The Toxicity of 3-Monochloro-1,2-Propanediol (+) on Activated T Cell in Mice
Shuang Guan1,2, Sitong Ma2, Linli Xu2, Tong Wang2, Mengrou Ren2, Xuming Deng1, Jing Lu1,2,*
1Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, Jilin 130062, People’s Republic of China
2Laboratory of Nutrition and Function Food, Jilin University, Changchun, Jilin 130062, People’s Republic of China

ABSTRACT
This study aimed to clarify the toxicity effects of 3-monochloro-1,2-propanediol(+) (3-MCPD(+)) on activated T cell in mice. The toxicity effects of 3-MCPD (+) on murine T lymphocyte responses were evaluated in vitro and in vivo. The data showed that 3-MCPD (+) markedly inhibited ConA-induced T lymphocytes proliferation, Th1/Th2 cytokines production, activated Ca2+/CaM/I-κB-NF-κB and Ca2+/CaM/CaN/NFAT signal transduction pathways in vitro. Furthermore, administration of 3-MCPD significantly inhibited T cell-mediated DTH response in vivo. These observations indicated that 3-MCPD (+) exhibited suppressive effects on activated mouse T cell in vitro and in vivo.

INTRODUCTION
3-MCPD is a number of a group of contaminants known as chloropropanols [1]. It was produced in the food the Processing and was thought to be a chloropropanols representative for its large amount of pollution and strong Toxicity. More recently, 3-MCPD has attracted renewed attention because of large amount of 3-MCPD fatty acid Esters were found in dietary especially in refined vegetable oils [2]. 3-MCPD fatty acid esters could release free 3-MCPD by hydrolysis in body. Combined exposure to 3-MCPD and 3-MCPD esters may lead to excessive dietary consumption of 3-MCPD. Thus, the hazard of 3-MCPD may greatly exceed the existing estimation. Previous studies showed that 3-MCPD itself has a variety of toxic effects such as genotoxicity [3,4] immunotoxicity [5], carcinogenicity [6] and infertility [7]. But there was no studies concern about the effect of 3-MCPD on mouse T cell.

It is known that T cell plays an important role in body immune system. They could extensively contact outside chemical poisons during the recirculation. So, T cell suppression may be an ideal early toxicity index before poisons making damage to organ. T cells activation, which triggering the T-cell receptor-CD3 complex in collaboration with stimulatory and adhesion receptors, is a complex process. Abundant studies have proved that Ca2+ influx was crucial for T cell activation upon antigen stimulation [8]. The alteration in intracellular calcium ([Ca2+]i) controls diverse cellular processes, including promoting translocation of transcription factors from the cytosol to the nucleus. The best known examples are those of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and NFAT (nuclear factor of activated T-cells) [9,10], which are the most relevant transcription factors regulating the expression of activation-associated and proinflammatory genes. They are activated by an increase of the cytosolic Ca2+ level and translocate to the nucleus, which induced expression of gene products mediating innate and adaptive immunity, thereby contributing to the control of cell proliferation, cytokine secretion and apoptosis [11].

In the study, we used concanavalin (ConA) as stimulant to study toxic effects and related mechanism of 3-MCPD on mouse T lymphocytes. Our works would be helpful to clear 3-MCPD immunotoxicity and lay foundation for finding sensitive toxicology biomarkers, which could be an early detection of the toxicity when expose to 3-MCPD.
METHODS

Reagents

3-MCPD(+) (As No. 57090-45-6, purity 98%) was provided by Bio tech inc in Shanghai. Di methyl sulfurous (DMSO), Concanavalin (ConA), Phosphorescence (CTX), 3-(4,5-methodological-2-cl)-2,5-impenetrability bromide (MTT), and Griefs reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,4-Trinitrotoluene (DNFB) was purchased from BD Bio-sciences Charming (CA, USA). Interleukin-2 (IL-2), Interferon gamma (IFN-γ), Interleukin-4 (IL-4) and Interleukin-6 (IL-6) ELISA kits were purchased from Bio legend. RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Estrogen-Gibbon (Grand Island, NY). Phosphate-specific antibodies for IκB as well as antibodies against p65 nuclear factor kB (NF-kB) IκB, β-actin, and nuclear factor of activated T cells 2 (NFAT2) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Flo-3/AM was from Bedtime Institute (Changchun, Jilin, China). Primary antibodies used for Western analysis Modulation (CaM), Calciferous (CaN), which were purchased from Cell Signaling (Beverly, MA), Peroration-conjugated Affinity goat anti-rabbit Egg (H+L) and Peroration-conjugated Affinity goat anti-rabbit Egg (H+L) were purchased from PTG (Chicago, IL, USA).

