Research Paper

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A randomized, double-blind, placebo-controlled study of daily cannabidiol for the treatment of canine osteoarthritis pain

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Abstract

Over the last 2 decades, affirmative diagnoses of osteoarthritis (OA) in the United States have tripled due to increasing rates of obesity and an aging population. Hemp-derived cannabidiol (CBD) is the major nontetrahydrocannabinol component of cannabis and has been promoted as a potential treatment for a wide variety of disparate inflammatory conditions. Here, we evaluated CBD for its ability to modulate the production of proinflammatory cytokines in vitro and in murine models of induced inflammation and further validated the ability of a liposomal formulation to increase bioavailability in mice and in humans. Subsequently, the therapeutic potential of both naked and liposomally encapsulated CBD was explored in a 4-week, randomized placebo-controlled, double-blinded study in a spontaneous canine model of OA. In vitro and in mouse models, CBD significantly attenuated the production of proinflammatory cytokines IL-6 and TNF-α while elevating levels of anti-inflammatory IL-10. In the veterinary study, CBD significantly decreased pain and increased mobility in a dose-dependent fashion among animals with an affirmative diagnosis of OA. Liposomal CBD (20 mg/day) was as effective as the highest dose of nonliposomal CBD (50 mg/day) in improving clinical outcomes. Hematocrit, comprehensive metabolic profile, and clinical chemistry indicated no significant detrimental impact of CBD administration over the 4-week analysis period. This study supports the safety and therapeutic potential of hemp-derived CBD for relieving arthritic pain and suggests follow-up investigations in humans are warranted.

Keywords: Osteoarthritis, Cannabidiol, Randomized trial, Liposomal encapsulation, TNF-α, IL-6

1. Background

Arthritis is a leading cause of pain, disfigurement, and disability in the United States where nearly one-quarter of all adults have received an affirmative diagnosis.[2] Although the incidence of rheumatoid arthritis has remained constant, osteoarthritis (OA) diagnoses have tripled since 2000 due to an aging population, increasing levels of obesity, and greater physician recognition of its prevalence. Accordingly, OA is a leading cause of chronic pain and disability among the elderly.[23] Irrespective of the precipitating cause, the pathology of joint destruction in arthritis is driven by an overlapping profile of pathologic inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-17, and IL-21.[26,46,49] In addition, pain, inflammation, and joint destruction among both etiologies are mediated by overlapping subsets of innate cell types, most prominently neutrophils.[16,45] Treatment of rheumatoid arthritis consists of both targeted and nonspecific immunosuppressive drug regimens (disease-modifying antirheumatic drugs), whereas treatment of OA consists of analgesics, nonsteroidal anti-inflammatory drugs, glucocorticoids, and joint replacement supplemented by a weight loss regimen, if applicable. In either case, pharmacomodulation is not curative and often accompanied by severe side-effects.[6,9,36] Because pain is the predominant symptom of OA, it is also the primary target of intervention. Recent reviews comparing the efficacy of pharmacotherapies for reducing OA pain conclude opioids are most effective, however, abuse potential limits utility. Overall, the effect size across all pharmacotherapies is small (0.39), signaling a need for additional treatments with novel and complementary mechanisms of action.[1,32,54,61]

The ubiquitous endocannabinoid system plays a role in many physiological and pathophysiological processes. Consistent with this, cannabis and its constituents are increasingly being recognized as bona fide pharmacologic agents with significant therapeutic potential. For example, cannabidiol (CBD), the major nontetrahydrocannabinol (THC) constituent of cannabis, can exert numerous biological effects through several different receptors and signaling pathways, including anti-inflammatory

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effects in both acute and chronic conditions.[10–13,20,25,43,47,51,57] Indeed, preclinical rodent models suggest the therapeutic potential of CBD in combating the underlying causes of both rheumatoid arthritis and OA.[15,35,37,48]

Although preclinical rodent models have provided evidence of efficacy for novel compounds to treat pain,[8,19,29] the clinical efficacy or safety of these compounds in human studies has been unsatisfying.[58,59] The late-stage failures of promising compounds in randomized studies have suggested a disconnect between the preclinical models used to study structural vs symptomatic aspects of disease.[17] Indeed, the initiating event and many of the pathological changes in the commonly used, chemically induced preclinical rodent models of chronic OA pain are not typical of human OA.[17,56] By contrast, spontaneous models, particularly domesticated canine models, are more appropriate for assessing OA pain treatments because they closely mimic the pathophysiology and pathogenesis of human OA pain.[17] In the present work, we determined the in vitro and in vivo effects of CBD on expression levels of shared, pathologic proinflammatory cytokines, and innate cell subsets in multiple model systems. Subsequently, the safety and efficacy of CBD were evaluated in a double-blind, placebo-controlled study in a spontaneous canine model.

2. Methods

2.1. Cannabidiol

Cannabidiol, provided by MedterraCBD (Irvine, CA), was isolated solely from hemp grown and extracted under the strict guidelines of the Kentucky Department of Agricultural Industrial Hemp pilot program. Subsequent analysis by third party (ProVerde Laboratories, Milford, MA) mass spectrometry confirmed the absence of Δ9-THC, other cannabinoid derivatives, and contaminants while further HPLC testing demonstrated CBD isolate purity of 99.9%. For all assays, CBD was solubilized in fractionated coconut oil. Liposomal CBD was produced using a sunflower lecithin (phosphatidylcholine) base. Each liposome was approximately 100 nm, allowing for encapsulation of 10 to 20 mg/mL CBD. Transmission electron microscopy was used to observe and confirm the stability of liposomal CBD concentration, size, and polydispersity after storage at 4°C for at least 3 months. Briefly, samples were placed on 150 mesh formvar-coated copper grids treated with poly-l-lysine for approximately 1 hour, then negatively stained with filtered aqueous 2% ammonium molybdate + 0.02% BSA, pH 7.0 for 1 minute. Stain was blotted dry from the grids with filter paper, and samples were allowed to dry. Samples were then examined in a JEM 1010 transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

2.2. Cell culture

Mouse RAW267.4 macrophage cells (ATCC, Manasas, VA), primary mouse splenocytes, human monocytic THP-1 cells (ATCC), and human PBMC were plated in a single well of a 6-well plate in 5-mL RPMI (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum at 5% CO_2 in a 37°C humidified incubator for either 2 (lipopolysaccharide [LPS]) or 4 hours (staphylococcal enterotoxin B [SEB]) before addition of CBD. Lipopolysaccharide and SEB concentrations used were determined by previous publications and/or empirical testing in cell culture. TNF-α levels in cell culture supernatants were determined using the TNF Flex Set immunoassay (BD Biosciences, San Jose, CA) as measured by an LSR II or Canto Violet flow cytometer (BD Biosciences) and analyzed with FlowJo version 10.0.00003 (Tree Star, Inc, Ashland, OR). All points were assayed in triplicate with at least 3 independent repetitions unless stated otherwise.

2.3. Mice

Approximately 342 female, 6- to 10-week-old C57BL/6 J mice with a weight range of 18 to 27 g were procured from Baylor College of Medicine or the Jackson Laboratory (Bar Harbor, ME) and maintained in accordance with the specific IACUC requirements of Baylor College of Medicine and in accordance with animal protocol AN-7942. Mice were housed under controlled standard conditions (23 ± 1°C, 55 ± 10% humidity and a 12-hour light/dark cycle) and provided standard laboratory chow and autoclaved water ad libitum.

2.4. Croton oil-induced ear inflammation model

All experiments were conducted between 10 AM and 3 PM, to avoid the influence of circadian variations in corticosteroid levels in the murine inflammatory response. Croton oil (2.5% in acetone) was topically applied (100 μL) to the right ear. Two hours after croton oil was applied, vehicle or 100 μL of 10 mg/mL CBD oil was topically applied to swollen and control ears. Two hours after these treatments, ear tissue samples were collected to determine myeloperoxidase (MPO) activity, and blood samples were collected by retrobleed to determine circulating TNF-α levels.

