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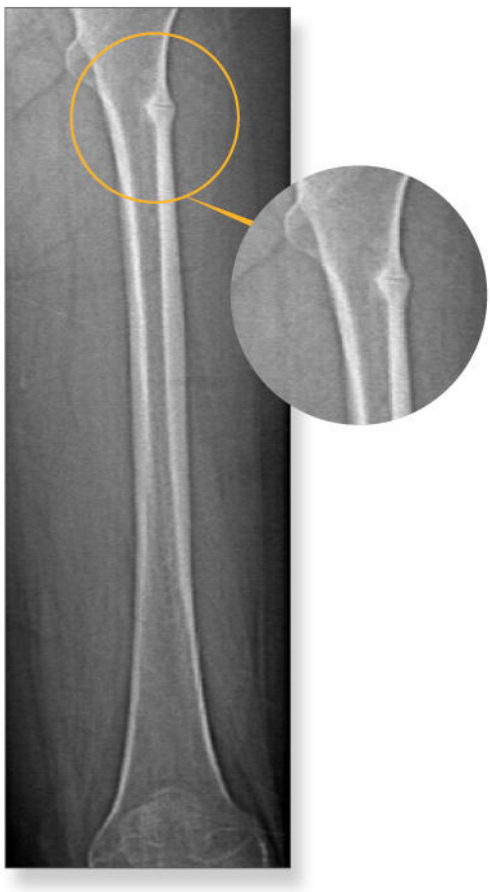

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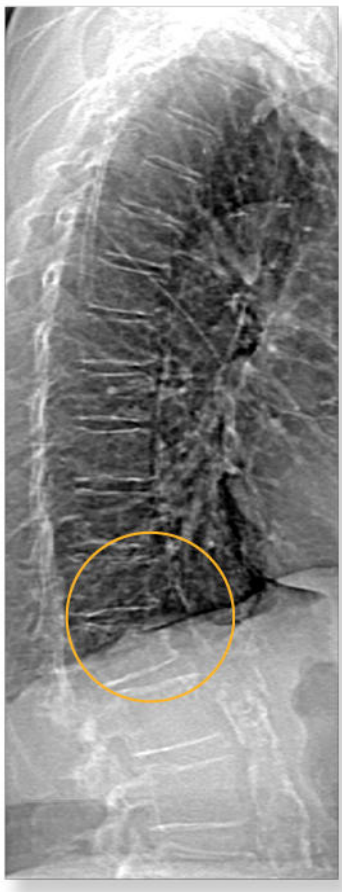

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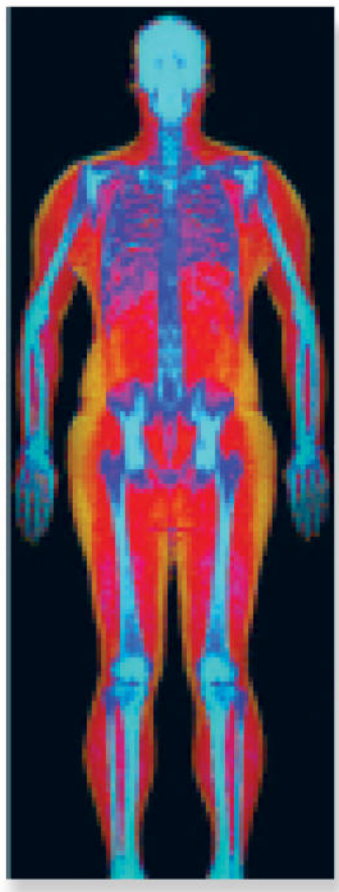

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ORIGINAL ARTICLE

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Cannabidiol, a Major Non-Psychotropic Cannabis Constituent Enhances Fracture Healing and Stimulates Lysyl Hydroxylase Activity in Osteoblasts

