The effects of vitamin E on NK cell activity and lymphocyte proliferation in treated mice by 2,3,7,8-tetrachlorodibenzo-p-dioxin

Xiao-Hui Wang, Xian-Qing Zhou, Jian-Ping Xu, Ying Wang, and Jing Lu

Department of Laboratory Animal Science, School of Basic Medical Sciences, Capital Medical University, Beijing, China

Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a widespread environmental contaminant, which induces wide variety of toxic and biochemical effects, while immune system is sensitively affected by TCDD. The previous study showed that TCDD decreased the neutrophil numbers, declined inflammatory chemokines and cytokines, suppressed the activity of cytotoxic T cell and the interleukin production, depressed the response of antibody to antigen and T lymphocyte transformation in mice, caused thymus atrophy, and suppressed the IgM secretion of B lymphoma cell line CH12.LX.

Vitamin E is an important immune promoting agent and antioxidant in both animal and human. vitamin C and E combination alleviated the decreases of spleen lymphocyte transformation and activity of nature kill cell (NK cell), antagonized the increase of serum and liver lipid peroxide levels, alleviated the decrease of superoxide dismutases (SOD) activity caused by cyclophosphamide in mice; Vitamin E and selenium combination partly retracted the declining of immunoglobin level and circulating immunecomplex (CIC) caused by malathion in chicken. Vitamin E or a combination of vitamin E with vitamin C promoted the non-specific immune function in soft-shelled turtles, declined or partly declined disadvantage effects from acid-stress. In vitro, vitamin E significantly decreased the IL-8 expression of human umbilical vein endothelial cells activated by LPS.

However, little information is available about the effects of vitamin E on spleen lymphocyte proliferation and NK cell activity in TCDD-treated mice. Our previous
studies showed vitamin E alleviated the turbulence of interleukin secreting and the change of spleen T cell subsets in mice caused by chronic TCDD-treated.\(^{[16]}\) The present study was designed to evaluate the effects of vitamin E on lymphocyte proliferation and NK cell activity in chronic and acute TCDD-treated mice, and to find the dose vitamin E which improved immune function in the TCDD-treated mice so as to alleviate the toxicity from TCDD.

**Materials and methods**

**Animals and husbandry**

Four-five week old clean ICR female mice were used in the present study, and were obtained from Beijing Vital River Laboratory Animal Technology Limited Corporation, Beijing, China. Each mouse was raised in a mouse box of 26cm × 15cm × 15cm individually. They were maintained at a constant temperature of 22 ± 2°C, and a humidity of 50 ± 5% with a 12:12 light/dark cycle. The commercial diets used were produced by Beijing Ke Ao Xiel Limited Corporation, (Saint Louis, USA) whose nutritional ingredient are protein 20.6%, fat 4.16%, carbohydrate 53.17%, crude fiber 4.92%, ash content 7.5%, moisture content 9.65%, respectively. All mice were given tap water ad libitum. TCDD (97% purity) was provided by Research Center for Eco-Environmental Sciences, the Chinese Academy of Sciences (Saint Louis, USA). Vitamin E (95% purity) was obtained from Sigma Company (Saint Louis, USA).

**Experimental design**

After 1 week of adaptation to laboratory conditions, healthy 45 female mice with an initial body weight of 15.4–24.9 g were selected. They were randomly assigned to five groups, and containing 9 mice each one. The levels of both TCDD and vitamin E were 0 and 0 (Control group I), 100 and 0 (experimental group I), 100 and 20 (experimental group II), 100 and 100 (experimental group III), and 100 ng/kg/d and 500 mg/kg/d (experimental group VI) in chronic TCDD-treated trial, respectively. Vitamin E and TCDD were given by gavage. TCDD was dissolve in corn oil, and given 2 hours before vitamin E was administrated. The mice in control group I received the corn oil of equal volume. All mice could take their food and drinking water freely during acclimation and experiment, and they were comfortable. After 4 weeks of the gavage experiment, all mice were killed by decapitation, and spleens were collected.

