Effects of paraquat on IL-6 and TNF-α in macrophages

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Abstract. Effects of paraquat (PQ) on interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in macrophages were investigated. Different concentrations of PQ were added to mouse macrophage RAW264.7 for culture. According to different concentrations of PQ, mice were divided into micro concentration (0.01 mmol/l), low concentration (0.1 mmol/l), medium concentration (1 mmol/l), high concentration (10 mmol/l), and control groups without PQ. Trypan blue solution was used for detecting cell viability, a microplate reader for detecting the fluorescence intensity of reactive oxygen species (ROS), ELISA for detecting the expression levels of IL-6 and TNF-α. The medium concentration and the high concentration groups had significantly lower cell viability than the other three groups ($P<0.050$). The high concentration group had significantly lower cell viability than the medium concentration group ($P<0.050$). At 1, 4 and 8 h, respectively, the medium and the high concentration groups had significantly higher ROS fluorescence intensity than the other three groups ($P<0.050$). The high concentration group had significantly higher ROS fluorescence intensity than the medium concentration group ($P<0.050$). There were significant differences in the expression levels of IL-6 and TNF-α at the 1st, 4th and 8th hour among the five groups ($P<0.050$). In the micro, the low, the medium and high concentration groups, the expression levels of IL-6 and TNF-α were the lowest at 1 h and the highest at 8 h, which were higher at 4 h than those at 1 h ($P<0.050$). PQ at a concentration of 1 mmol/l can produce toxicity to macrophages, and greatly increase the ROS fluorescence intensity, the expression levels of IL-6 and TNF-α. PQ poisoning is expected to be treated though IL-6 and TNF-α in the future.

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Introduction

Paraquat (PQ), a kind of 1,1'-dimethyl-4,4'-bipyridyl cationic salt, is commonly known as diguat and gramoxone. It is currently the most widely used organic heterocyclic contact defoliant and herbicide (1). PQ has an extremely high application value in agriculture, with a utilization rate of more than 70% in predominantly agrarian countries (2). However, with the high application rate of PQ, the subsequent impact is one of the most difficult problems in clinical practice. PQ is extremely toxic to humans and animals, and its poisoning route is very extensive, including breathing, skin contact, esophagus and intravenous injection (3). According to statistics, the number of PQ poisoning patients was over 650,000 worldwide in 2016 (4). It is increasing year by year comparing with statistics in previous years (5,6). The number of PQ poisoning patients is expected to exceed 1 million by 2025 (7). Besides, the mortality of PQ poisoning is extremely high. According to statistics, its clinical mortality is up to 60-80% (8). PQ poisoning easily causes multiple organ failure, which is one of the causes of its high mortality (9). Clinically, the increasingly serious problem of PQ poisoning has been the focus of research and continuous study of the pathogenesis and prevention and treatment of PQ is ongoing, but no significant breakthrough has been made yet. With the deepening of researches, studies in recent years have shown that oxidative stress-induced systemic inflammatory response syndrome (SIRS) may be the main pathogenic mechanism of PQ poisoning. Reactive oxygen species (ROS) produced by PQ in the body cause damage to organs through macrophages (10,11). Macrophages, a part of innate immune system in the body, play an important role in inflammatory repair and invasion resistance (12). Once they are abnormal, the immune system in the patient's body collapses, resulting in various damage. Therefore, in this study, different degrees of PQ poisoning mouse models were established, and the expression of inflammatory factors interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in macrophages were analyzed, to explore the effects of PQ on macrophages, thereby providing effective references and guidance for the clinical prevention and treatment of PQ poisoning.

Materials and methods

Animal data. Mouse macrophage RAW264.7 was purchased from Shenzhen Haodi Huatuo Biotechnology Co., Ltd. (HTX1568; Shenzhen, China), and cultured in cell culture

medium and incubator containing 10% fetal bovine serum (FBS; A3160801; Shanghai Mituo Biotechnology Co., Ltd., Shanghai, China). PQ was purchased from Shanghai Future Industrial Co., Ltd. (B3379; Shanghai, China) at concentrations of 0.01, 0.1, 1 and 10 mmol/l, respectively. All operations were done on a super clean station. The study was approved by the Ethics Committee of Hunan Provincial People's Hospital (Changsha, China). Signed informed consents were obtained from the patients or the guardians.

