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Therapeutic impact of orally administered cannabinoid oil extracts in an experimental autoimmune encephalomyelitis animal model of multiple sclerosis

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ABSTRACT

There is a growing surge of investigative research involving the beneficial use of cannabinoids as novel interventional alternatives for multiple sclerosis (MS) and associated neuropathic pain (NPP). Using an experimental autoimmune encephalomyelitis (EAE) animal model of MS, we demonstrate the therapeutic effectiveness of two cannabinoid oil extract formulations (10:10 & 1:20 – tetrahydrocannabinol/cannabidiol) treatment. Our research findings confirm that cannabinoid treatment produces significant improvements in neurological disability scoring and behavioral assessments of NPP that directly result from their ability to reduce tumor necrosis factor alpha (TNF-α) production and enhance brain derived neurotrophic factor (BDNF) production. Henceforth, this research represents a critical step in advancing the literature by scientifically validating the merit for medical cannabinoid use and sets the foundation for future clinical trials.

1. Introduction

Multiple sclerosis (MS) is a chronic, incurable disease characterized by inflammation and demyelination in the brain and spinal cord (SC) of the central nervous system (CNS). While the exact mechanism of the disease is unknown, it is widely considered as an autoimmune disease involving the infiltration of inflammatory cells through the blood-brain barrier into the CNS, resulting in the liberation of inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), which are involved in neuropathic pain (NPP) and neuronal destruction [1,2]. MS-induced NPP remains a compelling clinical problem to patients and healthcare providers as it responds poorly to conventional treatments [3].

Anecdotal reports of the beneficial medicinal properties associated with the plant *Cannabis Sativa* have led to a recent surge in investigative research to explore the potential benefits of cannabinoids that are linked to their ability to modulate the immune system and promote analgesia in chronic pain syndromes such as MS-induced NPP [4–6]. The key cannabinoid components of interest are Δ9-tetrahydrocannabinol (THC) [7], cannabidiol (CBD) and cannabichromene (CBC) [8]. With recent advances in biotechnology, it is possible to consistently adjust the THC/CBD ratios within the plant to achieve the specific desired effect and therapeutic outcomes while minimizing adverse effects [9]. Henceforth, the use of genetically altered plants by certain licensed producers in Canada allows for a tighter control over the consistent reproducibility of the differential production of the three main active constituents: THC, CBD and CBC.

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As such, the current research focused on confirming the potential therapeutic efficacy and specific molecular mechanisms by which interventional treatment with cannabinoids exerts their beneficial effects in MS associated NPP syndromes. Our results revealed that significant improvements in neurological assessments of MS induced neurological disability and behavioral assessments of NPP in EAE animals treated with the orally administered cannabinoids. In addition, we investigated the role of cannabinoids on the gene and protein expression of bio-markers, such as TNF-α and BDNF, in a rat EAE model. Moreover, we also determined the pharmacokinetic pattern of cannabinoid oil extracts. This research represents a critical step in scientifically validating the merit for cannabinoid use and sets the foundation for future pilot human clinical trials in patients suffering from MS.

2. Materials and methods

2.1. Experimental autoimmune encephalomyelitis (EAE) induction

Adolescent female Lewis rats 9–14 weeks of age (Charles River, Canada) were immunized subcutaneously with 500 μg myelin basic protein gp 69-88 in 1000 μl of Complete Freund's adjuvant at the lower back (0.2 ml/rat) at day 0 (Hooke Laboratories, Cat#. EK-3111, USA). Animals received two intraperitoneal injections of pertussis toxin at days 0 and 2. All animal experiments in this study were conducted according to protocols approved by the University of Manitoba Animal Protocol Management and Review Committee and in full compliance with the Canadian Council on Animal Care (Protocol Reference Number: 15-049/1/2 AC 11097).

2.2. Cannabinoid treatments

CanniMed Oil® Huile 10:10 (Lot# OL16007H) and 1:20 (Lot# OL16026J) were obtained from Prairie Plant Systems Inc (Saskatoon, Canada). A 215 mg/kg dose of the cannabinoid oil extracts is administered by oral gavage daily between day 6–18 post-disease based on the previous established in-house expertise with the EAE rat model of MS [10].

2.3. Neurological disability scoring (NDS)

NDS were conducted in accordance to our published criteria [11]. Animals were assessed daily for neurological disability until sacrificed. NDS were determined from mean clinical scores measured from a score of 0 (no disability) to 15 (maximal disability). The total score is the sum of the following individual scores obtained for each of the 6 specified clinical domains: *tail:* 0 = normal, 1 = weakness or partial paralysis, 2 = complete paralysis; *hind limbs and forelimbs:* 0 = normal, 1 = weakness, 2 = dragging or partial paralysis, 3 = complete paralysis; *bladder:* 0 = normal, 1 = incontinent. Only animals that progressed to the NDS of 4 or greater were included in the study.

