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Optimizing the Synergistic Effects of Cannabidiol and Δ⁹-Tetrahydrocannabinol for the Treatment of Neuropathic Pain in Mouse Behavioural Models

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Abstract

Introduction: The analgesic properties of CBD and THC in cannabis can potentially be leveraged for the treatment of neuropathic pain but have not been well investigated. Some commercial analgesics, such as opioids, have unfavourable side effects including addiction, which does not exist in cannabis. Combinations of CBD and THC may not only elicit stronger analgesic effects than single-compound drugs, but also curb the psychotropic effects commonly associated with THC. We present a novel protocol to find the ideal substance ratio in a CBD-THC mixture, which elicits maximum antinociception with the least psychotropic effect.

Methods: BALB/c mice will be assigned to 12 different treatment groups, representing 9 different ratios of CBD-THC mixtures, 2 positive controls (URB937 and sertraline hydrochloride), and 1 vehicle. Each mouse will be administered a compound via intraperitoneal injection and then subjected to behavioural testing. Chronic constriction injury and the Hargreaves' Test (HT) will be used to test nociceptive behaviour while the Tail Suspension Test (TST) will be used to test depression-like behaviour.

Expected Results: The ideal CBD-THC mixture will produce maximum withdrawal latency in the HT and maximum immobility time in the TST. Because the analgesic properties of combined CBD and THC still remain unclear in current literature, it is difficult to predict how withdrawal latency in the HT will change with varying CBD:THC ratios. Based on the psychotropic effects of THC, we expect increased THC concentrations to decrease immobility time in the TST.

Conclusion: By determining the optimal ratio of CBD:THC for maximal pain suppression and minimal psychotropic effects, our protocol may provide justification for an alternative non-addictive therapeutic for treating neuropathic pain. In order to increase the generalizability and translatability of the results in a clinical setting, future studies could benefit from changes in dosing strategies, routes of administration, supplemental observation methods, and experimental timeframes.

Keywords: cannabis; CBD-THC mixture; pain; neuropathy; nociception; analgesia; psychoactivity; behavioural test; mouse model; pharmacology

Introduction

It is estimated that up to 10% of individuals around the world suffer from neuropathic pain [1]. Neuropathic pain originates from damage to the somatosensory nervous system and is associated with complex symptoms, greater management difficulty, and inferior outcomes [2].

Although opioids have been at the forefront of pain management, some patients experience inadequate neuropathic pain relief and significant side effects such as addiction [3-5]. In recent years, the potential for non-opioid pain relief has been an important factor in the increase of medical cannabis use. Anecdotal reports and clinical trials have provided evidence of its analgesic properties and mechanisms of action [6-7]. Unlike synthetic analgesics, cannabis has complex properties that can be exploited to develop high efficacy treatments with minimal side effects.

Over 60 cannabinoids are found in cannabis that primarily interact with cannabinoid receptors, producing neuromodulatory effects via the endocannabinoid system [8]. The endocannabinoid system comprises cannabinoid receptors, endogenous cannabinoids, and enzymes that play important roles in the development and function of the central nervous system.

Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most well-studied exogenous cannabinoids. While both compounds bind to type 1 and 2 cannabinoid receptors, CB1 and CB2 respectively, as partial agonists, CBD exhibits a much lower affinity and also acts as a neutral antagonist [9-11]. THC is the primary psychotropic substance with effects that include antidepression and muscle relaxation [12]. CBD, on the other hand, has been central to medical cannabis use; it is non-psychotropic and has been associated with numerous therapeutic properties, in addition to reducing the psychotropic effects of THC [13].

Although CBD has become available for treating chronic pain in recent years, research supporting its efficacy is limited. Evidence for the treatment of most types of chronic pain is insufficient, and there exists minimal evidence suggesting CBD may alleviate neuropathic pain [14]. Identifying effective solutions for neuropathic pain can address its detrimental associated symptoms that include high pain intensity, pain hypersensitivity, and emotional distress [2].

The entourage effect describes the notion that greater health benefits are produced when all compounds from the cannabis plant work synergistically than when each substance works in isolation [15]. Antinociception is exhibited by both THC and CBD in animal models, but the substances may show enhanced efficacy when used in concert [16-17]. This effect is supported by the randomized controlled trial by Johnson et al., [18] which showed that compared to placebo and THC only, the CBD-THC mixture was more effective in reducing advanced cancer pain in patients who experience inadequate pain relief with opioids. We hypothesize that increasing the ratio of CBD:THC will result in correlated changes in antinociception and decreased psychoactivity, allowing us to identify a ratio that shows the greatest antinociception with the least psychoactivity.

