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Anti-inflammatory Effect of a Combination of Cannabidiol and *Morinda citrifolia* Extract on Lipopolysaccharide-stimulated RAW264 Macrophages

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Abstract. *Background/Aim: The inflammatory response plays an important role in the activation and progression of many inflammation-related diseases. Cannabis sativa and Morinda citrifolia have long been used in folk medicine to treat inflammation. Cannabidiol is the most abundant non-psychoactive phytocannabinoid in C. sativa and exhibits anti-inflammatory activity. The objective of this study was to examine the anti-inflammatory effect of cannabidiol in combination with M. citrifolia and compare its effects with those of cannabidiol alone. Materials and Methods: RAW264 cells stimulated with lipopolysaccharide (200 ng/ml) were treated with cannabidiol (0-10 μM), M. citrifolia seed extract (0-100 μg/ml), or a combination of both for 8 or 24 h. Following the treatments, nitric oxide production in the activated RAW264 cells and the expression of inducible nitric oxide synthase were assessed. Results: Our results showed that combination of cannabidiol (2.5 μM) and M. citrifolia seed extract (100 μg/ml) exhibited more efficient inhibition of nitric oxide production than cannabidiol treatment alone in lipopolysaccharide-stimulated RAW264 cells. The combination treatment also reduced the expression of inducible nitric oxide synthase. Conclusion: These results suggest that the anti-inflammatory effect of combined treatment with cannabidiol and M. citrifolia seed extract causes a reduction in the expression of inflammatory mediators.*

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Key Words: Cannabidiol, *Morinda citrifolia*, nitric oxide, RAW264 cells, anti-inflammatory.

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Inflammation occurs in response to injury and plays an important role in removing harmful stimuli (1). It is related to the progression of diseases, including pancreatitis, and cardiovascular and lung inflammatory diseases (2). The inhibition of the inflammatory response has become a therapeutic strategy for the treatment of these diseases.

Macrophages are important for the pathological process of inflammation. RAW264 cells, a murine macrophage-like cell line, stimulated by lipopolysaccharide (LPS), have frequently been used to investigate macrophage activation in *in vitro* models (3). Activated macrophages produce pro-inflammatory mediators such as interleukin-6 and nitric oxide (NO) (4). Traditional medicine continues to use medicinal plants as a substitute for modern medicines (5). It has been considered that plant-derived natural products or extracts include immunomodulators (6, 7).

Cannabis sativa L., commonly known as marijuana, and *Morinda citrifolia* L., known as noni, have been used as folk remedies by many cultures for centuries. The anti-inflammatory and antioxidant effects of natural products derived from plants have been extensively studied (8, 9). *C. sativa* plant contains more than 500 natural compounds and over 100 phytocannabinoids (10). Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) are the two most abundant cannabinoids present in cannabis. Although THC exhibits anti-inflammatory effects, the application of THC in clinical therapy has been limited mainly because of its psychotropic effects. In