2.4. Enzyme linked immunosorbent assay (ELISA)

Sandwich-style ELISA was performed using the Novex® rat TNF-α ELISA kit (Invitrogen, Cat#. KRC3011) and a BDNF Emax ImmunoAssay System (Promega, Cat#. G7611). TNF-α content was measured from standard curve runs for each plate (linear range of 0–200 ng/ml). BDNF protein concentration was interpolated from a standard curve with a range of 7.8–500 pg/ml.

2.5. Quantitative reverse transcriptase polymerase chain reaction (qRT- PCR)

The PCR reaction was performed using a SsoFast EvaGreen Supermix following manufacturers protocols (Bio-Rad, CA, USA) on a Real-Time PCR system (7500, Applied Biosystems, USA). TNF-α primers were forward: 5' -AGCCGATTTGCCATTTCATACCAG -3'; reverse: 5' -CACGCCAGTCGCTTCACAGAG -3′ at an annealing temperature of 60 °C. BDNF primers were forward: 5' -AGCTGAGCGTGTGTGACAGTATTAG -3'; reverse: 5' -GGGATTACACTTG GTCTCGTAGAAA -3′ at an annealing temperature of 57 °C. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH primers were forward: 5' -AAGAAGGTGGTGAAGCAGGCG -3'; reverse: 5' -AGACAACCTG GTCCTCAGTGTAGC -3'.

2.6. Dot Blot Analysis

The dot blot assay was performed as described previously [12]. Briefly, DNA samples were spotted on a nitrocellulose membrane at room temperature for 15 min. After incubation at 80 °C in an oven for 30 min, the membrane was blocked with 5% milk in phosphate-buffered saline with 0.1% Tween-20 for 1 h and incubated with primary antibodies (anti-5mc antibody, Millipore, USA; anti-5hmc antibody, Active Motif, USA) at 4 °C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA) for 1 h on the second day. The membrane was developed captured on autoradiographic films. Densitometry of methylene blue staining was used as a loading control to evaluate the densitometries of 5mc and 5hmc.

2.7. Thermal sensory testing

Withdrawal latencies to a radiant heat stimulus were assessed for each rat using a Model 336G Plantar/Tail Stimulator Analgesia Meter (IITC Life Sciences, USA) [13]. Region specific withdrawal responses consisted of licking the paws and flicking the tail in response to the heat stimulus. Withdrawal latencies were recorded three times for each paw and tail in seconds with a maximum of 20 s cut-off point programmed into the timer to prevent tissue damage. These latencies were then normalized to baseline values and presented as percentages.

2.8. Pharmacokinetic (PK) analysis of collected blood samples

2.8.1. Liquid Chromatography–Mass spectrometry (LC-MS) system and conditions

The LC-MS system was comprised of a Shimadzu HPLC and a LCMS-2020 mass spectrophotometer (Kyoto, Japan). Separation was achieved at ambient temperature (22 ± 1 °C) with a flow rate of 0.4 ml/min using a C18 Waters (5 μm, 250 × 4.6 mm) with UV detection at 220 nm. The mobile phase was prepared by mixing acetonitrile:water (70:30 v/v).

2.8.2. Preparation of standard solutions and blood collection

Working solutions of CBD, THC, and CBC were freshly prepared by diluting analytical standards (Sigma) in methanol to obtain a final concentration of 0.25, 0.5, 1and 5 μg/ml in rat serum.

Blood was collected from the saphenous vein at 1 h pre- and post- oral gavage from day 6 onward. On day 18, the collection time points were at 1h pre-treatment, and 1h, 4h, 8h and 12h post-treatment. Serum was collected by centrifuging the blood at 4000 rpm for 10 min at 4 °C.

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2.8.3. Treatment of serum samples for analysis

To a 50 μl aliqoute of rat serum sample, 0.5 ml of cold HPLC grade acetonitrile was added to precipitate plasma proteins, vortexed and centrifuged at 15,000 rpm for 10 min. The supernatant was transferred and evaporated to dryness using Savant SPD1010 SpeedVac Concentrator without heat (Thermo Fisher Scientific, USA). The residue reconstituted with 50 μl of acetonitrile was centrifuged at 15000 rpm for 5 min 10 μl of the supernatant was then injected into the LCMS system.

2.9. Statistical analysis

Statistics was performed using GraphPad Prism Software (San Diego, CA, USA). Statistical analysis was done using Two Way ANOVA, followed by Bonferroni post - hoc test.

