Paraquat Modulates Immunological Function in Bone Marrow-Derived Macrophages

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Abstract

Paraquat (PQ) is an herbicide commonly used worldwide. This herbicide is known to alter the human and animal immune systems. Many reports indicated that PQ impacts immune cell viability and functions. However, the underlying mechanism critical is still unknown. Therefore, the aim of this study was to evaluate effects of PQ on free radical production, oxidative stress, cell death, and pro-inflammatory gene expression of murine bone marrow-derived macrophages (BMDMs) from female C57BL/6NJcl mice *in vitro*. BMDMs were incubated with PQ at 0, 200, 400 μ M for 24 h. Intracellular reactive oxygen species (ROS) production, apoptosis, cell viability, nitric oxide, inducible nitric oxide synthase (iNOS), and IL-6 expression of murine BMDMs were measured. The results revealed that PQ treatments led to decrease the cell viability and induced apoptotic cell death in a dose-dependent manner. Additionally, PQ induced reactive oxygen species (ROS) generation. The mRNA expression level of pro-inflammatory mediator gene IL-6 and inducible nitric oxide synthase (iNOS) were elevated, while the level of lipid peroxides (MDA) production was unaltered by PQ treatment. Interestingly, PQ led to a decrease in nitric oxide production depends on its concentration. These phenomena indicated that PQ increased cellular ROS production which induced apoptosis, and the herbicide triggers production of iNOS and IL-6 in murine BMDMs.

1. 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 it constitutes a health risk (Baharuddin et al. 2011).

PQ causes damage to multiple organs, i.e. lung, liver and kidney by disrupting various biological functions (Dinis-Oliveira et al. 2008; Wu et al. 2018). PQ toxicity has a high mortality rate, nevertheless no specific medical therapy is available for it (Sun and Chen 2016). Tissue injury is mainly due to reactive oxygen species (ROS) causing irreversible oxidative damage to molecular structures, cell functions and exacerbated immune response (Wu et al., 2018). Recent studies have shown that PQ directly impacts on immunity and immune cell activities (Hassuneh et al 2012; Riahi et al. 2010) with effects such as decreased interferon (IFN)- γ production and phagocytic activity of monocytes in mice (Riahi et al. 2011). PQ also inhibits 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 potent inhibitors of neutrophil apoptosis and stimulates production of interleukin (IL)-6 and tumor necrosis factor (TNF)- α as well as inducing ROS through p38 MAPK/NF- κ B pathways leading to tissue injury (Huang et al. 2019; Wang et al. 2014). Moreover, PQ impairs phagocytic activity of monocytes and granulocytes (Jang et al. 2015), and induces apoptosis of mature CD4 T cells and B cells during a primary immune response. Thus, several studies mentioned above have shown that innate and adaptive immune cell functions were modulated after PQ exposure (Shao et al. 2019). Likewise, PQ also has influence on ROS production in

human neural progenitor cells and increased lung malondialdehyde (MDA) level (Chang et al. 2013; Toygar et al. 2015). These phenomena were in accordance with the study of Jang et al. (2015) who found that PQ alters 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 of macrophages to infectious disease, they produce various pro-inflammatory cytokines including IL-1, IL-6, IL-12 and TNF that have increased vascular permeability and inflammatory cell migration, resulting in inactivation or destruction of microorganisms (Arango Duque and Descoteaux 2014; Geissmann et al. 2010).

However, the immunotoxicity of PQ to macrophages is still poorly understood. As far as we know at present, there have not been any reports concerning potential immunomodulatory effects of PQ to murine BMDMs, which differ from macrophage cell lines in the cytokine profiles, the phagocytic process, and the ability to evoke the oxidative burst (Troupin et al. 2013). Therefore, the aim of this study was to evaluate effects of PQ on intracellular ROS production, malondialdehyde, apoptosis, cell viability, nitric oxide (NO), and iNOS and IL-6 expression of 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.

