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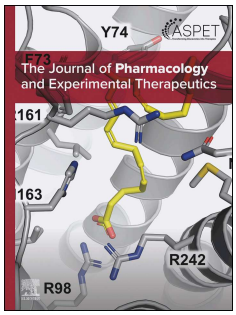

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PII: S0022-3565(25)39838-1

DOI: https://doi.org/10.1016/j.jpet.2025.103625

Reference: JPET 103625

To appear in: *The Journal of Pharmacology and Experimental Therapeutics*

Received Date: 17 October 2024

Revised Date: 29 April 2025

Accepted Date: 30 May 2025

Please cite this article as: Jenkins BW, Moore CF, Weerts EM, Cannabidiol (CBD) Interactions with Delta-9-Tetrahydrocannabinol (D9-THC) on Antinociception After Carrageenan-Induced Inflammatory Pain in Male and Female Rats, *The Journal of Pharmacology and Experimental Therapeutics* (2025), doi: https://doi.org/10.1016/j.jpet.2025.103625.

Cannabidiol (CBD) Interactions with Delta-9-Tetrahydrocannabinol (D9-THC) on Antinociception After Carrageenan-Induced Inflammatory Pain in Male and Female Rats

Bryan W. Jenkins, Catherine F. Moore, Elise M. Weerts*

Division of Behavioral Biology, Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

a) Running title: Cannabinoid combinations and acute inflammatory pain

b) *Corresponding author:

Elise M. Weerts, Ph.D.
Department of Psychiatry and Behavioral Sciences
Johns Hopkins Bayview Medical Campus
Behavioral Biology Research Center
5510 Nathan Shock Drive, Suite 3000
Baltimore, MD, 21224, USA
410-550-2781 (phone)
eweerts@jhmi.edu

c) Contents:

Text pages: 17
Tables: 1
Figures: 4
References: 64

Words in Abstract: 243/250
Words in Introduction: 710/750
Words in Discussion: 1753/1500

d) Nonstandard abbreviations: D9-THC = delta-9-tetrahydrocananbinol; CBD = cannabidiol; NSAIDs = nonsteroidal anti-inflammatory drugs; USP = United States Pharmacopeia; CB1R = cannabinoid type 1 receptor; CFA = complete Freund's adjuvant

e) Recommended section assignment: Behavioral Pharmacology

Abstract

Cannabis products used for pain typically contain delta-9-tetrahydrocannabinol (D9-THC) and cannabidiol (CBD) in varied amounts, but data on effects of specific cannabinoid formulations on different pain types are lacking. This study used the carrageenan-induced inflammatory pain model to test oral D9-THC, CBD, or their combination on acute edema and pain hypersensitivity. Male and female Sprague Dawley rats (n = 10-14 per sex/group) were pretreated (- 1 hr) with vehicle (sesame oil), D9-THC (1, 3, 10 mg/kg, p.o), CBD (10, 30, 100 mg/kg, p.o.), or select doses of D9-THC+CBD combinations prior to an intraplantar λ-carrageenan injection into the hind paw. The non-steroidal anti-inflammatory drug (NSAID) ketoprofen (10, 20 mg/kg, i.p.) or its vehicle (1:1:18 ethanol:cremophor:saline) was administered to a separate group as a positive control. Measurements were conducted at baseline, 1, 3, and 5 hours post carrageenan. Carrageenan produced edema and hypersensitivity to radiant heat (hyperalgesia) and mechanical pressure (allodynia). D9-THC alone sex- and dose-dependently decreased hyperalgesia and allodynia but not inflammation, with effects of D9-THC being greater in females than males, and the lowest D9-THC dose was pro-inflammatory in males. CBD alone did not affect pain sensitivity but had modest anti-inflammatory effects in males. Isobolographic and dose addition analyses indicated D9-THC+CBD was sub-additive relative to D9-THC alone. These data demonstrate that prophylactic oral D9-THC alleviates acute inflammatory pain with sex-dependent effects, and CBD diminishes D9-THC antinociception when combined. Findings suggest oral D9-THC is superior to CBD or combined D9-THC+CBD for acute inflammatory pain.

Significance Statement

Despite the popularity of cannabis for pain management, empirical data on how specific cannabinoid formulations affect acute inflammatory pain are limited. This study in rats found that pure D9-THC formulations were most effective at improving inflammatory pain compared to pure CBD or D9-THC+CBD combinations, and females were more sensitive than males to the antinociceptive effects of D9-THC.

Introduction

Pain relief is the most frequently cited reason for using medical cannabis, with many patients reporting that they use cannabis as a substitute for prescription opioids to manage their pain (Ogborne et al., 2000; Sexton et al., 2016; Reiman et al., 2017). Consequently, clinical trials have investigated cannabis or isolated cannabinoids, including delta-9-tetrahydrocannabinol (D9-THC), the primary psychoactive cannabinoid of cannabis, and cannabidiol (CBD), the second most abundant cannabinoid in cannabis, as potential therapeutic compounds for pain relief. Most studies to date have focused on chronic neuropathic pain-related outcomes (see review by Haleem and Wright (2020)). Overall, these trials report improvements in pain measures compared to placebo; however, results vary substantially by cannabinoid type and administration route. In contrast, there is little research on whether cannabinoid formulations may be useful for treating other types of pain, including acute inflammatory pain.

Inflammatory pain refers to spontaneous pain hypersensitivity occurring in response to injury and inflammation, including post-operative pain, traumatic pain, and tissue damage (e.g., burns). Associated symptoms include increased swelling (edema), indicating a pro-inflammatory response, and pain hypersensitivity to mildly painful stimuli (hyperalgesia) or typically non-painful stimuli (allodynia) (Jensen and Finnerup, 2014). Inflammatory pain is a complex condition that remains a significant clinical challenge due to the limited efficacy and/or poor tolerability of currently available treatment options. First-line treatments for managing symptoms are nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids (Park and Moon, 2010). NSAIDs can cause severe gastrointestinal bleeding and ulcers, and opioids have significant risks for dependence and overdose (Neuman et al., 2019; Wilson et al., 2020). As such, an increasing number of patients are seeking treatment alternatives (Pritchard et al., 2022). Ongoing efforts in cannabis-based drug development are focused on identifying novel formulations that may have therapeutic efficacy for different types of pain (Finn et al., 2021). Simultaneously, regulatory

changes in state laws legalizing cannabis for medical and recreational purposes have led to widespread availability of many cannabis products. These products are used by consumers to manage a variety of pain conditions, yet the empirical evidence supporting their use is limited.

Further limiting the development of cannabis-derived analgesics are the unwanted side effects of D9-THC (e.g., intoxication, cognitive and motor impairments). CBD, however, is non-intoxicating, and has been purported to augment the therapeutic effects of D9-THC and/or mitigate the adverse effects of D9-THC when co-administered (see review by Boggs et al. (2018)). Systematic testing of different dose combinations of D9-THC+CBD is required to evaluate whether specific formulations may improve inflammation and pain without producing adverse effects. Use of preclinical models provides a high-throughput platform to evaluate numerous cannabinoid dosing conditions under strict experimental control. Preclinical studies of the antinociceptive effects of exogenous cannabinoids (partially reviewed by Lotsch et al. (2018)) have demonstrated that both D9-THC and CBD alone can decrease pain and inflammation in rodent models of arthritis (Malfait et al., 2000; Cox and Welch, 2004), multiple sclerosis (Mecha et al., 2013; Feliu et al., 2015), and inflammatory pain (Costa et al., 2004a; Britch et al., 2020; Britch and Craft, 2021). There is some evidence in neuropathic pain models that D9-THC and CBD may act synergistically to produce increased antinociception (Casey et al., 2017).