3. Results

3.1. Neurological disability score (NDS)

At EAE day 7 neurological deficits began to be displayed in some animals in the form of tail weakness (Fig. 1A). By EAE day 8, all animals started to display clinical signs of neurological disability. Statistical analysis revealed significant decreases in severity of NDS on day 10–13 between EAE cannabinoid (10:10 formulation) treated and EAE untreated groups. In addition, results revealed a 2-day delay in an onset of peak NDS in cannabinoid treated (10:10) EAE animals. Significant decreases were also seen for EAE cannabinoid (1:20 formulation) compared with EAE untreated groups at 10 - 13 dpi. In addition, results revealed a 1-day delay in an onset peak NDS is seen in cannabinoid treated (1:20) EAE animals.

3.2. Behavioral analysis for neuropathic pain (NPP)

3.2.1. Tail results

With respect to the tail, there was statistically significant decrease in tail withdrawal latency at 11 dpi identified in the EAE treated with cannabinoid (10:10) relative to the EAE untreated animals (Fig. 1B). Although the withdrawal latencies in the EAE treated with cannabinoid (1:20) at day 11 to day 13 were markedly lower than that of EAE untreated animals, no significant reductions in withdrawal latency of the tail were noted at any time points between the EAE treated with cannabinoid (1:20) and EAE untreated or NC animals.

3.2.2. Right rear paw results

With respect to the rear right paw, the withdrawal latency for EAE treated with cannabinoid (10:10) was significantly lower at 12 dpi and marked lower at 13 dpi than that of the EAE untreated animals (Fig. 1C). Although there was a marked decrease in withdrawal latency in the EAE treated with cannabinoid (1:20) relative to EAE untreated animals at 13 dpi, overall there were no significant reductions in withdrawal latency of the right hind limb noted at any time points between the EAE treated with cannabinoid (1:20) and EAE untreated or NC animals.

3.2.3. Left rear paw results

With respect to the rear left paw, although the withdrawal latency in the EAE treated with cannabinoid (10:10) was markedly lower than that of EAE untreated animals between 11 and 13 dpi, these reductions in withdrawal latency never reached significance between the EAE treated with cannabinoid (10:10) and EAE untreated or NC animals. Interestingly, at 13 dpi, there was a significant reduction and marked reduction at 14 dpi in withdrawal latency in the EAE treated with cannabinoid (1:20) relative to that depicted in the EAE untreated animals (Fig. 1D).

3.3. TNF-α gene and protein analysis

3.3.1. Cannabinoid 10:10 formulation treatment results

Our results of qRT-PCR analysis of TNF-α mRNA expression in the SC show a significant reduction at 15 dpi between EAE treated with (10:10) cannabinoid formulation versus EAE untreated groups. Additionally, significant elevations in TNF-α mRNA expression were also seen at 15 dpi in EAE untreated animals relative to NC animal (Fig. 2A).

Our results also indicate significant elevations of TNF-α protein expression at 12 dpi between EAE untreated and NCs with corresponding significant reductions in TNF-α noted in EAE treated with (10:10) cannabinoid formulation versus EAE untreated (Fig. 2B). Significant reductions in TNF-α protein were also found at 18 dpi between EAE treated with (10:10) cannabinoid formulation as compared to TNF-α protein identified in the EAE untreated group.

3.3.2. Cannabinoid 1:20 formulation treatment results

Significant reductions in TNF-α mRNA expression were seen at 15 dpi in the EAE treated with (1:20) cannabinoid formulation versus EAE untreated groups (Fig. 2C). Significant reductions in TNF-α protein were also found at 12 and 18 dpi between EAE treated with (1:20) cannabinoid formulation as compared to TNF-α protein identified in the EAE untreated group (Fig. 2D).

3.4. BDNF gene and protein analysis

3.4.1. Cannabinoid 10:10 formulation treatment results

There was a significant increase in the BDNF mRNA expression at 18 dpi in the EAE animals treated with cannabinoid (10:10) relative to that of EAE untreated animals and NC control animals (Fig. 2E).

There was a significant increase in the BDNF protein expression in the EAE untreated and EAE treated with cannabinoid (10:10) as compared to the NC (Fig. 2F) at 12 dpi. This significant increase in BDNF protein expression was maintained at 15 dpi in the EAE treated with cannabinoid (10:10) relative to EAE untreated and relative to NC animals. Furthermore, this significant increase in BDNF protein expression was sustained in the EAE cannabinoid treated (10:10) animals over that of EAE untreated animals at 18 dpi.