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ABSTRACT

Cannabinoid ligands regulate bone mass, but skeletal effects of cannabis (marijuana and hashish) have not been reported. Bone fractures are highly prevalent, involving prolonged immobilization and discomfort. Here we report that the major non-psychoactive cannabis constituent, cannabidiol (CBD), enhances the biomechanical properties of healing rat mid-femoral fractures. The maximal load and work-to-failure, but not the stiffness, of femurs from rats given a mixture of CBD and Δ^9-tetrahydrocannabinol (THC) for 8 weeks were markedly increased by CBD. This effect is not shared by THC (the psychoactive component of cannabis), but THC potentiates the CBD stimulated work-to-failure at 6 weeks postfracture followed by attenuation of the CBD effect at 8 weeks. Using micro–computed tomography (μCT), the fracture callus size was transiently reduced by either CBD or THC 4 weeks after fracture but reached control level after 6 and 8 weeks. The callus material density was unaffected by CBD and/or THC. By contrast, CBD stimulated mRNA expression of *Plod1* in primary osteoblast cultures, encoding an enzyme that catalyzes lysine hydroxylation, which is in turn involved in collagen crosslinking and stabilization. Using Fourier transform infrared (FTIR) spectroscopy we confirmed the increase in collagen crosslink ratio by CBD, which is likely to contribute to the improved biomechanical properties of the fracture callus. Taken together, these data show that CBD leads to improvement in fracture healing and demonstrate the critical mechanical role of collagen crosslinking enzymes. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: FRACTURE HEALING; COLLAGEN CROSSLINKING; LYSYL HYDROXYLASE; CANNABIDIOL; μCT; FTIR

Introduction

Since its discovery almost a decade ago, the skeletal cannabinoid system has attracted substantial attention. Although cannabinoid ligands attenuate and rescue ovariectomy-induced bone loss,[1] the effect of cannabinoids on fracture healing has not been reported. Because of the high incidence of both cannabis use and bone fractures it is likely that many fracture patients consume cannabis, which may have beneficial or adverse effects on the healing process. Cannabis contains a very high number of chemical entities with different biological activities.[2] The major constituents of cannabis, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), have been characterized for a wide range of activities in human and animal studies. Furthermore, they exhibit, individually or jointly, most of the effects attributed to whole cannabis preparations. THC is by far the main psychoactive ingredient of cannabis. It also has orexigenic, analgesic, and antiemetic effects. By contrast CBD has no psychoactivity, and is primarily anti-inflammatory (reviewed in Kogan and Mechoulam[3]). Studies carried out in the past decade suggest that a mixture of equal amounts of THC and CBD may be advantageous for the treatment of pain and

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*YG and IB co-directed the project.
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multiple sclerotic spasticity.[4–6] Given the key role of THC and CBD in cannabinoid science and medicine, and because the composition of whole cannabis preparations depends on factors such as genetic strain and cultivating conditions of the cannabis plant as well as the method of preparation,[7] the present study has assessed the possible role of purified THC and CBD in fracture healing.

Materials and Methods

Study design

The study consists of three major experiments. In experiment 1 we tested the effects of individually administered THC or CBD on the structural and mechanical properties of fracture healing. In experiment 2 we analyzed the effect of a mixture of equal amounts of CBD and THC on the mechanical properties of the fracture callus. In both experiments 1 and 2, the molecular composition of the specimens after 8 weeks was assessed using Fourier transform infrared (FTIR) spectroscopy. In experiment 3 we measured the effect of THC and CBD on the expression of osteoblastic enzymes that catalyze collagen crosslinking.