Inflammatory pain can be modeled in rats by administering an intraplantar injection of λ-carrageenan. Carrageenan is an irritating substance that induces transient inflammation and swelling and produces pain hypersensitivity, which typically peaks within 2 to 3 hours post injection and subsides by 24 hours. The aim of the current study was to systematically investigate the acute effects of D9-THC and CBD, alone or combined in select dose combinations, on measures of inflammation (edema) and pain (hyperalgesia, allodynia) in male

and female rats using the carrageenan-induced paw inflammation procedure. There is evidence that females may be disproportionately affected by pain compared to males, and responses to medications can be sex dependent (Mogil, 2020; Osborne and Davis, 2022; Britch and Craft, 2023; Salis et al., 2024). Preclinical research on sex as a biological factor in pain is critically needed. We hypothesized that D9-THC and CBD alone would produce antinociceptive and anti-inflammatory effects, and that D9-THC+CBD co-administration would have synergistic antinociceptive and anti-inflammatory effects. We further hypothesized that females would show more severe inflammatory pain symptoms and would be more sensitive to the effects of these cannabinoids than males.

Materials and Methods

Animals

Adult male and female Sprague Dawley rats (N = 321 total, 51% female; Charles River, Wilmington, MA) were same-sex pair-housed in wire- and filter- topped plastic cages (27 × 48 × 20 cm) with standard enrichment and *ad libidum* food and water access. Diet was a corn-based chow (Teklad product no. 2018, Global 18% Protein Rodent Diet; Harlan, Indianapolis, IN). The vivarium was on a 12-hr reverse light cycle (lights off at 8:00 AM) and was humidity and temperature controlled. All animals were handled and habituated to procedures prior to experimentation. All procedures used in this study were approved by the Johns Hopkins Institutional Animal Care and Use Committee. The facilities adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and met the American Association for Laboratory Animal Science standards.

Drugs

Ampules of concentrated (−)-trans-D9-THC (50–200 mg/ml in 95% USP ethyl alcohol) were provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program; ethanol was evaporated using nitrogen gas prior to use. Pure hemp-derived CBD isolate was obtained from Albany Molecular Research Inc. (Rensselaer, NY). CBD was confirmed to not contain D9-THC or other contaminants by independent testing. D9-THC and CBD were mixed in 100% USP sesame oil (Spectrum Chemical; New Brunswick, NJ) and administered via oral gavage (1 ml/kg, p.o.). D9-THC doses (1, 3, 10 mg/kg) were selected based on our past data showing antinociceptive effects of 10 mg/kg D9-THC in acute pain with 1 and 3 mg/kg having subthreshold effects (Moore et al., 2021; Moore and Weerts, 2022). As 10 mg/kg D9-THC was shown to produce significant side effects (e.g., neurocognitive impairment) in our prior study (Moore et al., 2023), we chose lower THC doses for the D9-THC+CBD combinations. CBD doses (10, 30, 100 mg/kg) were selected to permit investigation of a range of ecologically relevant, CBD dominant ratio combinations (e.g., 1:10, 1:30, 3:10). Specific dose combinations tested were 1 mg/kg D9-THC+10 mg/kg CBD, 1 mg/kg D9-THC+30 mg/kg CBD, 3 mg/kg D9-THC+10 mg/kg CBD, and 3 mg/kg D9-THC+30 mg/kg CBD. Ketoprofen (Sigma Aldrich, St. Louis, MO) was mixed in 1:1:18 ethanol:cremophor:saline and administered at a dose of 10 or 20 mg/kg (i.p.) per prior publications (Herrero et al., 1997; Aguilar-Carrasco et al., 2014). An equivalent volume (1 ml/kg) of sesame oil (D9-THC and CBD vehicle, p.o.) or ethanol:cremophor:saline (ketoprofen vehicle, i.p.) was administered to control groups.

Induction of transient inflammatory pain

We used standard methods for carrageenan-induced transient inflammatory pain in rats (Winter et al., 1962; Rock et al., 2018). λ-carrageenan (Sigma Aldrich) was mixed in physiological saline to yield a 1% w/v solution for injection. Rats were briefly anaesthetized using isoflurane (3-5%) prior to receiving an intraplantar injection of 0.1 ml of carrageenan solution into their left or right hind paw (counterbalanced) using a 27-G needle.

Evaluation of edema

Measurements (mm) of hind paw thickness (edema) were collected using an electronic digital caliper (Control Company, Fisher Scientific, Hampton, NH) as a proxy measure to assess the magnitude of inflammatory response to carrageenan across the dorsoventral axis of the paw. Measurements were taken at baseline (i.e., immediately prior to carrageenan injection) and again at specific intervals post-injection (Figure 1A).

Evaluation of thermal hyperalgesia

The Hargreaves procedure was performed to assess thermal hyperalgesia (Hargreaves et al., 1988; Rock et al., 2018). Prior to baseline testing, rats were habituated to the apparatus for 10 min. Rats were placed in clear plastic cubicles atop an elevated glass platform under which sat a movable infrared light generator connected to the computerized plantar test apparatus (Ugo Basile 37370, Italy). Infrared light was focused on the midplantar region of the target paw until an elicited withdrawal behavior (i.e., a nocifensive hind paw flexion reflex) triggered a beam-break. Paw withdrawal latency was measured to the nearest 0.1s across 3 trials, >1 minute apart. The intensity of the heat source was set at 55% based on preliminary tests showing average baseline latencies of 10-12s. A maximum cut off time of 30s was set to prevent tissue damage; testing ceased if the maximum cut off time was reached. Carrageenan-induced hyperalgesia was demonstrated as a shorter withdrawal latency when compared to baseline.

Evaluation of mechanical allodynia

The von Frey test was performed to assess mechanical allodynia as in our prior study (Moore and Weerts, 2022). Prior to baseline testing, rats were habituated to the apparatus for >10 min and to the procedure. Rats were placed in clear plastic cubicles atop a mesh floor, through which nylon monofilaments were applied to the target hind paw in a sequence of increasing

force (9 filaments used, 0.6–15.0 g range of force, beginning with 2.0 g). Filaments were applied perpendicularly with enough force to cause a slight buckling against the paw. The presence or absence of a withdrawal response was recorded after each filament application. If a response did not occur, the next strongest filament was applied; if a response did occur, the next weakest filament was applied. This up-down method was repeated four times after the first change in response, and the 50% threshold for paw withdrawal was calculated using the individual response pattern and the force of the last von Frey filament tested (Dixon, 1991; Chaplan et al., 1994). Carrageenan-induced allodynia was demonstrated as a decrease in the withdrawal threshold from baseline.

Experimental design

Male and female rats were randomly assigned to experimental groups (n =10-14 per sex/group). Baseline pain testing (8:15 AM) was completed before rats were administered vehicle or test drug (9:00 AM). One hour later, rats received carrageenan injections (10:00 AM) as described above and returned to their home cage. Rats then underwent pain testing and edema measurements at 1-, 3-, and 5-hours post-carrageenan (Figure 1A). Experimenters were blinded to the treatment groups. Testing was completed in the following order: 1) Hargreaves testing, 2) edema measurement, 3) von Frey testing. All pain testing was completed under light (not dark) conditions to facilitate data collection.

Data analysis

Outcome variables analyzed were paw thickness (mm) for edema, withdrawal latency (sec) for Hargreaves testing, and withdrawal threshold (g) for von Frey testing. Raw vehicle control data were used to confirm successful model induction. Model induction was evaluated for each outcome measure in vehicle (p.o.) treated control data using one-way repeated measures

ANOVAs in GraphPad Prism v10.2.3 with sex as the between-subject factor and time as the within-subject factor.