2. Materials And Methods

2.1 Experimental design

The experimental protocol was approved by the Animal Ethics Committee of Mahasarakham University (IACUC-MSU 042/2019). This experiment adopted a completely randomized design. Bone marrow-derived macrophage (BMDMs) cells were incubated with PQ at 0, 200, and 400 μ M (Paraquat dichloride hydrate, Sigma-Aldrich) for 24 h. Each concentration of PQ composed was replicated in triplet and intracellular ROS production, malondialdehyde, cell viability, apoptotic cell death, NO, and iNOS and IL-6 expression of BMDMs were measured.

2.2 Animal, bone marrow-derived macrophages isolation, culture and identification

Female 9-week-old C57BL/6NJcl mice were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. Mice were euthanized with carbon dioxide and cervical dislocation. Bone marrow-derived macrophages were generated from precursors in the presence of M-CSF as previously described (Davis 2013). Briefly, bone marrow cells were isolated from the tibias and femurs of 9-week-old mice. The legs of mice 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). The bones were then cut at both ends

and the bone marrow cavity was flushed out with a 25-gauge needle and syringe filled with RPMI 1640 until the bone cavity appeared white. The bone marrow cells were centrifuged for 5 min at 1,500 rpm (4°C), the supernatant was discarded and the cells were then added to red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO) for removing red blood cells. Bone marrow cells were incubated at room temperature for 5 min, and then centrifuged for 5 min at 1,500 rpm (4°C). The cell pellet was resuspended into the BMDMs growth medium. Cells were cultured in RPMI 1640 media (Gibco BRL, Life Technologies), containing 10% FBS (Invitrogen), 1% penicillin-streptomycin (Gibco BRL, Life Technologies) and 20 ng/ml recombinant mouse macrophage colony stimulating factor (M-CSF; BioLegend, San Diego, USA) in tissue culture wells and cultivated at 37°C and 5% CO₂. The culture media was exchanged every 48 h and non-adherent cells in the media were removed. After 7 days, the adherent cells were harvested for the experiment.

The phenotypes of BMDMs were identified by cell morphology and cell-specific surface markers. During cell culture, mouse 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 BMDMs were examined by using flow cytometry. Briefly, BMDMs were incubated with anti-CD16/32 Fc blocker for 15 min at 4°C, 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 FACSCelesta (BD Biosciences, Franklin Lakes, NJ) and the data analyzed using FlowJo Software. It was found that the expression of CD11b⁺F4/80⁺ double-positive cells of BMDMs was greater than 98% (Fig. 1b).

2.3 Laboratory investigation

2.3.1 Cell viability

Cellular viability was assessed by using the MTT assay. MTT solution was prepared by dissolving 50 mg of MTT powder in 10 ml PBS. Briefly, the cells were seeded in 96-well plates (1×10⁴ cells/well) for 24 h, and then incubated with PQ at 0, 200, 400 µM for 24 h. Following incubation, the supernatant of each well was removed and then 100 µl of FBS-free culture medium was added. Subsequently, the cells were incubated with MTT (5 mg/ml) at 37°C and 5% CO₂ 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) in the well plate. Absorbance was measured at 450 nm with a Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland).

2.3.2 Apoptosis

The apoptosis of BMDM was evaluated by using FITC Annexin V Apoptosis Detection Kit (Biolengend, San Diego, CA) and analyzed using flow cytometry (the FACSCelesta, BD Bioscience) and FlowJo software. Briefly, after co-culturing with PQ at 0, 200, 400 µM for 24 h, the cells of BMDMs 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⁻).

2.3.3 Intracellular ROS production

Intracellular ROS was measured using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay kit (Abcam, #ab113851) according to the manufacturer's instruction. Briefly, 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 μ M) 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. Cells were again washed with PBS. The fluorescence intensity was measured using FACSCelesta (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo Software.