Animals and standard fracturing

Closed, unilateral, mid-diaphyseal femoral fractures were performed in male Sprague Dawley rats (Harlan, Rehovot, Israel) weighing 300 g on average at the time of fracture. The procedure, as described,[8] involves using a 1.1-mm-diameter intramedullary pin (Sulzer Orthopaedics Ltd., Baar, Switzerland) for prefracture fixation. Fracture standardization was confirmed radiographically immediately following the surgery and animals with nonstandard fractures were euthanized and excluded. In experiment 1, the animals were randomly divided into three treatment groups, receiving 5 mg/kg/day THC, CBD, or ethanol/emulphor/saline vehicle (VEH) intraperitoneally, commencing immediately after the fracturing surgery. In experiment 2, the animals were randomly divided into two treatment groups, receiving a mixture of equal amounts of THC and CBD (5 mg/kg/day each) or VEH. In experiment 1, the fractured femur was analyzed ex vivo 2, 4, 6, and 8 weeks postoperatively by micro–computed tomography (μCT), mechanical testing. Animals in experiment 2 were evaluated biomechanically. In both experiments, the 8-week groups were examined with FTIR spectroscopy.

At euthanasia, the fractured femurs were separated and transferred for 48 hours to phosphate-buffered formalin and then kept in 70% ethanol as reported.[9] The experimental protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, The Hebrew University of Jerusalem.

μCT analysis

Qualitative and quantitative analysis on femurs with radiographically standard fractures was carried out as described (Fig. 1*A*) (Gabet and colleagues[10]) using a desktop system (μCT 40; Scanco Medical AG, Brüttisellen, Switzerland) at 15-μm nominal resolution. This was preceded by pin removal. Specimens in which the pins could not be removed without damaging the callus were discarded. To delineate the callus periphery, the specimens were coated with a thin layer of fine dental varnish (Copalite Dental Cavity Varnish; Cooley & Cooley Ltd., Houston, TX, USA) containing 0.1% barium sulfate (Shanghai Yuantai Chemical Products, China). For the mineralized callus analysis, the cortex and radio-opaque label were digitally extracted using tracing and

