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Toxicity effects of Cannabidiol (CBD) on immune cells

Kanyaruck Jindaphun[1] Nuchjira Takheaw[1,2] Witida Laopajon[1,2] Supansa Pata[1,2] Watchara Kasinrerk[1,2*]

[1]Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.
[2]Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.

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ABSTRACT

Background: Cannabis extract has a long history of being used in the treatment and prevention of several medical conditions. The utilization of cannabis extracts, whether for medical or localized purposes, is widely observed. In cannabis extract, cannabidiol (CBD) is one of the most important non-psychoactive compounds. Several studies have demonstrated that CBD has several benefits in the treatment of various medical conditions. Nevertheless, CBD has also been demonstrated to suppress both innate and adaptive immune responses. Despite CBD has claimed to have many benefits, the toxicity of CBD is often pointed out and discussed. Nonetheless, the data on the toxicity effects of CBD on immune cells are limited.

Objectives: In this study, we aimed to investigate the toxicity effects of various concentrations of CBD on immune cells, including CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes.

Materials and methods: Various concentrations of peripheral blood mononuclear cells (PBMCs) were treated with various concentrations of CBD or relative concentrations of methanol as a diluent control for 12, 24, and 48 hrs. Cell morphology was observed using flow cytometry. The percentage of cell death in the treated cells was determined by cell viability assay. In addition, the toxic effects of CBD on PBMC sub-populations were determined by staining with fluorochrome-conjugated zombie viability dye and fluorochrome-conjugated monoclonal antibodies specific to each cell sub-population. Then, the percentage of cell death in each sub-population was assessed using flow cytometry.

Results: CBD at concentrations of 40 and 80 µM showed toxicity effects on PBMCs. At these concentrations, CBD induced both cell morphological changes and cell death. While 20 µM CBD induced different effects, ranging from none to mild and high toxicity. The toxicity of CBD at 20 µM concentration depends on the individual. In contrast, CBD at ten µM and below showed no toxicity to PBMCs. The observed toxic effects of CBD occurred in all sub-populations of PBMCs, including CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes.

Conclusion: CBD has toxicity effects on immune cells. These effects depend on CBD concentrations, PBMC concentrations, and the duration of CBD exposure. Our findings emphasize the importance of awareness for CBD users when consuming CBD.

Introduction

Recently, several natural plant extracts have been getting attention for their potential in treating various medical conditions. Cannabis extract has become popular in the treatment of various conditions, e.g., epilepsy, anxiety, depression, and cancer.[1] Plant cannabis, or phytocannabinoids, contains many components, possibly up to 500 by some estimates.[2] Delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are two major components

∗ **Corresponding contributor.**
Author's Address: *Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.*
E-mail address: watchara.k@cmu.ac.th
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present in cannabis extract.[3] THC is widely recognized as a psychoactive compound and is often associated with several adverse effects.[4] Nevertheless, THC is still used in the medical field. The FDAs of several countries have approved THC for the reduction of chemotherapy-induced nausea and vomiting, as well as for appetite stimulation.[5] CBD was also demonstrated to have several important positive impacts on many diseases, such as diabetes, Parkinson's disease, Alzheimer's disease, and cancer, but without psychotropic effects.[6]

CBD is a naturally occurring cannabinoid. It contains 21 carbon atoms, with the formula $C_{21}H_{30}O_2$, and a molecular weight of 314.464 g/mol.[7] CBD has a wide range of biological effects.[6] The effects of CBD are mediated through several receptors expressed throughout the body, i.e., cannabinoid receptors of the endocannabinoid system, 5-HT1A serotonin receptors, and TRPV1 channels.[8] The effect of CBD is predominantly observed within two major systems, including the nervous system and the immune system.[9-11] In the nervous system, CBD has demonstrated significant benefits in experimental models of various neurological disorders, such as seizures, epilepsy, Parkinson's disease, and Alzheimer's disease.[12,13] In the immune system, the induction of immunosuppression by CBD has been reported.[11] CBD inhibited both innate and adaptive responses. It had a strong suppressive effect on the release of innate cytokines (IL-1 and TNF) and could induce apoptosis in primary human monocytic cells.[14,15] In adaptive immune cells, CBD inhibited IFN-γ production and induced apoptosis in T cells.[16,17] As a result, CBD is often used as an anti-inflammatory drug to treat a variety of inflammatory conditions.[18,19]

Even though CBD has exhibited a range of advantageous effects, research in the clinical trial phase of CBD is still limited and controversial. There is only one CBD product (Epidiolex®, GW Pharmaceuticals) approved by the FDAs in 2018 for the treatment of rare pediatric seizure disorders.[20] The toxicity of CBD has also been focused on and highlighted as the significance of dosage considerations. At high doses, CBD exhibits various toxicities on multiple systems within the body, including reduced fertility, hormonal alteration, severe hypotension, and the potential to induce damage to liver cells (hepatocellular damage).[21-23] However, data on the toxic effect of CBD on immune cells is limited.