2.3.4 Nitric oxide

NO concentration was measured using a Griess reagent. Briefly, BMDMs were cultured in RPMI 1640 culture medium at 37°C for 24 h, and then 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% H₃PO₄) were mixed and incubated for 15 min at room temperature. 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). A fresh RPMI 1640 culture media was used as a control blank.

2.3.5 Malondialdehyde

Malondialdehyde was measured by using a thiobarbituric acid reactive substance (TBARS) assay. Briefly, after incubating BMDMs in PQ at each concentration level for 24 h, the supernatants were collected. 100 μ l of supernatant was added into the test tubes containing 450 μ l 0.9% NaCl, 200 μ l 0.12M thiobarbituric acid and 1000 μ l 10% (w/v) trichloroacetic acid and then boiled for 30 min. The test tube was then cooled using running tap water and the mixture was centrifuged at 1100 g for 10 min and 200 μ l of the reaction mixture transferred to a 96-well plate and the absorbance of the mixture was measured at 532 nm. The amount of TBARS was estimated using MDA as the standard (Potter et al. 2011).

2.3.6 iNOS and IL-6 expression

Total RNA was isolated using a Nucleospin RNA kit (Macherey-Nagel Duren, Germany) according to the manufacturer's instructions. After extraction, the total concentration of RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The absorbances ratios 260/280 nm of RNA between 1.8-2.0 was used to assess the purity of RNA for subsequent analyses. Complementary DNA (cDNA) was synthesized using 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. GAPDH was used as an endogenous control. The PCR reaction was initiated at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C 15 sec, and extension at 72°C for 20 sec. The melt-curve analysis was performed to check a specific product. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4 Statistical analysis

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

3. Results

3.1 PQ decreases the viability of BMDMs

To determine the effect of PQ on the cell viability of BMDMs, cells were incubated with PQ at different concentrations (0, 200, 400 μ M) for 24 h. Cell viability was measured with the MTT assay. As demonstrated by Fig. 2, PQ treatment reduced BMDMs cell viability in a dose-dependent manner. Viability of BMDMs cells incubated with PQ at 200 and 400 μ M was significantly lower than that of the control group ($P < 0.05$). Viability of BMDMs cells incubated with PQ at 400 μ M was significantly lower than that of at 200 μ M ($P < 0.05$).

3.2 PQ induces apoptosis in BMDMs in a concentration-dependent manner

To investigate whether PQ-induce cytotoxicity involves apoptosis, we assessed flow cytometric analysis of PQ treated BMDMs by Annexin V-FITC/PI double staining. Compared with the untreated cells, PQ increased the rate of cells in both early apoptosis and late apoptosis. As indicated by Annexin V-FITC positive cells, significantly increased after BMDMs were exposed to PQ at 200, 400 μ M for 24 h, with apoptotic ratios of 25.7% and 48%, respectively (Fig. 3) in a dose-dependent manner.

3.3 PQ induces ROS generation in BMDMs

We next generated BMDMs and further investigated the generation level of ROS after exposure to PQ. Various concentrations of PQ were added. After 24 h incubation, PQ-induced intracellular ROS level was examined by DCF-DA assay and flow cytometry. As shown in Fig. 4, ROS production of BMDMs incubated with PQ at 400 μ M was significantly higher than that of at 200 μ M ($P < 0.05$).

3.4 Effect of PQ on NO production of BMDMs

We next determined the accumulation of nitrite, as a stable metabolite product of NO, in activated BMDMs was measured. BMDMs were cultured in the absence and presence of PQ with varying amounts (0, 200, 400 μ M) for 24 h. Then the released NO was examined by Griess assay. Surprisingly, NO of

BMDMs incubated with PQ at 200 and 400 μ M was significantly lower than that of the control group ($P < 0.05$) (Fig. 5).

3.5 The lipid peroxidation is independently associated with the concentration of PQ

To investigate malondialdehyde (MDA), a stable end-product of membrane lipid peroxidation, we measured the level of malondialdehyde (MDA) in the PQ-treated BMDMs for 24 h. As seen in Fig. 6, malondialdehyde levels of BMDMs incubated with PQ at 0, 200 and 400 μ M were not significantly different ($P > 0.05$).