To evaluate treatment effects, all positive control and test drug data were transformed to absolute change from baseline scores for the 1 hr, 3 hr, and 5 hr timepoints. Data for each outcome measure were analyzed using separate three-way repeated measures ANOVAs with sex and treatment as between-subject factors and time as the within-subject factor. For ketoprofen and D9-THC or CBD alone conditions, 'treatment' referred to the dose of test drug, which was analyzed against their respective vehicle control. For D9-THC+CBD combination conditions, 'treatment' referred to the dose of CBD, which was analyzed against the matched D9-THC alone condition (e.g., 1 D9-THC + 0 CBD, 1 D9-THC + 10 CBD, 1 D9-THC + 30 CBD). Initial three-way repeated measures ANOVAs yielded main effects of sex ($p < 0.05$). Data were then disaggregated by sex and separate two-way ANOVAs were run on each outcome measure for each sex. Statistical analyses were performed using JASP v0.18.1 (for initial three-way ANOVAs) and GraphPad Prism (for subsequent within-sex two-way ANOVAs and for multiple comparisons analyses). Greenhouse-Geisser corrections were used where Mauchly's test of sphericity indicated that the assumption of sphericity was violated. Where main or interaction effects were observed, Dunnett's post hoc tests were performed to isolate treatment effects. Significance was accepted at $p < 0.05$ for a two-tailed test. All test statistics are reported in tables in the supplementary material (Supplemental Tables 1 – 3).

To further illustrate cannabinoid effects, arithmetic differences between i) D9-THC or CBD data and vehicle control data or ii) D9-THC+CBD combination data and matched D9-THC alone data were computed and visualized using radar plots. The radar plots provide a summative profile of the relative change in outcome across sexes and timepoints that is produced by varying cannabinoid doses and ratios. The x axes are the polygonal axes that define the plot boundaries

and data range; the y axes are the radial axes projecting from the center point with each axis representing a different treatment condition. Along the y axes, a value of x = 0 means that there is no difference (i.e., no outcome change) between treatment and comparison groups; a value of x > 0 (toward the outermost x axis) indicates an increase; a value of x < 0 (towards the inner center point) indicates a decrease.

Drug combination analysis

The combined effects of D9-THC+CBD were evaluated using isobole and dose addition analysis as previously described (Porreca et al., 1990; Tallarida et al., 2003; Tallarida, 2016; Hayduk et al., 2024). Von Frey data from the time of peak effects (1 hr) were used for the analysis as this measure showed the greatest response magnitude. Data were transformed to percent change from vehicle (% vehicle). Dose response curves for D9-THC alone, CBD alone, and D9-THC as a function of CBD dose were plotted and linear regressions were computed to calculate ED50 values for each condition. An ED50 could not be computed for CBD alone data because this condition had no effect on pain measures. Linear regression of the D9-THC alone data was constrained to the 1 and 3 mg/kg doses to match the D9-THC+CBD combinations. ED50s from D9-THC alone and D9-THC+CBD combinations were plotted and visually compared on an isobologram to evaluate additivity. The line of additivity was set as a vertical line at the D9-THC ED50 value (due to the lack of CBD effect) and the D9-THC+CBD combination ED50s were compared against this line of additivity. If the combination ED50s underwent a leftward shift relative to the line of additivity, the observed effect was considered synergistic; if they underwent a rightward shift, the observed effect was considered sub-additive. Due to the between-subject design, ED50 values were computed from group-level data which resulted in a single value for each condition and limited statistical analysis.

Dose addition analysis was also performed to compare the observed D9-THC+CBD combination ED50 (z_{mix}) to the predicted combination ED50 (z_{add}). Because we designed the study to evaluate D9-THC+CBD ratio combinations with ecological relevance to human cannabis use, dose elements were not scaled in a fixed proportion. Thus, we defined our z_{mix} value as the ED50 of the D9-THC+CBD combinations with a fixed ratio (i.e., 1:10, with the 1+10 and 3+30 D9-THC+CBD conditions). z_{add} was calculated using the following formula:

$$z_{add} = \frac{\mathrm{A}}{1 - \rho_{\mathrm{A}}}$$

Where A is the ED50 of D9-THC and ρ_{A} is the proportion of D9-THC in the 1:10 combination. If the observed combination ED50 was greater than the expected ED50 ($z_{mix} > z_{add}$), the interaction was considered sub-additive; if the observed ED50 was lesser than the expected ($z_{mix} < z_{add}$), the interaction was considered synergistic.

Results

Induction of edema and pain hypersensitivity by carrageenan

In vehicle controls, intraplantar carrageenan produced acute inflammatory pain comprising hind paw edema, thermal hyperalgesia, and mechanical allodynia lasting at least 5 hrs (Figure 1B-D). Main effects of time on paw thickness, withdrawal latency, and withdrawal threshold were observed (p's < 0.001; see Supplemental Table 1 for full results). Post hoc testing showed that between baseline and post-carrageenan timepoints, edema was increased at all timepoints in both male and female rats (p's < 0.001); withdrawal latencies were decreased in male rats at 5 hr (p = 0.008) and female rats at 3 hr and 5 hr (p's < 0.01); withdrawal thresholds were decreased in male and female rats at all time points (p's < 0.05).

Effects of the NSAID ketoprofen on carrageenan-induced edema and pain hypersensitivity

Expectedly, ketoprofen decreased edema in males and females (Figure 1E; Supplemental Table 2, interaction of treatment x time, $p < 0.05$). Specifically, at the 5 hr timepoint, edema was decreased in males that received 20 mg/kg ($p = 0.009$) and in females that received either 10 or 20 mg/kg (p's < 0.05). Ketoprofen also decreased thermal hyperalgesia and mechanical allodynia in females only (Figure 1F,G): effects of treatment on withdrawal latency and threshold were observed (p's < 0.05; Supplemental Table 3). Both doses increased withdrawal latencies at the 3 hr and 5 hr timepoints compared with vehicle (p's < 0.05). The 10 mg/kg dose also increased withdrawal thresholds at the 3 hr and 5 hr timepoints (p's < 0.05).

Cannabinoid effects on edema

D9-THC alone had dose-dependent effects on edema in males and females (Figure 2; Supplemental Table 2, interaction of treatment x time, $p < 0.05$). In males, 1 mg/kg D9-THC increased edema ($p = 0.039$) at the 5 hr timepoint (Figure 2C). In females, 3 and 10 mg/kg D9-THC produced non-significant decreases in edema (3: $p = 0.060$, 10: $p = 0.056$) at the 5 hr timepoint (Figure 2C). CBD alone had anti-inflammatory effects in males only (Figure 2C; main effect of dose, $p < 0.05$), where 10 mg/kg CBD decreased edema ($p = 0.049$) at the 5 hr timepoint. CBD did not affect edema in females. Co-administration of CBD with 1 mg/kg D9-THC did not affect edema as no statistical differences were observed between the 1 mg/kg D9-THC+CBD combinations and 1 mg/kg D9-THC alone. Co-administration of CBD with 3 mg/kg D9-THC increased edema compared to D9-THC alone: there was a main effect of treatment (i.e. CBD dose) on edema in females (Supplemental Table 3; $p < 0.001$). Both dose combinations increased edema compared to D9-THC alone in females at the 1 hr, 3 hr, and 5 hr timepoints (p's < 0.05; Figure 2A-C). Radar plots (Figure 2D-F) were used to visualize average differences between cannabinoid dose conditions and the respective comparison group (i.e., vehicle controls for D9-THC/CBD alone doses, denoted by the white background, and matched D9-THC alone dose for D9-THC+CBD combination doses, denoted by the light gray

background). Comparison groups are represented by the 0 axis (an average difference of 0). Sexes are shown in different shades (blue = males; orange = females) to visualize sex differences in drug effects on edema. An increase or decrease from the 0 axis along the dose condition axis represents an increase or decrease in paw thickness, respectively, for that specific dose condition.