In this study, we investigated the toxicity effects of CBD on immune cells. The examinations were composed of various factors, including PBMC concentrations, CBD concentrations, and CBD exposure time. Our study indicated the toxicity effects of CBD on immune cells. These results might raise caution to the CBD user.

Materials and methods

Reagents, antibodies, and cells

Cannabidiol (CBD) solution of 1 mg/mL concentration in methanol (catalog number C-045-1ML) was purchased from Cerilliant (Round Rock, Texas, USA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, New York, USA). Ficoll-Hypaque solution was purchased from Robbins Scientific Corporation (Sunnyvale, California, USA). 7-AAD solution, BV510 conjugated Zombie dye, FITC-conjugated anti-CD3 mAb, PerCP-conjugated anti-CD4 mAb, BV785-conjugated anti-CD8 mAb, PE-conjugated anti-CD14 mAb, FITC-conjugated anti-CD19 mAb, and PECy5-conjugated anti-CD56 mAb were purchased from BioLegend (San Diego, California, USA).

PBMCs were isolated from the buffy coat of healthy donors obtained from the Thai Red Cross Society (Regional Blood Center X, Chiang Mai, Thailand) or heparinized blood of healthy blood donors using Ficoll-Hypaque gradient centrifugation.

Toxicity effects of CBD on PBMCs

The PBMCs were adjusted into a concentration of $2x10^6$ and $8x10^6$ cells/mL in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 40 mg/mL gentamicin, and 2.5 mg/mL amphotericin B (10% FBS-RPMI). The PBMCs were plated in a 96-well V-bottom plate, and various concentrations of CBD (0, 10, 20, 40, and 80 µM) or a relative methanol concentration as a diluent control were added. The cells, in total volume of 100 µL, were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 12, 24, and 48 hrs. After incubation, the cells were harvested and monitored for cell morphological changes by flow cytometry. Cells were also stained with 7-AAD 0.5 µg/mL. The percentage of dead cells (7-AAD+ cells) was analyzed by BD Accuri C6 plus flow cytometry (BD Bioscience, San Jose, California, USA).

Toxicity effects of CBD on sub-populations of PBMCs

The PBMCs at a concentration of $2x10^6$ cells/mL were incubated with 20 µM CBD or a relative concentration of methanol in total volume of 100 µL in a 96-well V-bottom plate in a 5% CO_2 incubator at 37 °C for 24 hrs. After cultivation, the cells were harvested and stained with the BV510-conjugated zombie dye for 15 min at room temperature in a dark box. Then, the cells were washed twice with PBS containing 1% BSA and 0.1% NaN_3 (FACS) buffer. Next, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.1% saponin, 5% FCS, and 0.1% NaN_3 in PBS to allow the antibodies to access the intracellular targets. After that, the cells were blocked by incubation with 10% AB serum in 0.1% saponin, 5% FCS, and 0.1% NaN_3 in PBS for 30 min at 4 °C. Blocked cells were stained with fluorochrome conjugated antibodies against CD3, CD4, CD8, CD14, CD19, or CD56 (for identifying the cell sub-populations) for 30 minutes at 4 °C. Then, the cells were washed twice with 0.01% saponin, 5% FCS, and 0.1% NaN_3 in PBS. After washing, the cells were fixed with PBS containing 1% paraformaldehyde. The stained cells were then analyzed by BD FACSCelesta flow cytometry (BD Bioscience, San Jose, California, USA) to assess the percentage of dead cells (zombie dye+ cells).

Statistical analysis

Data were analyzed using GraphPad Prism 9 software. An unpaired t-test was used to compare data.

Results

Toxicity effects of CBD on PBMCs

To investigate the toxicity effects of CBD on PBMCs, various concentrations of PBMCs ($2x10^6$, and $8x10^6$ cells/mL) were treated with various concentrations of CBD (0, 10, 20, 40, and 80 µM) or relative concentrations of methanol as a diluent control. The treated PBMCs were incubated for 12, 24, and 48 hrs and monitored for cell morphological changes and cell death by flow cytometry.

Upon treatment with CBD at 40 and 80 µM, PBMCs at all concentrations showed morphological change after being incubated for all incubation times (Figure 1A and 1B). The morphological change was not observed in the control group, which was treated with relative concentrations of methanol diluent (Figure 1A and 1B). These findings indicated that 40 and 80 µM of CBD exhibited cytotoxic effects on PBMCs. We subsequently performed a viability assay to investigate cell death in each condition. As predicted, 40 and 80 µM of CBD could induce cell death in treated PBMCs compared with diluent controls (Figure 1C). It is worth noting that the methanol control did not induce cell death compared with the medium control. The toxicity effect of CBD was dose-dependent. CBD at 80 µM showed a higher toxicity than at 40 µM. Longer exposure time induced more cell death. Taken together, our findings suggest that CBD at concentrations of 40 and 80 µM have cytotoxic effects on PBMCs.