2.5. Lipopolysaccharide-induced inflammation model

Lipopolysaccharide (200 ng) was administered intraperitoneally. Two hours after LPS administration, mice were injected intraperitoneally with CBD (1, 10, or 100 μg) or administered either CBD (100 μg) or 18.3% methyl salicylate/16% menthol (Ben-Gay; Johnson & Johnson, New Brunswick, NJ) topically at the LPS injection site. Two hours after treatments, blood samples were collected by retro-orbital bleed to determine cytokine and neutrophil levels.

2.6. Tissue myeloperoxidase activity

In brief, ear tissue samples (4-mm punch) collected at 1, 2, 3, or 4 hours after croton oil was applied were homogenized in MPO assay buffer (Abcam, Cambridge, MA) per the manufacturer's instructions. Samples and MPO assay buffer were equilibrated to room temperature before use, and samples were diluted 1:5 in assay buffer. Groups were assayed in triplicate in individual wells in 50 μL of reaction mix for 2 hours at room temperature before addition of 2 μL of stop mixture. Subsequently, 50 μL of TMB developer substrate was added and incubated for 10 minutes, and the output was measured by spectrophotometry at OD_{412} nm.

2.7. Cytokine and neutrophil analysis

Mice were bled retro-orbitally at specified intervals. Blood samples were mixed with 0.5M EDTA to prevent clotting then pelleted to extract the serum. Red blood cells in the cell pellet were lysed by suspension in ammonium chloride (Sigma-Aldrich, St. Louis, MO) per the manufacturer's instructions. The remaining white blood cells were then stained for neutrophils by CD45-

APC-Cy7, CD11b-APC, Ly6G-FITC, and CD115-PE (all from BioLegend, San Diego, CA) before analysis by flow cytometry. The serum was subsequently analyzed for various cytokines using the BD flex set (BD Biosciences). In brief, sera were diluted with supplied buffer per manufacturer's instructions, incubated with the appropriate capture antibody/bead for 1 hour at room temperature, incubated with the detection antibody/bead for another hour at room temperature, washed, centrifuged, resuspended in flow buffer, and analyzed by flow cytometry.

2.8. Bioluminescence imaging

Mice were subcutaneously injected with 5×10^5 luc2$^+$ KRAS tumor cells near the hindquarters 24 hours before experimentation. Subsequently, mice were subcutaneously injected with 100 μL either 10-mg/mL naked D-luciferin (Regis Technologies, Morton Grove, IL) or 10 mg/mL liposomally encapsulated D-luciferin near the forequarters on the ipsilateral side. Mice were then analyzed continuously by IVIS imaging (Caliper Life Sciences, Waltham, MA) for 2 hours.

2.9. Human subjects bioavailability trial design

A longitudinal crossover study to compare the bioavailability of liposomal vs naked CBD was approved and performed under an IRB-approved protocol under the auspices and guidance of the Institute for Regenerative and Cellular Medicine (IRCM, Santa Monica, CA). After provision of informed consent, subjects were randomized regarding the order of which to receive an isolate of either naked CBD or liposomally encapsulated CBD. At first study visit, peripheral blood was drawn after overnight fasting to measure the baseline CBD blood levels. Subjects then orally ingested an amount of isolate equivalent to 10 mg CBD in either naked or liposomally encapsulated form. One hour after the product was ingested, a second blood draw was taken to determine circulating levels of CBD. Two weeks later at the second study visit, the same procedure was followed with the exception that the study subject was administered the converse form of delivery not received at the first study visit. Subjects were eligible for inclusion if (1) between the ages of 25 and 70, (2) able to read and sign the informed consent and stay compliant with study requirements and schedule, (3) not taking any other CBD product concurrently, and (4) in good general health. Patients with terminal illnesses were prohibited from study participation. Bioavailability ratio of liposomally encapsulated CBD to naked CBD administration was calculated using an LOQ value of 0.05 ng/mL (limit of detection) if naked CBD administration produced undetectable levels of circulating CBD.

2.10. Osteoarthritis veterinary trial design

Canine veterinary studies were performed with oversight as stipulated by Baylor College of Medicine IACUC protocol AN-7705. The study population consisted of client-owned dogs presenting to Sunset Animal Hospital (Houston, TX) for evaluation and treatment of lameness due to OA. Owners completed a brief questionnaire to define the affected limb(s), duration of lameness, and duration of analgesic or other medications taken. Dogs were considered for inclusion in the study if they (1) received an affirmative diagnosed of OA by a veterinarian and (2) demonstrated signs of pain according to assessment by their owners, detectable lameness on visual gait assessment, and painful joint(s) upon palpation. Complete blood count (CBC) and serum chemistry were performed at presentation to rule out other underlying disease. Dogs were excluded by the attending study veterinarian if they exhibited evidence of uncontrolled renal, endocrine, neurologic, or neoplastic disease or were undergoing physical therapy. No cases of OA were related to trauma, and no animals with end-stage disease were enrolled. All other medications were discontinued at least 2 weeks before enrollment, and dogs were not allowed to receive any medications during the 4-week study period except the study medication. Large (>20 kg, mean 41 ± 15 kg) domestic canines were enrolled in the 4-week, randomized placebo-controlled trial in which both owner and veterinarian were blinded. After provision of informed owner consent, 20 study subjects were randomly assigned 1:1:1:1 to 1 of 4 groups: placebo, 20 mg/day (0.5 mg/kg) naked CBD, 50 mg/day (1.2 mg/kg) naked CBD, or 20 mg/day liposomal CBD. Simple randomization was achieved by providing the blinded study drug regimens to the veterinary investigator in a randomized numerical order labeled 1 to 20 as assigned by the rolling of a die. After randomization, aggregate average weight of each study group remained within one SD of all other study groups. Blood was collected for CBC and clinical chemistry at initiation and at day 30 of treatment. Before treatment initiation and at day 30, each dog was evaluated by the study veterinarian who assessed locomotion because it related to walking, running, and assuming a standing position from both a sitting and lying down position on a 5-point scale (1 = best) during physical examination. Owners also evaluated dogs before treatment and at weeks 4 and 6 using the Helsinki Chronic Pain Index, a validated, 11-item assessment of treatment response in dogs with OA pain scored ordinally on a scale from 0 to 4.[18]

2.11. Statistical analysis

Data are expressed as the mean ± SD unless otherwise specified. Student's *t*-test was used for pairwise comparisons, and one-way analysis of variance followed by post hoc Tukey–Kramer was used for analysis of multiple comparisons. Normality of data was determined by Q-Q plot. Statistical significance was defined as $P < 0.05$ unless stated otherwise. Sample sizes for mouse, canine, and human experiments were based on power analysis indicating that a difference in mean value ($\Delta\mu$) as small as 0.25-fold could be detected with a power of 0.8 and type I error rate (α) of 0.05 with a sample size of 4 subjects assuming a SD (σ) of 0.33. Given this calculation, we chose a sample size of 5 subjects for all experimental groups to permit even greater statistical discernment power ($<\Delta\mu$ of 25%) and/or to accommodate greater variance ($\sigma >$ 1/3 SD) between groups.