Animals

BALB/c female mice weighing 18–22 g were purchased from Jilin University Experimental Animal Center and acclimatized for 1 week before use. Rodent laboratory chow and tap water were provided audibility and maintained under controlled conditions with a temperature of 24 ± 1°C, humidity of 40–80%, and a 12-h light/12-h dark cycle. All of the procedures were in strict accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the Policy of Animal Care and Use Committee of Jilin University.

Cell preparation

BALB/c female mice were sacrificed and their spleens were aseptically removed and ground by passing through a sterile plastic strainer under aseptic conditions. After centrifuging the cells twice at 1000 rpm for 5 min, erythrocytes were Elysee in Eris–NH4Cl and the cell pellets were washed twice with RPMI-1640 medium. Then cells were re-suspended in complete RPMI-1640 medium. Cell counts were performed using a hydrometer and cell viability was determined using the pedantry-blue dye exclusion technique and in all cases cell viability was higher than 95%. The culture media were maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

T cell purifications

T cells were purified from whole splenocyte preparations using the Pan T Cell Isolation Kit according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, splenocytes were collected and a single cell suspension was generated in MACS buffer (PBS, 0.5% BSA and 2 mM EDTA). Cells were then incubated with antibody cocktail and magnetic beads that allow for negative selection of T cells in the presence of a magnetic column. The purity of mouse CD3 T cells was consistently more than 95%.

T cell cytotoxicity assay

T cell were plated at a density of 2 × 10⁶ cells/mL onto 96-well plates (Costar USA) containing 100 uL of RPMI 1640 complete medium (triplicate wells). Then the cells were treated with multiple concentrations of 3-MCPD(+) (0–32 mM). After 44 h, 20 uL of MTT (5 mg/mL) was added to each well and the cells were further incubated for 4 h at 37 °C with 5% CO₂. Cell-free supernatants were then removed and resolved with 150 μL/well DMSO. The optical density was measured at 570 nm on a microplate reader.

T cell proliferation assay

T cell (2 × 10⁶ cells/mL) collected as described above were treated with 3-MCPD(+) (terminal concentration: 1, 2 or 4 mM) plus ConA (terminal concentration: 5 ug/mL) and ConA alone for 48 h. After 44 h, 20 uL of MTT (5 mg/mL) was added to each well and the cells were further incubated for 4 h at 37 °C with 5% CO₂. The plate was centrifuged at 1800 rpm for 10 min and supernatants were then removed and resolved with 150 μL/well DMSO. The optical density was measured at 570 nm on a microplate reader.

Determination of cytokines

T cells (2 × 10⁶ cells/mL) were incubated with 3-MCPD(+) (terminal concentration: 1, 2 or 4 mM) plus ConA (terminal concentration: 5 ug/mL) and ConA alone for 48 h. The concentrations of cytokine IFN-α, IL-2, IL-4, and IL-6 in the supernatants were measured by ELISA according to the manufacturer’s instructions (Biolegend, Inc, Camino Santa Fe, Suite E San Diego, CA, USA).

RT-PCR Analysis

T cells were prepared as above. Total RNA was extracted from the ConA-activated T cell using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then pooled for the RT-PCR analysis. The total RNA was reverse-transcribed using the BioRT cDNA first strand synthesis kit (Bioer Technology) according to the manufacturer’s recommendations.

Primers used in this study were as follows: β-actin (forward primer 5’-ATCATGTGTGGACCTCACA-3’ and reverse primer 3’-CATGCTTGTGCATCTGCTG-5’).
primer 5’-CATCTCTTCGCTGGAAGTCCA-3’), IFN-r (forward primer 5’-TCTGAGCAATGAACGCACAGC-3’ and reverse primer 5’-TCTCAGAAAGTCCACCA-3’), IL-2 (forward primer 5’-CTACACGGAAGACAGC-3’ and reverse primer 5’-TCCTCAGAAAGTCCACCA-3’), IL-4 (forward primer 5’-TCGGCATTTGGAAGAGGTC-3’ and reverse primer 5’-GAAAAGCCCGAACGGCTTTG-3’), IL-6 (forward primer 5’-TCCAGTTGCCTTCTTGGGAC-3’ and reverse primer 5’-GTGTAATTAAGCCTCCGACTTG-3’). The cDNA served as the template in a 20-µL reaction mixture and was processed using an initial step at 94°C for 5 min followed by 30-35 amplification cycles (94°C for 30 s; 55-60°C for 30 s; and 72°C for 40 s) and 72°C for 10 min. Final PCR products were separated on 2% agarose gels. Transcription amounts were normalized against the β-actin transcript.