3.4.2. Cannabinoid 1:20 formulation treatment results

qRT-PCR results revealed that EAE cannabinoid treated (1:20) had significant elevations of mRNA expression for BDNF at 18 dpi relative to EAE untreated animals and relative to NC animals (Fig. 2G). No significant differences were noted in BDNF protein expression at 12, 15 or 18 dpi between EAE untreated and EAE cannabinoid treated (1:20) (Fig. 2H).

3.5. Dot blot analysis

Dot blot analysis and methylene blue staining was conducted to find the relative levels of 5-methylcytosine (5mc) and 5-hydroxymethylcytosine (5hmc) in the NC, EAE, and EAE treated with cannabinoid (10:10) animals at 12 dpi and 15 dpi to determine if the changes in BDNF gene and protein could be correlated with the respective changes in 5mc and 5hmc. No significant differences were found in 5mc levels between EAE untreated and EAE treated with cannabinoid (10:10) at 12 or 15 dpi relative to each other or to NC's (Fig. 3A and B). However, at 12 and 15 dpi, significant reductions in 5hmc were found in EAE untreated relative to NC animals, while no significant differences in 5hmc levels were

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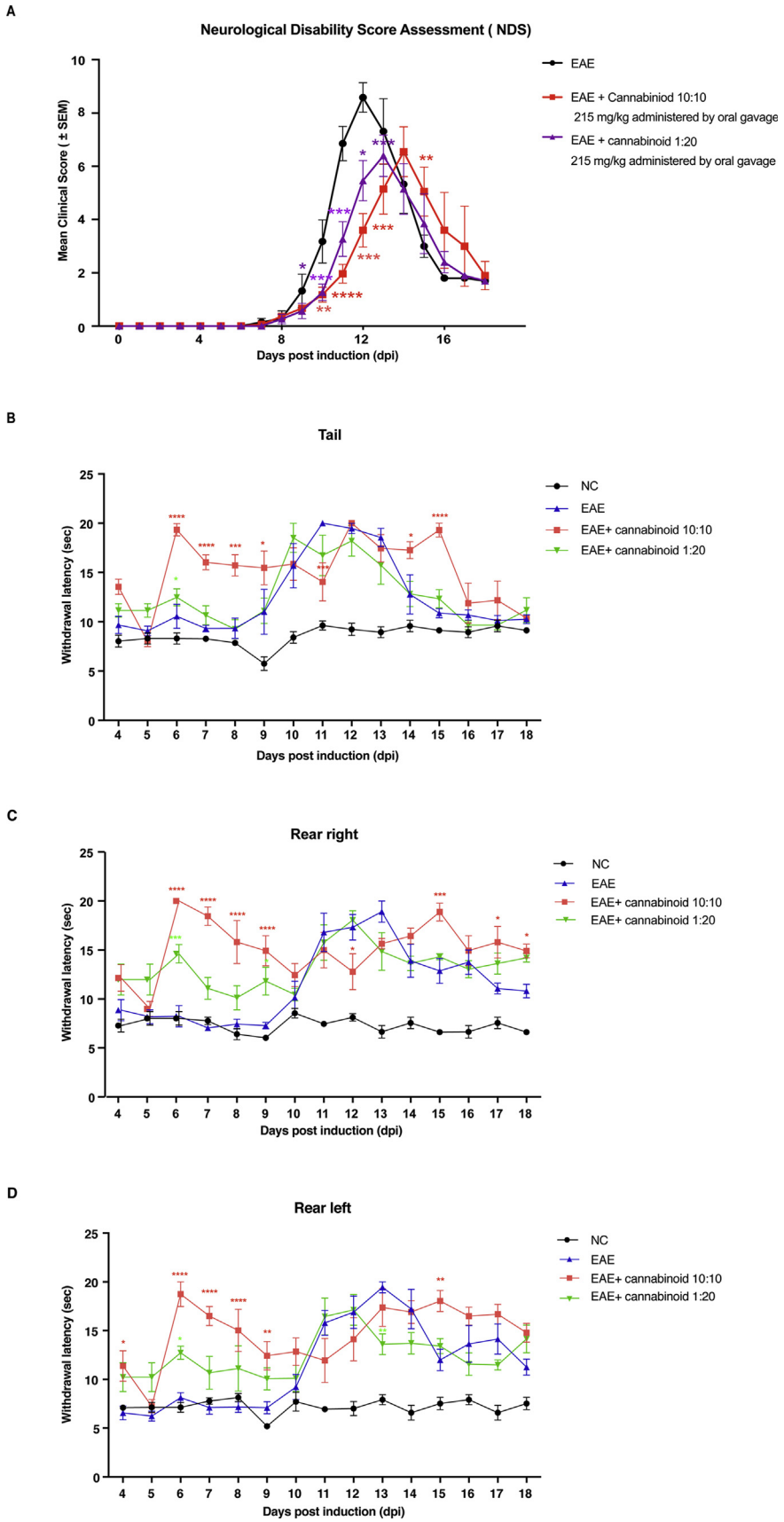

Fig. 1. Cannabinoid improved neurological disability score and showed benefits in behavioral analysis. (A) Neurological disability score assessment. (B-D) Behavioral analysis - thermal testing. Thermal sensitivity of three specific anatomical sites involving that of the tail (B), right (C) and left (D) hind paws were measured. (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ respectively).

