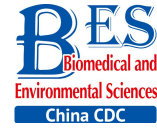


Letter to the Editor



Early Changes of Peripheral Blood Lymphocyte Subpopulations in Patients with Occupational 2,4-dinitrophenol Poisoning*

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2,4-dinitrophenol (DNP), an organic compound which frequently used in industry, is considered to have high toxicity. This study aimed to investigate the early changes of lymphocyte subpopulations in patients with occupational 2,4-DNP poisoning. Totally 9 patients with acute occupational 2,4-DNP poisoning and 30 healthy volunteers as control were enrolled. The patients received immediately comprehensive supportive treatments, including large-dose glucocorticoid and repeated hemoperfusion (HP). The ratio of CD4⁺/CD8⁺ T cells were significantly higher in patients upon admission compared to healthy controls ($P < 0.01$); however, counts of total lymphocytes, CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, B (CD19⁺), and natural killer (NK) cells (CD16⁺CD56⁺) were significantly reduced (all $P < 0.001$). The NK cell count was negatively correlated with initial plasma 2,4-DNP concentration ($r = -0.750$, $P = 0.026$). Thus, acute occupational 2,4-DNP poisoning was accompanied by immediate complex immune cell reactions, especially NK cells might play important role in severe 2,4-DNP poisoning.

2,4-dinitrophenol (DNP) is an organic compound that widely used as a chemical intermediate for making dyes, other organic chemicals, and wood preservatives in the manufactures^[1]. It is also used to make photographic developer, explosives, and insect control substances as well. In addition, 2,4-DNP was once used as a weight-loss drug which had an ability to increase basal metabolic rate greatly. However, it has since been banned by the United States Food and Drug Administration because of its serious side effects, including hyperthermia, tachycardia, and even death^[1]. And yet, 2,4-DNP poisoning is still reported due to illicit use, as well as exposure due to its wide use in industry^[2]. Although

most cases of 2,4-DNP poisoning reported are caused by oral ingestion, we investigated 9 occupational cases in this study due to direct skin and respiratory tract contact. The toxicity of 2,4-DNP is thought to result from the uncoupling of oxidative phosphorylation, but a definitive mechanism remains to be determined^[2]. The lymphocyte expression research would promote a better understanding of the differential function of immune cell subsets in an immune response of 2,4-DNP poisoning. Very few studies have evaluated the lymphocyte subsets of 2,4-DNP poisoned patients up to now. Thus, in the present study, we analyzed early variations of peripheral blood lymphocyte subpopulations in patients and compared them with initial blood 2,4-DNP concentrations.

This research was carried out in accordance with the principles of the *Declaration of Helsinki* and was approved by the Ethics Committee of First Affiliated Hospital, School of Medicine, Zhejiang University. Nine patients including 8 males and 1 female (mean age 44.3 ± 15.5 years, range 25-64 years), who all worked at a same chemical factory in East China, were directly exposed to 2,4-DNP yellow powder (direct contact with respiratory tract and skin) without any protection, lasting for 5-6 work hours. The asymptomatic incubation period from exposure was 2-30 h (mean time 17.1 h). After that, heavy perspiration was the first symptom in all patients, accompanying by fever, fatigue, and skin redness. The mean oral temperature was 39.2 °C (range 38.6-40.7 °C), and the mean pulse rate was 106 beats/min (range 95-144 beats/min). The 2,4-DNP contaminated parts of the skin had no feeling of pain in all patients. These 9 patients were diagnosed as having acute occupational 2,4-DNP poisoning based on their toxic exposure history, hygienic

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investigation at the exposure site, clinical manifestations, laboratory examination and poison identification made by Zhejiang Provincial Center for Disease Control and Prevention (Zhejiang CDC). The initial plasma 2,4-DNP concentrations was (19.27 ± 13.18) $\mu\text{g/mL}$ (range 2.01-41.88 $\mu\text{g/mL}$), which measured in all the patients. Patients were excluded in this research if they: 1) had tumor, trauma, or basic diseases (such as cardiac diseases, diabetes, and autoimmune diseases) before poisoning; 2) received immune suppressive treatment in the past three months. After physical exams, these 9 patients were all included in this study.