Cannabinoid effects on thermal hyperalgesia

D9-THC alone dose-dependently decreased thermal hyperalgesia in male and female rats (Figure 3; Supplemental Table 2, main effect of treatment, $p < 0.05$). In females, all D9-THC doses increased withdrawal latency at the 1 hr and 3 hr timepoints, and 3 and 10 mg/kg continued to increase withdrawal latencies at the 5 hr timepoint (p's < 0.05; Figure 3A-C). Post hoc effects were not detected in males. CBD alone did not produce effects on withdrawal latency in either males or females. Similarly, the D9-THC+CBD dose combinations did not produce different effects compared to D9-THC alone. Radar plots (Figure 3D-F) were used to visualize average differences between cannabinoid dose conditions and respective comparison groups (i.e., vehicle controls for D9-THC/CBD alone doses, denoted by the white background, and matched D9-THC alone dose for D9-THC+CBD combination doses, denoted by the light gray background). Comparison groups are represented by the 0 axis (an average difference of 0). Sexes are shown in different shades (blue = males; orange = females) to visualize sex differences in drug effects on thermal hyperalgesia. An increase or decrease from the 0 axis along the dose condition axis represents an increase or decrease in withdrawal latency, respectively, for that specific dose condition.

Cannabinoid effects on mechanical allodynia

D9-THC alone dose-dependently decreased mechanical allodynia and females were more sensitive to these effects (Figure 4; Supplemental Table 2, interactions of treatment x time and

treatment x sex, p's < 0.05). The 3 and 10 mg/kg D9-THC doses increased withdrawal threshold at the 1 hr and 3 hr timepoints in males (p's < 0.05) and at the 1 hr, 3 hr, and 5 hr timepoints in females (p's < 0.05; Figure 4A-C). CBD alone did not produce effects on withdrawal threshold in either sex. The combination of CBD with 1 mg/kg D9-THC did not modify D9-THC effects on withdrawal threshold (no main effect nor interactions with treatment). However, combining 10 mg/kg CBD with 3 mg/kg D9-THC blunted the improvement in allodynia seen with 3 mg/kg D9-THC alone (interaction of treatment x time, p < 0.05). The combination of 3 mg/kg D9-THC + 10 mg/kg CBD decreased withdrawal threshold in males at the 3 hr timepoint (p = 0.017) and in females at the 1 hr and 3 hr (p's < 0.05) timepoints compared to D9-THC alone (Figure 4A,B). Radar plots (Figure 4D-F) were used to visualize average differences between cannabinoid dose conditions and respective comparison groups (i.e., vehicle controls for D9-THC/CBD alone doses, denoted by the white background, and matched D9-THC alone dose for D9-THC+CBD combination doses, denoted by the light gray background). Comparison groups are represented by the 0 axis (an average difference of 0). Sexes are shown in different shades (blue = males; orange = females) to visualize sex differences in drug effects on mechanical allodynia. An increase or decrease from the 0 axis along the dose condition axis represents an increase or decrease in withdrawal threshold, respectively, for that specific dose condition.

Drug combination analysis

Isobole analysis was used to evaluate the potential additivity of D9-THC+CBD combinations relative to D9-THC alone effects using empirical ED50 values. The ED50 values were determined to be 1.23 mg/kg for oral D9-THC alone, 1.74 mg/kg for D9-THC combined with 10 mg/kg CBD, and 1.37 mg/kg for D9-THC combined with 30 mg/kg CBD. Thus, ED50 values for the D9-THC+CBD combinations lay to the right of the line of additivity, indicating that D9-THC+CBD combinations produced sub-additive effects compared to THC alone (Figure 5). Further dose addition analysis was performed to compare the observed combination ED50

(z_{mix}) with the predicted combination ED50 (z_{add}) using tested dose combinations with fixed proportions. For these data, z_{mix} was determined to be 2.15 mg/kg and z_{add} was determined to be 1.353 mg/kg. Since $z_{mix} > z_{add}$, combined D9-THC+CBD was determined to sub-additive.

Discussion

The key findings of this study are summarized in Table 1. Oral D9-THC dose-dependently decreased hyperalgesia and allodynia associated with acute inflammatory pain, and effects were greater in females. Decreased hyperalgesia was accompanied by modest (non-significant) decreases in edema in females. The 1 mg/kg D9-THC dose was subeffective for pain but pro-inflammatory in males. CBD alone did not affect pain measures at any dose tested, although 10 mg/kg CBD decreased inflammation in males. Co-administration of 10 mg/kg CBD reduced the antiallodynic effects of 3 mg/kg D9-THC in both sexes. In females only, CBD co-administered with 3 mg/kg D9-THC increased edema relative to D9-THC alone. Notably, drug combination analyses showed that D9-THC+CBD combinations were sub-additive. Ketoprofen produced similar antinociceptive effects to oral D9-THC in this study, but also reduced edema, with greater effects in females.

The current study extends past preclinical research showing single doses of oral D9-THC or nabilone decreased acute carrageenan-induced thermal hyperalgesia in male rats (Conti et al., 2002; Rock et al., 2018). In the current study, D9-THC decreased thermal hyperalgesia in both sexes, but was only statistically significant in females. Greater variability in our male responses likely contributed to these differences between studies. Differences in test parameters (e.g., thermal intensity and time of testing) with Rock et al. or drug (e.g., nabilone vs. THC) and species (Wistar vs. Sprague Dawley rats, see our previous work (Moore et al., 2021)) with Conti et al. may have also contributed to small differences in effect magnitudes. Importantly, our findings are consistent with recent human laboratory studies. In studies of experimentally-

induced acute inflammatory pain, smoked cannabis dose-dependently decreased capsaicin-evoked pain sensitivity (Wallace et al., 2007) although administration of a single oral dose of cannabis extract did not (Kraft et al., 2008). In other acute pain studies, smoked cannabis (controlled for D9-THC dose) and oral dronabinol decreased experimentally-induced pain sensitivity in the cold-pressor test in healthy participants (Cooper et al., 2013), whereas oral CBD did not (Arout et al., 2022). Additional multi-dose laboratory and placebo controlled clinical trials are needed to determine the effects of oral cannabinoids on acute inflammatory pain in humans.

Similar doses of oral D9-THC and nabilone previously decreased carrageenan-induced inflammation in male rats (Sofia et al., 1973; Conti et al., 2002; Rock et al., 2018). In the current study, D9-THC produced sex- and dose-specific effects on inflammation (edema): D9-THC was pro-inflammatory at the low dose in males, while higher doses produced modest (non-significant) anti-inflammatory effects in females. While pain alleviation involves both pro- and anti-inflammatory signaling (Linher-Melville et al., 2020), our data show that decreased inflammation after D9-THC was associated with decreased pain only in females. In males, the lowest D9-THC dose was pro-inflammatory but did not affect pain measures and higher D9-THC doses decreased pain without affecting inflammation. These data suggest the inflammatory effects are dose-related and dissociable from the antinociceptive effects of D9-THC.

The radar plots, showing cannabinoid effects on inflammatory pain relative to their respective comparison group (vehicle controls or THC alone), further inform our results beyond statistical analysis. For example, relative to vehicle, 1 mg/kg D9-THC increased inflammation in males at the 5 hr timepoint, whereas 3 and 10 mg/kg D9-THC decreased inflammation, possibly revealing biphasic dose effects of D9-THC on inflammation. Biphasic effects of cannabinoids were observed for pain-related outcomes in human and rodent behavioral studies (Casey et al.,

2017; Shustorovich et al., 2024) and for pro-inflammatory cytokine activity in cultured cells (Berdyshev et al., 1997; Verhoeckx et al., 2006). Biphasic effects of cannabinoids are observed across a variety of measures, including anxiety, motor behavior, food-maintained behavior, and emesis (Katsidoni et al., 2013; Bruijnzeel et al., 2016; DeVuono and Parker, 2020; Salviato et al., 2021; Moore and Weerts, 2022). Additional studies with full dose-response curves are needed to evaluate potential biphasic effects of D9-THC on inflammation and determine whether similar effects are observed for CBD or other cannabinoids.