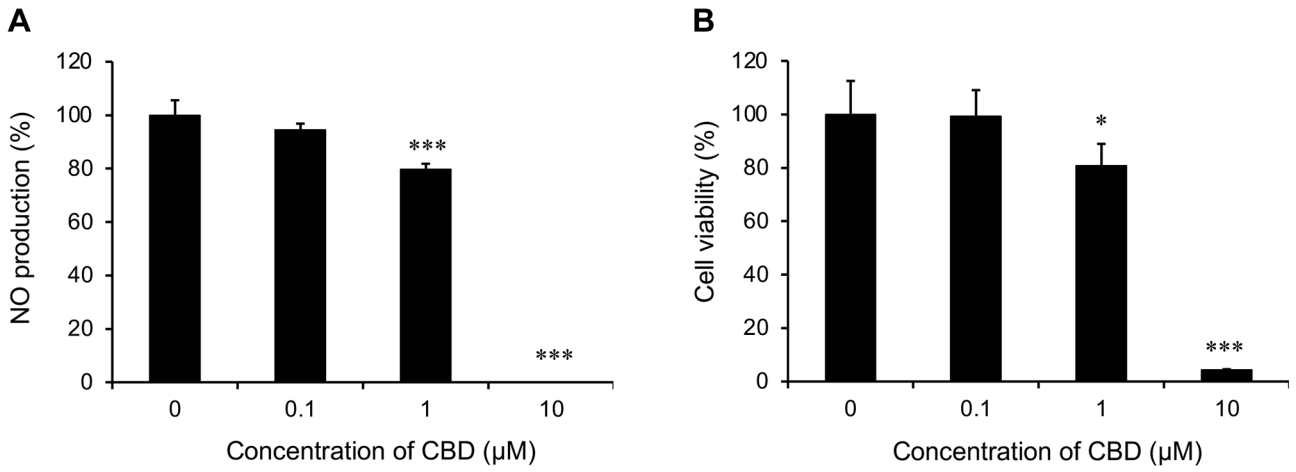

Figure 1. *Effect of cannabidiol (CBD) on nitric oxide production and cell viability of lipopolysaccharide (LPS)-stimulated RAW 264 cells. Cells were incubated with the indicated concentrations of CBD and 200 ng/ml LPS for 24 h. The final concentration of the samples incubated with LPS is indicated. A: The level of nitric oxide (NO) in the culture medium was measured using the Griess reaction. B: Cell viability was measured using a Cell Counting Kit-8. Data are presented as the mean±standard deviation (n=3-4). Significantly different at: *p<0.05 and ***p<0.001 compared to samples without CBD.*

contrast, CBD exerts anti-inflammatory and antioxidant effects without causing any psychotropic effects (11).

M. citrifolia has been used in the treatment of many diseases, including diabetes, cancer, and hypertension (12-14). *M. citrifolia* seed extract (MCS-ext) has been reported to have human leukocyte elastase-inhibitory, tyrosinase-inhibitory, antioxidant, and anti-inflammatory properties (15, 16).

In this study, we evaluated the anti-inflammatory activity of combination treatment with CBD and MCS-ext, in comparison with CBD alone, in lipopolysaccharide (LPS)-stimulated RAW264 cells.

Materials and Methods

Cell culture experiment. RAW264 cell line was obtained from RIKEN BRC (Ibaraki, Japan). This cell line was cultured in Eagle's minimum essential medium with L-glutamine and phenol red supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), streptomycin (100 μg/ml), penicillin (100 U/ml), and 1% minimum essential medium with non-essential amino acids (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The experiments were performed using a modified version of previously described protocols (15, 17). Briefly, RAW264 cells were plated in Dulbecco's modified Eagle's medium (high glucose) without L-glutamine and phenol red containing 0.5% fetal bovine serum, 2 mM L-glutamine (FUJIFILM Wako Pure Chemical Corporation), streptomycin (100 μg/ml), and penicillin (100 U/ml) and cultured overnight at 37˚C in a humidified atmosphere with 5% CO_2. MCS-ext (Oryza Oil & Fat Chemical Co., Ltd., Aichi, Japan) was added to the medium 0.5 h before CBD (2.5 μM) (Cayman Chemical, Ann Arbor, MI, USA) and LPS from *Escherichia coli* O111 (200 ng/ml) (FUJIFILM Wako Pure Chemical Corporation) addition. The final concentration of MCS-ext in samples incubated with LPS was 0-100 μg/ml. After 24 h treatment with the mixture, NO accumulation in the cell culture medium was measured using the Griess method. The absorbance at 550 nm was measured with a microplate reader. The cell viability was assessed by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The absorbance was measured at 450 nm using a microplate reader. The final concentration of dimethyl sulfoxide was adjusted to be less than 0.15%.

RNA isolation and real-time reverse transcriptase–polymerase chain reaction. MCS-ext (100 μg/ml) was added to the medium 0.5 h before CBD (2.5 μM) and LPS (200 ng/ml) addition and the cells were incubated with the mixture for 8 h. The RNA from the cells was then isolated using Isogen II (Nippon Gene Co., Ltd., Tokyo, Japan). The RNA was reverse-transcribed into cDNA with FastGene Scriptase II cDNA Synthesis (NIPPON Genetics Co., Ltd.). Real-time reverse transcriptase–polymerase chain reaction was performed in a Thermal Cycler Dice Real-Time System (Takara Bio Inc., Shiga, Japan) with EvaGreen (Biotium, Inc., Fremont, CA, USA) and Taq DNA Polymerase (New England BioLabs, Ipswich, MA, USA). The primers were as followed: Mouse β-actin (*Actb*): sense and antisense, 5'-CGG TTC CGA TGC CCT GAG GCT CTT-3' and 5'-CGT CAC ACT TCA TGA TGG AAT TGA-3'; mouse inducible nitric oxide synthase (*Nos2*) sense and antisense, 5'-TGG AGC CAG TTG TGG ATT GTC-3' and 5'-GGT CGT AAT GTC CAG GAA GTA G-3'. The $2^{-\Delta\Delta Ct}$ method was used to normalize the relative mRNA expression levels to that of *Actb*.