**Fluorescence measurement**

The method for photochemical measurements using an inverted fluorescence microscope (Motic AE31) is feasible. The special instrument was used for testing the changes of intracellular calcium. Purified mouse T cells were treated by 3-MCPD (+) (terminal concentration: 1, 2 or 4 mM) plus ConA (terminal concentration: 5 µg/mL) and ConA for 30 min. According to the number of cells of each sample, added fluo-3/AM to the suspension to estimate [Ca²⁺]i in the cytoplasm. The cells were incubated with Fluo-3/AM for 30 min before any measurement. Fluo-3/AM was measured 480-500.

**Western blot analysis**

T cells (2 × 10⁶ cells/mL) were pre-incubated with different concentrations of 3-MCPD (+) (1, 2 or 4 mM) in 6-well plates at 37°C for 60 min followed by 30 min incubation with ConA (final concentration 5 µg/mL). Cytoplasmic protein was lysed by Cytoplasmic Extraction Reagents (Beyotime, Jiangsu, China) according to the manufacturer’s instructions. Nuclear protein was lysed by corresponding Nuclear Protein Extraction Reagents (Beyotime, Jiangsu, China). Protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany) before storage at -80°C. Nuclear and cytoplasmic proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Western blotting was performed with the appropriate antibodies. The proteins were visualized by enhanced chemiluminescence (ECL).

**DNFB-induced delayed type hypersensitivity (DTH) response**

Six-week-old female BALB/c mice were divided into 6 groups, each consisting of ten mice. On days 1 and 2, BALB/c mice were initially sensitized with 20 mL of 5% DNFB dissolved in acetone–olive oil (4:1) on the shaved abdominal skin of recipients. Beginning on the day of immunization, the immunized mice were daily gavage-fed water or 3-MCPD (+) in water at dose of 10, 20, 40 mg/Kg/day for 6 consecutive days, or CTX (20 mg/kg in 2% DMSO) for 6 days once daily. The control groups received the same volume of saline. The DTH reaction that was evaluated by the increase in the ear patch weight (8-mm punches) between the left and right ear was measured 36 h after the second challenge.

**Statistical analysis**

All the data were presented as the mean ± standard S.D. Differences between the groups were analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was examined using one-way analysis of variance (ANOVA). A P value of <0.05 was considered to be statistically significant.

**RESULTS**

**Effect of 3-MCPD (+) on T cell cytotoxicity**

The effect of 3-MCPD (+) on T cell cytotoxicity was determined by MTT assay after incubating cells for 48 h. 3-MCPD (+) did not significantly affect the viability of T cell treated with concentrations ranging from 0 to 4 mm (data not shown). Based on this observation, we chose 1, 2 and 4 mm as experiment doses.

**Effect of 3-MCPD (+) on T lymphocytes**

In our study, proliferation was tested by MTT assay. As shown in Figure 1.

3-MCPD (+) could dose-dependently suppressed ConA-induced T lymphocytes proliferation at the concentrations of 1–4 mm. These results indicated that 3-MCPD suppressed ConA-induced T lymphocytes proliferation.

Effect of 3-MCPD (+) exposure on Th1 and Th2 cytokine secretion and mRNA expression in vitro. In this study, we used ConA as T cell mitogens, and selected IL-2, IFN-r as Th1 cytokines and IL-4, IL-6 as Th2 cytokines to test the effect of 3-MCPD (+) on cytokines production in vivo. As shown in Figure 2.

Treatment of mouse splenocytes with ConA alone resulted in significant increase of cytokine production compared to control. We found that the four cytokine levels in the cell supernatant treated with 1, 2 or 4 mm of 3-MCPD(+) significantly decreased...
compared to those of ConA group in dose-dependent manner. Moreover, Total RNA was extracted from the T lymphocytes treated by 3-MCPD (+) with Trizol. We tested IL-2, IL-4, IL-6, IFN-γ mRNA levels by PCR assay. The data showed the same currency Figure 3.

Note: Significant differences from control group were indicated by ###P<0.01 versus control group; *P<0.05 or **P<0.01 versus ConA group.

Figure 1. Effects of 3-MCPD (+) on T cell proliferation. T cells collected from mice were treated with 3-MCPD (+) (terminal concentration: 1, 2 or 4 mm) plus ConA (terminal concentration: 5 ug/mL) and ConA (5 ug/ml) for 48 h. The T cell proliferation was determined by MTT assay. The results were from three independent experiments and presented as mean ± standard deviation.

Note: ##p<0.01 vs. Control group, *P<0.05 or **P<0.01 vs. Con A group.