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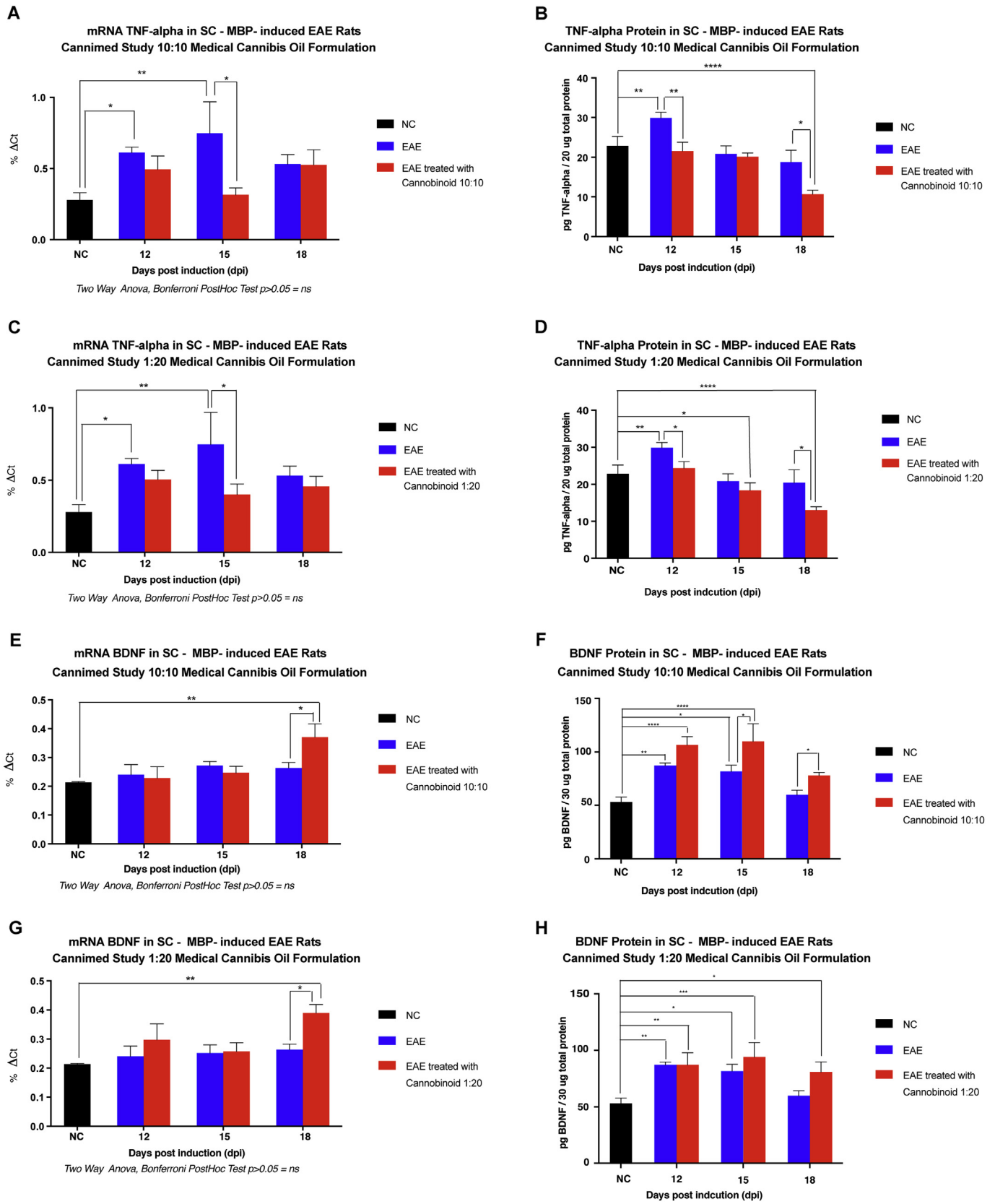

Fig. 2. TNF-α and BDNF gene and protein analysis. Comparative qRT-PCR analysis of TNF-α (A and C) and BDNF (E and G) mRNA expression was conducted between the NC, EAE untreated, and EAE treated with 2 cannabinoid formulations (10:10 and 1:20) at 12, 15, and 18 dpi. Data is presented as the fold difference in relative mRNA expression determined as the ratio of TNF-α (BDNF) mRNA/GAPDH mRNA (%ΔCt)
The protein expressions of TNF-α (B and D) and BDNF (F and H) in the rat SC were analyzed by ELISA at 12, 15, and 18 dpi. Data in B and D are presented in pg TNF-α/20 μg total protein. Data in F and H are presented in pg BDNF/30 μg total protein. (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 respectively).