There was a big controversy on the effectiveness and side effect of Dantrolene therapy for 2,4-DNP toxicosis, so supportive managements are the best treatment option until now^[3]. All patients received immediately comprehensive supportive treatments which includes electrocardiogram monitoring, cleaning the polluted skin, cutting off the contaminated hair and nails, physical cooling, administering isotonic saline solution, maintaining electrolyte and acid-base balance, glucocorticoid and blood purification treatment^[4-5]. A dosage of 500 mg/d of methylprednisolone was given to all the patients for 3 d, decreased gradually thereafter, and stopped at day 7. Hemoperfusion (HP) was applied to the patients within 6 h after admission and once a day (4-6 h) for 6 d. Nine patients in this study were all recovered and discharged at day 20-25. There was no sequela evident at the three-month follow-up.

Thirty healthy volunteers were recruited as a control group, including 20 males and 10 females (mean age 38.3 ± 10.7 years, range 20-65 years). The permission to use clinical data in this study was obtained by written informed consent from the patients/participants or their next of kin, and all the data were analyzed anonymously.

Blood samples from patients were collected within 30 min of admission, before glucocorticoid and HP were given, and again on the 3th, 5th, and 9th day after admission. Blood samples from control volunteers were collected only once. A conventional hemogram was used to determine the number of leukocytes, and total lymphocyte counts were obtained using an automatic blood cell analyzer. Peripheral blood mononuclear cells were prepared for detection of differential lymphocytes. A flow cytometer (FC500, FACS Calibur; Beckman-Coulter Corp., Brea, CA, USA) was used to identify the following lymphocyte subpopulations: CD3⁺ (total T

cells), CD3⁺CD4⁺ (T-helper-inducer cells), CD3⁺CD8⁺ (T-cytotoxic-suppressor cells), CD19⁺ (B cells), and CD16⁺CD56⁺ [natural killer (NK) cells]. All antibodies used for flow cytometry were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The absolute cell counts of lymphocyte subpopulations were calculated according to standard flow cytometry criteria for lymphocyte subpopulation identification and the total lymphocyte counts obtained in conventional hemogram.

Whole blood samples (1 mL) collected from patients upon admission before treatment were centrifuged at 3500 $\times g$ for 5 min. Then, 200 μL of the supernatant was mixed with 400 μL of acetonitrile, vortexed and centrifuged at 3500 $\times g$ for 5 min. The resulting supernatant (400 μL) was mixed with 500 μL mobile phase (10 mmol/L formic ammonium and acetonitrile, 9:1) and filtered through a membrane before analysis. Plasma 2,4-DNP concentrations were measured using ultrahigh performance liquid chromatography/ tandem mass spectroscopy (UPLC-MS/MS) (Waters, Milford, Massachusetts, USA). The quantitation limit for plasma 2,4-DNP concentration was 0.01 $\mu\text{g/mL}$.

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Enumeration data, such as gender, were analyzed with Fisher's exact test. A one-sample Kolmogorov-Smirnov test was used to evaluate the data distribution normality. Normally distributed data, such as age and plasma toxin concentration, were represented as mean \pm standard deviation (SD). The difference of ages of two groups was analyzed by independent sample *t*-test. The total sample size was not large, and part immune cell subpopulations of patients were not normally distributed. Thus, the cell counts of lymphocyte subpopulations were represented as median (range). A Mann-Whitney *U* test was used to compare the immune cell subpopulations between patients and healthy controls. Correlation analysis was performed using a Spearman's coefficient. Statistical significance was set at $P < 0.05$.

The contaminated hands and feet of all 9 patients were dyed yellow, or even black, without pain or other sensory dysfunction. Heavy perspiration accompanied with fever was the characteristic symptom indicated by all patients. There were no significant differences between patients and healthy controls with regard to age or gender ($P = 0.184$, $P = 0.399$, respectively). The comprehensive supportive treatments were effective, and all patients were recovered and

discharged on day 20-25, at which time plasma 2,4-DNP concentrations were all undetectable.