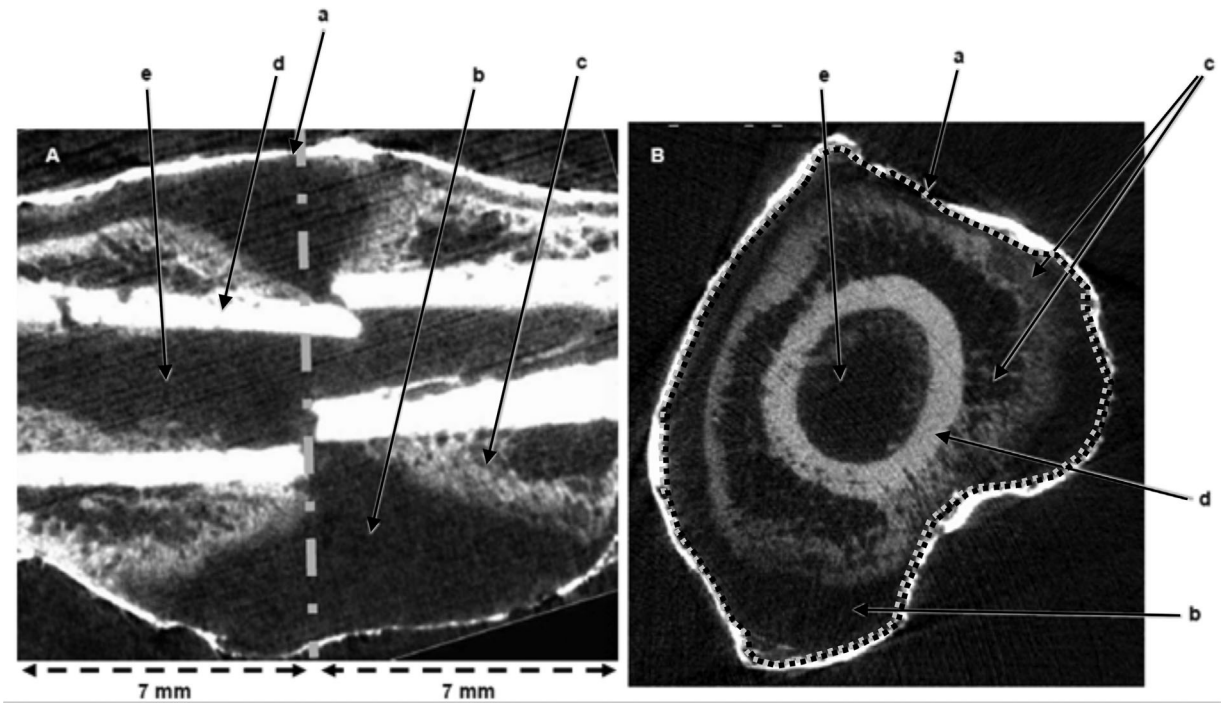

Fig. 1. Two-dimensional μCT images of callus 4 weeks after fracturing. (*A*) Mid-longitudinal plane. (*B*) Cross-sectional plane. Dashed line = fracture line; a = barium sulfate containing varnish delineating the outer callus border; b = unmineralized callus; c = mineralized callus; d = preexisting femoral diaphyseal cortex; e = medullary cavity.

multi-threshold segmentation (Fig. 1*A, B*). The tissue material density (degree of mineralization) of the mineralized callus was determined using calibrated measurement records as reported.[11] The number of calluses included in the analysis are shown in Supplemental Table 1.[11]

Biomechanical testing

Following μCT image acquisition, the varnish was removed with acetone and the specimens rehydrated in PBS overnight.[9] The femurs were subjected to four-point bending using a Zwick materials testing machine (Zwick Z005; Zwick, Ulm, Germany) using a 1-N static preload. All other parameters were prepared as described.[12] Tests were carried out to failure at 5 mm/min. Work-to-failure was calculated as the area under the force–displacement curve up to the maximum load. Stiffness was defined as the slope of the linear part of the curve prior to yielding. Specimens with failure plane outside the callus were discarded. Supplemental Table 1 provides the number of femurs per group included in the biomechanical report.

Primary osteoblast culture

Newborn mouse calvarial osteoblasts (NeMCOs) were prepared from 5-day-old mice by successive collagenase digestion[13] and seeded at 1600 cells/cm^2 in αMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum. The cells were grown for 4 days at 37°C and 5% CO_2. THC or CBD were added when the culture confluence reached ~80%. RNA was extracted 24 hours later.

Real-time RT-PCR

RNA was isolated using TRI reagent kit (Molecular Research Center Inc, Cincinnati, OH, USA), followed by 1-bromo-3-chloropropane extraction and isopropyl precipitation. The mRNA expression of genes catalyzing collagen crosslinking was analyzed by real-time RT-PCR using Roche Designer Assay (Roche Diagnostica, Mannheim, Germany). Data were normalized to GAPDH. Assay ID of mouse genes used is shown in Table 1. Differences between treatments higher than twofold were considered significant (contingent on statistical significance).

FTIR spectroscopy

Specimens from the 8 weeks postfracture groups in experiments 1 and 2 were prepared for FTIR spectroscopy through dehydration and embedding in epoxy. Bone samples were sectioned longitudinally through the femur and callus. Longitudinal centerpiece sections of 3 μm were cut and placed on BaF_2 windows. The measurements were done with a Bruker 66V FTIR spectrometer coupled to a Bruker Hyperion 3000 IR microscope using a focal plane array (FPA) detector (128 × 128) at Beamline D7 (MAX-III, Max-IV Laboratory, Lund, Sweden). The FPA detector consists of 128 × 128 elements and covers an area of 340 × 340 μm^2. Based on the light microscope image, two areas of callus bone and one cortex area were chosen for analysis using 128 repeated scans and a spectral resolution of 4 cm^{-1}. The infrared spectra were collected at the range of 800 to 3800 cm^{-1}. Mineral-to-matrix ratio (phosphate peak [900 to 1200 cm^{-1}]/amide I peak [1585 to 1720 cm^{-1}]), [14] acid phosphate substitution (APS; 1127/1096 cm^{-1}), [15] and collagen crosslink ratio (ratio of mature to immature collagen crosslinks; ie, 1660/1690 cm^{-1})[16] were determined after removing the spectrum of the epoxy employing custom-made MATLAB code (MATLAB, v 7.6.0; MathWorks Inc. Natick, MA, USA).[17]