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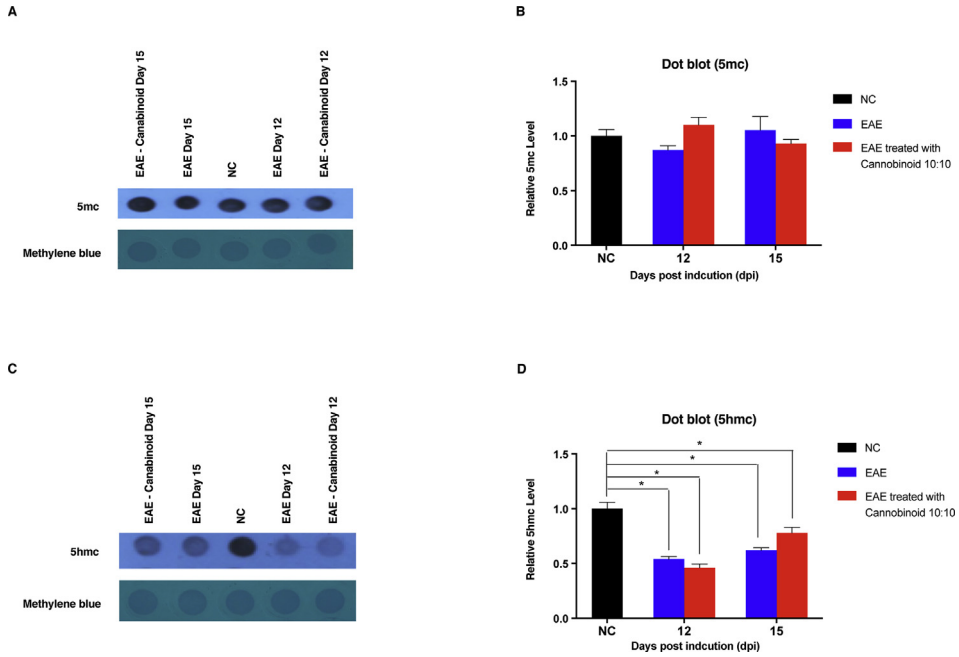

Fig. 3. Dot blot analysis. Dot blot staining and analysis indicate relative 5-mc (A, B) and 5-hmc (C, D) levels in rat SC.

identified between EAE untreated and EAE treated with cannabinoid (10:10) (Fig. 3C and D).

3.6. Serum drug levels

According to the product information for the 1:20 formulation, the amount of total CBD is 21.9 ± 0.6 mg/ml, while total THC is only 1.0 ± 0.1 mg/ml, which means the amount of CBD is 20 times higher than that of THC. Additionally, in the 10:10 formulation, the amount of total CBD was 10.2 ± 0.5 mg/ml, while total THC was 9.9 ± 0.5 mg/ml, which means the amount of CBD was approximately the same as that of THC.

3.6.1. Cannabinoid 10:10 formulation treatment results

Following the treatment administration with the 10:10 cannabinoid oil formulation, results revealed a significant increase in THC concentration in serum on days 7, 8, 9, 13 and 16. In addition, THC serum levels of 4 h post-drug administration following the last day of treatment (day 18) was statistically elevated (Fig. 4A). Marked increases of the CBD concentrations in serum were also seen after treatment (days 7, 8, 9, 13, 16), however, these increases did not reach statistical significance. CBC levels were undetectable at all days assessed in the EAE cannabinoid treated (10:10). In order to obtain a crude elimination rate constant for THC and CBD, we used regression analysis to approximate the elimination phase. Using the time points of 4 and 8 h the Kel for THC was calculated to be 0.776 ± 0.542 hrs^{-1} while the Kel for CBD was 0.181 ± 0.001 hrs^{-1} (n = 2). Half-life for THC was then calculated to be 0.9 h while CBD was 3.8 h.