The lymphocyte subpopulations were detected on day 1 (on admission), 3, 5, and 9 during hospitalization. The absolute cell counts of total lymphocytes, total T cells (CD3⁺), T-helper-inducer cells (CD3⁺CD4⁺), T-cytotoxic-suppressor cells (CD3⁺CD8⁺), B cells (CD19⁺), NK cells (CD16⁺CD56⁺)

and the ratio of CD4⁺/CD8⁺ T cells of patients were all tested significantly different than controls on the admission day (all *P* < 0.01), in which the ratio of CD4⁺/CD8⁺ T cells were significantly higher compared to healthy controls (*P* < 0.01); however, counts of total lymphocytes, CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, B, and NK cells were significantly reduced (all *P* < 0.001) (Table 1, Figure 1).

Table 1. Cell Counts of Lymphocyte Subpopulations in Peripheral Blood of Patients with Acute 2,4-DNP Poisoning and Healthy Controls [M (*P*₂₅ *P*₇₅)]

Cell Counts (×10 ⁹ /L)	Healthy Controls (n = 30)	Patients (Days since admission)				
		Day 1 (n = 9)	<i>P</i> value	Day 3 (n = 9)	Day 5 (n = 9)	Day 9 (n = 9)
Total lymphocytes	2.34 (1.13-3.47)	0.55 (0.09-1.13)	< 0.001 ^a	0.94 (0.29-1.67)	0.64 (0.30-1.84)	1.19 (0.65-1.37)
CD4 ⁺ /CD8 ⁺ ratio	1.63 (0.83-2.36)	2.40 (0.98-5.17)	0.001 ^a	2.68 (1.08-8.44)	3.33 (1.09-9.84)	2.17 (0.88-5.18)
CD3 ⁺	1.58 (0.85-2.51)	0.36 (0.05-0.76)	< 0.001 ^a	0.55 (0.22-0.97)	0.38 (0.18-1.26)	0.68 (0.45-0.75)
CD3 ⁺ CD4 ⁺	0.92 (0.44-1.33)	0.19 (0.03-0.32)	< 0.001 ^a	0.33 (0.14-0.65)	0.26 (0.12-0.62)	0.18 (0.09-0.40)
CD3 ⁺ CD8 ⁺	0.55 (0.29-1.08)	0.08 (0.01-0.33)	< 0.001 ^a	0.14 (0.04-0.30)	0.10 (0.02-0.57)	0.17 (0.09-0.36)
B	0.27 (0.10-0.53)	0.08 (0.02-0.30)	< 0.001 ^a	0.11 (0.02-0.27)	0.12 (0.04-0.42)	0.19 (0.07-0.36)
NK	0.32 (0.09-0.74)	0.12 (0.01-0.23)	< 0.001 ^a	0.13 (0.02-0.40)	0.08 (0.01-0.17)	0.18 (0.10-0.46)

Note. Data are presented as Median (range). ^avs. control. NK = natural killer.