Table 1. Real-Time RT-PCR Assay ID for Collagen Crosslinking Genes

Gene symbol	Gene name	Assay ID
Gapdh	Glyceraldehyde 3-phophate dehydrogenase	307884
Lox	Lysyl oxidase	316750
Loxl1	Lysyl oxidase-like 1	316755
Loxl2	Lysyl oxidase-like 2	316748
Loxl3	Lysyl oxidase-like 3	316741
Loxl4	Lysyl oxidase-like 4	316753
Plod1	Lysyl hydroxylase 1	316746
Plod2	Lysyl hydroxylase 2	316757
Plod3	Lysyl hydroxylase 3	316743
P4ha1	Prolyl 4-hydroxylase alpha polypeptide I	316745

Statistical analysis

Differences between time/treatment groups were analyzed by ANOVA. When significant differences were indicated by ANOVA, group means were compared using the Fisher-least significant difference (LSD) test for pairwise comparisons.

Results

In most cases fractures heal through the formation of a callus that provides initial bridging over the fracture gap[12] (Fig. 1*A*). In experiment 1, μCT analysis revealed that 4 weeks after fracture, the callus size was approximately 26% smaller in rats administered either THC or CBD compared with animals administered VEH only. This decrease included both the mineralized and the unmineralized callus but did not persist in the 6-week and 8-week time points (Fig. 2), suggesting a transient enhancement of the healing process.

Four-point bending of the same femoral specimens showed that CBD markedly enhanced the biomechanical properties of the healing femurs after 8 weeks, when most of the cartilaginous callus was replaced by bone (Figs. 2 and 3). This enhancement consists of respective ~35% and ~50% increases in the maximal force and work-to-failure with no significant effect on stiffness, and was not shared by THC. If any, THC reduced the stiffness compared to CBD (Fig. 3). Importantly, CBD did not influence the ultimate displacement at failure (Fig. 3), which indicates that the increased work-to-failure (ie, toughness) is due entirely to increased strength (maximum force). Neither the VEH-treated nor THC-treated fractures reach the same levels of strength. Also, the CBD or THC treatments did not affect body weight (data not shown), a factor that could potentially influence osteogenesis.[18]

A combination of THC and CBD is not only a hallmark of cannabis, but a mixture of equal amounts of these ingredients has been recommended for therapeutic use.[6] Hence, experiment 2 tested their combined effect on the biomechanics of the healing bone. In this experiment we avoided structural analysis of the callus, because in experiment 1 the microstructural changes did not survive the entire follow-up period and failed to correlate with the biomechanical results. The addition of THC increased the maximal force slightly more than CBD alone (compare Figs. 3 and 4) as well as the stiffness. However, it