Our observation that 10 mg/kg CBD had only a modest anti-inflammatory effect in males without affecting pain differs from some studies showing oral CBD dose-dependently (5–40 mg/kg) decreased carrageenan-induced edema and thermal hyperalgesia in male rats (Costa et al., 2004a; Costa et al., 2004b). Costa et al. used a curative/corrective model (CBD post-inflammatory pain induction), vs the prophylactic/preventative model (CBD pre-inflammatory pain induction) used in our study, which may explain the discrepant findings. Prophylactic models have been reported to produce greater cannabinoid antinociception than curative models (Guindon et al., 2007). Evaluating prophylactic use of cannabinoids for pain management has translational value for clinical contexts in which cannabinoids are being considered for prevention of inflammatory pain (e.g., peri-operative pain, chronic pain flare-ups, etc.). Using methods like those in the present study, prophylactic oral CBD (10 mg/kg) did not affect edema in male rats (Rock et al., 2018). Rat studies using Complete Freund's Adjuvant (CFA) to induce paw inflammation suggest that repeat dosing may improve the therapeutic efficacy of CBD for inflammatory pain (Britch et al., 2020; Britch and Craft, 2023; Craft et al., 2023). Altogether, effects of oral CBD on acute inflammatory pain remain equivocal.

Evidence from our study indicates that CBD alone was largely ineffective, but combining CBD with D9-THC had sub-additive effects on acute inflammatory pain responses. This result is

consistent with past literature (Greene et al., 2018; Benredjem and Pineyro, 2023), including a prior study using the CFA model of persistent inflammatory pain in rats (Britch and Craft, 2023). Sub-chronic exposure to intraperitoneal D9-THC or CBD alone decreased hyperalgesia and allodynia in CFA rats; however, D9-THC+CBD dose ratio combinations (3:1, 1:1, 1:3 D9-THC:CBD) increased pain compared to D9-THC alone (Britch and Craft, 2023). While a few studies report D9-THC+CBD synergy on pain sensitivity (Casey et al., 2017; Casey et al., 2022), most report no interactions or antagonistic (i.e., sub-additive) effects in acute and chronic pain models (Welburn et al., 1976; Sanders et al., 1979; Varvel et al., 2006; Booker et al., 2009; Abraham et al., 2020; Mitchell et al., 2021; Sepulveda et al., 2022).

The alteration of D9-THC antinociception by CBD may involve pharmacokinetic interactions, as CBD can alter D9-THC hepatic metabolism (Qian et al., 2019; Bansal et al., 2020; Bansal et al., 2022; Bansal et al., 2023; Zamarripa et al., 2023). It is possible that CBD shifted the D9-THC parent-metabolite ratio to favor the lesser potent parent molecule (as previously reported in rats (Greene et al., 2018)), resulting in decreased antinociception relative to D9-THC alone; however, pharmacokinetics of THC or CBD were not evaluated in this study. Changes in the time course of D9-THC antinociception could also suggest altered D9-THC metabolism. The radar plots show that the decreased antinociception produced by the 3 mg/kg D9-THC+CBD combinations relative to D9-THC alone at 1 and 3 hr was followed by increased antinociception by these combinations at 5 hr. This mirrors past reports that showed CBD increased D9-THC antinociception starting 4-6 hr after administration (Britch et al., 2017). Notably, the oral doses in the present study produced cannabinoid plasma levels comparable to other rodent studies (Baglot et al., 2021; Moore et al., 2021) and to human levels after oral dosing (Grotenhermen, 2003; Newmeyer et al., 2017; Moore et al., 2023). Continued investigation is required to further define the temporality of D9-THC+CBD sub-additivity reported in the current study.

Sex differences were apparent throughout our study where females were more sensitive than males to the effects of D9-THC. In several past studies, female rats had greater improvements in CFA-induced hyperalgesia and allodynia than males after acute intraperitoneal D9-THC (Craft et al., 2013; Britch et al., 2020; Britch and Craft, 2021), whereas male rats had greater improvements in edema compared to females (Craft et al., 2013). Male rats also required more D9-THC than female rats to have an effect (Britch and Craft, 2021). Repeated sub-chronic dosing and increased tolerance to D9-THC resolved differences (Britch et al., 2020). These sex differences may be attributed to greater levels of D9-THC active metabolites produced in female vs male rats (Tseng et al., 2004; Britch et al., 2017; Greene et al., 2018). Understanding these sex differences is essential to inform clinical pain management in patients using cannabinoid-based medications. Current expert consensus dosing guidelines for pain management with cannabis so far do not consider potential sex differences in cannabinoid effects on pain outcomes (Allan et al., 2018; Hauser et al., 2018; Banerjee and McCormack, 2019; Bhaskar et al., 2021; Bell et al., 2024). We also observed that ketoprofen produced greater antinociceptive and anti-inflammatory effects in females compared to males. Ketoprofen was previously shown at lesser doses to have greater antinociceptive effects in females compared to males, whereas edema was decreased more in males than females (Craft et al., 2021). Together, these data further demonstrate pain and inflammation outcomes are sex- and dose-dependent.

While the current study had many strengths, some caveats are noteworthy. Mainly, the present study did not evaluate effects of cannabinoids on motor behavior (e.g., sedation), which may have affected the evoked pain responses. However, there were minimal effects on motor behavior at these oral doses of D9-THC and CBD alone, as well as some of the combinations tested (3 mg/kg D9-THC + 10 mg/kg CBD), in our prior studies (Moore et al., 2021; Moore and Weerts, 2022; Moore et al., 2023). Also, if sedative effects were affecting our pain responses, then withdrawal latencies and thresholds with our D9-THC+CBD combination doses should

increase; we observed the opposite, confirming sedative motoric effects were not affecting our pain responses. Future examinations of pain-suppressed behaviors (e.g., decreases in wheel running, feeding, etc.) may provide a more complete picture of the potential therapeutic effects of cannabinoids on acute inflammatory pain.

In summary, the current study demonstrates that oral D9-THC, but not oral CBD, is efficacious for ameliorating acute inflammatory pain symptoms. Our data suggest that the antinociceptive effects of D9-THC-containing cannabis products on acute inflammatory pain are mainly due to D9-THC content (in alignment with conclusions from Harris et al. (2019)). Additionally, we show that combining CBD with D9-THC worsens D9-THC antinociception. Taken together, our data and existing preclinical studies do not support the use of CBD alone, CBD-dominant, or balanced D9-THC:CBD formulations to prevent inflammation and inflammatory pain. Moreover, evidence increasingly refutes the notion that combining CBD with D9-THC affords an added therapeutic benefit for inflammatory pain and/or mitigates the side effects of D9-THC (Britch and Craft, 2023; Englund et al., 2023; Zamarripa et al., 2023; Gorbenko et al., 2024). Given the sex differences we observed, future investigations should prioritize including both sexes to explore differences in pain symptom expression, pro-inflammatory signaling, and potential underlying mechanisms.

Acknowledgements

The authors thank the NIDA Drug Supply Program for providing the D9-THC, Blanca Bravo and Alma Hausker for contributing to data collection, and Dr. Austin Zamarripa for assistance with the drug combinations analyses. Financial support for this work was provided by the Department of Psychiatry and Behavioral Sciences at Johns Hopkins University School of Medicine.