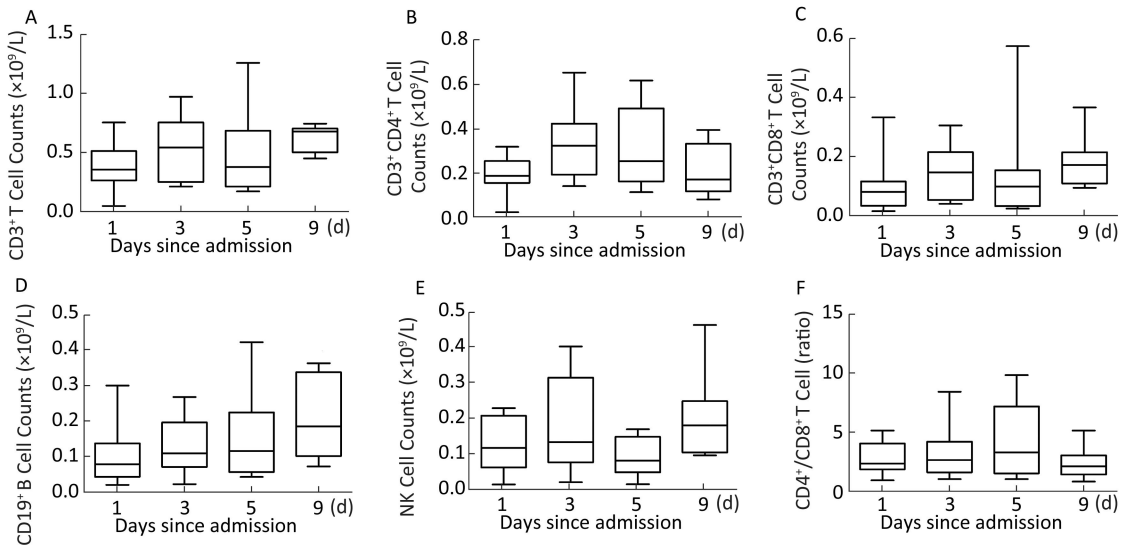


Figure 1. Dynamic changes of lymphocyte subpopulations in patients. Immune cell counts from peripheral blood of patients with acute 2,4-DNP poisoning on day 1, 3, 5, and 9 (since admission).

Due to the effects of large-dose glucocorticoid and repeated HP treatment on lymphocyte subpopulation changes in patients with occupational 2,4-DNP poisoning, we only demonstrated the cell counts of lymphocyte subpopulations on day 3, 5, 9, but didn't analysis these data.

Correlation analysis between initial toxin concentration and lymphocyte subpopulations revealed that only NK cell count on the admission day was significantly and negatively correlated with the initial plasma 2,4-DNP concentration ($r = -0.750$; $P = 0.026$) (Figure 2).

The 2,4-DNP molecule (184.1 molecular weight) is lipophilic with a $pK_a = 4.09$, and can thus be absorbed through the acidic stomach lining and passively permeate cell membranes through water channels. Most cases of 2,4-DNP poisoning occur following conscious ingestion, though 2,4-DNP can readily enter the body through epidermal and respiratory routes, as demonstrated in the patients described in the present study^[1]. After absorption, 2,4-DNP rapidly distributes to the liver, where it is primarily metabolized, as well as to the lungs and kidneys, with the highest concentrations detected in blood. Less toxic metabolites, such as 2-amino-4-nitrophenol and 4-amino-2-nitrophenol, which may be conjugated to glucuronic acid or sulfate, reach their highest levels approximately 30 min following oral administration^[6]. Theoretically, 2,4-DNP is a protoplasmic poison, which can act directly on cellular metabolism by inducing oxidation and inhibiting phosphorylation, leading to the

uncoupling of oxidative phosphorylation. Then, the cellular energy generated from the mitochondria is released directly as heat and results in uncontrolled transepidermal water loss and thermogenesis, which were in accordance with clinical manifestations of patients^[4].

Clinically, inflammation from 2,4-DNP exposure was accompanied by low numbers of total lymphocytes, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells in peripheral blood, possibly indicating two causes: long-term and high-intensive suppressed immune responses, or overactive immune responses. Considering the pathogenesis and clinical manifestations, we thought that occupational 2,4-DNP poisoning potentially showed the overactive immune responses. 2,4-DNP can rapidly distribute to various tissues, inducing a large infiltration of various immune cells into tissues, thus reducing the number of these lymphocyte subsets present in peripheral blood^[6]. We still need further study to better understand the mechanism of 2,4-DNP toxicity in uncoupling of oxidative phosphorylation process. High-dose glucocorticoid therapy can successfully inhibit inflammation and alleviate toxic damage to the body, especially for the severe cases^[7]. Furthermore, the counts of lymphocyte subpopulations remained relatively low levels in our patients after 9 d. It demonstrated that the exhausted immune cells in blood were unable to be completely supplemented at day 9. This incomplete recovery may also have been a result of the immune inhibitory role of glucocorticoid (continuous used until