Here we describe an experimental protocol to investigate the effects of CBD and THC as they manifest in inhibiting nociception in mice. This protocol uses chronic constriction injury (CCI) to stimulate chronic neuropathic pain followed by injections of URB937 or sertraline hydrochloride controls, and CBD-THC mixtures in varying ratios. The Hargreaves' Test (HT) and the Tail Suspension Test (TST) will be used to quantify antinociceptive and psychotropic effects, respectively.

Rodents are common models for preclinical drug validations and pain behaviour assessments. Their genetic, physiological, and anatomical similarities to humans have made them a preferred animal model for translational research [19]. The BALB/c mouse strain are well-suited for behavioural testing as these mice are generally less active [20].

CCI, developed by Bennett and Xie [21], is a widely-used model for investigating chronic neuropathic pain by mimicking peripheral nerve injury. Pain hypersensitivity results from neuropathic pain and is often quantified by the latency of withdrawal from a heat source, as measured by the HT [20]. The combination of CCI with the HT allows us to measure hyperalgesia, or the increased response to painful stimuli, caused by neuropathic pain. CCI followed by hypersensitivity testing provides a model to investigate the effectiveness of different CBD:THC ratios for treating neuropathic pain [22].

The psychotropic effects of THC are primarily manifested through its antidepressant properties. Thus, we employ the TST, a technique commonly used in the screening of antidepressant drugs, to assess the psychotropic properties elicited by cannabis [23]. The TST was developed by Steru et al. [24] and functions by suspending mice by the tail, during which behavioural analysis is completed by measuring the time each animal spends mobile. Suspending mice by their tails subjects them to short-term stress, which can be reversed by antidepressants. In the assessment of cannabis, decreased mouse immobility time correlates with antidepressant effects.

Unique positive controls utilized in our protocol are URB937 and sertraline hydrochloride (also known as Zoloft) for assessments of antinociceptive and psychotropic effects, respectively. We use URB937 as a standard for maximum antinociceptive effects with minimal or no psychotropic effects. URB937 inhibits peripheral fatty acid amide hydrolases and thus decreases the production of pain signalling molecules [25]. We use sertraline hydrochloride, a common antidepressant drug, as a standard for maximum psychotropic effects. It is a selective serotonin reuptake inhibitor that alters neurotransmitter levels in the central nervous system [26].

Methods

1. Model Organism

Healthy female and male BALB/c mice (22 ± 2 g) aged 16 weeks with no known prior health conditions will be housed in standard holding cages in a laboratory facility with controlled temperature (22 ± 1 °C) and 12-hour light/dark cycle [27-28]. They will be provided with *ad libitum* access to water and standard chow [29]. Mice will be allowed to habituate in their holding room conditions for at least seven days prior to experimentation.

2. Drug Administration

Substances will be delivered via injection directly into the peritoneal cavity of the animals. Not only are intraperitoneal (i.p.) injections minimally invasive, but they are also capable of safely and consistently administering larger volumes of fluids where they can be rapidly absorbed [30].

From two days prior to injection, each mouse will be handled for one minute and accustomed to the i.p. injection procedure daily using injections of 0.5 mL saline at 6 hours into the light cycle ± 30 minutes [29].

3. Dosage Optimization of Positive Controls

In order to obtain accurate results, the appropriate dosage levels must be determined for the two positive controls [31]. URB937 and sertraline hydrochloride will be administered at increasing concentrations and the results will be used to construct dose-response curves. The response will be represented by withdrawal time (seconds) in the HT for URB937 and immobility time (seconds) in the TST for sertraline hydrochloride. For each substance, a sigmoidal curve will be fitted to the data and the lowest dosage that produces maximum response will be identified. In the identified optimal dosages, URB937 and sertraline hydrochloride will be used as positive controls.

4. Experimental Groups

Sample Size. G Power software will be used for sample size calculations to ensure an appropriate power. The variability within the samples will be measured using standard deviation calculations. It is also necessary to account for the expected attrition or death of animals throughout the experiments: [corrected $n = n / (1 - (\%\ \text{attrition}/100))$].