Methods
Detection of cell viability. Mouse macrophage RAW264.7 was placed in a 75 cm^2 culture flask. When the contact rate of cell growth reached 80%, cells were separately placed in 15 (25 cm^2) culture flasks and cultured again until the contact rate reached 80%. Then, the supernatant was aspirated and cell culture medium containing 0.5% FBS was added, and incubated in a cell incubator for 24 h. The next day, culture flasks were taken out. Different concentrations of PQ were separately added to 4 culture flasks as: The micro concentration (0.01 mmol/l), the low (0.1 mmol/l), the medium (1 mmol/l) and the high concentration groups (10 mmol/l). The remaining 3 culture flasks were continuously added with the culture medium without 0.5% FBS as the control group. Then, all the culture flasks were placed in a cell incubator. One culture flask was taken out from each group at 1, 4 and 8 h of incubation. The supernatant was aspirated, centrifuged at 3,000 x g for 5 min at 20°C and frozen for testing. The trypan blue solution was used for detecting the cell viability.

Detection of ROS. The cell suspension was added to a 96-well black-bottomed cell culture plate. When the contact rate of cell growth reached 80%, the supernatant was aspirated. Then, cell culture medium containing 0.5% FBS was added, incubated in a cell incubator for 24 h. The next day, the culture plate was taken out. Different concentrations of PQ were separately added to 4 cell culture wells as the micro concentration (0.01 mmol/l), the low (0.1 mmol/l), the medium (1 mmol/l) and the high concentration groups (10 mmol/l). The culture medium without 0.5% FBS was added as the control group. After that, the culture plate was placed in a cell incubator, which was taken out and washed 3 times with deionized water at 1, 4 and 8 h of incubation, respectively. 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany QN1289-JUI) was added and the plate was returned to the cell incubator. After 30 min of incubation, the microplate reader (485 nm excitation wavelength, 525 nm emission wavelength; Bio-rad, Hercules, CA, USA) was used for detecting the ROS fluorescence intensity.

Detection of IL-6 and TNF-α. ELISA was used for detecting the expression levels of IL-6 and TNF-α in the five groups. IL-6 kit was purchased from American Boao Pike Biological Co., Ltd. (45-IL6HU-E05), TNF-α kit from Diken Trading Co., Ltd. (BE45471; Shanghai, China). All operations were done in strict accordance with the kit instructions.

Outcome measures. The cell viability, the ROS fluorescence intensity and IL-6 and TNF-α expressions in the micro

concentration, the low, the medium, the high concentration and the control groups at 1, 4 and 8 h, respectively, were evaluated.

Statistical analysis. SPSS 24.0 statistical software (IBM Corp., Armonk, NY, USA) was used for analyzing and processing the data. Measurement results were expressed as mean ± standard deviation, and t-test was used for comparison between the two groups. Count results were expressed as ratio, and Chi-square test was used for comparison between the two. The analysis of variance (ANOVA) of repeated measurements and Fisher's test was used for the comparison among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. There was no significant difference in the cell viability at the 1st hour among the five groups (P>0.050), but there was a significant difference at 4 and 8 h (P<0.050). At 1 h, there was no significant difference in the cell viability among the micro concentration, the low concentration and the control groups (P>0.050), and between the medium and the high concentration groups (P>0.050). The medium and the high concentration groups had significantly lower cell viability than the other three groups (P<0.050). At 4 and 8 h, there was no significant difference in the cell viability among the micro concentration, the low concentration and the control groups (P>0.050). The medium and the high concentration groups had significantly lower cell viability than the other three groups (P<0.050). The high concentration group had significantly lower cell viability than the medium concentration group (P<0.050). There was no significant difference in the cell viability among 1, 4 and 8 h in the micro concentration, the low concentration and the control groups (P>0.050). In the medium and the high concentration group, the cell viability was the highest at 1 h and the lowest at 8 h, which was lower at 4 h than that at 1 h (P<0.050; Table I).

ROS. There was no significant difference in the ROS fluorescence intensity at 1 h among the five groups (P>0.050), but there was a significant difference at 4 and 8 h (P<0.001). At 1, 4 and 8 h, respectively, there was no significant difference in the ROS fluorescence intensity among the micro concentration, the low concentration and the control groups (P>0.050). The medium and the high concentration groups had significantly higher ROS fluorescence intensity than other three groups (P<0.050). The high concentration group had significantly higher ROS fluorescence intensity than the medium concentration group (P<0.050). There was no significant difference in the ROS fluorescence intensity among 1, 4 and 8 h in the micro concentration, the low concentration and the control groups (P>0.050). In the medium and the high concentration groups, the ROS fluorescence intensity was the lowest at 1 h and the highest at 8 h, which was higher at 4 h than that at 1 h (P<0.050; Table II).