3. Results

3.1. Cannabidiol reduces proinflammatory TNF-α secretion in vitro

It has been widely reported that CBD possesses significant anti-inflammatory properties in a variety of different experimental systems.[27] To validate that the CBD used for these studies might potentiate anti-inflammatory effects relevant to arthritis, 2 different inflammatory stimuli were applied to 4 different relevant cell populations including a mouse monocyte cell line, a human monocyte cell line, primary mouse PBMC, and primary human PBMC. As illustrated in **Figure 1**, both LPS and SEB induced log-fold elevations in TNF-α secretion in comparison with untreated or CBD-only treated controls from RAW267.4 mouse cells, primary mouse PBMC, THP-1 human cells, and primary human PBMC. However, concurrent application of 100 ng/mL CBD in

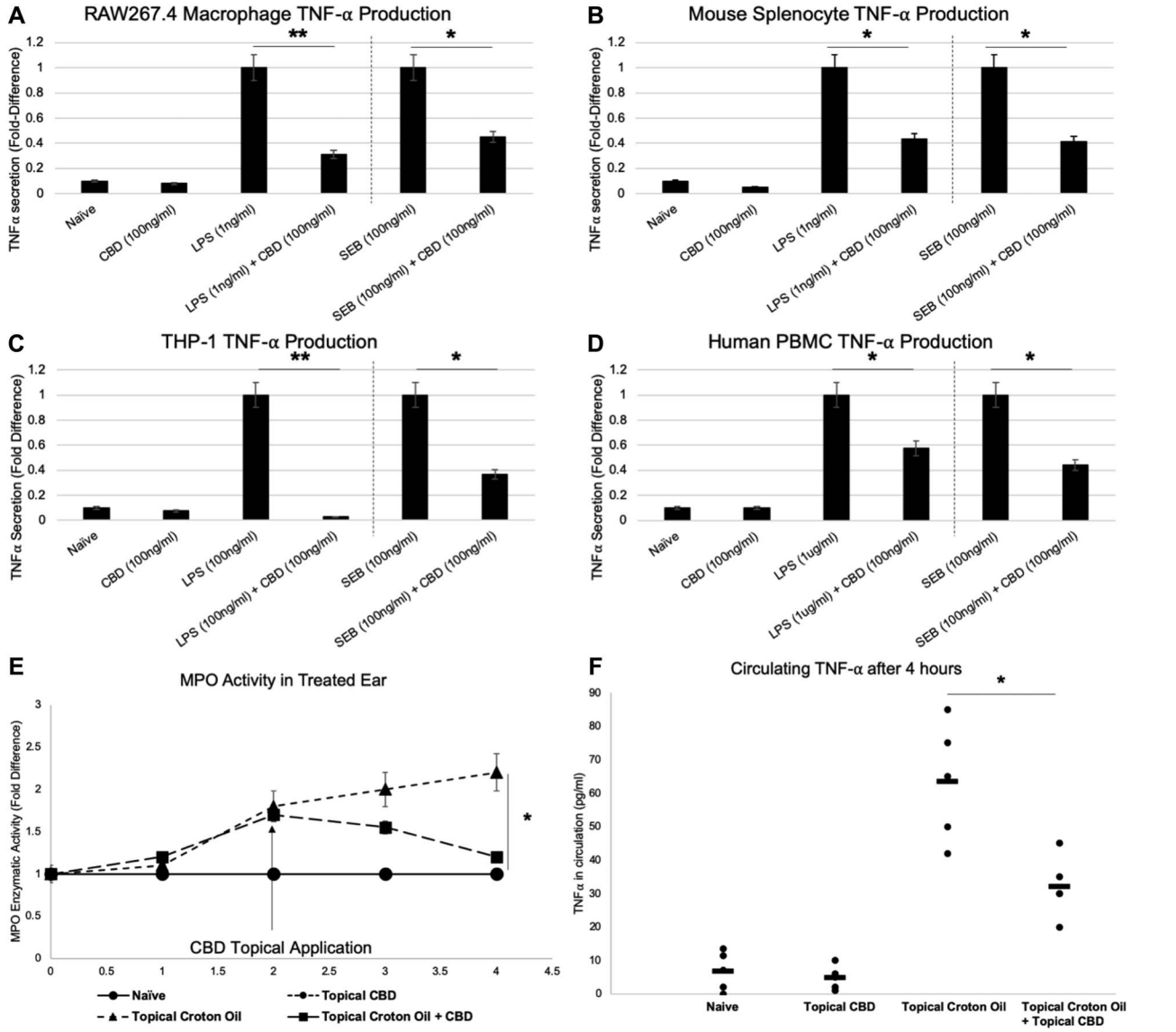

Figure 1. CBD reduces hallmarks of arthritis-related inflammation in vitro. A total of 5×10^6 cells of the specified type were plated in triplicate in 6-well plates in 5-mL RPMI + 10% FBS followed by addition of either 1-ng/mL LPS for 4 hours or 100-ng/mL SEB for 6 hours with or without the addition of 100-ng/mL CBD after 2 hours. After the incubation period, the media were analyzed using the BD TNF-α Flex set. (A) TNF-α levels in murine RAW267.4 macrophage cell line. (B) TNF-α levels in primary mouse splenocytes. (C) TNF-α levels in human THP-1 monocyte cell line. (D) TNF-α levels in primary human PBMC. Representative experiment of 3 shown. Error bars ± SD. *$P < 0.05$, **<0.01 by Student's two-tailed *t* test for all (A–D). Cohorts of female mice were also treated on one ear with 100-μL 2% croton oil-acetone, and ear edema was allowed to occur for 1 to 4 hours. At 2 hours, mice were treated on the swollen ear with either 100 μL of vehicle or 100 μL of 10-mg/mL CBD oil. In addition, a group of untreated mice also received 100-μL CBD oil (E). At each time point indicated, 4-mm biopsies from the most central portion of swelling were obtained, homogenized, and measured for myeloperoxidase (MPO) activity by ELISA. (F) After 4 hours, each cohort was retro-orbitally bled for analysis of circulating TNF-α concentrations using the BD TNFα Flex set. Each cohort consisted of n = 5 mice. Representative experiment of 3 shown. Error bars ± SD. *$P < 0.05$ by Student's two-tailed *t* test. CBD, cannabidiol; LPS, lipopolysaccharide.

conjunction with LPS treatment induced a 42% (primary human PBMC) to 97% (human THP-1 cells) reduction in TNF-α secretion. Similarly, concurrent application of 100 ng/mL CBD in conjunction with SEB treatment induced a 55% (RAW267.4 mouse cells) to 63% (human THP-1 cells) reduction in TNF-α secretion (**Figs. 1A–D**, *$P < 0.05$ or **$P < 0.01$).

3.2. Cannabidiol induces broad anti-inflammatory effects in vivo

Encouraged by the in vitro data, we next used 2 different mouse inflammatory models to analyze the impact of CBD on local and systemic inflammation in vivo. We first used the croton oil model in which topical administration of croton oil to the ear of a mouse induces an inflammatory reaction that includes edema, erythema, neutrophil influx, and the production of proinflammatory TNF-α.[28] Two hours after application of 2.5% croton oil ± topical application of 1 mg CBD, local MPO activity (a proxy for neutrophil influx) was measured. As indicated in **Figure 1E**, MPO activity in the treated ear was reduced over 80% (*$P < 0.05$) with concurrent application of CBD. Four hours after croton oil application, levels of circulating TNF-α were assessed. As shown in **Figure 1F**, circulating TNF-α was decreased by 50% among mice to which croton oil + CBD had been applied in comparison with croton oil alone (*$P < 0.05$). Cannabidiol treatment also significantly reduced the development of edema.

When administered intraperitoneally, LPS induces an inflammatory response that includes increased expression of proinflammatory TNF-α and IL-6, 2 cytokines relevant to the pathogenesis of arthritis. In this model system, 200 ng of LPS was administered intraperitoneally. Two hours later, mice were then treated intraperitoneally with increasing doses of CBD (1, 10, or 100 μg) or topically with a single CBD dose of 100 μg. After an additional 2 hours, the impact of CBD treatment on circulating cytokine levels was assessed. As indicated, intraperitoneal administration of CBD reduced circulating TNF-α and IL-6 levels in a dose-responsive fashion, and 100 μg of topically applied CBD generated an anti-inflammatory effect similar to that of 100 μg injected intraperitoneally (**Fig. 2A/B**). Interestingly, systemic administration of CBD alone increased levels of anti-inflammatory IL-10 in the absence of inflammatory stimulus, an effect that was significantly potentiated in the presence of LPS (**Fig. 2C**). Application of 18.3% methyl salicylate/16% menthol (Ben-Gay) made no significant impact on any of these cytokine concentrations. In contrast to alterations in proinflammatory and anti-inflammatory cytokine levels, significant changes to circulating neutrophil chemoattractants CXCL1 (KC) and CXCL2 (MIP-2) were not observed (**Fig. 2D/E**). Nonetheless, circulating neutrophil levels were reduced up to 60% among LPS-treated mice to which CBD had been administered (**Fig. 2F**).