3.6 PQ induces expression of IL-6 and iNOS in BMDMs

To further assess the effects of PQ on BMDMs, we next examined the expression of IL-6 and iNOS as proinflammatory mediators. BMDMs were stimulated with PQ (0, 200, 400 μ M) for 24 h and the levels of IL-6 and iNOS mRNA expression were measured by quantitative real-time PCR. As shown in Fig. 7, iNOS expression of BMDMs with PQ at 200 and 400 μ M was significantly higher than that of the control group ($P < 0.05$) (Fig. 7a). IL-6 expression of BMDMs incubated in PQ at 400 μ M was significantly higher than that of at 200 μ M and the control group ($P < 0.05$) (Fig. 7b).

4. Discussion

Generally, ROS play a key role in normal cellular functions in physiological processes (Milkovic et al. 2019). Excessive production of intracellular ROS might lead to oxidative damage to DNA, including apoptosis (Finkel 2003) and may conduce a pathologic condition (Milkovic et al. 2019). This was in accordance with the report of Chang et al. (2013) who 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 cause induction of intracellular ROS generation (Cristóvão et al. 2009). In the present study, we found that PQ at 400 μ M induced BMDMs to produce higher levels of ROS than that of the control group. In addition, there were more apoptotic cells of BMDMs incubated with PQ at 200 and 400 μ M compared to the control group. These finding were similar to the report of Doonan et al. (2008) who found that lower concentrations of PQ exposure resulted in the induction of morphological apoptotic changes. In addition, Jang et al. (2015) studied the effect of PQ on RAW264.7 cells and found that PQ increased the intracellular ROS level on RAW264.7 in a dose-dependent manner, which could result in apoptosis through a caspase-dependent mitochondrial pathway.

Cell viability is an important parameter for indicating cellular activity. Chemical agents may cause toxicity in cells through different pathways. The cell viability in vitro can be measured using the MTT colorimetric assay, which is widely used to evaluate cellular metabolic activity (Aslantürk 2018). In this study, we found that PQ at 200 and 400 μ M decreased the BMDMs cell viability to a lower value than in the control group. This finding was in accordance with the study of Yang et al. (2007) who found that PQ caused

reduction of viability of human SY5Y neuroblastoma cell line in a dose-dependent manner. Furthermore, the decrease of cell viability after incubation with PQ in the present study was also in harmony with the report of (González-Polo et al. 2004) who 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 ROS production.

Modulation of iNOS mRNA resulted in regulation of NO production (Xue et al. 2018). Excess NO generation has been implicated in cytotoxic mediation in pathophysiological processes (Lu et al. 2003). It promotes an increased rate of apoptosis in many cell types including macrophages (Gotoh et al. 2002). Berisha et al. (1994) reported that PQ-induced lung tissue damage depended on the amount of NO-related activity. In this study, we found that iNOS expression of BMDMs incubated with PQ at 200 and 400 μ M was higher than that of the control group. While NO of BMDMs incubated in PQ at 200 and 400 μ M was lower than that of the control group. These findings indicated that PQ induced increased expression of iNOS. However, NO level of BMDMs was decreased when the concentration of PQ increased. The reduction of NO was similar to the report of Fukushima et al. (2002) who found that PQ effectively inhibited NO synthesis by using NO synthase transferred electrons to synthesize superoxide resulting in a decreased amount of NO. Moreover, NO was 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 malondialdehyde (MDA), a lipid peroxidation biomarker (Ogata and Manabe 1990) of BMDMs incubated in PQ (200 and 400 μ M) was not different from the control group. The finding was similar to reports of Ogata et al. (1990) who found that PQ-mediated toxicity in pulmonary macrophages does not result from lipid peroxidation. It is probable that BMDMs damage during incubation in PQ may not occur by 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 peroxynitrite formation resulting in decreased MDA. Conversely, Liu et al. (2018) 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 confirms the ability of the PQ to induce SH-SY5Y cell damage from oxidative stress (Yang and Tiffany-Castiglioni 2005).