IL-6 and TNF-α. There was a significantly difference in the expression level of IL-6 at 1, 4 and 8 h among the five groups (P<0.050). There was no significant difference in the control group among the 1, 4 and 8 h (P>0.050). In the micro, low, medium and the high concentration groups, the

Table I. Comparison of cell viability (%).

Time (h)	Micro concentration	Low concentration	Medium concentration	High concentration	Control	F	P-value
1	90.43	89.14	81.04[b,c]	79.54[a-d]	89.43[d,e]	0.247	0.167
4	92.64	91.33	74.16[a-c]	53.72[a-d]	88.69[d,e]	2.721	0.032
8	90.27	91.28	67.25[a-c]	19.64[a-d]	90.74[d,e]	16.652	<0.001

[a]P<0.050, compared to the cell viability at the 1st hour in the same group; [b]P<0.050, compared to the cell viability in the micro concentration group at the same time point; [c]P<0.050, compared to the cell viability in the low concentration group at the same time point; [d]P<0.050, compared to the cell viability in the medium concentration group at the same time point; [e]P<0.050, compared to the cell viability in the high concentration group at the same time point.

Table II. Comparison of ROS fluorescence intensity.

Time (h)	Micro concentration	Low concentration	Medium concentration	High concentration	Control	F	P-value
1	324.61±117.14	315.84±137.62	406.51±176.56[a-c]	429.64±232.55[a-d]	314.56±142.62[d,e]	0.333	0.117
4	316.27±126.08	318.65±127.61	436.16±189.65[a-c]	467.56±265.99[a-d]	309.48±134.34[d,e]	5.515	<0.001
8	314.66±128.64	317.16±131.57	476.41±207.91[a-c]	549.56±307.74[a-d]	291.64±135.48[d,e]	10.572	<0.001

[a]P<0.050, compared to the ROS fluorescence intensity at the 1st hour in the same group; [b]P<0.050, compared to the ROS fluorescence intensity in the micro concentration group at the same time point; [c]P<0.050, compared to the ROS fluorescence intensity in the low concentration group at the same time point; [d]P<0.050, compared to the ROS fluorescence intensity in the medium concentration group at the same time point; [e]P<0.050, compared to the ROS fluorescence intensity in the high concentration group at the same time point; ROS, reactive oxygen species.

Table III. Comparison of IL-6.

Time (h)	Micro concentration	Low concentration	Medium concentration	High concentration	Control	F	P-value
1	4.85±1.54	7.12±1.63	9.26±3.84	14.77±2.96	3.04±0.52	4.443	0.004
4	19.36±3.49[a]	26.71±6.27[a]	46.34±6.02[a]	58.27±8.07[a]	3.36±0.60	49.143	<0.001
8	82.63±8.24[a,b]	120.76±9.81[a,b]	153.69±6.32[a,b]	219.55±8.96[a,b]	3.49±070	341.527	<0.001

[a]P<0.050, compared to the expression level of IL-10 at the 1st hour in the same group; [b]P<0.050, compared to the expression level of IL-10 at 4 h in the same group; IL-6, interleukin-6.

expression level of IL-6 was the lowest at 1 h and the highest at 8 h, which was higher at 4 h than that at 1 h (P<0.050). At 1, 4 and 8 h, respectively, the expression level of IL-6 was the lowest in the control group, followed by the micro, low and medium concentration groups, and the highest in the high concentration group (P<0.050; Table III and Figs. 1-3). There was a significant difference in the expression level of TNF-α at 1, 4 and 8 h among the five groups (P<0.050). There was no significant difference in the control group among 1, 4 and 8 h (P>0.050). In the micro, low, medium and the high concentration groups, the expression level of TNF-α was the

