019): Short overview of ROS as cell function regulators and their implications in therapy concepts. Cells **8**.

Naik, E. and Dixit, V. M. (2011): Mitochondrial reactive oxygen species drive proinflammatory cytokine production. J. Exp. Med. **208**, 417–420.

Ogata, T. and Manabe, S. (1990): Correlation between lipid peroxidation and morphological manifestation of paraquat-induced lung injury in rats. Arch. Toxicol. **64**, 7–13.

Potter, T. M., Neun, B. W. and Stern, S. T. (2011): Assay to detect lipid peroxidation upon exposure to nanoparticles. Methods Mol. Biol. **697**, 181–189.

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. Immunot. **8**, 39–45.

Shao, Y., Zhao, Y., Zhu, T., Zhang, F., Chang, X., Zhang, Y. and Zhou, Z. (2019): Paraquat preferentially induces apoptosis of late stage effector lymphocyte and impairs memory immune response in mice. Int. J. Environ. Res. Publ. Health **16**.

Sun, B. and Chen, Y. G. (2016): Advances in the mechanism of paraquat-induced pulmonary injury. Eur. Rev. Med. Pharmacol. Sci. **20**, 1597–1602.

Toygar, M., Aydin, I., Agilli, M., Aydin, F. N., Oztosun, M., Gul, H., Macit, E., Karslioglu, Y., Topal, T., Uysal, B. and Honca, M. (2015): The relation between oxidative stress, inflammation, and neopterin in the paraquat-induced lung toxicity. Hum. Exp. Toxicol. **34**, 198–204.

Trouplin, V., Boucherit, N., Gorvel, L., Conti, F., Mottola, G. and Ghigo, E. (2013): Bone marrow-derived macrophage production. J. Visualized Experiments: JoVE (81), e50966–e50966.

Tsai, W. T. (2013): A review on environmental exposure and health risks of herbicide paraquat. Toxicol. Environ. Chem. **95**, 197–206.

Virág, L., Jaén, R. I., Regdon, Z., Boscá, L. and Prieto, P. (2019): Self-defense of macrophages against oxidative injury: fighting for their own survival. Redox Biol. **26**, 101261.

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

Wu, M., Zhou, C., Li, M., Yu, H., Zhao, D., Xue, W., Qin, L. and Peng, A. (2020): Depletion of NK cells attenuates paraquat-induced acute lung injury by manipulating macrophage polarization. Int. Immunopharm. **86**, 106698.

Wu, Q., Xu, Q., Jian, X., Wang, H., He, X., Gao, B., Wang, K. and Kan, B. (2018): A new sight for paraquat poisoning from immunology. Immunopharmacol. Immunotoxicol. **40**, 269–272.

Wynn, T. A., Chawla, A. and Pollard, J. W. (2013): Macrophage biology in development, homeostasis and disease. Nature **496**, 445–455.

Yang, W. and Tiffany-Castiglioni, E. (2005): The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: relevance to the dopaminergic pathogenesis. J. Toxicol. Environ. Health. A. **68**, 1939–1961.