The vitamin E dosage which antagonized TCDD toxicity was selected depending on chronic TCDD-treated trial above, and farther studied the effect of the vitamin E in acute TCDD-treated trial. 24 female mice 4–5 weeks old were selected in the acute TCDD-treated trial, and whose initial body weight was from 20.3 g–28.5 g. They were randomly assigned to three groups, which is control group II, experimental group V and experimental VI, respectively; and containing 8 mice each. Both experimental group V and experimental VI were given TCDD 30µg/kg by gavage, and then the experimental VI was given vitamin E 100 mg/kg/d for next 3 days, while the control group II and experimental group V received the corn oil of equal volume. The other conditions were same as chronic TCDD-trial above. After 4 days, the mice were killed, and spleens were collected.

**The assay of NK cell activity**

The activity of NK cells was measured by lactate dehydrogenase (LDH) release method as described by Wu at al.\(^{[17]}\) using YAC-1 cells target cells. The spleen lymphocytes were adjusted to the concentration of 1 × 10⁵ cells/ml using complete RPMI-1640 medium, which was as effect cells. The YAC-1 cells cultured for 48h were washed three times, and then adjusted to the concentration of 1 × 10⁵ cells/ml using complete RPMI-1640 medium. In a 96-well U-bottom plate, 100 µl lymphocytes suspension per well was added, and followed by 100µl YAC-1 cells at a ratio of effector 10 : target 1 (E/T = 10:1). At the same time, 100 µl YAC-1 cells suspension and 100 µl culture solution were add at a single well as a spontaneous release, and 100 µl YAC-1 cells suspension and 100 µl 1% Nonidet P40 (1%NP2 40) solution add at other well as maximum release, respectively. They incubated at 37°C and 5% CO₂ for 18h, and then centrifugated at 1500rpm for 5min, and 100 µl upper solution per well was extracted and added to the other a 96-well plate, and incubated 37°C and 5% CO₂ for 10 min, and then 100 µl LDH was added per well.

After response at room temperature under dark for 15 min, 1 M citric acid 30 µl was added per well so as to end the enzyme facilitating reaction. Then the absorbance of mixture per well (A) was immediately determined at 570 nm wavelength by BIO-RAD 550 Microplat Reader. The experiments were carried out triplicates. The NK cell activity was calculated according to the formula: \(\frac{([A_{\text{of experimental release}} - A_{\text{of spontaneous release}}])}{([A_{\text{of maximum release}} - A_{\text{of spontaneous release}}])} \times 100\%\).

**The assay of lymphocyte proliferation**

The proliferation of spleen lymphocyte was measured by MTT method as described by Bai at al.\(^{[18]}\)

The spleen each mouse was collected under germfree, and triturated in a glass utensil with 10ml RPMI-1640 medium. Then 1 ml lymphocyte separate solution (Ficoll
solution) and 2 ml spleen cell suspension were added in a germfree centrifuge tube, centrifugated at 2000 rpm for 20 min. The spleen lymphocytes were collected in other germfree centrifuge tube, and followed by 5 ml RPMI-1640 medium to be washed by centrifugating at 1500 rpm for 15 min for three times.

Then the spleen lymphocytes from mice were suspended in RPMI-1640 medium containing 10% calf serum, and then diluted to the concentration of $2 \times 10^6$ cells/ml using this medium. Trypan blue exclusion assays were used to test the viability of spleen lymphocytes, and the survival rate was more than 95%. In a 96-well culture plate, 100 μl lymphocyte suspension was added, followed by 100 μg/ml Con A 5 μl for stimulation. After they were co-incubated at 37°C and 5% CO$_2$ for 68 h, 70 μl/well mixture liquid was thrown away, and 70 μl RPMI-1640 medium except calf serum and 5 mg/ml MTT solution 5 μl were added, and then incubated at 37°C and 5% CO$_2$ for 4 h again.