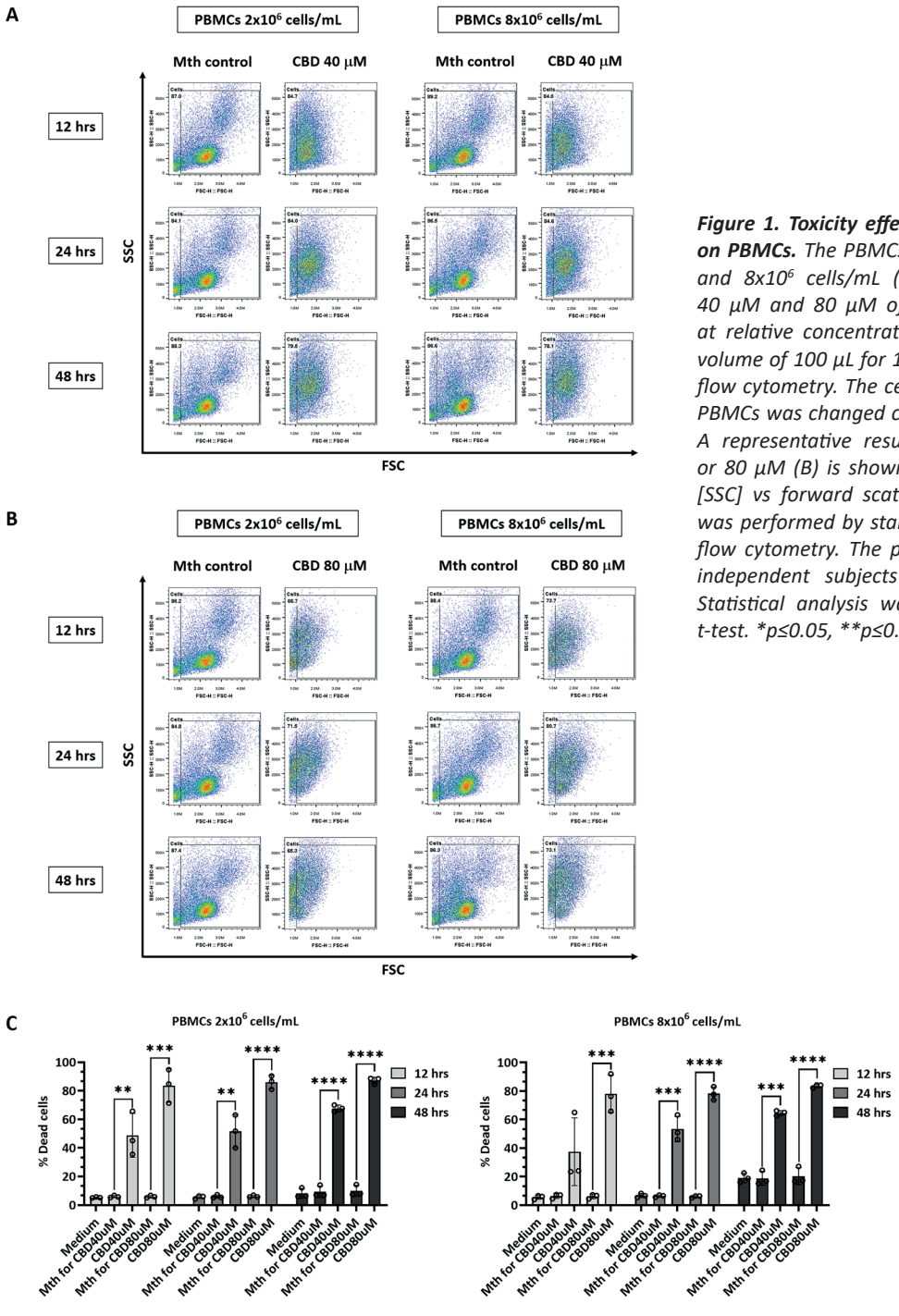

Figure 1. Toxicity effects of CBD at 40 µM and 80 µM on PBMCs. *The PBMCs (N=3) at concentrations of $2x10^6$ and $8x10^6$ cells/mL (as indicated) were treated with 40 µM and 80 µM of CBD or methanol (Mth control) at relative concentrations as a diluent control in total volume of 100 µL for 12, 24, and 48 hrs and analyzed by flow cytometry. The cell morphology of all three treated PBMCs was changed compared to the methanol control. A representative result treated with CBD 40 µM (A) or 80 µM (B) is shown in the scatter plots (side scatter [SSC] vs forward scatter [FSC]). (C) The viability assay was performed by staining with 7-AAD and analyzed by flow cytometry. The percentages of dead cells of three independent subjects in each condition are shown. Statistical analysis was performed using an unpaired t-test. *p≤0.05, **p≤0.01, ***p<0.0005, ****p<0.0001.*