Statistical analysis. Analysis of variance followed by Dunnett's multiple comparison test was carried out. The significance of differences was analyzed using Student's *t*-test. In all cases, statistical significance was accepted at $p<0.05$.

Results

Effect of CBD on NO production. Firstly, we examined whether CBD treatment inhibited NO production in activated (LPS-stimulated) RAW264 cells. RAW264 cells were treated with

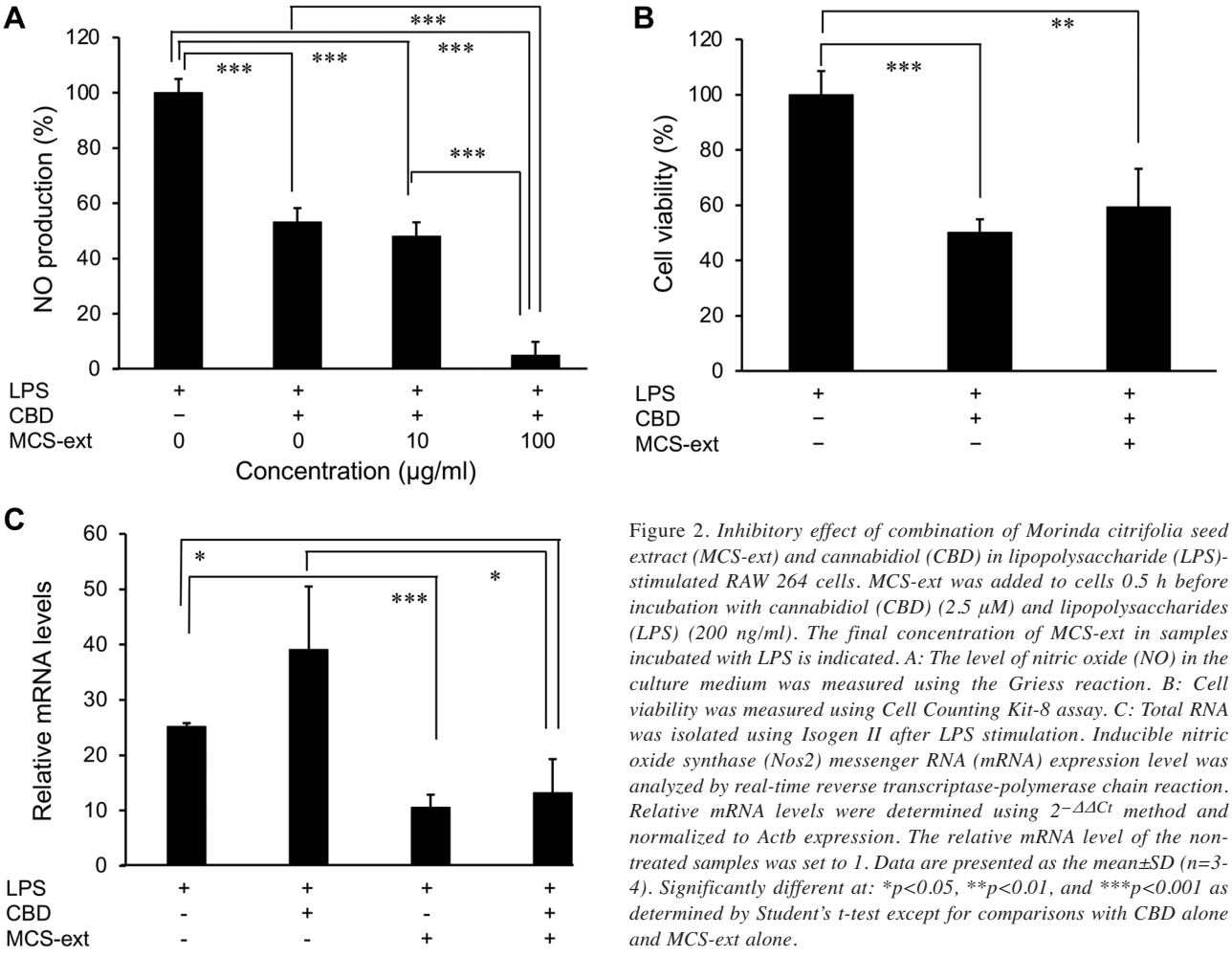

Figure 2. *Inhibitory effect of combination of Morinda citrifolia seed extract (MCS-ext) and cannabidiol (CBD) in lipopolysaccharide (LPS)-stimulated RAW 264 cells. MCS-ext was added to cells 0.5 h before incubation with cannabidiol (CBD) (2.5 μM) and lipopolysaccharides (LPS) (200 ng/ml). The final concentration of MCS-ext in samples incubated with LPS is indicated. A: The level of nitric oxide (NO) in the culture medium was measured using the Griess reaction. B: Cell viability was measured using Cell Counting Kit-8 assay. C: Total RNA was isolated using Isogen II after LPS stimulation. Inducible nitric oxide synthase (Nos2) messenger RNA (mRNA) expression level was analyzed by real-time reverse transcriptase-polymerase chain reaction. Relative mRNA levels were determined using $2^{-\Delta\Delta Ct}$ method and normalized to Actb expression. The relative mRNA level of the non-treated samples was set to 1. Data are presented as the mean±SD (n=3-4). Significantly different at: *p<0.05, **p<0.01, and ***p<0.001 as determined by Student's t-test except for comparisons with CBD alone and MCS-ext alone.*

CBD and LPS for 24 h, and NO production was measured using the Griess reagent system. CBD suppressed NO production at concentrations ranging from 1 to 10 μM and dose-dependently reduced LPS-induced NO production (Figure 1A). We also examined whether CBD treatment of LPS-stimulated RAW264 cells induced cytotoxic effects. Treatment with CBD reduced the viability of LPS-stimulated RAW264 cells as its concentration increased (Figure 1B). Therefore, the inhibitory effect of CBD on NO production may be related to cell viability. These results suggest that it is not appropriate to simply increase CBD content to expect the inhibitory effect on NO production.