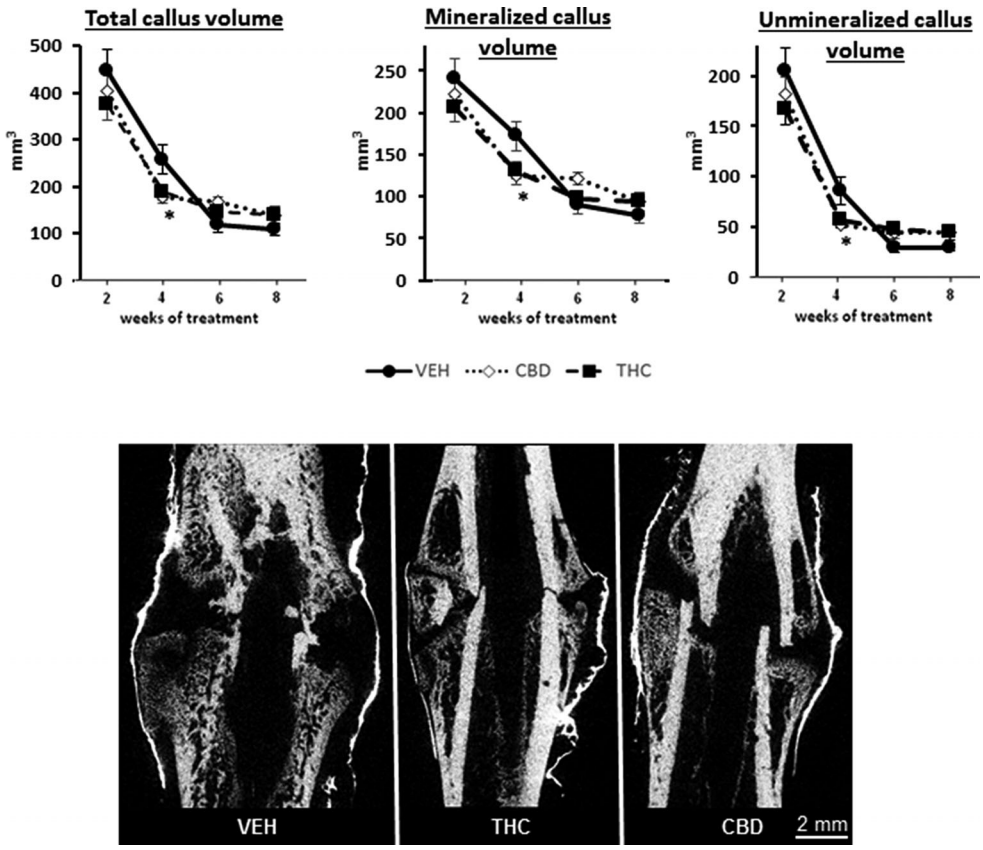

Fig. 2. Effect of individual cannabis ingredients on fracture callus size. (Top) Quantitative 3D μCT measurements. Quantitative data are mean ± SE obtained in 5 to 13 rats per condition. *$p < 0.05$ versus VEH-treated rats. (Bottom) Representative 2D μCT mid-callus images obtained from rats with median values of total callus volume 4 weeks after fracture. VEH = vehicle; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol.