The PBMCs treated with 20 µM of CBD did not show cell morphological change after being incubated for 12 hrs (Figure 2A). However, the morphological change was observed after 24 and 48 hrs of incubation, compared with the diluent controls (Figure 2A). The 48-hr incubation time showed higher morphological changes than the 24-hr incubation period. Subjects treated with 20 µM CBD at the 24-hr incubation period, however, exhibited a different CBD effect on cell morphology. The morphological changes were found in nine independent subjects (called CBD-sensitive subjects) out of sixteen tested subjects, ranging from mild to high alterations (Figure 2B). However, seven out of sixteen tested subjects have no morphological change when compared with the diluent controls. These seven subjects were called CBD-resistant subjects (Figure 2B). Therefore, upon treatment with 20 µM CBD, the tested subjects could be categorized as CBD-sensitive (56.3%) and CBD-resistant individuals (43.7%).

We further confirmed CBD-induced cell death using a 7-AAD cell viability assay. 20 µM CBD induced PBMC death was observed at 24- and 48-hr incubation times, but not at 12-hr incubation, in comparison with diluent control (Figure 2C). Our findings suggested that 20 µM of CBD might have different effects on PBMCs individually. Using CBD at this concentration is cautionary due to its cytotoxic effect in some subjects.

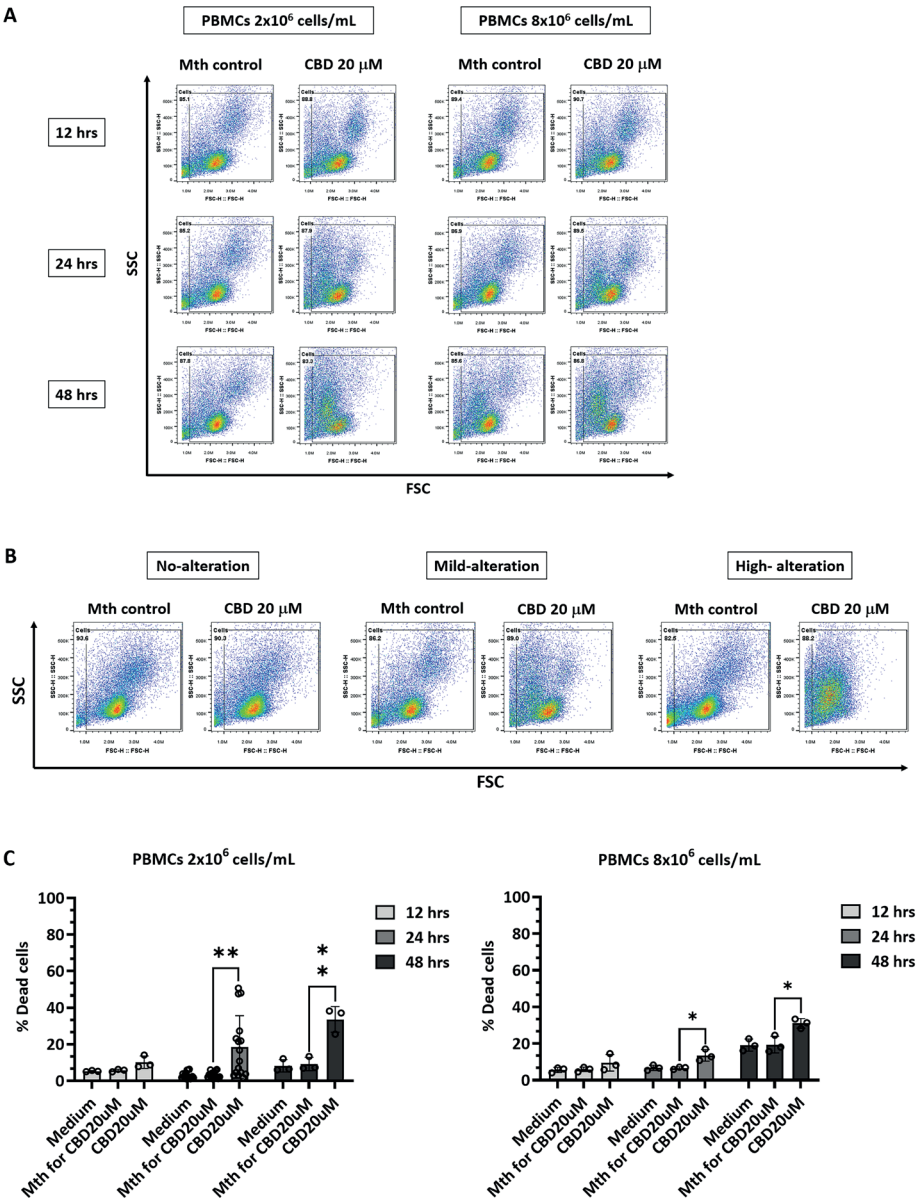

Figure 2. Toxicity effects of CBD at 20 µM on PBMCs. *The PBMCs (N=3) at concentrations of 2x10⁶ and 8x10⁶ cells/mL (as indicated) were treated with 20 µM CBD or methanol (Mth control) at relative concentrations as a diluent control in 100 µL total volume for 12, 24, and 48 hrs. The 24-hr PBMCs treatment at a concentration of 2x10⁶ cells/mL was performed with sixteen independent subjects. The cell morphology was investigated using flow cytometry. (A) The morphological change appeared after incubation for 24 and 48 hrs, but not for 12 hrs. (B) The patterns of morphological change in CBD-treated PBMCs at 2x10⁶ cells/mL incubated for 24 hrs are displayed. The extent of morphological change, ranging from no alteration to mild alteration and high alteration of a representative result, is shown. (C) The cell viability was determined using 7-AAD viability assay and analyzed by flow cytometry. The percentages of dead cells in each condition are shown. Statistical analysis was performed using an unpaired t-test. *p≤0.05, **p≤0.01.*

The cytotoxic effects of CBD at 10 µM concentration were also determined. The PBMCs at all concentrations tested upon treatment with 10 µM CBD did not show any morphological changes after being incubated for 12, 24, and 48 hrs, in comparison with the diluent controls (Figure 3A). The cytotoxic effects of CBD at 10 µM were determined by cell viability assay. The percentage of dead cells in the treated PBMCs showed no statistically significant differences compared with the relative concentrations of methanol (Figure 3B). Therefore, these results indicated that 10 µM of CBD did not have a cytotoxic effect on PBMCs.