At the end of the incubation period, 100 μl dimethylsulfoxide (DMSO) was added at each well. The absorbance of mixture was determined at 570 nm wavelength by BIO-RAD 550 Microplat Reade. The experiments were carried out in triplicate. The ConA was not added in the three blank control wells. The lymphocyte proliferation ability was estimated by absorbance.

**Statistical analysis**

The data were analyzed by SPSS 11.5 statistics software. In the chronic TCDD-treated trial, the effect of different dosage vitamin E treatments (experimental group I–IV) were determined by one-way analysis (ANOVA), followed by LSD’s multiple range test to detect significant differences between groups. The differences of between control group I and experimental group I in the chronic TCDD-treated trial, control group II and experimental group V, and experimental group V and experimental group VI in the acute TCDD-treated trial were all determined by independent-sample T test, respective. All values were mean ±SD. The results were considered significant at $P<0.05$.

**Results**

**The change of spleen NK cell activity in chronic TCDD-treated mice**

The chronic TCDD treatment had no significant effects on spleen NK cell activity in mice ($P>0.05$). The NK cell activity had no significant difference among experimental group I–IV. Although the NK cell activity had a increasing tendency with the increase of vitamin E dosage within the range of 0–100 mg/kg, there were no significant difference among experimental group I–IV (Fig. 1B).

**The change of spleen lymphocyte proliferation in chronic TCDD-treated mice**

The chronic TCDD treatment had no significant effects on spleen lymphocyte proliferation in mice ($P=0.057$).
Although the lymphocyte proliferation in experimental group I had a decreasing tendency compared to control group I, there was no significant difference between control group I and experimental group I (Fig. 2A).

There were no significant effects of vitamin E on spleen lymphocyte proliferation in chronic TCDD-treated mice ($P > 0.05$). The lymphocyte proliferation increased with the increase of vitamin E among experimental group I–III, and reached a peak in experimental group III. The lymphocyte proliferation in the experimental group IV had a decrease tendency compared with the experimental group III, but there were no significant difference (Fig. 2B).

The change of spleen NK cell activity in acute TCDD-treated mice

Acute TCDD treatment had significant effect on spleen NK cell activity in mice ($P = 0.011$). The NK cell activity in the experimental group V was notably lower than that of control group II (Fig. 3A). Vitamin E had significantly effects on spleen NK cell activity in acute TCDD-treated mice ($P = 0.012$). The experimental group VI had significant higher NK cell activity than the experimental group V (Fig. 3B).

The change of spleen lymphocyte proliferation in acute TCDD-treated mice

Acute TCDD treatment notably suppressed the spleen lymphocyte proliferation in mice ($P < 0.00$). The lymphocyte proliferation in the experimental group V was significantly lower than that of control group II (Fig. 4A).

Vitamin E notably improved the spleen lymphocyte proliferation in acute TCDD-treated mice ($P = 0.012$). The lymphocyte proliferation in the experimental group VI was significantly higher than that of the experimental group V (Fig. 4B).

Figure 2B. The effect of vitamin E on spleen lymphocyte proliferation in chronic TCDD-treated mice (M±SD). (The letters with absolute different superscript are significant difference [$P < 0.05$].)

Figure 3A. The effect of acute TCDD treatment on spleen NK cell activity in mice (M±SD). (*represent significant difference compared to control group II.)

Figure 4A. The effect of acute TCDD treatment on spleen lymphocyte proliferation in mice (M±SD). (*represent significant difference compared to control group II.)

Figure 3B. The effect of vitamin E on spleen NK cell activity in acute TCDD-treated mice (M±SD). (*represent significant difference compared to experimental group V.)

Figure 4B. The effect of vitamin E on spleen lymphocyte proliferation in acute TCDD-treated mice (M±SD). (*represent significant difference compared to experimental group V.)
Discussion

**The effects of TCDD treatment on NK cell activity and lymphocyte proliferation in mice**

NK cells are lymphocytes which can directly kill kinds of tumor cells and virus infecting cells, which do not need antigen stimulation nor MHC limit. The NK cell play a important role for immune surveillance and killing target cells, and it is considered one of the first defense mechanisms of the immune system against an invading pathogen. Lymphocyte play a important role in controlling the response of acquired immunity, and many substance such as mitogen PHA and ConA could result in T lymphocytes transformation, and cause non-specific proliferation by stimulating T lymphocyte. The lymphocyte proliferation represented the ability of cellular immunity to a certain extent.