3.6.2. Cannabinoid 1:20 formulation treatment results

In the 1:20 cannabinoid formulation, results showed significant increases in concentrations of CBD in serum only on days 6, 7, 13, 14, 16 and 18. Results demonstrated consistently higher CBD levels at all time points assessed relative to THC and CBC, level of which were undetectable in serum post-treatment (Fig. 4B). Interestingly, the peak temporal serum levels of CBD were similar in nature to the peak THC time points levels with the 10:10 cannabinoid formulation. Using the 4- and 8-h data points for CBD the calculated Kel was 0.235 ± 0.099 hrs^{-1} (n = 5) with a half-life of 2.9 h.

4. Discussion

Our pre-clinical research findings demonstrate significant improvements in NDS and behavioral assessments of MS induced NPP in EAE animals treated with the orally administered medical grade cannabinoids.

Based on the NDS data, both cannabinoid oral oil formulations (10:10 & 1:20) were able to partially reverse the disease progression after EAE induction as evident by their ability to delay the onset of peak neurological disability and reduce the peak severity. However, the 10:10 cannabinoid formulation appears to have a potential advantage to delay time to peak NDS by 2 days compared to the 1 day for the 1:20 cannabinoid formulation.

Our behavioral analysis assessment results showed that interventional treatment with cannabinoids could help improve (lessen) the withdrawal latency compared to that of EAE untreated animals. Additionally, the 10:10 cannabinoid formulation appears to have a potential advantage over the 1:20 formulation as evident by its ability to have a greater therapeutic impact on more domains of pain assessment. However, during the pre-EAE attack phase (6 - 10 dpi), both cannabinoids treated EAE groups (10:10 & 1:20) have higher withdrawal latency times than that of EAE untreated animals. These initial longer withdrawal latencies occurred due to the psychoactive side effects that are associated with the use of medical cannabinoids [14,15]. This effect particularly observed in the treatment with the 10:10 formulation during this same period was as expected due to its higher content of THC relative to the low THC levels observed in the 1:20 cannabinoid formulation (Fig. 4).

Our results of the TNF-α gene and protein analysis then suggested that cannabinoid interventional treatment reduces the overall liberation of TNF-α protein in SC during an immune system mediated attack that contributes to the delay in onset of peak NDS and to the reduced overall severity of NDS. Several studies have demonstrated the beneficial role of BDNF in re-myelination and myelin repair and its key role in MS induced NPP [10,16,17].

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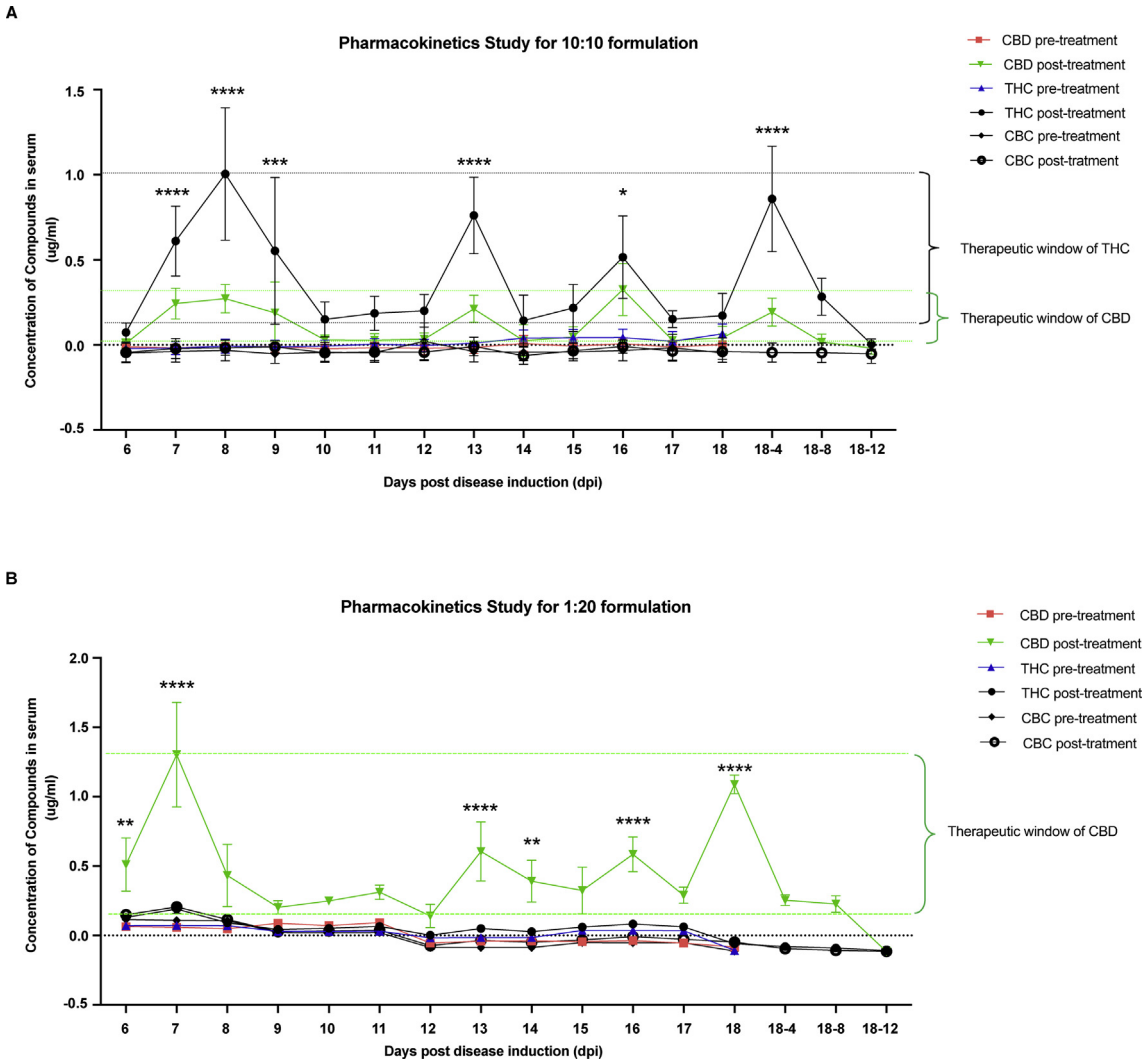