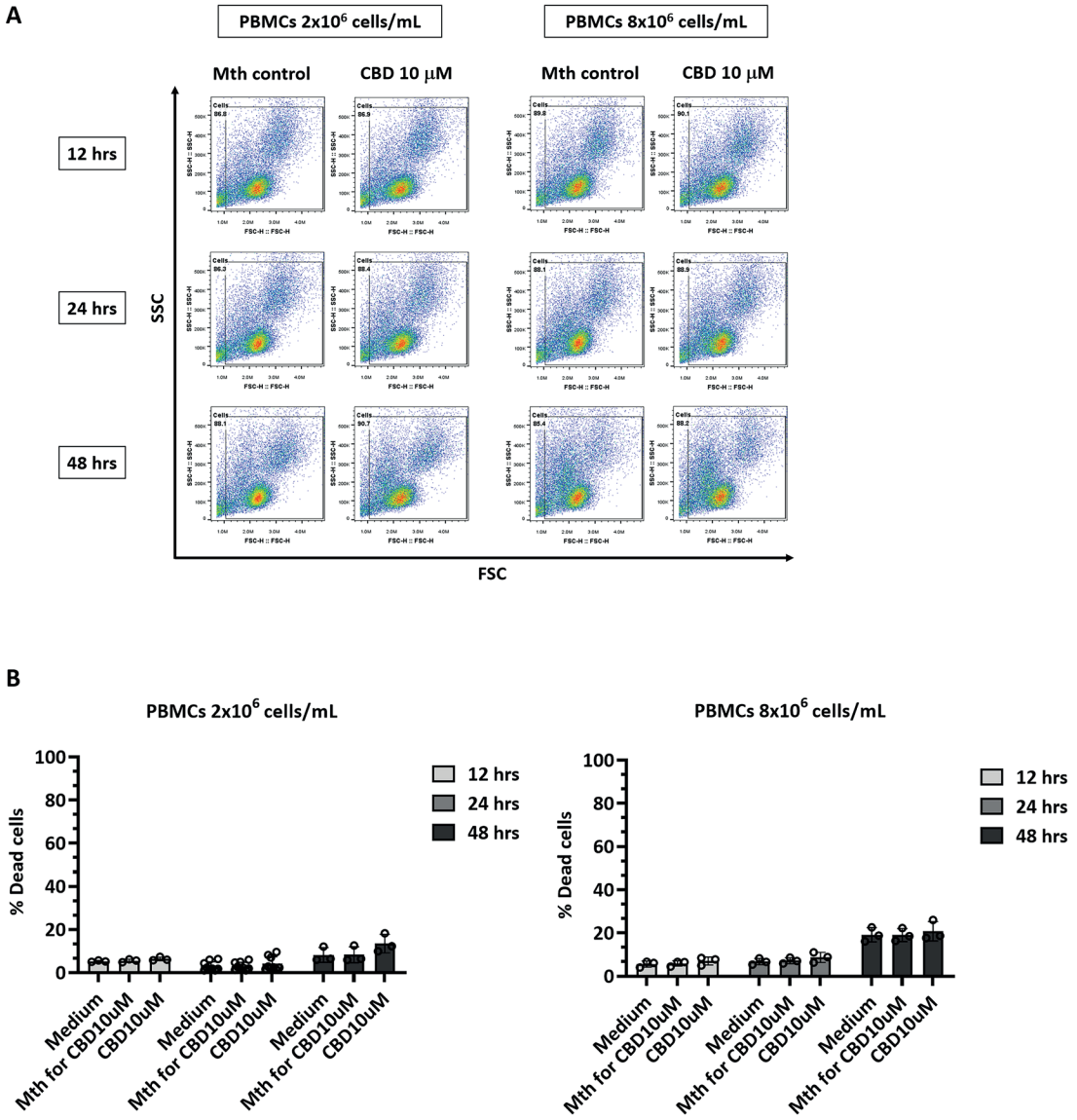

Figure 3. Toxicity effects of CBD at 10 µM on PBMCs. *The PBMCs (N=3) at concentration of 2x10^6 and 8x10^6 cells/mL (as indicated) were treated with 10 µM CBD or methanol (Mth control) in total volume of 100 µL for 12, 24, and 48 hrs. The 24-hr CBD treatment of PBMCs at a concentration of 2x10^6 cells/mL was performed with nine independent subjects. Then, the cells were analyzed by flow cytometry. (A) A representative result treated with 10 µM CBD is shown in the scatter plots (side scatter [SSC] vs forward scatter [FSC]). The morphology of all treated PBMCs did not show morphological changes compared with methanol control. (B) The cell viability was investigated using 7-AAD viability assay and analyzed by flow cytometry. The percentages of dead cells of all independent subjects in each condition are shown. Statistical analysis was performed using an unpaired t-test. No statistically significant differences were observed.*

As CBD at 10 µM was not toxic to the cells, we investigated the toxicity effects of CBD at concentrations of 1, 2, and 5 µM. In this study, PBMCs at a concentration of $2x10^6$ cells/mL with 24 hrs of incubation time were selected as a representative condition. As predicted, CBD at these concentrations was not toxic to the PBMCs of any study subjects (Figure 4). This confirms the non-toxic effects of CBD at concentrations below 10 µM.