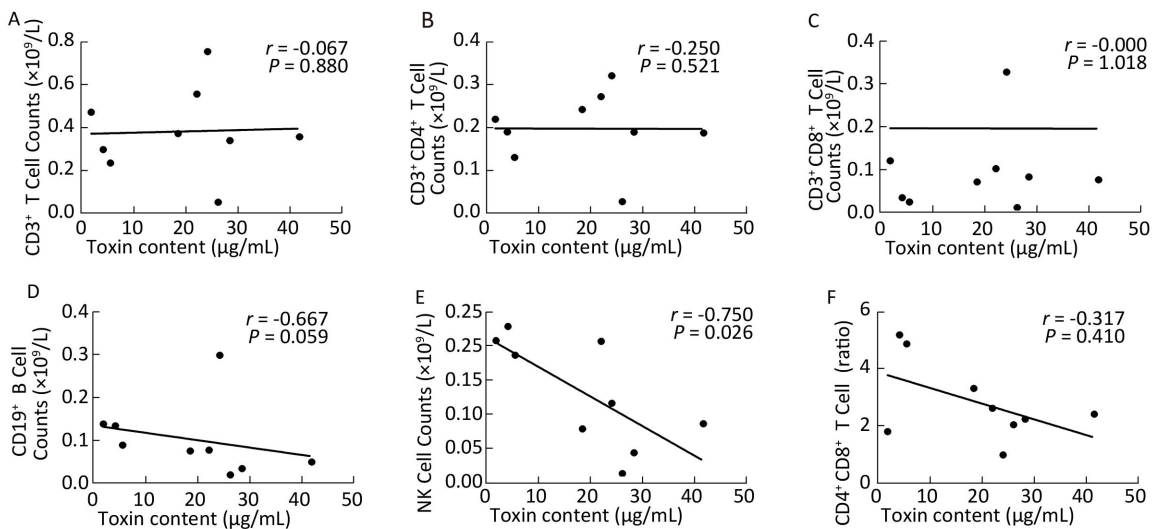


Figure 2. Correlations between initial plasma 2,4-DNP concentrations and immune cell subpopulations.

day 7), which can suppress the expression of Fc receptors and T cell growth factor and induce lymphocyte apoptosis, mainly through regulation of gene transcription.

The ratio of CD4⁺/CD8⁺ T cells was significantly increased in patients with acute occupational 2,4-DNP poisoning on admission. This result suggested that immune disorder may occur during the acute phase of poisoning, resulted from higher toxin concentrations in tissue driving stronger active T cell responses, such as the infiltration of cytotoxic CD3⁺CD8⁺ lymphocytes.

NK cells play an important immune roles in many disease (including tumor and infection), not only through direct killing of target cells but also through the cytokine IFN- γ secreted by NK cells^[8]. But the researches of NK cells in cases with poisoning were very limited. Previous studies have shown reduced ratios of NK cells in Formaldehyde exposed workers and mice compared with controls^[9-10]. In the present study, lower NK cell counts in peripheral blood of patients were accompanied with higher initial plasma toxin concentrations. Thus, NK cell counts in peripheral blood may be a good supplementary marker for the severity of patients with acute occupational 2,4-DNP poisoning.

This clinical study had several limitations. First, the sample size was not large and the randomization was not implemented nicely, based on cohort study. Second, it was not possible to exclude the influence of glucocorticoid completely in the results presented here, as all patients received treatment. The best study design, which tracks the lymphocyte subpopulations counts longitudinally to clarify the role of lymphocyte subsets in severe 2,4-DNP poisoning, is to monitor dynamically the patients without any treatment. However, it will be considered ethically unacceptable.

Exposure to 2,4-DNP promptly lowers lymphocyte counts in peripheral blood, which contributes to the initiation of inflammation. These pathologic phenomena may be indications for glucocorticoid therapy. Fortunately, the patients responded well to the treatments with no evidence of glucocorticoid-related complications. And the lymphocyte counts were recovered to the normal level when the patients discharged on day 20-25 after admission. We considered the fact that short-term and high dose administering of glucocorticoid may suppress the toxic effects of

2,4-DNP without promoting uncontrollable and unpredictable risks.

In conclusion, large-dose glucocorticoid and repeated HP therapy had a remarkable healing effect without significant side effects. It is important to note that all 9 patients who accepted this comprehensive treatments recovered favorably without significant complications. Thus, although the effects of this treatment strategy on the immune system need to be further validated in animal models and the optimal treatment programs of glucocorticoid and HP for patients need further discussion, we still recommend it for the cases of acute occupational 2,4-DNP poisoning.

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Contributorship Statement Y.Q.L conceived the experiments and performed the treatments. J.K.J and L.H.G collected samples and conducted the experiments. L.H.G analyzed the data. Y.Q.L, J.K.J, and W.F contributed to the writing of the manuscript. All authors reviewed the manuscript.

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