Fig. 4. Serum drug levels. Blood was collected from EAE animals that received interventional treatment with the 10:10 (A) or 1:20 (B) cannabinoid oral oil formulations at 1 h pre- and post-oral gavage from day 6 to day 18.

Henceforth, our current research also examined the temporal changes in BDNF expression at the gene and protein levels in EAE animals treated with cannabinoids. Our findings suggested that once the initial BDNF protein was released (12-15 dpi), the mRNA expression for BDNF was then subsequently activated at 18 dpi to replace the BDNF that was liberated at the time period of the peak EAE immune system mediated attack. As a result, we conclude that treatment of EAE animals with the cannabinoids contributed to the critical and timely release of BDNF protein and enhanced BDNF gene expression that in part facilitated the resultant onset delay and reduced overall severity of the NDS relative to EAE untreated animals.

A recent publication showed an increase in BDNF gene and protein expression that is directly associated with 5hmc abundance in BDNF promoter region [12]. As such, we conducted research to determine the effects of EAE induction and cannabinoid treatment (10:10 formulation) on regulating the 5hmc/5mc ratio. Our findings demonstrate that treatment of EAE animals with the cannabinoid did not alter the levels of 5mc and 5hmc relative to NC or EAE untreated animals (Fig. 3A and B). However, our research did confirm that EAE induction was responsible for the significant reduction of 5hmc at 12 and 15 dpi (Fig. 3C and D). Overall, the changes for 5mc and 5hmc do not account for the relative changes in BDNF gene and protein expression changes reported in the EAE treated with cannabinoid (10:10). As such, further research is warranted to confirm how cannabinoids may increase expression of BDNF to promote myelin repair during an EAE-induced immune system attack.

In our study, a validated HPLC-MS method was applied to investigate the THC, CBD, and CBC concentrations in serum to maximize the therapeutic effect and minimize drug side effect of each new formulation currently on the market for human consumption. Our study showed that 10:10 formulation treatment resulted in a higher concentration of THC in serum, while the concentration of CBD was significantly higher than the other two components (THC and CBC) in the 1:20 formulation treatment group. The results are consistent with the concentration of composing compounds. An interesting pattern for absorption was observed in the present study for the two cannabinoid formulations. Levels of component showed a circadian like pattern peaking at days 7–8, 13, 16 and 18. An explanation for this pattern is not readily apparent and needs to be verified using higher drug levels and longer sampling times.

THC and CBD have been shown to follow a two-compartment

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open model with peak concentrations following oral administration ranging from 1 to 5 h in humans [18]. The elimination rates allowed us to calculate drug half-life, showing that THC is eliminated much faster, approximately four times that of CBD. It is recognized to obtain reliable pharmacokinetic parameters for THC, CBD, and CBC using these formulations, higher oral doses and longer sampling times are required. Nevertheless, the present preliminary elimination rates and half-life allow us to design proper kinetic animal studies for subsequent application to human clinical trials.

In summary, the successful application of this pre-clinical research can be extrapolated to human clinical trials in patients with MS and associated NPP thereby representing significant promise in improving patient's quality of life and reducing costs to the health care system.

Conflicts of interests

The authors declare no competing interests.

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Transparency document

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