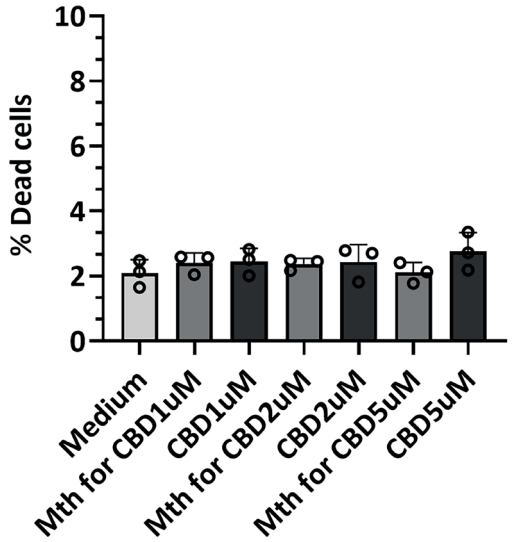

Figure 4. Toxicity effects of CBD at 1, 2, and 5 µM on PBMCs. *The PBMCs (N=3) at a concentration of $2x10^6$ cells/mL were treated with 1, 2, and 5 µM of CBD, or methanol (Mth) or medium alone with total volume of 100 µL for 24 hrs. The treated cells were stained with 7-AAD and analyzed by flow cytometry. The percentage of dead cells in each condition is shown. Statistical analysis was performed using an unpaired t-test. No statistically significant differences were observed.*

Toxicity effects of CBD on PBMC sub-populations

To get insight into CBD's cytotoxic effects on PBMCs, the toxicity of CBD in specific sub-populations of PBMCs, including CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes, was studied. The PBMCs at a concentration of $2x10^6$ cells/mL were incubated for 24 hrs with 20 µM CBD or relative concentrations of methanol (diluent control). After cultivation, the cells were stained with fluorochrome-conjugated zombie dye, which indicated cell death. They were intracellular immunofluorescence stained with fluorochrome-conjugated monoclonal antibodies specific to each cell sub-population. The percentage of dead cells in each PBMC sup-populations was measured.