3.3. Liposomal packaging of cannabidiol enhances bioavailability in vivo

Although CBD clearly displayed a role in regulating inflammation in both in vitro and in vivo murine models, the relatively low bioavailability of this hydrophobic molecule when administered orally may reduce its effectiveness. To potentially improve absorption of hydrophobic CBD isolate, we packaged it within liposomes, a vehicle delivery system previously shown to improve uptake of other hydrophobic compounds.[30] Using sunflower lecithin as a base, phosphatidylcholine liposomes approximately

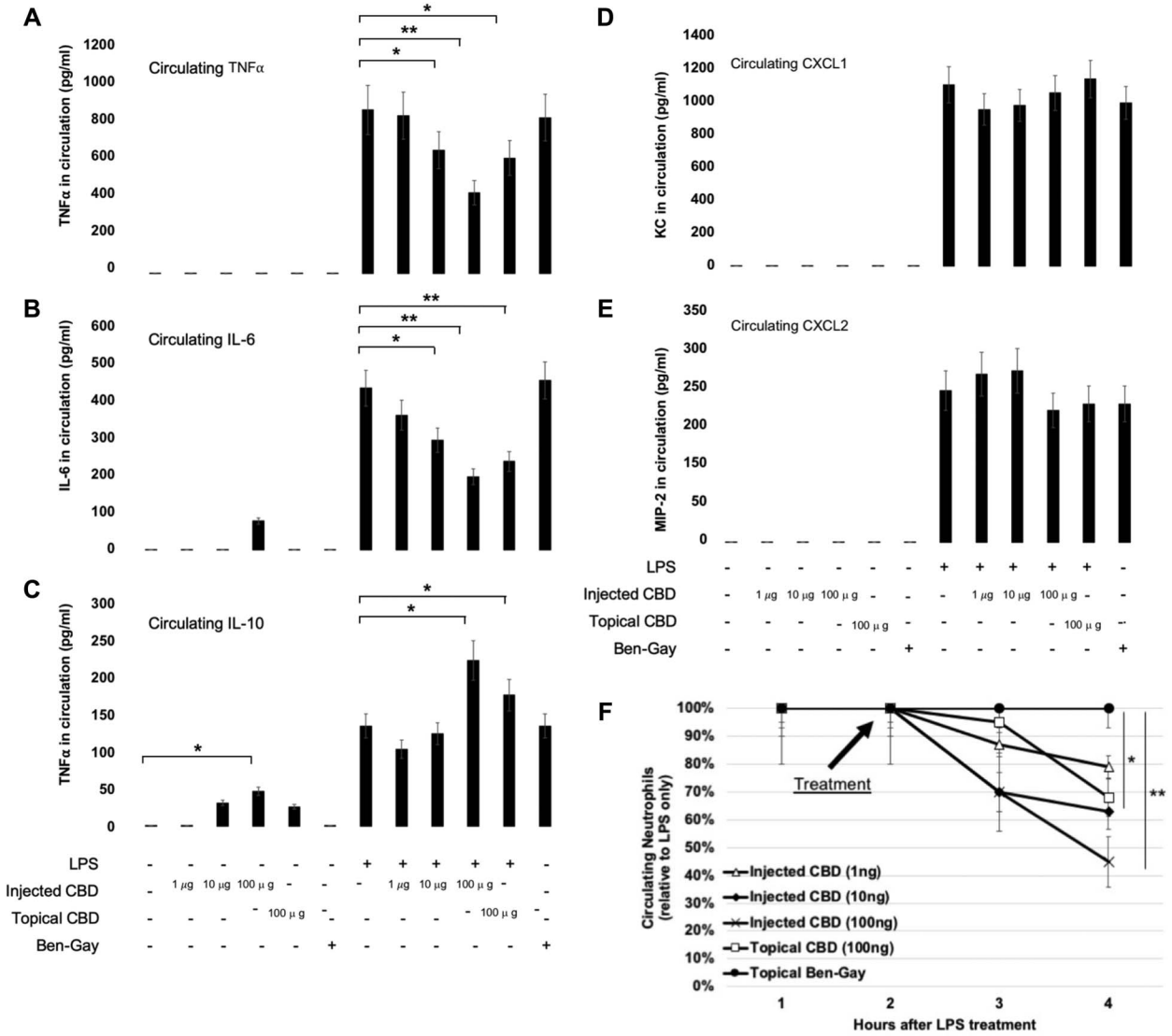

Figure 2. Intraperitoneal CBD administration reduces inflammatory cytokines and circulating neutrophils in an in vivo LPS inflammatory model. Cohorts of female mice were treated intraperitoneally with 200-ng LPS for 2 hours and subsequently administered intraperitoneal CBD, intraperitoneal PBS control, topical CBD, or topical Ben-Gay control as indicated. After an additional 2 hours, mice were retro-orbitally bled and circulating cytokines were analyzed with the appropriate BD Flex set. (A) TNF-α. (B) IL-6. (C) IL-10. (D) CXCL1. (E) CXCL2. (F) Flow cytometry analysis of the cellular portion was performed each hour to determine relative number of neutrophils ($CD115^{neg}$ $CD11b^{+}$ $Ly6G^{+}$) in circulation. Representative experiment shown. Error bars ± SD. *$P < 0.05$, **<0.01 by one-way ANOVA. CBD, cannabidiol; LPS, lipopolysaccharide.

100 nm in diameter and loaded with 10 to 20 mg/mL CBD were produced. Electron microscopy demonstrated that liposomal CBD was stable at both room temperature and 4°C and between pH 5 to 9 for a period of 3 months (**Fig. 3A**).

To compare the bioavailability of molecules encapsulated within this liposomal containment system to that of naked molecules, we developed a proof-of-principle system using liposomally encapsulated D-luciferin and a luciferase-expressing tumor cell line. In this assay system, 5×10^5 luc2$^+$ tumor cells were implanted subcutaneously near the hindquarters of C57BL/6 mice. Twenty-four hours later, D-luciferin (100 μL @ 10 mg/mL) or liposomally encapsulated D-luciferin (100 μL @ 10 mg/mL) were administered subcutaneously near the forequarters. Luminescence was then monitored for a continuous 2-hour period by IVIS with post hoc photon measurement at the target serving as a proxy for substrate absorption into circulation and bioavailability. As shown in **Figures 3B and C**, liposomal packaging of D-luciferin significantly enhanced both the speed and magnitude at which this substrate was able to reach the tumor site and induce photon emission, resulting in a full log-fold enhancement of peak emissions at 60 minutes after D-luciferin administration (*$P < 0.05$, **$P < 0.01$ for time points indicated). Next, using the LPS acute inflammatory model, 200-ng LPS was administered intraperitoneally, and circulating TNF-α was assayed every 30 minutes and plotted as a percentage of preadministration TNF-α. Two hours after introduction of LPS, mice were orally gavaged with 100-μL 10-mg/mL CBD, 10-mg/mL liposomal CBD, 10-mg/mL liposomal D-luciferin, or PBS. As shown in **Figure 3D**, orally administered liposomal CBD began to significantly reduce rising TNF-α levels within an hour of administration, whereas an additional hour was required before orally administered naked CBD significantly reduced rising TNF-α levels in comparison with negative controls. Moreover, although both naked and liposomally encapsulated CBD administered orally significantly reduced relative levels of circulating TNF-α below those of the negative controls, liposomally encapsulated CBD made a significantly greater impact on such levels (*$P < 0.05$, **$P < 0.01$ at 4 hours after CBD administration).