lowest at 1 h and the highest at 8 h, which was higher at the 4 h than that at 1 h (P<0.050). At 1 and 4 h, the expression level of TNF-α was the lowest in the control group, followed by the micro, low and the medium concentration groups, and the highest in the high concentration group (P<0.050). At 8 h, there was no significant difference in the expression level of TNF-α between the medium and the high concentration groups (P>0.050), which was significantly higher than that in the other three groups (P<0.050). In the other three groups, the expression level of TNF-α was the lowest in the control group, which was higher in the micro concentration group than
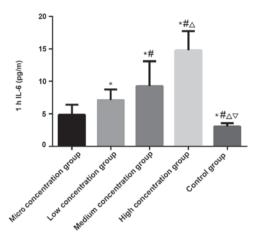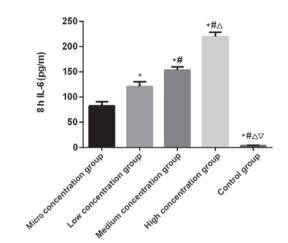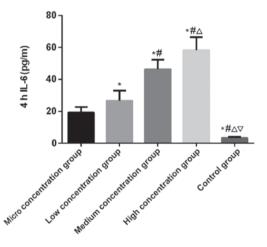
Figure 1. Expression level of IL-6 at 1 h. The expression level of IL-6 was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group, and higher in the high concentration group than that in the medium concentration group. *P<0.050, compared to the expression level of IL-6 in the micro concentration group; #P<0.050, compared to the expression level of IL-6 in the low concentration group; △P<0.050, compared to the expression level of IL-6 in the medium concentration group; ▽P<0.050, compared to the expression level of IL-6 in the high concentration group; IL-6, interleukin-6.

Figure 3. Expression level of IL-6 at 8 hr. The expression level of IL-6 was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group, and higher in the high concentration group than that in the medium concentration group. *P<0.050, compared to the expression level of IL-6 in the micro concentration group; #P<0.050, compared to the expression level of IL-6 in the low concentration group; △P<0.050, compared to the expression level of IL-6 in the medium concentration group; ▽P<0.050, compared to the expression level of IL-6 in the high concentration group; IL-6, interleukin-6.
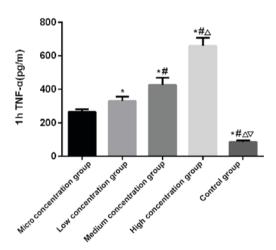

Figure 2. Expression level of IL-6 at 4 h. The expression level of IL-6 was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group, and higher in the high concentration group than that in the medium concentration group. *P<0.050, compared to the expression level of IL-6 in the micro concentration group; #P<0.050, compared to the expression level of IL-6 in the low concentration group; △P<0.050, compared to the expression level of IL-6 in the medium concentration group; ▽P<0.050, compared to the expression level of IL-6 in the high concentration group; IL-6, interleukin-6.

Figure 4. Expression level of TNF-α at 1 h. The expression level of TNF-α was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group, and higher in the high concentration group than that in the medium concentration group. *P<0.050, compared to the expression level of TNF-α in the micro concentration group; #P<0.050, compared to the expression level of TNF-α in the low concentration group; △P<0.050, compared to the expression level of TNF-α in the medium concentration group; ▽P<0.050, compared to the expression level of TNF-α in the high concentration group; TNF-α, tumor necrosis factor-α.

that in the control group, and higher in the low concentration group than that in the micro concentration group (P<0.050; Table IV and Figs 4-6).

Discussion

As the most common organic heterocyclic herbicide in agriculture, PQ is low in price, with high herbicidal efficiency. It can be quickly degraded in the soil and has no pollution to the environment, so it is widely used (13). Since the first

PQ poisoning death in the 1960s, PQ poisoning has begun to increase (14). It has many infection routes, with strong toxicity and rapid poisoning time (15). This is also an important reason for clinical attention. At present, there is no effective method for treating acute PQ poisoning, so research on its pathogenesis are especially important. It is a major research focus and difficulty in the development of effective treatments through knowing the mechanism of PQ poisoning. With the deepening of research, more and more studies have proved that

Table IV. Comparison of TNF-α.

Time (h)	Micro concentration	Low concentration	Medium concentration	High concentration	Control	F	P-value
1	264.19±17.24	329.62±27.96	426.52±42.17	659.18±48.37	86.37±10.24	126.437	<0.001
4	756.33±24.26[a]	868.04±30.58[a]	2,019.86±106.40[a]	3,632.81±227.06[a]	92.33±9.68	450.713	<0.001
8	2,677.68±164.07[a,b]	4,022.86±149.81[a,b]	6,158.27±269.34[a,b]	6,094.56±254.76[a,b]	90.72±10.53	519.526	<0.001

[a]$P<0.050$, compared to the expression level of TNF-α at the 1st hour in the same group; [b]$P<0.050$, compared to the expression level of TNF-α at 4 h in the same group; TNF-α, tumor necrosis factor-α.