Each animal will be matched by sex and randomly assigned to one of four types of treatment groups:

a) Cannabinoid groups 1-9 with different ratios of CBD:THC (Table 1)
b) URB937 group to generate the maximum nociceptive effect
c) Sertraline hydrochloride group to generate the maximum antidepressant effect
d) Vehicle only

Table 1. Cannabinoid treatment groups and their prescribed CBD:THC ratios

	CBD:THC			
CBD < THC	0:1	1:10	1:5	1:2
CBD = THC	1:1			
CBD > THC	2:1	5:1	10:1	1:0

All drugs will be suspended in 100% ethanol and then mixed with Cremophor EL (CrEL) and saline in a ratio of 1:1:18 (drug/ethanol : CrEL : saline). CrEL is a solubilizer and emulsifier used to effectively dissolve drugs with low water solubility [32]. Every 6 mg of cannabinoid will be suspended in 1 mL of 100% ethanol [33]. The total dosage of cannabinoids is 3 mg/kg.

Within each treatment group, animals will be matched once more by sex and randomly assigned into two subgroups of equal size. Each subgroup will then be subjected to only one behavioural test, either the nociceptive or the depression-like behaviour test.

5. Nociceptive Behaviour

Chronic Constriction Injury (CCI) Model. CCI will be performed by operatively placing constrictive ligatures around the sciatic nerve in anesthetized mice according to the protocol developed by Austin et al. [22] The wounds will be closed by sutures in the muscle and staples in the skin. Sham-operated animals will serve as controls and receive only sciatic exposure without ligation of the nerve. Mice will be given 24 hours to recover from the surgery before undergoing the HT.

Hargreaves' Test (HT). The mice will be repeatedly left in these enclosures for 45 minutes every day for 2 days before the experiment and will be placed in the testing enclosure for 1 hour right before to acclimatize them. Following the i.p. injection, the HT will be performed as described by Cheah et al. [20] The animal's hind paw will be exposed to a heat source and timed until a withdrawal response is evoked. Since withdrawal due to heat is typically accompanied by checking or licking of the paws, trials ending in voluntary movement will be discarded. Withdrawal times (seconds) will be recorded for multiple mice to obtain an average time for each treatment group. After each use, the enclosure will be thoroughly sanitized.

6. Depression-Like Behaviour

Tail Suspension Test (TST). The mice will be placed in the testing room for 1 hour to acclimatize. Following the i.p. injection, the TST will be performed as described by Can et al [23]. Mice will be suspended upside-down by the tail and the time each animal spends mobile will be measured. The time of mobility (seconds) will be recorded for multiple mice to obtain an average time for each treatment group. Mobility is defined as any movement of the hind legs or any indication of a desire to escape, excluding pendulum swinging motions. Immobility time will be recorded as mobility time subtracted from total observation time. Again, after each use, the enclosure will be thoroughly sanitized.

7. Statistical analysis

Hypothesis Testing and Effect Size. If differences are observed between the nociceptive and depression-like data, a one-way ANOVA will be performed to determine statistical significance. The assumption conditions for a one-way ANOVA will be confirmed using Levene's test for equal variance between groups and the Shapiro-Wilk test for normal distribution within groups [34]. If a correlation is found, a χ^2 test will be performed on the data to verify the extent of the correlation between observed nociception response and cannabis formulation ratios. Additionally, the effect size will be calculated using Cohen's d test to determine if the results are biologically and clinically significant [35].

Reducing Type 1 Error Rate. Post-hoc analysis (e.g., the Tukey method) will be performed on the data after statistical analysis of the results [36].

Confounders. Any confounding variables identified after experimentation can be accounted for using a multivariate regression model (e.g., ANCOVA).

Table 2. Recording template for behavioural test results for each treatment group

	Negative Control	Positive Control		CBD:THC								
	Vehicle	**URB937**	**Sertraline hydrochloride**	**0:1**	**1:10**	**1:5**	**1:2**	**1:1**	**2:1**	**5:1**	**10:1**	**1:0**
Hargreaves Test (sec)			N/A									
Tail Suspension Test (sec)		N/A										

Results

Behavioural test results for each treatment group will be recorded using the template provided in Table 2.