Data Availability Statement

The data that support the findings of this study will be kept for at least 6 years post-publication and are available on request from the corresponding author.

Authorship Contributions

Participated in research design: Jenkins, Moore, Weerts

Conducted experiments: Jenkins, Moore

Performed data analysis: Jenkins, Moore

Wrote or contributed to the writing of the manuscript: Jenkins, Moore, Weerts

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Footnotes

a) This work was supported by funding from the Department of Psychiatry and Behavioral Sciences at Johns Hopkins University School of Medicine. A subset of the single-dose D9-THC and CBD data was supported in part by funds from MyMD Pharmaceuticals, Inc. and MIRA Pharmaceuticals, Inc.

b) BWJ has no conflicts to report. CFM and EMW received funds from MyMD Pharmaceuticals, Inc. and MIRA Pharmaceuticals, Inc for contract preclinical research investigating a novel cannabinoid. Neither MyMD or MIRA had any input regarding the current study. EMW received funds from Cultivate Biologics LLC, and Canopy Growth Corp. for clinical research projects unrelated to this paper.

Figures legends

Figure 1. Carrageenan induced acute inflammatory pain comprising paw edema, thermal hyperalgesia, and mechanical allodynia in untreated and ketoprofen-treated male and female rats. Experimental timeline for pain induction and pre- and post-treatment measures of inflammation and pain (**A.**). In male and female vehicle control rats, acute inflammatory pain was evidenced by increased paw thickness (**B.**) and decreased Hargreaves withdrawal latency (**C.**) and von Frey threshold (**D.**) measures from baseline across the 1-, 3-, and 5-hour post-carrageenan timepoints. Both ketoprofen doses decreased paw thickness (**E.**) in males and females. Ketoprofen increased withdrawal latency (**F.**) in females. 10 mg/kg Ketoprofen increased withdrawal threshold in females (**G.**). Data shown as Mean ± SEM. Vertical dotted line represents carrageenan injection timepoint. Veh = vehicle. D9-THC = delta-9-tetrahydrocannabinol. Keto = ketoprofen. * = $p < 0.05$ compared to vehicle. + = $p < 0.05$ compared to baseline. × = $p < 0.05$ between sexes.

Figure 2. Cannabinoid effects on edema. Top panels (**A-C**) depict absolute change from baseline scores (△ in paw thickness) across timepoints: 1 mg/kg D9-THC was pro-inflammatory in males at the 5-hour timepoint (**C**). 10 mg/kg CBD was modestly anti-inflammatory in males at the 5-hour timepoint (**C**). 3 mg/kg D9-THC combined with 10 and 30 mg/kg CBD was pro-inflammatory in females compared to 3 mg/kg D9-THC alone across all timepoints (**A-C**). Data shown as Mean ± SEM. * = $p < 0.05$ compared to vehicle. # = $p < 0.05$ compared to matched D9-THC alone condition. Bottom panels (**D-F**) depict radar plots showing differences in edema between treatment and comparison groups after varying cannabinoid doses and ratios across timepoints; blue shaded area represents males, orange shaded area represents females; the unshaded right half identifies differences between D9-THC or CBD alone condition and vehicle control data, the shaded left half identifies differences between D9-THC+CBD conditions and

matched D9-THC alone data; the “0” axis (x = 0) represents no arithmetic difference between treatment and comparisons groups, an increase toward the outer edge of the plot (x > 0) is an increase in paw thickness up to +1 mm (outer edge), and a decrease toward the center of the plot (x < 0) is a decrease in paw thickness down to -1 mm (center point). D9-THC = delta-9-tetrahydrocannabinol. CBD = cannabidiol.

Figure 3. Cannabinoid effects on thermal hyperalgesia. Top panels (**A-C**) depict absolute change from baseline scores (△ latency) across timepoints: all doses of D9-THC increased withdrawal latency in females across all timepoints (**A-C**). 3 and 10 mg/kg D9-THC increased withdrawal latency in males across all timepoints (**A-C**). Data shown as Mean ± SEM. * = p < 0.05 compared to vehicle. Bottom panels (**D-F**) depict radar plots showing differences in hyperalgesia between treatment and comparison groups after varying cannabinoid doses and ratios across timepoints; blue shaded area represents males, orange shaded area represents females; the unshaded right half identifies differences between D9-THC or CBD alone condition and vehicle control data, the shaded left half identifies differences between D9-THC+CBD conditions and matched D9-THC alone data; the “0” axis (x = 0) represents no arithmetic difference between treatment and comparisons groups, an increase toward the outer edge of the plot (x > 0) is an increase in withdrawal latency up to +15 sec (outer edge), a decrease toward the center of the plot (x < 0) is a decrease in withdrawal latency down to -5 sec (center point). D9-THC = delta-9-tetrahydrocannabinol. CBD = cannabidiol.

Figure 4. Cannabinoid effects on mechanical allodynia. Top panels (**A-C**) depict absolute change from baseline scores (△ withdrawal threshold) across timepoints: 3 and 10 mg/kg D9-THC increased withdrawal threshold in both males and females with effects in females being greater than those in males (**A-C**). D9-THC effects resolved in males by the 5-hour timepoint

(**C**). the 3 mg/kg D9-THC + 10 mg/kg CBD dose combination decreased withdrawal threshold in males and females compared to D9-THC alone (**A-C**). Data shown as Mean ± SEM. * = $p < 0.05$ compared to vehicle. # = $p < 0.05$ compared to matched D9-THC alone condition. × = $p < 0.05$ between sexes. Bottom panels (**D-F**) depict radar plots showing differences in allodynia between treatment and comparison groups after varying cannabinoid doses and ratios across timepoints; blue shaded area represents males, orange shaded area represents females; the unshaded right half identifies differences between D9-THC or CBD alone condition and vehicle control data, the shaded left half identifies differences between D9-THC+CBD conditions and matched D9-THC alone data; the "0" axis (x = 0) represents no arithmetic difference between treatment and comparisons groups, an increase toward the outer edge of the plot (x > 0) is an increase in withdrawal threshold up to +10 g (outer edge), a decrease toward the center of the plot (x < 0) is a decrease in withdrawal threshold down to -5 g (center point). D9-THC = delta-9-tetrahydrocannabinol. CBD = cannabidiol.

Figure 5. Drug combination analysis. Isobologram depicting ED50s (plus symbols) of D9-THC combined with 10 mg/kg or 30 mg/kg CBD relative to the ED50 of D9-THC alone, defined as the line of additivity. ED50s were computed from Von Frey data at time of peak effects (1 hr timepoint) transformed to percent change from vehicle (% vehicle). An ED50 could not be computed for CBD alone data because this condition had no effect on pain measures. The ED50 values were determined to be 1.23 mg/kg for oral D9-THC alone, 1.74 mg/kg for D9-THC combined with 10 mg/kg CBD, and 1.37 mg/kg for D9-THC combined with 30 mg/kg CBD. The combined ED50s thus fell to the right of the line of additivity, indicating that they produced sub-additive effects on antinociception.

Table 1. Summary of results across sex, drug condition, and outcome measures. Edema was measured as paw thickness (mm). Hyperalgesia was measured as change in withdrawal latency (sec). Allodynia was measured as change in withdrawal threshold (g). Down arrow = decreased inflammation/pain. Up arrow = increased inflammation/pain. Dash = no effect of treatment. D9-THC or CBD alone conditions were compared to the vehicle control condition. D9-THC+CBD combined conditions were compared to the D9-THC alone conditions. D9-THC = delta-9-tetrahydrocannabinol. CBD = cannabidiol.