In this experiment, four independent subjects were performed. The observed morphological changes were significant in two independent subjects (N3, N4), and the other two independent subjects (N1, N2) were modulate-change observed (Figure 5A). The study on cell viability in the specific sub-populations of PBMCs showed that CBD could induce cell death in all PBMC sub-populations, i.e., CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes. Surprisingly, CD4 T cells were less affected, with almost statistically significant difference compared with diluent control (Figure 5B). Our findings suggest that the cytotoxic effect of CBD affects all PBMCs sub-populations.

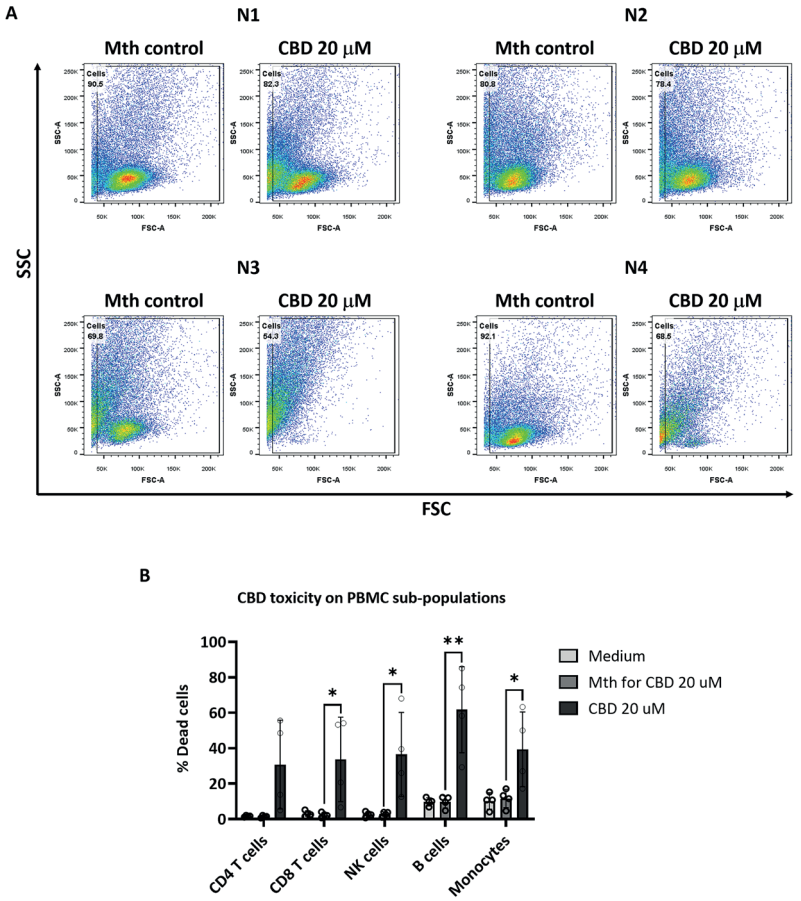

Figure 5. CBD cytotoxic effects on PBMC sub-populations. *The PBMCs (N=4) at a concentration of 2x10^6 cells/mL were treated with 20 µM CBD or methanol (Mth control) as diluent control in 100 µL total volume for 24 hrs. The cells were analyzed by flow cytometry. (A) The morphological change was observed in all test subjects. The cell morphology is shown in the scatter plots (side scatter [SSC] vs forward scatter [FSC]). (B) The treated cells were stained with BV510-conjugated zombie dye to assess cell viability. The percentage of dead cells of PBMC sub-populations, including CD4 T cells (CD3$^+$/CD4$^+$), CD8 T cells (CD3$^+$/CD8$^+$), NK cells (CD3$^-$/CD56$^+$), B cells (CD19$^+$), and monocytes (CD14$^+$), are shown. Statistical analysis was performed using an unpaired t-test. *p≤0.05, **p≤0.01.*

Discussion

In several countries, including Thailand, cannabis extracts and CBD were suggested to be used in the treatment of various diseases, including central nervous system diseases, inflammatory diseases, cardiovascular disorders, metabolic syndrome-related disorders, and cancer.[1,12,18,19,24,25] The use of cannabis extracts in treatment, however, usually comes without control of CBD concentration or an understanding of CBD toxicity effect. In a previous study, various CBD concentrations, ranging from 0.03 to 100 µM, were used to evaluate its impact on immune cells.[11] The majority of published articles suggest that CBD has immunosuppressive effects. [14-17] Nevertheless, some of these studies did not investigate the direct cytotoxicity of CBD on immune cells.[18,26] The data of the toxicity effect of CBD on immune cells is limited. We speculated that CBD may have a toxic effect on immune cells, suppressing immune cell functions. In the present study, we investigated whether CBD was toxic to immune cells. To investigate the toxicity effects of CBD, various concentrations of PBMCs were treated with various concentrations of CBD over various exposure times. Our study would answer the toxicity effect of CBD.