Since URB937 is predicted to elicit antinociceptive effects without psychotropic properties, we expect mice treated with URB937 to demonstrate maximum withdrawal time in the HT with no effect in the TST. In contrast, because sertraline hydrochloride is an antidepressant that elicits psychotropic properties without antinociceptive effects, we expect mice treated with sertraline hydrochloride to have a minimum immobility time in the TST with no effect in the HT.

Depending on the relative analgesic efficacies of THC and CBD, withdrawal time in the HT may vary with changes in CBD:THC ratio. In the TST, increasing levels of CBD will likely increase immobility time, while increasing the THC content will likely decrease immobility time.

Discussion

The optimal CBD:THC ratio will be indicated by maximum withdrawal latency in the HT and maximum immobility time in the TST after treatment. The CCI model causes neuropathic pain in mice and is shown to effectively induce pain hypersensitivity, which results in shorter withdrawal latency when the hind limb is exposed to a heat stimulus [2]. Analgesic compounds such as URB937 and cannabis inhibit nociception and decrease hypersensitivity; thus, we expect the withdrawal latency to increase in the HT for these compounds. In our protocol, the administration of URB937 specifically provides the strongest antinociception and should cause the highest withdrawal latency. THC and CBD both have pain-relieving effects, though THC also has psychotropic properties that large doses of CBD can mediate [13,37]. Since past studies have not quantified the relative analgesic effects of THC and CBD, it is difficult to predict the exact change in mice behaviour upon modifying CBD:THC ratios.

As mice normally exhibit immobility in response to short-term stress, any reduction in immobility time in the TST upon drug administration indicates antidepressant-like effects [38].

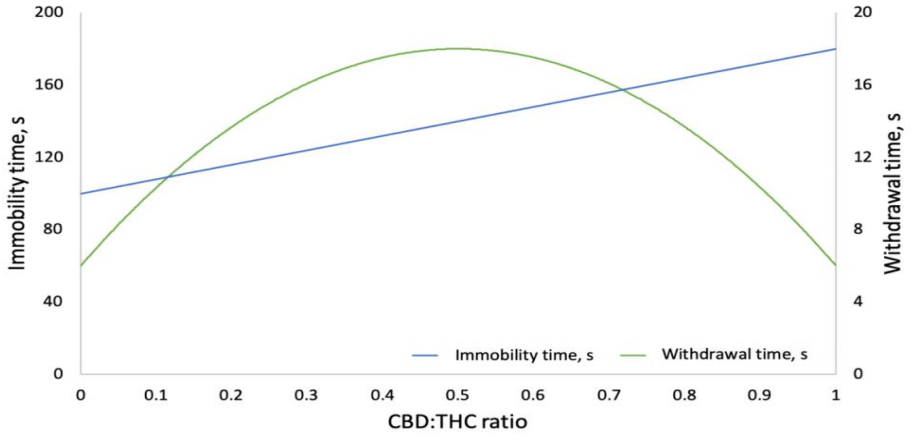

Figure 1. Predicted trends in TST (immobility time) and HT (withdrawal time) results for corresponding CBD:THC ratios

The psychotropic effects of THC include antidepression in humans; thus the psychotropic effects of THC are indicated by suppressing depression-like behaviour in mice. Sertraline hydrochloride has antidepressant properties while THC induces psychotropic effects that include antidepression. Thus, both substances should decrease immobility time in the TST. Because sertraline hydrochloride has the strongest antidepressant effects, we expect its administration to work as a positive control and produce the lowest time of immobility. Since mice normally exhibit immobility in response to short-term stress, any reduction in immobility time in the TST upon drug administration indicates antidepressant-like effects [38].

In data analysis, we can find the optimal CBD:THC ratios by plotting the withdrawal latency and immobility time separately, then interpolating data points and performing mathematical analyses. These steps will allow us to elucidate the immobility time and withdrawal latency that correspond, respectively, to the minimum psychotropic and maximum antinociceptive effects.

Our protocol has several notable strengths. Compared to other similar behavioural tests, such as the grimace scale and sucrose preference test, the HT and the TST are less subjective and more quantitative [39-40]. These advantages allow for not only higher versatility in statistical analysis but also results with greater reliability. Subjecting each animal to only one treatment group effectively prevents both the development of drug tolerance from consecutive drug administrations and undesired performance variations caused by unnecessary stress. Our protocol is the first to characterize the synergistic effects of THC and CBD on both nociception and psychoactivity in an animal model. It can therefore provide an essential step in the development of cannabis as a therapeutic for neuropathic pain.