Figure 5. Expression level of TNF-α at 4 h. The expression level of TNF-α was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group, and higher in the high concentration group than that in the medium concentration group. *$P<0.050$, compared to the expression level of TNF-α in the micro concentration group; #$P<0.050$, compared to the expression level of TNF-α in the low concentration group; △$P<0.050$, compared to the expression level of TNF-α in the medium concentration group; ▽$P<0.050$, compared to the expression level of TNF-α in the high concentration group; TNF-α, tumor necrosis factor-α.
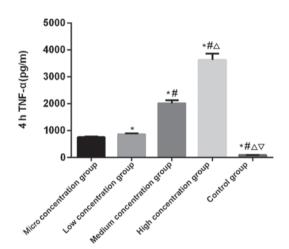

Figure 6. Expression level of TNF-α at 8 h. The expression level of TNF-α was the lowest in the control group, which was higher in the low concentration group than that in the micro concentration group, and higher in the medium concentration group than that in the low concentration group. There was no significant difference between the high concentration group and the medium concentration group. *$P<0.050$, compared to the expression level of TNF-α in the micro concentration group; #$P<0.050$, compared to the expression level of TNF-α in the low concentration group; △$P<0.050$, compared to the expression level of TNF-α in the medium concentration group; ▽$P<0.050$, compared to the expression level of TNF-α in the high concentration group; TNF-α, tumor necrosis factor-α.

PQ poisoning is mainly related to various tissue cell injuries in the body caused by inflammatory cells (16-18).

The anti- and pro-inflammatory effects of the body maintain a dynamic equilibrium under normal circumstances. The invasion of PQ causes significant overexpression of pro-inflammatory mediators, which shows multiple organ dysfunction and failure, resulting in death in patients (19). During the inflammatory response, IL-6 and TNF-α are two representative inflammatory factors. As a B cell growth factor, IL-6 is secreted by activated immune cells such as lymphocytes and macrophages. It accelerates inflammatory injury by promoting the inflammatory activation and differentiation of cells, and promotes neutrophil respiratory outbreaks and degranulation to produce oxygen free radicals, thereby aggravating tissue and organ injuries (20). TNF-α, an inflammatory factor secreted by endothelial cells and mononuclear macrophages, activates nuclear factor-κB (NF-κB) to mediate the expression of a series of inflammatory factors,

thereby promoting neutrophil degranulation and the release of lysosomes, and aggravating the damage to patients (21). There are currently few studies on IL-6 and TNF-α in PQ. Therefore, in the experiment, different concentrations of PQ poisoning mouse macrophage RAW264.7 models were established, and the expression of IL-6 and TNF-α was detected, to explore the mechanism of action of PQ on inflammatory cells.

The results of this experiment showed that there was no significant difference in the cell viability among the micro concentration, the low concentration and the control groups, but the medium concentration and the high concentration groups had significantly lower cell viability than the other three groups. It suggests that PQ at a concentration of 1 mmol/l has an impact on the environment in the body. It is speculated that PQ can be reduced by enzymes after entering macrophages, thereby forming PQ-electrons (22). At this time, PQ-electrons bind to oxygen ions in macrophages to provide electron donors for NADPH, thereby accelerating the conversion of oxygen

into active oxygen radicals in the body. As a result, the normal oxidative phosphorylation process and energy synthesis in the body are reduced, causing cell failure and metabolic dysfunction. There were no significant differences in the ROS fluorescence intensity, IL-6 and TNF-α among the micro concentration, the low concentration and the control groups, but the medium concentration and the high concentration groups had significantly higher ROS fluorescence intensity, IL-6 and TNF-α than the other three groups. It proves that PQ poisoning has an effect on the body at a concentration of 1 mmol/l. It is speculated that PQ induces macrophages to produce ROS after entering macrophages, thereby promoting activated macrophages to synthesize and release a large amount of pro-inflammatory mediators, proteases and chemokines. At this time, the expression of ROS in cells is greatly increased. ROS can induce the release, infiltration and activation of IL-6 and TNF-α through NF-κB, and accelerate the damage of the environment in the body, finally leading to organ dysfunction and failure. The findings of He *et al* (23) in the study on the effects of PQ on alveolar epithelial cells are basically consistent with the results of this experiment, which can support the views of this experiment.

Macrophage RAW264.7 in mice is different from that in the human body, and the sample size of this experiment is small. Therefore, the statistical analysis of big data cannot be performed. Human experiments will be conducted as soon as possible, and the sample size will be enlarged to improve the experimental results.

In summary, PQ at a concentration of 1 mmol/l can produce toxicity to macrophages, and greatly increase the ROS fluorescence intensity and the expression levels of IL-6 and TNF-α. PQ poisoning is expected to be treated through IL-6 and TNF-α in the future.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JH and NN were responsible for detection of cell viability and ROS. JH and WZ performed ELISA. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hunan Provincial People's Hospital (Changsha, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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