We found that CBD at 40 and 80 µM showed strong toxicity in PBMCs. These concentrations induced cell morphological changes of PBMCs in all tested subjects. The results indicated that CBD at these concentrations might disrupt the cell membrane, causing a change in cell morphology.[27] CBD at 40 and 80 µM concentrations could induce PBMC death, particularly at low concentrations of PBMCs and long exposure time to CBD. A low PBMC:CBD ratio induced a higher percentage of cell death, indicating a higher concentration of CBD per cell led to higher cell toxicity.

Moreover, the toxicity effect of CBD followed a dose-dependent manner. This suggested that CBD at 40 µM and higher was toxic to immune cells; when using CBD for medical treatment, the dosage of CBD needs to be carefully considered. Several in-house cannabis extracts, which had an unknown concentration of CBD, were discovered to be widely in use in several countries. This, therefore, poses a danger to the users.

CBD at 20 µM, nevertheless, showed a different effect. At 20 µM, CBD toxicity appeared in some subjects, called CBD-sensitive persons. We found that nine out of sixteen subjects (56.3%) were sensitive to CBD. Cell

morphological change and cell death could be seen in these sensitive subjects. However, 43.7% of tested subjects were not affected by CBD at this concentration. The different observed effects may be because of the levels of CBD receptors, and the membrane fragility of each subject may not be the same.[27] Furthermore, it might be influenced by other biological factors, such as age, sex, blood group, genetics, etc. However, this study did not clarify these influences due to limitations restricted by human ethics. According to the results, we suspected that CBD at 20 µM might be the cytotoxic threshold dosage. Using CBD at 20 µM, therefore, needed to be individually considered.

In contrast, CBD at concentrations of 10 µM and lower did not show any cytotoxic effect on PBMCs in all tested subjects. Neither cell morphological change nor cell death was seen at all concentrations of PBMC:CBD ratio and any exposure times at these CBD concentrations. Importantly, the subjects tested with CBD at ten µM were also included in the 20 µM treatment group. Whether classified as CBD-sensitive or CBD-resistant based on the effects of CBD at 20 µM, no morphological changes and cell death were observed when these subjects were exposed to CBD at ten µM. Our results align with the previous study, which demonstrated that the toxicity of CBD was approximately in the range of 20-22 µM, depending on the cell type. The highest concentration of CBD that did not cause cell death was 13 µM.[28] In our study, we demonstrated that CBD concentrations at ten µM or lower would be an appropriate condition for any future studies involving CBD effect.

As mentioned, CBD at a high dose has a cytotoxic effect on PBMCs. We asked whether this effect was on all PBMC sub-populations or a specific cell sub-population. We, therefore, investigated the toxicity of CBD on PBMC sub-populations, including T cells, B cells, NK cells, and monocytes. In this study, 4 CBD-sensitive subjects with varied responses to 20 µM CBD were chosen.

We found that in strong 20 µM CBD-sensitive persons, the high toxicity of CBD was observed in all PBMC sub-populations, including CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes. While, in individuals who are less sensitive to CBD, low cytotoxicity of CBD was observed in all PBMC sub-population. The mechanism of CBD cell toxicity is still unproven in this study.

To the best of our knowledge, this is the first time the toxicity effect of CBD on PBMCs has been demonstrated. Our results of the toxicity of CBD are in line with the previous studies, which reported that CBD could induce immune suppression, both in innate and adaptive immune responses.[14-17] The effects of CBD on the suppression of immune cell function may be due to the toxicity of CBD that affects both innate immune cells, monocytes and NK cells, and adaptive immune cells, T and B lymphocytes if the high concentration of CBD were used in the study. The researchers who perform experiments on the function of CBD need to monitor the toxicity of CBD on the tested cells concurrently in their research.

Conclusion

In this study, we investigated the toxic effects of CBD on each PBMC sub-population. Our study indicated that CBD had toxicity effects on immune cells in a dose-dependent manner. The toxicity effect of CBD was influenced by CBD concentrations, PBMC concentration, and exposure time. Specifically, the percentage of dead cells increased with a high CBD:cell concentration ratio and an extended exposure time. The obtained results are a caution for CBD users to be aware of the concentration of CBD being consumed.

Conflict of interests

The authors declare no conflict of interest.

Ethics approval

The human ethics of this study was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (study code; AMSEC-66EX-013).

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