Figure 2. Effect of 3-MCPD (+) exposure on cytokines production in cell culture supernatant. The levels of cytokines IL-2, IFN-γ, IL-4 and IL-6 were analyzed by ELISA the values are the mean ± standard deviation of three independent experiments.

Note: A representative Western blot is shown in here. ##P<0.01 versus control group; *P<0.05 or **P<0.01 versus ConA group.

Figure 3. Effect of 3-MCPD (+) exposure on Th1 and Th2 cytokines mRNA expression. IL-2, IFN-γ, IL-4 and IL-6 mRNA expressions were tested by RT-PCR. Shown in the down panel were from three independent experiments. A representative Western blot is shown in here.

Effects of 3-MCPD (+) on [Ca2+]i in T cells. In the study, we used Fluo-3 AM to examine the effects of 3-MCPD (+) on intracellular Ca2+ mobilizations in mouse T cell. As shown in Figure 4.

The level of intracellular Ca2+ increased significantly in ConA group. As the 3-MCPD (+) concentration at 1, 2 or 4 mm,
intracellular free Ca\textsuperscript{2+} fluorescence decreased dramatically. Effects of 3-MCPD (+) on NFAT pathway. In the experiment, we examined the effect of 3-MCPD (+) on Con A-induced NFAT2 by western blot. As shown in Figure 5.

Note: The results were from three independent experiments and presented as mean±standard deviation of three independent experiments. ##P<0.01 versus control group; *P<0.05 or **P<0.01 versus ConA group.

Figure 4. Effects of different concentration of 3-MCPD (+) on the [Ca\textsuperscript{2+}] in mouse T lymphocytes. T Cells were pretreated with 20 μM Fluo-3-AM and measured by a Confocal Laser Microscope. The fluorescence intensity in ConA group was increased compared with the control group, and 3-MCPD (+) could diminish the fluorescence intensity. The results were from three independent experiments and presented as mean±standard deviation of three independent experiments.

Note: Significant differences with control group were designated as ##P<0.01 versus control group; *P<0.05 or **P<0.01 versus ConA group.

When T cells were stimulated with ConA, the expression of CaM, CaN and NFAT2 were increased compared with the control group and 3-MCPD (1, 2 or 4 mm) suppressed the expression of CaM, CaN and NFAT2 in a dose-dependent manner.

Figure 5. Effects of 3-MCPD (+) on NFAT pathway. The protein from mouse T cells was extracted according to the manufacturer’s instructions. (A) CaM, CaN; (B) nucleic NFAT2 protein expression were tested by Western blot-analysis. Shown in the down panel are means ± S.D. These results were from three independent experiments.
Effects of 3-MCPD (+) on NF-κB pathway

In the study, we examined the effect of 3-MCPD (+) on the ConA-induced production of NF-κB, p-IκB, IκB by Western blot analysis. The result showed that NF-κB and phosphorylation of IκB in purified mouse T cells increased after ConA administration but was significantly inhibited by 3-MCPD treatment. Meantime, IκB decreased after ConA treatment but was significantly increased by 3-MCPD in a dose-independent manner Figure 6.

Figure 6. Effects of 3-MCPD (+) on NF-κB pathway. The protein from mouse T cells was extracted according to the manufacturer's instructions. (A) IκB, P-IκB, and (B) nucleic NF-κB protein expression were tested by Western blot-analysis. Shown in the down panel are means ± S.D. These results were from three independent experiments.

Effect of 2,3-DCP on DNFB-induced DTH response in mice

To test the immunotoxicity activity of 3-MCPD (+) on T-cell-mediated immune responses in vivo, we analyzed the effects of 3-MCPD (+) on DNFB-induced DTH responses in BALB/c mice. As shown in Figure 7

Figure 7. Effects of 3-MCPD on DNFB-induced DTH reaction in BALB/c mice. A: The ear swelling (Δmears) was calculated as the difference between the weights of untreated and DNFB-treated ear punches 36 h after challenge. The values are presented as means ± S.D. (n=10).

The ear swelling was significantly increased in DTH group compared with control group. The result indicated that DNFB-induced...
DTH model was successful. 3-MCPD (+) at doses of 10, 20 or 40 mg/kg also significantly reduced the ear swelling in DNFB-induced mice compared with the DTH group. The immunotoxicity effect of high dose (40 mg/kg) was stronger than positive control drug CTX.

**DISCUSSION**

It is well known that activated T cell plays an important role in process of body defense. Suppressing T-cell-mediated immune responses can be clearly detected in many diseases including cancer and tumor [12]. In the present study, we focused on the toxic effects of 3-MCPD on activated T-cell both in vitro and in vivo. We hope our study can provide a contribution to prevention-control of 3-MCPD.