Effect of CBD in combination with MCS-ext on NO production. To examine the anti-inflammatory effect of CBD in combination with natural product extracts, we used *M. citrifolia* seed extract (MCS-ext). Various natural extracts have been reported to exert anti-inflammatory effects. We have previously reported the anti-inflammatory effect of MCS-ext in LPS-stimulated RAW264 cells *in vitro* (15). Noni juice has also been reported to exhibit anti-inflammatory and antioxidative effects and reduce carrageenan-induced paw edema (18) and lung inflammation sensitized by ovalbumin (19) in animal models of inflammation. MCS-ext (0-100 μg/ml) was added to the medium and cells were incubated for 0.5 h. We next added LPS and CBD to the medium and NO production was measured after 24 h. We found that the combination of CBD and MCS-ext led to a greater reduction than CBD alone (Figure 2A). However, the cell viability did not decrease when LPS- and CBD-stimulated RAW264 cells were treated with 100 μg/ml MCS-ext (Figure 2B). These results indicated that the addition of MCS-ext beforehand can be expected to have a higher inhibitory effect on NO production than the addition of CBD alone, without an increase in cytotoxicity.

Additionally, we examined whether combination treatment with CBD and MCS-ext modulates the expression of inducible nitric oxide synthase, and whether this is related to NO production in LPS-stimulated RAW264 cells. Activated macrophages produce pro-inflammatory mediators, such as inducible nitric oxide synthase, which synthesizes NO using L-arginine as a substrate (4, 20). MCS-ext (100 μg/ml) was

added to cells 0.5 h before incubation with CBD and LPS for 8 h. The expression level of *Nos2* messenger RNA in LPS-induced cells was enhanced compared to that of non-treated cells. However, MCS-ext alone and the combination of CBD and MCS-ext inhibited expression of *Nos2* mRNA of LPS-stimulated RAW264 cells (Figure 2C). CBD treatment alone caused no significant change in *Nos2* mRNA expression in LPS-stimulated.