Encouraged by these data, we sought to validate enhanced bioavailability of liposomally encapsulated CBD in healthy human volunteers under the auspices of an IRB-approved and monitored human crossover study. In brief, after provision of informed consent, healthy human volunteers were randomized to receive 10-mg oral CBD in either a naked or liposomally encapsulated formulation. Circulating CBD levels were determined from preadministration and 1-hour postadministration blood draws. At a second study visit, this procedure was repeated in the same volunteer using the converse delivery method (ie, naked vs liposomally encapsulated), and a bioavailability ratio was calculated. For instances in which naked CBD administration produced undetectable levels of circulating CBD, the bioavailability ratio was calculated using an LOQ value of 0.05 ng/mL (limit of detection). Among the 5 study volunteers for whom data were available, the bioavailability of liposomally encapsulated CBD was 17.1 ± 16-fold greater than that of naked CBD at 1-hour postadministration (*$P < 0.05$). Furthermore, although 2 of 5

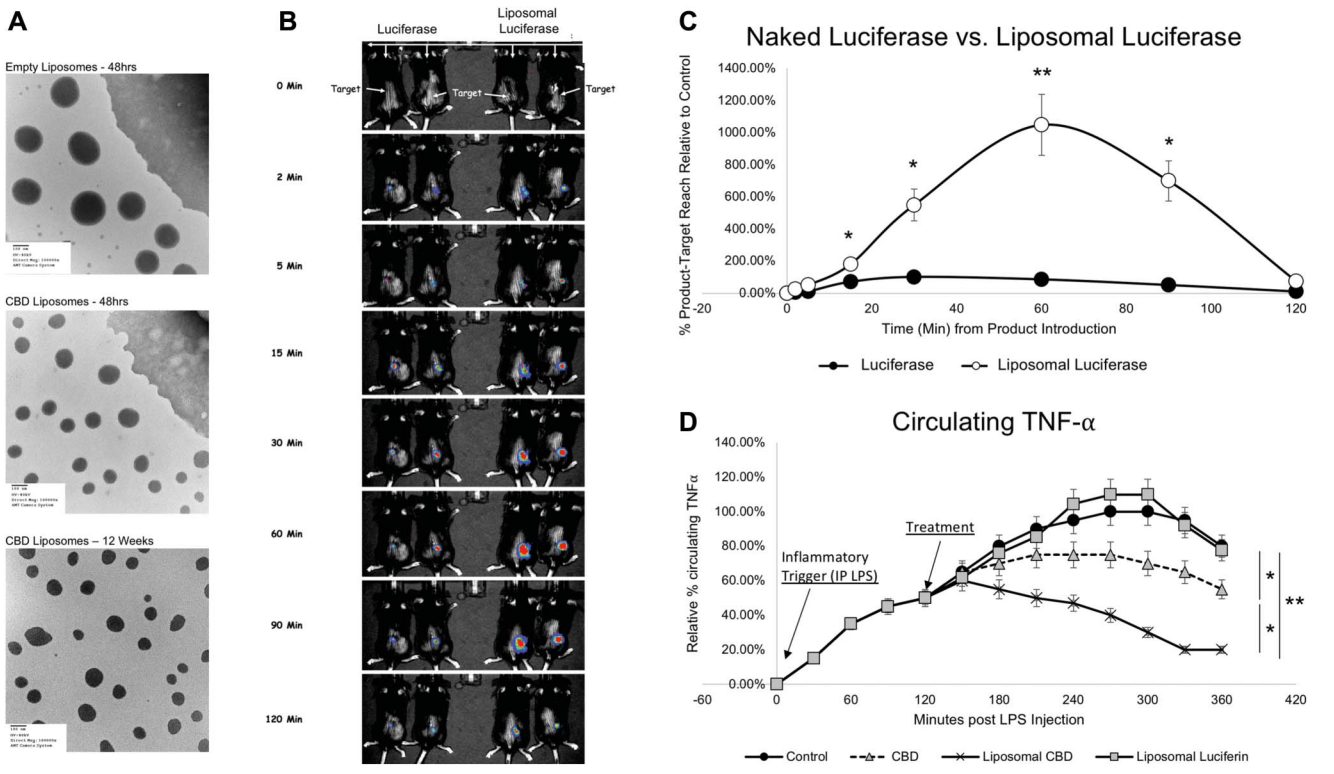

Figure 3. Liposomal encapsulation of small molecules enhances bioavailability. Sunflower lecithin (phosphatidylcholine) was used as a base to make liposomes approximately 100 nm in size that could encapsulate small molecules at a concentration of 10 to 20 mg/mL and retain polydispersity and size for at least 3 months at 4°C. (A) Stable size and polydispersity observed by transmission electron microscopy (TEM). (B) Cohorts of mice were implanted subcutaneously injected with 500,000 luc2$^+$ cells near the hindquarters. (B/C) Twenty-four hours later, D-luciferin (100 μL, 10 mg/mL) or D-luciferin liposomes (100 μL, 10 mg/mL) were applied subcutaneously near the forequarters, and animals were continually imaged by IVIS for 2 hours with subsequent photon measurement at the target serving as a proxy for absorption and bioavailability. (D) The ability of liposomal CBD to reduce TNF-α production relative to controls and naked CBD was determined. For B and C, n = 5 mice per cohort. Representative experiment of 3 shown. For D, n = 8 mice per cohort. Representative experiment of 2 shown. Error bars ± SD. *$P < 0.05$, **$P < 0.01$ by Student's two-tailed *t* test. CBD, cannabidiol.

subjects exhibited undetectable circulating CBD levels after oral administration of naked CBD isolate, all 5 subjects exhibited detectable levels of circulating CBD levels after oral administration of liposomally encapsulated CBD (**Table 1**).

3.4. Short-term administration of cannabidiol to domestic canines diagnosed with osteoarthritis is safe and improves quality of life

Although there exists a variety of different preclinical mouse models of arthritis, as noted, these model chemical and pathologic features of the disease have been poorly predictive in determining symptomatic or therapeutic responses.[17] In an effort to better model treatment efficacy, we conducted a randomized, double-blind, placebo-controlled trial among large (>20 kg; mean = 41 ± 15 kg) outbred canines with an affirmative veterinary diagnosis of OA and experiencing decreased mobility and quality of life. After diagnosis and provision of owner informed consent, animals were enrolled and randomly provided with identical medication bottles which contained one of 4 treatments including 10-mg/mL naked CBD, 25-mg/mL naked CBD, 10-mg/mL liposomal CBD, or a placebo consisting only of fractionated coconut oil. Baseline and day 30 CBC and metabolic panel as well as alanine aminotransferase (ALT) and alkaline phosphatase (ALKP) were also determined. Symptomology was assessed by the attending study veterinarian through clinical examination on days 0 and 30 and by each animal's owner on study days 0, 30, and 45 using the Helsinki Chronic Pain Index assessment.[18] Characteristics of each enrolled animal are provided in Supplementary Table 1 (available at http://links.lww.com/PAIN/B3). As shown in **Figure 4A/B**, owner assessment of animal symptomology was not significantly altered by administration of placebo or 20-mg/day naked CBD; however, administration of 50-mg/day naked CBD or 20-mg/day liposomal CBD generated statistically significant reductions in pain symptomology (**$P < 0.01$), an effect that remained statistically significant (*$P < 0.05$) for at least 15 days after cessation of therapy. With some variability, veterinarian clinical examination largely matched that of the owner's assessment with generally no improvements observed among animals administered placebo or 20-mg/day naked CBD, and significant improvements noted among all 4 assessment categories (sitting to standing, lying to standing, walking, and running) among dogs who received 50-mg/day naked CBD and 20-mg/day liposomal CBD as evidenced by group compilation raw assessment scores (**Figs. 5A–D**) or a secondary analysis (**Fig. 6**) that considered only whether symptomology in a given study participant worsened, remained the same, or improved over the course of therapy (*$P < 0.05$, **$P < 0.01$, ****$P < 0.001$). No sex differences with regard to treatment efficacy were observed, and there were no significant alterations to CBC, metabolic panel, or ALT/ALKP values over the course of the study in any group (**Figs. 7A–C** and **Table 2**).

Table 1

Naked vs liposomally encapsulated circulating CBD levels in healthy volunteers.