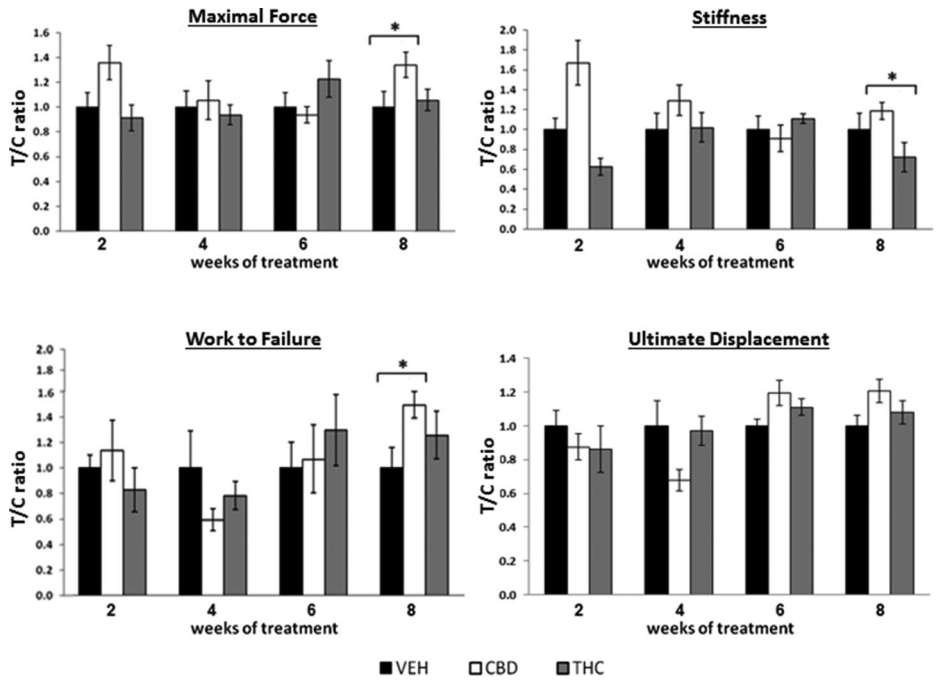

Fig. 3. Effect of individual cannabis ingredients on fracture callus biomechanical properties. Data are mean ± SE obtained in 5 to 12 rats per condition (except $n = 3$ in the 6-week THC group). *$p < 0.05$. VEH = vehicle; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol; T/C ratio = THC/CBD ratio.

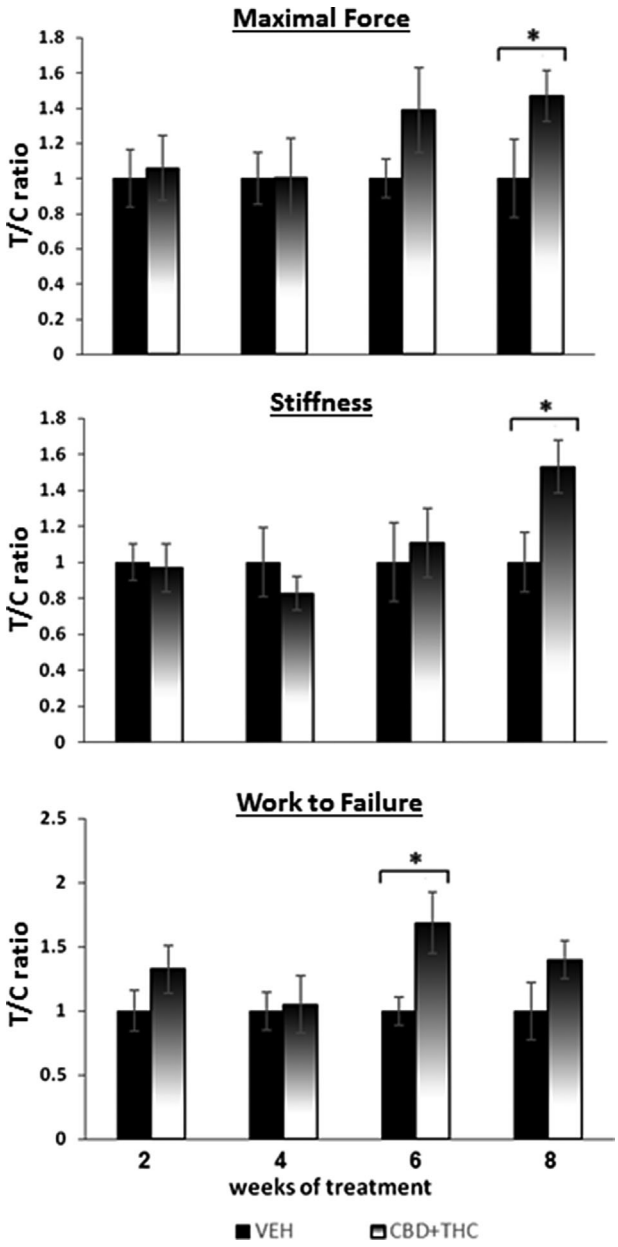

Fig. 4. Effect of THC-CBD mixture on fracture callus biomechanical properties. Data are mean ± SE obtained in 8 to 13 rats per condition. *$p < 0.05$. VEH = vehicle; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol; T/C ratio = THC/CBD ratio.

eliminated the CBD-induced increase in work-to-failure at 8 weeks (Fig. 4). Nevertheless it had a marked stimulatory effect on this parameter at 6 weeks (Fig. 4).