Discussion

Recently, foods, drinks and creams containing CBD have become widely available around the world. CBD is used for its relaxant, antioxidant, and anti-inflammatory effects. In this study, we focused on the anti-inflammatory effects and suggested the potential significance of a combination of CBD with other materials. Our results demonstrate that CBD and MCS-ext appear to exhibit an anti-inflammatory effect in LPS-stimulated RAW264 cells.

Previous studies have found that CBD induced apoptosis of HL-60 cells, human myeloblastic leukemia cells (at 1-8 μg/ml or 3.226 μM) (21), primary human monocytic cells (at 4-16 μM) (22, 23), and human and murine leukemia cells (at 1.25-10 μM) (24). In our study, CBD reduced cell viability at 1-10 μM (Figure 1B). These results indicate that a high concentration of CBD alone potentially has not only an anti-inflammatory effect but also causes cell damage.

To maintain the anti-inflammatory effect of CBD with low cytotoxicity, we evaluated the anti-inflammatory effect of MCS-ext in combination with a low concentration of CBD in LPS-stimulated RAW264 cells. The combination of CBD and MCS-ext (100 μg/ml) inhibited NO production in LPS-stimulated RAW264 cells more than CBD alone (Figure 2A). The combination of CBD and MCS-ext did not reduce cell viability compared to treatment with CBD alone (Figure 2B). However, the combination of CBD and MCS-ext treatment significantly reduced NO production compared to CBD treatment. Thus, the significant anti-inflammatory effect in LPS-stimulated RAW264 cells treated with the combination of CBD and MCS-ext is unrelated to the reduction in cell viability. MCS-ext alone and in combination with CBD suppressed *Nos2* expression in LPS-stimulated RAW264 cells (Figure 2C). The detailed mechanism of inhibition of NO production caused by the combination of MCS-ext and CBD is unclear. Inducible nitric oxide synthase is known to be regulated by nuclear factor-κB (NF-κB) and becomes active after degradation of the inhibitory protein, I-κB (25, 26). It has been reported that compounds from Hawaiian noni fruit juice suppress NO production through the inhibition of the IKKα/β, I-κBα, and NF-κB p65 signaling pathways in LPS-stimulated macrophages (27). Therefore, MCS-ext might reduce NO production through the inhibition of the NF-κB signaling pathway. CBD treatment did not lead to a significant decrease in inducible nitric oxide synthase expression in LPS-stimulated macrophages compared to dimethyl sulfoxide treatment (28). CBD has potential antioxidant activity because cation free radicals have several resonance structures in which unpaired electrons are mainly distributed on the ether and alkyl moieties, as well as the benzene ring (29). The decrease in NO may be related to the scavenging capacity of CBD. In the present study, we measured only NO production as a pro-inflammatory mediator to examine the anti-inflammatory effect of the combination of CBD and MCS-ext in LPS-stimulated RAW264 cells. Activated macrophages secret various cytokines such as tumor necrosis factor, and interleukins 1 and 6 (30). It is important to further investigate the inhibitory effects of CBD and MCS-ext combination on other pro-inflammatory mediators in LPS-stimulated macrophages. Future studies are necessary to elucidate the molecular mechanism underlying the anti-inflammatory effects of CBD and MCS-ext combination treatment in LPS-stimulated macrophages. Furthermore, in general, natural products, including *M. citrifolia L.* may be affected by several environmental and genetic factors including the area and variety cultivated. Because our studies performed only one set of MCS-ext samples in this study, further studies are required. Finally, the combination of CBD and MCS-ext might be a new, potential anti-inflammatory treatment option against inflammation-related disorders.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Conceptualization and supervision, T.T.; Methodology, T.T., M.K., Y.H., N.T., A.U., F.I., T.Y. and Y.I.; Formal analysis, T.T. and M.K.; writing—original draft preparation, T.T.; writing—review and editing, T.T., M.K., Y.H., N.T., A.U., F.I., T.Y. and Y.I. All Authors have read and agreed to the published version of the article.

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