Subject	Naked CBD		Liposomal CBD		Ratio
	Pre (ng/mL)	Post (ng/mL)	Pre (ng/mL)	Post (ng/mL)	
1	0.00	0.87	0.00	5.90	6.8
2	0.00	0.00	0.00	0.87	17.4
3	0.00	0.14	0.10	2.00	13.6
4	0.00	0.00	0.19	2.40	44.2
5	0.00	0.45	0.00	1.60	3.6
Averages		0.29 ± 0.37		2.55 ± 1.95	17.1 ± 16.1

CBD, cannabidiol.

4. Discussion

Arthritis is a painful degenerative condition that impacts the lives of almost a quarter of all Americans, with OA in particular accounting for 60% of all-cause arthritis diagnoses.[2,23] Because current treatment regimens are not curative and can be accompanied by significant comorbidities,[6,9,36] the present studies were undertaken to validate whether the recently legalized supplement CBD might positively impact the symptomology of this degenerative condition. We first validated the widely reported anti-inflammatory effects of CBD administration both in vitro and in vivo, demonstrating substantial impact on inflammatory cytokines and innate immune cell subsets relevant to the pathophysiology of arthritis. After additional experimentation that established greater bioavailability of liposomally encapsulated vs naked CBD in both mice and humans, we demonstrated the short-term clinical efficacy of CBD in a double-blind, placebo-controlled veterinary study in which neither owner nor veterinarian knew the content of the study medications. In this study, neither animals given placebo nor animals given a low daily dose of naked CBD responded to therapy in any significant fashion. Conversely, animals given a high dose of naked CBD or a low dose of liposomally encapsulated CBD experienced significant improvements in quality of life scores as documented by both owner and veterinarian assessments. In this setting, administration of CBD was not associated with any significant alterations to circulating lymphocyte subsets, clinical chemistry values, or assessed metabolic parameters.

In vitro and in vivo studies focused on important pathologic mechanisms applicable to a wide variety of arthritis etiologies. We found that CBD significantly reduced LPS- and SEB-induced production of TNF-α in human and mouse cell lines and PBMC, consistent with the results of previous studies.[3,51,60] Similarly, in a croton oil-induced murine model of inflammation, we found that topical administration of CBD significantly reduced TNF-α production and MPO activity, the latter of which is consistent with previous reports of systemic CBD administration in mice.[4,50] Consistent with previous in vivo studies, we demonstrated that CBD also significantly reduced LPS-induced proinflammatory cytokine[33,37] and neutrophil production,[40] while increasing anti-inflammatory IL-10 production in a dose-responsive fashion.[33] Given the wide variety of grades, formulations, and suppliers of commercially available CBD, it was important to validate and characterize the functional activity of the CBD isolate planned for use in subsequent veterinary studies. In those studies, the finding that 50 mg/day of naked CBD-improved treatment outcomes is consistent with a previous study in dogs with OA[14]; however, this is the first report of a randomized, double-blind, placebo-controlled study that uses a spontaneous model for assessing the potential therapeutic effects of CBD for treating OA pain and increasing quality of life. As in humans, the pathogenesis of canine OA involves changes in all tissues of the synovial joint.[5,24,31,34,38] The dominant symptom of OA for both humans and dogs is pain, and the current therapeutic goal for both species is management of that pain and

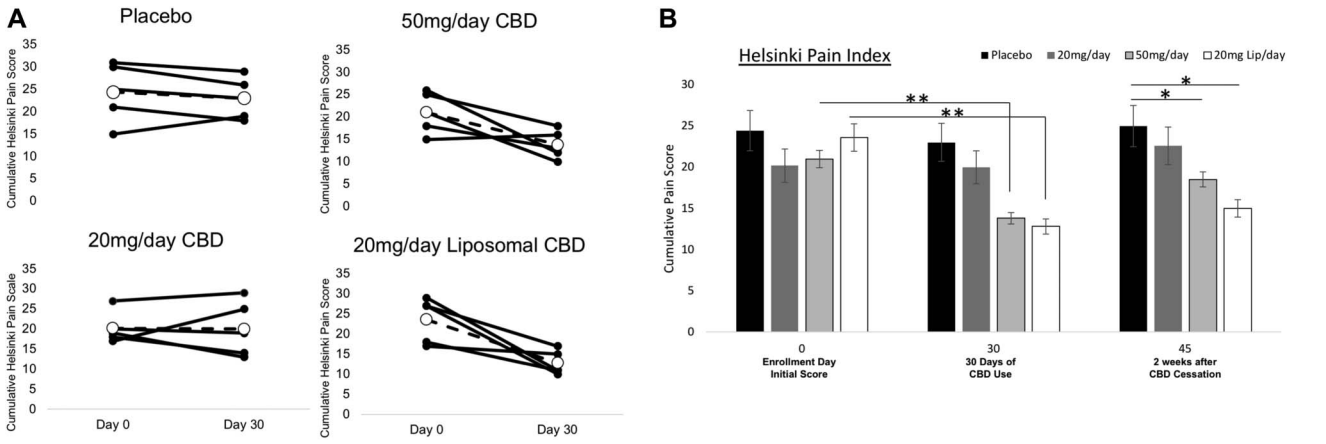

Figure 4. Daily administration of CBD for 30 days improves owner-perspective quality of life scores among large dogs with affirmative diagnosis of osteoarthritis. Twenty large domestic canines with affirmative diagnosis of osteoarthritis were enrolled in a double-blind, placebo-controlled randomized study. Animals were administered coconut oil placebo, 20-mg/day naked CBD, 50-mg/day naked CBD, or 20-mg/day liposomal CBD. Owners assessed their animals by means of the Helsinki Chronic Pain Index (HPCI) on days 0, 30, and 45. (A) Individual HPCI values were plotted for each study cohort on days 0 and 30. (B) Cohort HPCI values were plotted on days 0, 30, and 45. Error bars ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's two-tailed *t* test. CBD, cannabidiol.

associated movement deficits.[7] Thus, an extrapolation of these findings suggest that CBD could be useful for treating pain and improving quality of life in humans with an affirmative diagnosis of OA and/or other inflammatory conditions that might be ameliorated by a reduction in proinflammatory cytokines and pathologic neutrophil activity.

The absorption of CBD administered by smoking, vaporization, buccal spray, or oral ingestion is highly variable and results in extremely inconsistent pharmacokinetic profiles when investigated.[21,39,42,44,52] Cannabidiol also shows limited oral bioavailability due to poor aqueous solubility and extensive first-pass metabolism.[22,41,55] Although the current study did not assess pharmacokinetic parameters among canine study participants, the effect of liposomal CBD on LPS-induced TNF-α production in mice provides an objective measure of its pharmacodynamic drug action and suggests a greater