The present study showed that the chronic TCDD treatment resulted in decrease tendencies of NK cell activity and lymphocyte proliferation. The results suggested that chronic TCDD treatment partly suppressed the cellular immunity in mice. TCDD significantly decreased thymic cellularity as well as induced changes in T-cell subsets in pregnancy mice; impaired B lymphocyte line CH12.LX secreting IgM, suppressed splenocyte adhesion and adaptive immune response, increased T-cell death and AhR expression in CH12.LX in vitro.

Our previous study showed that the acute TCDD increased serum L-1α level and the percentage of spleen CD8+ T lymphocyte, and declined serum IL-2 level and the percentage of spleen CD4+ T lymphocyte in mice. But Esser et al. showed that no differences in the frequencies of B-cells, T-cells, or NK-cells were detectable after a single dose of TCDD given to young mice. The reason of various results above could be relative to the different sensitivity of immune index to TCDD, the used TCDD dosage, and individual difference in animals.

Acute exposure to TCDD could suppress adaptive immunity. TCDD mediated lymphoid atrophy, suppressed antibody levels, and enhanced interleukin-2 production. The present study showed that the acute TCDD-treatment notably declined NK cell activity and lymphocyte proliferation in mice. The results suggested that immune toxicity from acute TCDD-treatment were more severe compared to chronic TCDD treatment, which implied that short-term TCDD treatment produced severe toxicity when its dosage was enough big.

The toxic effects from TCDD are known to be mediated by the aryl-hydrocarbon receptor (AhR). AhR was an important modulator of the development and function of the immune system. AhR activation altered different elements of the immune system at different times during development by affecting different tissue targets. It caused long-lasting functional alterations in the developing immune system.

Activation of the AhR must occur within the first 3 days of an immune response, and that CD4+ T cells are primary targets in mice. AhR activation in the T-lineage cells was directly involved in thymocyte loss and skewed differentiation, and suppressed the immunization-induced increase in both T cells and B cells.

Most toxic effects induced by TCDD were mediated by binding to the AhR which binds together with a second protein, ARNT, to the response elements of a number of target genes, and thus modulated gene expression.

The activated AhR induced the expression of various genes having xenobiotic responsive elements (XREs) in their enhancer regions, such as the gene for cytochrome P-450 1A1 (CYP1A1). TCDD interfered with physiological signaling of the AhR, leading to cell-specific changes in gene transcription and cell differentiation, and thereby produced toxic effects.

The expression of genes in the AhR-dependent pathway, including AhR, aryl-hydrocarbon receptor nuclear translocator (ARNT), CYP1A1, and CYP1B1 transcripts, and the CYP1A1-associated 7-ethoxyresorufin-O-deethylase (EROD) activity was measured in lymphocytes.

But Dong et al. showed that TCDD did not increase the AhR expression, while enhanced the expression of CYP1A6 mRNA, CYP1A7 mRNA and CYP1A protein in Xenopus laevis. The immune system is sensitively affected by TCDD, while the precise mechanism of how TCDD acts in each immune cell type remains to be determined, and need to be study farther.