The release of ROS, which is produced by macrophages, provides key signaling-transducing molecules and mediators of inflammation that plays an important role during the progression of inflammatory condition (Virág et al. 2019) via triggering the up-regulation of pro-inflammatory molecules such as IL-6, TNF- α , ROS and NO whereby distinct molecular pathways (Arango Duque and Descoteaux 2014; Geissmann et al. 2010; Naik and Dixit 2011; Wynn et al. 2013). In addition, Naik et al. (2011) reported that mitochondrial ROS influences the balance of regulators, which cause an increase in transcription of proinflammatory cytokines such as IL-6. IL-6, 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, IL-6 expression of BMDMs incubated with PQ at 400 μ M was higher than that

of at 200 μ M and the control group. The increase of IL-6 expression of BMDM after incubating with PQ in this study is similar to the report of Hu et al. (2017) who found that during PQ exposure, PQ induced alveolar macrophages to produce IL-6 and caused pulmonary fibrosis. This finding indicates that PQ can trigger macrophages to produce IL-6.

Declarations

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Author contributions

P.S designed and performed all experiments. J.W assisted with cell culture experiments. W.A assisted with TBARS and Griess assay. P.S and W.A. wrote the manuscript text. All authors reviewed and approved the final manuscript.

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Data availability

All data and materials in this study are available for publication

Compliance with ethical standards

Conflict of interest

There are no conflicts of interest to declare.

Ethics approval

Ethical approval for all the procedures was given by the Animal Ethics Committee of Mahasarakham University (IACUC-MSU 042/2019).

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Morphology of BMDMs and expression of F4/80+ and CD11b+ on the cell surface of BMDMs. (a.) Morphology of BMDMs on days 2, 4, and 7 of culture period; (b.) Expression of F4/80+ and CD11b+ on BMDMs cell surface on day 7 of culture. The proportion of CD11b+F4/80+ double-positive cells was greater than 98%.

Figure 2

PQ induced cytotoxicity in BMDMs. BMDMs exposed to various concentrations of PQ (200, 400 μM) for 24 h, and cell viability was measured using the MTT assay. The values with different letters are significantly different ($P < 0.05$).

Figure 3

PQ induced apoptotic cell death in BMDMs. Apoptotic cells of BMDMs that incubated with PQ at 0, 200 and 400 μM for 24 h, (a) Detection of apoptosis by staining with annexin V-FITC and PI. (b) Percentage of apoptotic cells that incubated with PQ at 0, 200 and 400 μM . The values with different letters are significantly different ($P < 0.05$).

Figure 4

PQ markedly increased levels of ROS in BMDMs. ROS production of BMDMs incubated with PQ at 0, 200 and 400 μM for 24 h. (a); Detection of ROS using carboxy-H2DCFDA dye. (b). Quantified ROS levels were expressed as % change in ROS production (fluorescence intensity, % of control). The values with different letters are significantly different ($P<0.05$)

Figure 5

PQ effects on BMDMs NO production. NO in the supernatant of BMDMs incubated with PQ at 0, 200 and 400 μM for 24 h. The values with different letters are significantly different ($P<0.05$).

Figure 6

Effect of paraquat treatment on the lipid peroxidation in BMDMs. Malondialdehyde level in the supernatant of BMDMs incubated with PQ at 0, 200 and 400 μM for 24 h. The values with different letters are significantly different ($P < 0.05$).

Figure 7

Effects of PQ on the mRNA expression of proinflammatory mediators in BMDMs. iNOS (a), IL-6 (b) expression of BMDMs incubated with PQ at 0, 200 and 400 μ M for 24 h. The values with different letters are significantly different ($P<0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.pptx](#)