First, we tested lymphocyte proliferation by MTT assay, which was the most widely used method to assess immunological function. ConA is a plant mitogen, which only stimulates T lymphocytes proliferation [13]. In the experiment, 3-MCPD (+) dose-dependently suppressed ConA-induced splenocyte proliferation at the concentrations of 1-4 mm Figure 1. These results indicated that 3-MCPD (+) suppressed ConA-induced T lymphocytes proliferation. Cytokines play a critical role as messenger molecules in the immune system. They are also the potential targets for immunomodulation. It is clear that Th1 cells produce IL-2, IFN-γ, IL-12 and impel cell-mediated immune responses, whereas Th2 cells cytokines produce IL-4, IL-6 and IL-10 and promote humoral immune responses. The two types of cytokines work together to modulate the immune function. In this study, we found that the four cytokine levels in the cell supernatant treated with 1, 2 or 4 mm of 3-MCPD (+) were significantly decreased compared to those of ConA group in a dose-dependent manner. Moreover, we extracted Total RNA from the T lymphocytes treated with 3-MCPD (+) using Trizol and tested IL-2, IL-4, IL-6, IFN-γ mRNA levels by PCR assay. The data showed the same trend as cytokines.

Intracellular Ca2+ is a quintessential intracellular messenger. Many cell functions such as mature, differentiation, and signal transduction are regulated by change of [Ca2+] [14,15]. In the study, we used Fluo-3 AM to examine the effects of 3-MCPD (+) on intracellular Ca2+ mobilizations in mouse T cell. The data indicated that 3-MCPD (+) strongly inhibited the intracellular free Ca2+ levels in T cells.

NFAT family plays a crucial role in T cell activation. Many studies have revealed that NFAT activation depends on a Ca2+/calmodulin/calcineurin pathway. Calmodulin (CaM) is a key regulator of numerous cellular processes and is the predominant intracellular receptor for Ca2+ signals [16]. The calcium/CaM complex regulates several downstream targets including protein kinases and phosphatases. Calcineurin (CaN) is a serine/threonine phosphatase enzyme that is expressed in many types of tissues such as immune cells, muscle cells and neurons and regulates NFAT phosphorylation. Once activated by stimuli, NFAT translocates from the cytosol into the nucleus, and then regulate the transcription of genes, including cytokines (IL-2, IFN-γ, TNF-α) and regulatory enzymes such as COX-2 [17]. In the experiment, we examined the effect of 3-MCPD (+) on Con A-induced NFAT2 by western blot. The data showed 3-MCPD (+) suppressed the expression of CaM, CaN and NFAT2 in a dose-dependent manner.

NF-κB is also Ca2+ dependent transcription factor that responsible for the activation of immune response especially in T cell. The Calcium/Calmodulin-dependent protein kinase II (CaMKII) is also an important downstream target of Ca2+ signaling pathway, which is activated by calcium-binding protein CaM. NF-κB activation depends on a Ca2+/calmodulin/CaM/KII pathway [18]. NF-κB mostly participates in the regulation of genes in inflammation, immunity, cell proliferation, cell apoptosis and other process of physiology and pathology. Normally NF-κB is sequestered in the cytoplasm by interacting with inhibitory IκB molecules. Once stimulated, NF-κB-IκB is activated by phosphorylation of conserved serine residues of IκB. Then IκB is ubiquitinated and degraded, which results in

NF-κB migrating to the nucleus and turning on transcription. Thus, the phosphorylation of IκB plays an important role in the activity of NF-κB [19,20]. In the study, we examined the effect of 3-MCPD (+) on the ConA-induced production of NF-κB, p-IκB and IκB by Western blot analysis. The result showed that NF-κB and phosphorylation of IκB in purified mouse T cells were increased after ConA administration but significantly inhibited by 3-MCPD (+) treatment. Meanwhile, IκB was decreased after ConA treatment but was significantly increased by 3-MCPD (+) in a dose-independence manner.

**CONCLUSION**

This work has characterized the toxicity of 3-MCPD (+) on ConA induced mouse T cell activation. The results showed that 3-MCPD (+) could inhibited ConA-induced T cell activation. The underlying mechanism may be through suppressing of Ca2+/CaM/NF-κB and Ca2+/CaM/CaN/NFAT signal transduction pathways. During the experiment, no obviously signs of toxicity were
observed in the 3-MCPD(+)-treated mice on the basis of body weight and examination of individual organs (data no shown), which indicated that low dose of 3-MCPD(+) has done harm to mice immune system before other organ damage.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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