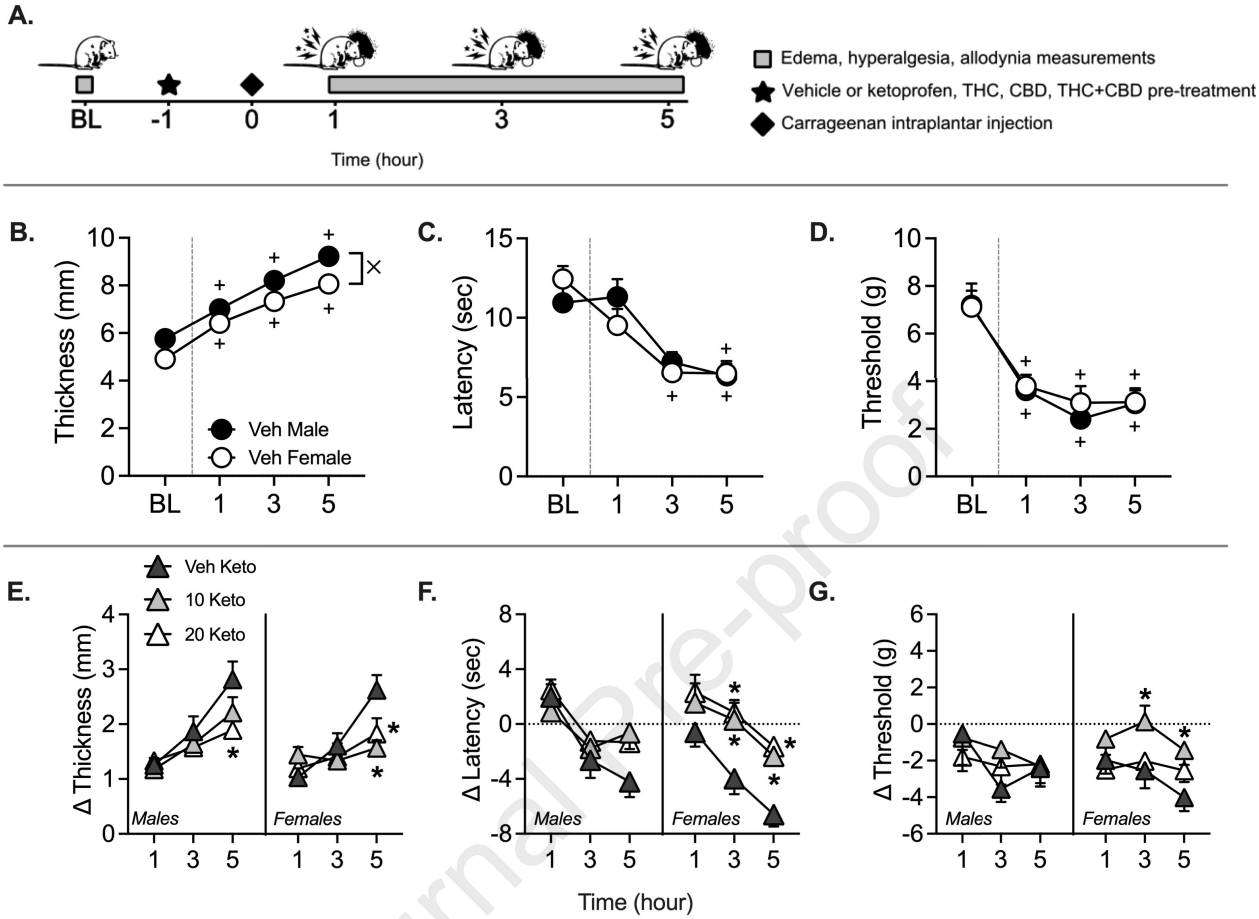
Table 1. Summary of results across sex, drug condition, and outcome measures.

Groups	Dose (mg/kg)	Edema		Hyperalgesia		Allodynia	
		Males	*Females*	*Males*	*Females*	*Males*	*Females*
Vehicle(s)	0	–	–	–	–	–	–
Ketoprofen	10	–	↓	–	↓	–	↓
	20	↓	↓	–	↓	–	–
THC alone	1	↑	–	–	↓	–	–
	3	–	–	–	↓	↓	↓
	10	–	–	–	↓	↓	↓
CBD alone	10	↓	–	–	–	–	–
	30	–	–	–	–	–	–
	100	–	–	–	–	–	–
THC+CBD	1+10	–	–	–	–	–	–
	1+30	–	–	–	–	–	–
THC+CBD	3+10	–	↑	–	–	↑	↑
	3+30	–	↑	–	–	–	–

Table 1. Summary of results across sex, drug condition, and outcome measures.

Groups	Dose (mg/kg)	Edema		Hyperalgesia		Allodynia	
		Males	*Females*	*Males*	*Females*	*Males*	*Females*
Vehicle(s)	0	–	–	–	–	–	–
Ketoprofen	10	–	↓	–	↓	–	↓
	20	↓	↓	–	↓	–	–
THC alone	1	↑	–	–	↓	–	–
	3	–	–	–	↓	↓	↓
	10	–	–	–	↓	↓	↓
CBD alone	10	↓	–	–	–	–	–
	30	–	–	–	–	–	–
	100	–	–	–	–	–	–
THC+CBD	1+10	–	–	–	–	–	–
	1+30	–	–	–	–	–	–
THC+CBD	3+10	–	↑	–	–	↑	↑
	3+30	–	↑	–	–	–	–

Figure 1.



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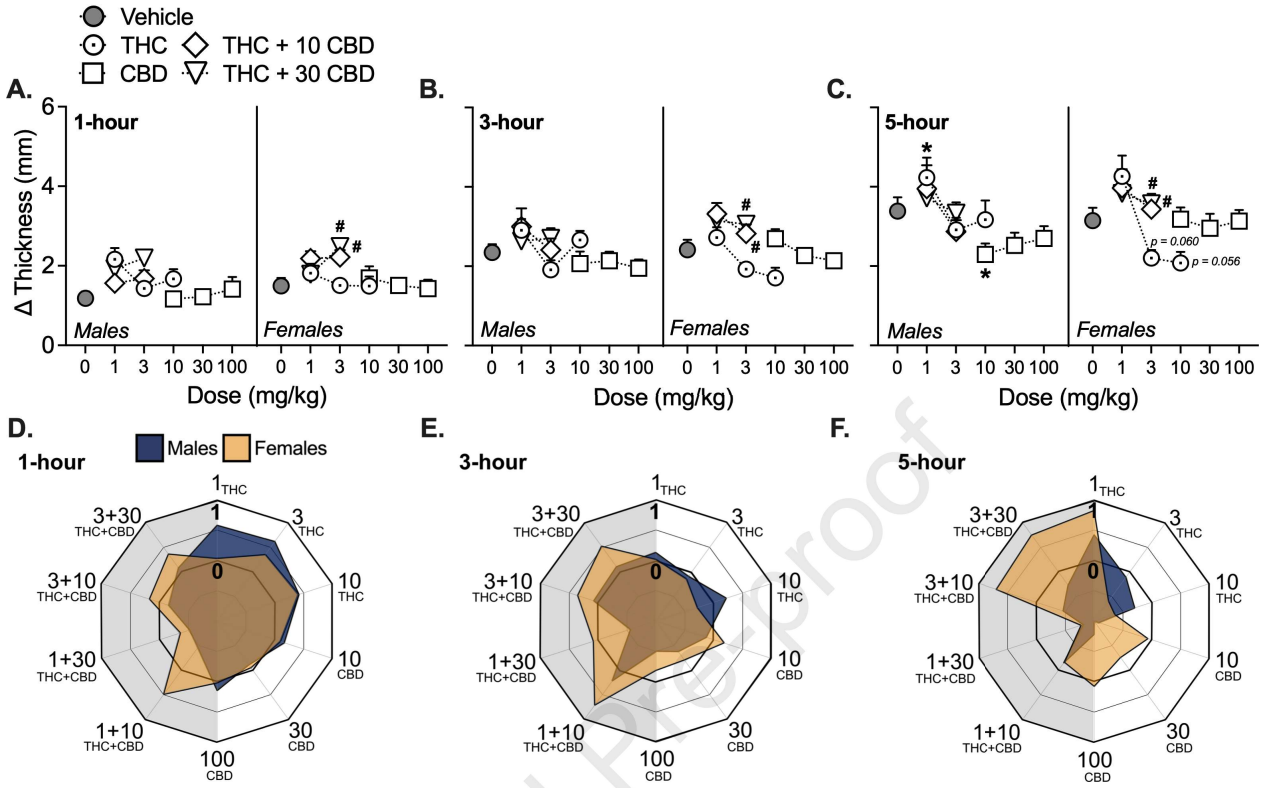