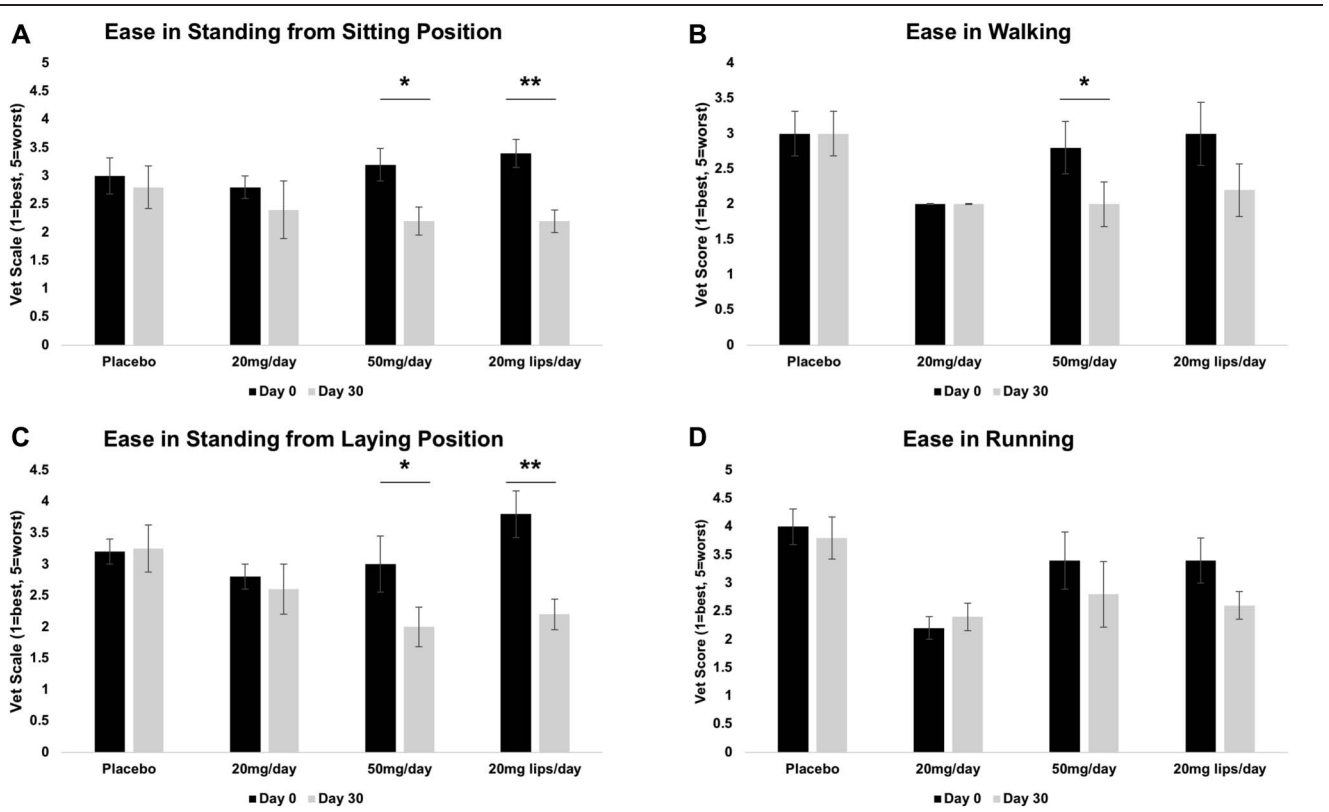

Figure 5. Daily administration of CBD for 30 days improves veterinarian-perspective subset quality of life scores among large dogs with affirmative diagnosis of osteoarthritis. Study enrolled canine subjects were scored by the (blinded) study veterinarian on days 0 and 30 using a scale of 1 (best) to 5 (worst) for 4 different movements consisting of sitting to standing (A), lying to standing (B), walking (C), and running (D). Subset scale data comparing day 0 and day 30 scores for each task are shown by cohort. Error bars ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's two-tailed *t* test. CBD, cannabidiol.

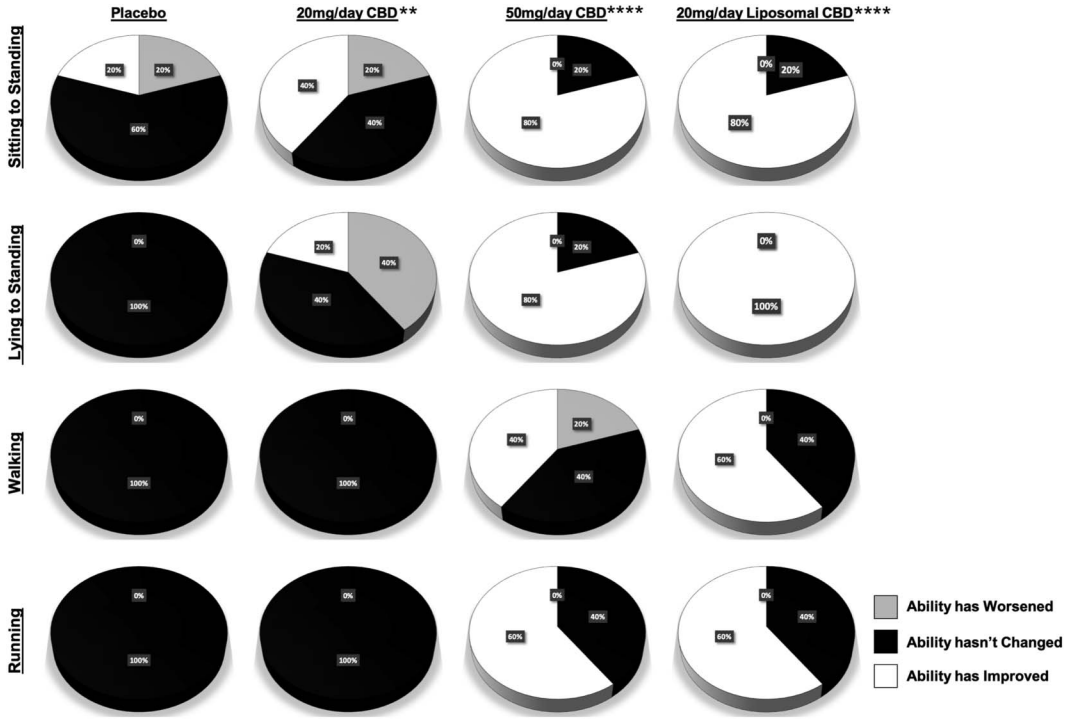

Figure 6. Daily administration of CBD for 30 days improves veterinarian-perspective overall quality of life scores among large dogs with affirmative diagnosis of osteoarthritis. Study-enrolled canine subjects were scored by the (blinded) study veterinarian on days 0 and 30 using a scale of 1 (best) to 5 (worst) for 4 different movements consisting of sitting to standing, lying to standing, walking, and running. Data are represented as pie charts indicating percent of each cohort that showed improvement, worsening, or no change in condition for the animals enrolled in each study group. $^{**}P < 0.01$, $^{****}P < 0.001$ by Pearson's χ^2. CBD, cannabidiol.

bioavailability than naked CBD. Although previous studies regarding the bioavailability of liposomal CBD are not found in the literature, a single study of Δ^9-THC, the main psychoactive constituent in cannabis, reported that liposomal encapsulation improved bioavailability in rats in comparison with administration of the naked molecule.[53] Based on these animal studies, we performed an IRB-approved crossover study in healthy human volunteers to validate approved bioavailability of CBD after liposomal encapsulation. The data demonstrated a 17-fold increase in bioavailable circulating

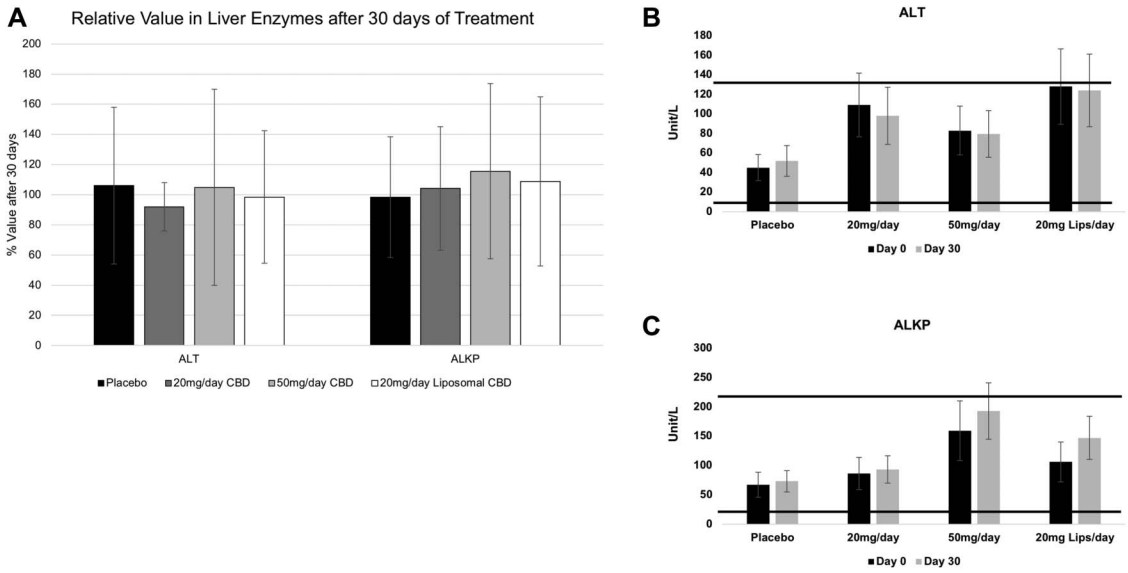

Figure 7. Daily administration of CBD for 30 days does not alter alanine aminotransferase (ALT) or alkaline phosphatase (ALKP) levels. Blood was drawn from animals we enrolled in the clinical study on days 0 and 30, and Chem10 analysis was performed. (A) Relative changes in circulating ALT and ALKP values over the 30-day period. (B, C) Specific changes in circulating ALT and ALKP values over the 30-day period. Dark horizontal lines outline normal range. Error bars ± SD. No statistically significant changes were observed. CBD, cannabidiol.