**The effects of vitamin E on spleen NK cell activity and lymphocyte proliferation in TCDD-treated mice**

Vitamin E, a lipid-soluble antioxidant vitamin, is important for normal function of the immune cells. The vitamin E significantly improved the cytolytic activity of NK cell in patients with colorectal cancer; aged people; mice; boosted delayed-type hypersensitivity skin response and antibody production in response to vaccination, enhanced proliferation of T cells and blood neutrophil functions in aged people; increased cell division and IL-2 production by naive T cells, improved the percentage of old CD4+ T cells capable of forming an effective immune synapse in aged animals and humans. The vitamin E-induced enhancement of immune functions in the aged was associated with significant improvement in resistance to influenza infection in aged mice, and a reduced risk of acquiring upper respiratory infections in nursing home residents. While vitamin E-deficiency impaired the proliferative response of spleen lymphocytes from adult rats. Vitamin E has been shown to decrease immunosenescence, improve immune function, and may be neuroprotective.
The present study showed that spleen NK cell activity and lymphocyte proliferation had increase tendencies with the increase of vitamin E dosage within the range of 0–100 mg/kg in chronic TCDD-treated mice, and lymphocyte proliferation in the group given vitamin E 100 mg/kg was significantly higher than that of chronic TCDD-treated group. In the acute TCDD-treatment trial, vitamin E 100 mg/kg significantly alleviated the decreases of NK cell activity and lymphocyte proliferation caused by TCDD. The results suggested that vitamin E alleviated partly alleviated the immune toxicity from TCDD.

What the antagonistic action of vitamin E against TCDD could carry out through the following ways:

1. Vitamin E suppressed the activated target gene expression of AhR induced by TCDD. As we already know, TCDD produced toxic effects through activating AhR, and the target gene of AhR was CYP 450 gene. TCDD upregulated the transcription and expression of CYP1A1, CYP1A2, CYP1B1 gene by AhR.\(^{(37)}\)

The study from Wejheden et al.\(^{(38)}\) showed that TCDD improved the expressions of AhR and CYP1A1 in osteoblasts, increased the mRNA levels of CYP1A1, CYP1A2 and CYP1B1 in human thymus cell lines MCF10A.\(^{(39)}\) While vitamin E alleviated the increases of mRNA levels of CYP450, 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase gene.\(^{(40)}\)

Our study also showed that vitamin E partly antagonized the mRNA increases of P450 1A1, 1A2, and 1B1\(^{(41)}\) caused by acute TCDD-treatment 200, and alleviated the increase of P450 1A2 mRNA caused by chronic TCDD – treatment.\(^{(42)}\)

2. Vitamin E inhibited oxidation stress caused by TCDD. Both T cells and NK cells are affected by a multitude of mechanisms of which the generation of reactive oxygen species is of major importance.\(^{(31)}\) Vitamin E could decrease TCDD toxicity through inhibited levels of oxidative stress caused by TCDD. The most biologically active form of vitamin E is alpha-tocopherol (alpha-TOC). In cellular membranes, alpha-TOC prevents lipid peroxidation by scavenging free radicals and functioning as an antioxidant.

Herpes simplex virus encephalitis (HSE) results in increased levels of oxidative stress, including the production of reactive oxygen species, free radicals, and neuroinflammation. Vitamin E deficient had more severe symptoms of encephalitis than vitamin E adequate in infected the mice intranasally with HSV-1.\(^{(43)}\) Vitamin E alleviated lipid peroxidation caused by aflatoxin in mice\(^{(44)}\); vitamin E succinate single or combination with ellagic acid all suppressed brain oxidation stress caused by TCDD rates\(^{(45, 46)}\). Our study showed that vitamin E declined the decrease of liver antioxidase gene transcription in acute TCDD-treated mice\(^{(41)}\).

Conclusion

It seems that the immunotoxicity caused by acute TCDD with big dosage is more severe compared to that from chronic TCDD, and vitamin E 100 mg/kg was optimum dosage in the present experiment. The immunotoxicity caused by TCDD can be antagonized by vitamin E, which provides a way for preventing or alleviating the toxicity from TCDD.

Acknowledgments

This projects was supported by the National Natural Science Foundation of China (No. 30770345).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

2. Vorderstrasse, B.A, Lawrence, B.P. Protection against lethal challenge with streptococcus pneumoniae is conferred by aryl hydrocarbon receptor activation but is not associated with an enhanced inflammatory response. Infect. Immun. 2006, 74(10), 5679–5686.