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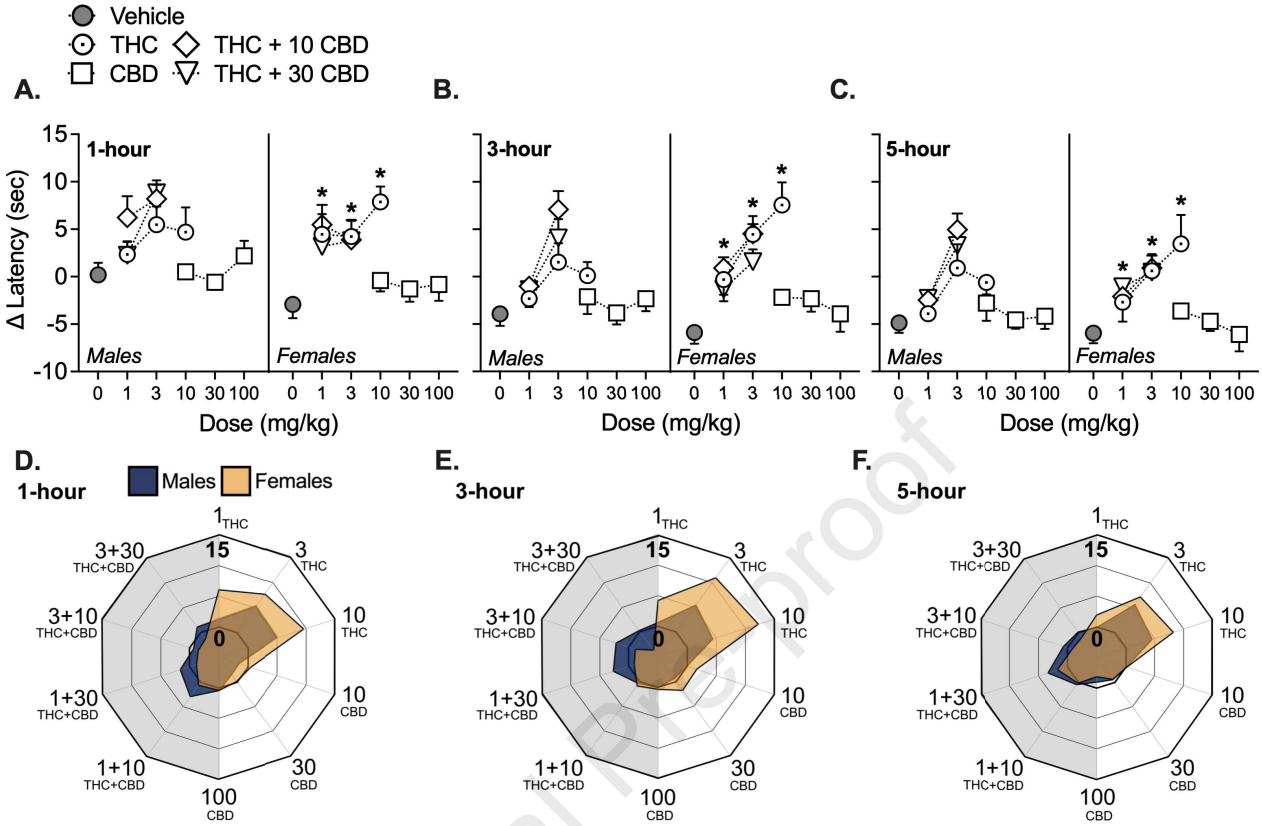

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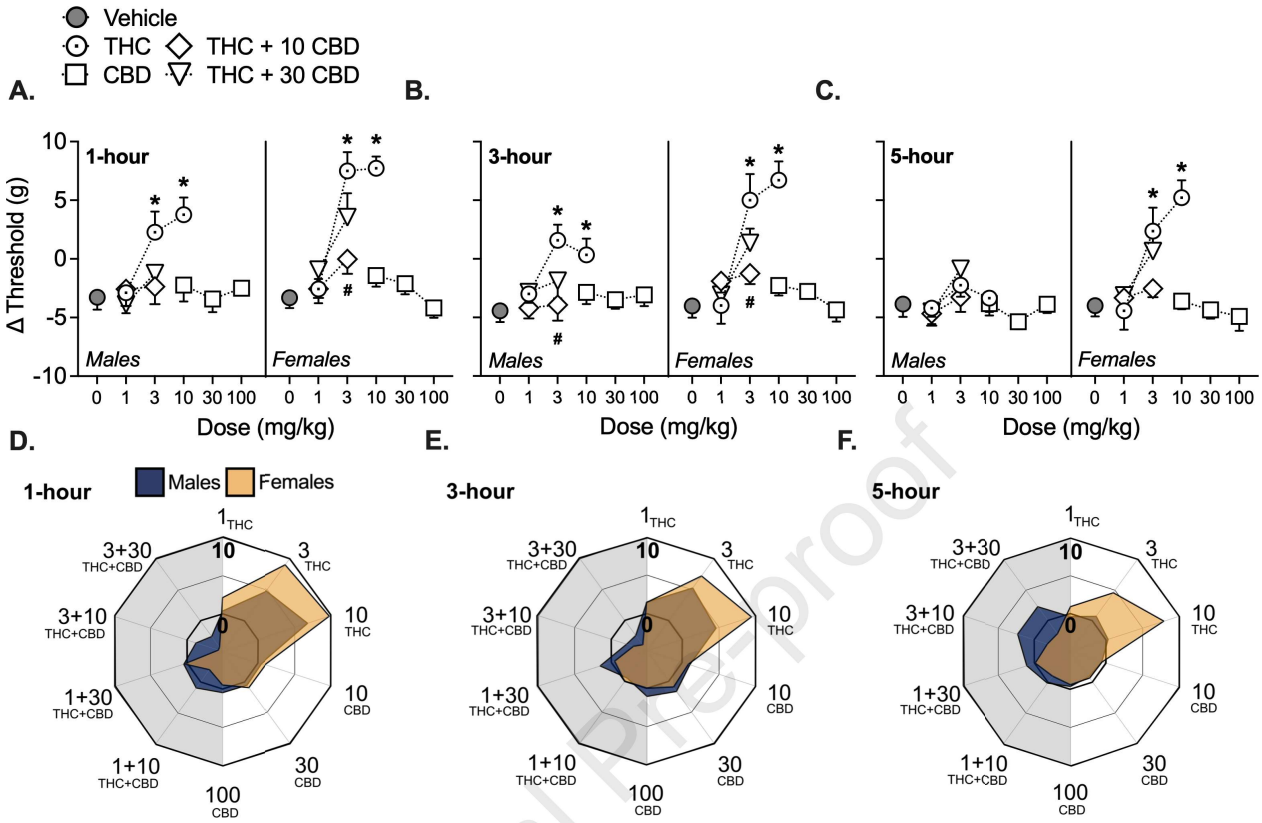

Figure 5.