Table 2

Veterinary study CBC, metabolic panel, and clinical chemistry values.

	Day 0 WBC (K/uL)	Day 30 WBC (K/uL)	Day 0 Lymphocytes (K/uL)	Day 30 Lymphocytes (K/uL)	Day 0 Neutrophils (K/uL)	Day 30 Neutrophils (K/uL)	Day 0 Basophils (K/uL)	Day 30 Basophils (K/uL)	Day 0 Eosinophils (K/uL)	Day 30 Eosinophils (K/uL)
Placebo	8.84 (±2.52)	9.1 (±2.01)	1.34 (±0.51)	1.6 (±0.68)	6.45 (±2.25)	6.2 (±1.75)	0.015 (±0.007)	0.017 (±0.009)	0.535 (±0.38)	0.596 (±0.24)
20 mg/day	9.496 (±1.11)	10.168 (±3.01)	1.486 (±0.50)	1.628 (±0.68)	6.954 (±0.81)	7.446 (±2.36)	0.014 (±0.008)	0.024 (±0.005)	0.4 (±0.2)	0.564 (±0.3)
20 mg lips/day	9.77 (±1.71)	8.89 (±1.35)	1.68 (±0.73)	1.95 (±0.82)	6.74 (±1.75)	5.875 (±1.31)	0.0225 (±0.005)	0.0175 (±0.003)	0.67 (±0.45)	0.452 (±0.25)
50 mg/day	8.39 (±1.07)	9.25 (±1.74)	1.72 (±0.78)	1.91 (±0.82)	5.44 (±0.95)	5.99 (±1.66)	0.0125 (±0.003)	0.03 (±0.01)	0.495 (±0.19)	0.46 (±0.38)

	Day 0 RBC (K/uL)	Day 30 RBC (K/uL)	Day 0 HCT (%)	Day 30 HCT (%)	Day 0 HGB (g/dL)	Day 30 HGB (g/dL)	Day 0 Platelets (K/uL)	Day 30 Platelets (K/uL)
Placebo	6.34 (±1.01)	7.1 (±1.15)	47.8 (±6.25)	44.3 (±9.1)	15.2 (±2.34)	15.8 (±2.1)	242.0 (±47.2)	248.5 (±42.2)
20 mg/day	7.71 (±0.41)	7.79 (±0.48)	50.36 (±3.29)	50.46 (±4.84)	17.2 (±1.12)	17.1 (±1.36)	253.4 (±45.8)	259.8 (±68.8)
20 mg lips/day	7.38 (±0.88)	7.41 (±0.73)	50.82 (±4.2)	49.82 (±5.12)	16.97 (±1.76)	17.25 (±1.43)	261.0 (±42.1)	272.0 (±23.4)
50 mg/day	7.08 (±0.79)	6.74 (±1.35)	48.25 (±7.11)	44.0 (±7.45)	16.32 (±2.27)	14.75 (±2.45)	387.5 (±42.48)	427.0 (±53.35)

	Day 0 Glucose (mg/dL)	Day 30 Glucose (mg/dL)	Day 0 Creatinine (mg/dL)	Day 30 Creatinine (mg/dL)	Day 0 BUN (mg/dL)	Day 30 BUN (mg/dL)
Placebo	98.0 (±7.53)	105.5 (±9.25)	1.25 (±0.19)	1.28 (±0.22)	13.0 (±4.24)	14.5 (±4.1)
20 mg/day	103.8 (±14.24)	102.6 (±7.89)	1.34 (±0.38)	1.34 (±0.34)	18.6 (±6.22)	18.6 (±4.39)
20 mg lips/day	119.5 (±32.1)	102.25 (±8.31)	1.125 (±0.17)	1.175 (±0.377)	15.20 (±6.16)	15.0 (±8.32)
50 mg/day	107.25 (±6.75)	114.0 (±10.23)	1.375 (±0.35)	1.5 (±0.42)	21.25 (±4.5)	16.5 (±0.7)

	Day 0 Albumin (G/dL)	Day 30 Albumin (G/dL)	Day 0 Globulin (G/dL)	Day 30 Globulin (G/dL)	Day 0 A:G ratio	Day 30 A:G ratio
Placebo	3.225 (±0.41)	3.4 (±0.38)	3.62 (±0.52)	3.65 (±0.42)	0.9 (±0.12)	0.93 (±0.1)
20 mg/day	3.08 (±0.14)	3.3 (±0.24)	3.94 (±0.16)	3.7 (±0.44)	0.82 (±0.18)	0.92 (±0.16)
20 mg lips/day	3.52 (±0.34)	3.52 (±0.18)	3.575 (±0.36)	3.425 (±0.46)	1.0 (±0.14)	1.05 (±0.13)
50 mg/day	3.375 (±0.377)	3.5 (±0.075)	4.05 (±0.33)	4.05 (±0.22)	0.825 (±0.15)	0.8 (±0.1)

	Day 0 ALT (U/L)	Day 30 ALT (U/L)	Day 0 ALKP (U/L)	Day 30 ALKP (U/L)
Placebo	52.0 (±19.7)	63.25 (±15.65)	76.5 (±15.2)	81.2 (±23.5)
20 mg/day	109.2 (±78.7)	85.2 (±54.5)	86.4 (±59.7)	95.2 (±67.8)
20 mg lips/day	128.6 (±86.28)	124.6 (±90.25)	106.25 (±44.9)	147.5 (±46.4)
50 mg/day	83.25 (±29.57)	79.5 (±40.79)	129.9 (±21.2)	138.5 (±25.4)

ALKP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CBC, complete blood count; HCT, hematocrit; RBC, red blood cells; WBC, white blood cells.

CBD after oral administration of the liposomal formulation as compared to the naked isolate.

5. Conclusions

In summary, we demonstrate here that the widely available supplement CBD exerts robust and quantifiable anti-inflammatory properties in experimental systems. These experimental results were translatable in a randomized, double-blind, placebo-controlled trial in a spontaneous canine model of OA. In this assessment, administration of liposomally encapsulated or high-dose naked CBD (but not low-dose naked CBD or placebo) was associated with significant improvements to quality of life as quantitated by both owner and veterinarian. The results suggest that clinical studies in humans may be warranted in a variety of different etiologies and disease stages of arthritis.

Conflict of interest statement

Institutional policy requires W.K. Decker, M.M. Halpert, and V. Konduri to declare their ownership stakes in Diakonos Research, Ltd, an unrelated immuno-oncology company. In addition, M.M. Halpert is a paid scientific advisor for Medterra CBD. The remaining authors have no conflicts of interest to declare.

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Author contributions: C.D. Verrico wrote the article and analyzed data. S. Wesson designed and performed experiments, analyzed data, and contributed critical research tools. V. Konduri analyzed data. C.J. Hofferek performed experiments. J. Vazquez-Perez performed experiments. E. Blair designed and performed experiments, analyzed data, and contributed critical research tools. K. Dunner analyzed data. P. Salimpour analyzed data and provided critical research tools. W.K. Decker analyzed data, wrote the article, and provided critical research tools. M.M. Halpert designed and performed experiments, analyzed data, wrote the article, and provided critical research tools.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B3.

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