However, this protocol has potential limitations that provide opportunities for additional research. Animal models of human cognitive processes and pain rely on indirect outward manifestations through behaviour in assays such as the HT and the TST [39-41]. Therefore, although animal models have a high utility in the development of therapies for clinical applications, their representativeness is imperfect [39-41]. Even though CCI causes neuropathic pain and corresponding hypersensitivity, it may not accurately represent the clinical manifestation of pain and may introduce experimenter bias. Hence, extensions of our protocol should consider supplementing the CCI model with additional observations of operant pain and spontaneous behaviour [22]. Additionally, humans and mice have opposite sleep-wake cycles, thus these differences could be investigated to better understand the patterns of medicinal cannabis use by humans [42]. Moreover, common methods of cannabis administration also include intravenous injection, inhalation of vaporized cannabis, and sublingual tinctures [43]. Future experiments could include other methods of cannabis administration [42]. Lastly, since our protocol comprises short-term experiments completed in a controlled environment, results may not be generalizable to clinical cannabis use where patients often present with complex comorbidities and have diverse cannabis use patterns [44]. To further validate safety and efficacy, future clinical assessments of CBD-THC mixtures are required.

Conclusions

In the treatment of neuropathic pain, many current treatment options rely on opioid use, but opioids can lead to complications and adverse effects, like addiction. In contrast, cannabis-derived therapeutics bypass the unfavorable properties normally associated with opioids, while potentially having enhanced efficacy. Here we present a novel protocol to find the ideal substance ratio in a CBD-THC mixture, which elicits maximum analgesia for treating neuropathic pain with the least psychotropic effect. We described a methodology that uses assays for antinociception and suppression of depression-like behaviour in mice. Establishing the optimal ratio of CBD:THC for pain suppression may be an important step to address the public health issue of opioid addiction in the treatment of neuropathic pain.

List of Abbreviations Used

ANCOVA: analysis of covariance
ANOVA: analysis of variance
BALB/c: bagg albino laboratory-bred mouse, strain C
CB1: cannabinoid receptor type 1
CB2: cannabinoid receptor type 2
CBD: cannabidiol
CCI: chronic constriction injury
CrEL: cremophor EL
HT: hargreaves' test
i.p.: intraperitoneal
THC: Δ^9-tetrahydrocannabinol
TST: tail suspension test

Conflicts of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be interpreted as actual or potential conflicts of interest.

Ethics Approval and/or Participant Consent

All procedures involving animal experiments will be reviewed by and ethical approval will be obtained from the University of Toronto University Animal Care Committee (UACC) as well as from the Canadian Council on Animal Care (CCAC). A submission of the Animal Use Protocol to the Faculty of Medicine Local Animal Care Committee (LACC) will also be made for full committee review. The approved protocol will be subject to Post Approval Review (PAR) in order to ensure the highest level of care for research animals and adherence to regulatory requirements.

Authors' Contributions
PQD, FCC, ST, and LRX jointly contributed to the conception of this protocol and drafting of the manuscript. The final version of the manuscript was reviewed and approved by all authors.

Acknowledgements
The authors would like to thank the Scinapse Undergraduate Science Case Competition (USCC) for providing the opportunity to innovate a solution for a prominent and ongoing problem in the Canadian population.

Funding
This study was funded through the generous support of the following Departments and Unions in the University of Toronto:

- Arts & Science Students Union (ASSU)
- Department of Pharmacology & Toxicology
- Department of Physiology
- University of Toronto Students' Union (UTSU)

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: O'Llenecia Walker, Sonya Kouthouridis
Article Dates: Received Jun 01 20; Accepted Jul 03 20; Published Jul 16 20

Citation
Please cite this article as follows:
Chang F-C, Ding PQ, Tam S, Xu LR. Optimizing the synergistic effects of cannabidiol and Δ^9-tetrahydrocannabinol for the treatment of neuropathic pain in mouse behavioural models. URNCST Journal. 2020 Jul 16: 4(6).
https://urncst.com/index.php/urncst/article/view/195
DOI Link: https://doi.org/10.26685/urncst.195