Because the CBD-induced strength enhancement of the healing fracture could not be explained on structural volumetric grounds, we assessed the effect of CBD and THC on the bone material properties. Referring back to the μCT scans from experiment 1, we analyzed the material density of the mineralized matrix. However, this analysis showed no differences between VEH-treated, THC-treated, and CBD-treated rats (Fig. 5). Therefore, the improvement of callus strength and unaltered material density suggested that the effects of CBD or

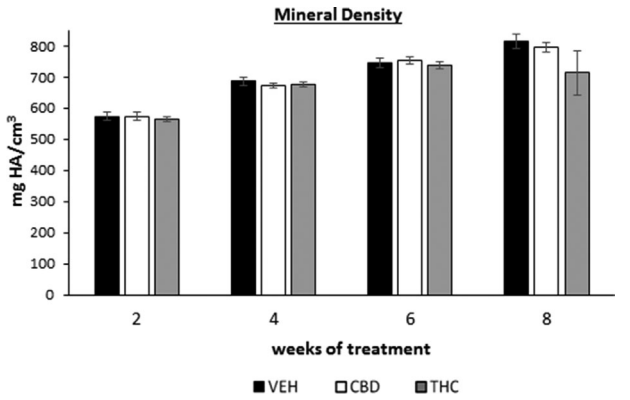

Fig. 5. Cannabis components do not affect mineral density. THC and CBD administered individually, 5 mg/kg/day each. Data are mean ± SE obtained in 5 to 13 rats per condition. VEH = vehicle; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol; HA = hydroxyapatite.

THC involve changes in the organic matrix, such as the degree of collagen crosslinking, known to affect flexibility and toughness of bone.[19] Indeed, in primary osteoblast cultures, CBD selectively stimulated the mRNA levels of the lysyl hydroxylase PLOD1 (experiment 3; Fig. 6, Supplemental Fig. 1), an enzyme that hydroxylases lysine residues intracellularly, the first step in pyridinoline and pyrrololine crosslink formation.[20] This stimulation occurred at 1×10^{-10} M to 1×10^{-12} M CBD concentrations but was reversed at higher concentrations, an effect typical for cannabinoid ligands (Fig. 6). THC stimulated the osteoblastic mRNA levels of the lysyl hydroxylase PLOD2; however, this stimulation was biologically significant only at 1×10^{-8} M concentration (Fig. 6). The effect of THC on the other mRNA transcripts was below the twofold threshold determined for biological significance (Supplemental Fig. 2).

To further demonstrate that CBD enhances the mechanical properties of the callus by altering the molecular composition of the tissue, FTIR spectroscopy measurements were performed in the callus tissue of the same specimens that were tested in four-point bending (experiments 1 and 2, 8-week groups). The newly formed bone tissue was compared between the groups and to the preexisting cortical bone (Fig. 7*A*). As expected, the mineral-to-matrix ratio was lower and the acid phosphate substitution (APS) was higher in the callus tissue compared to the cortex over all treatment groups (Supplemental Table 2). When comparing the callus tissue between the groups, no differences were found in the degree of mineralization, which substantiates our μCT measurements (Supplemental Table 2, Supplemental Fig. 5). Importantly, we found that the collagen crosslink ratio was significantly higher in the CBD compared to the VEH group (Fig. 7*C*). However, both the THC and the combined CDB+THC groups did not differ significantly from their respective controls. Additionally, the APS into the hydroxyapatite (which negatively correlates with tissue maturity[15]) was higher in the THC group compared to VEH (Supplemental Table 2).

Discussion

Bone fractures of all types are among the most common injuries affecting millions of individuals of all ages and both genders worldwide. Healing of bone fractures is affected by multiple

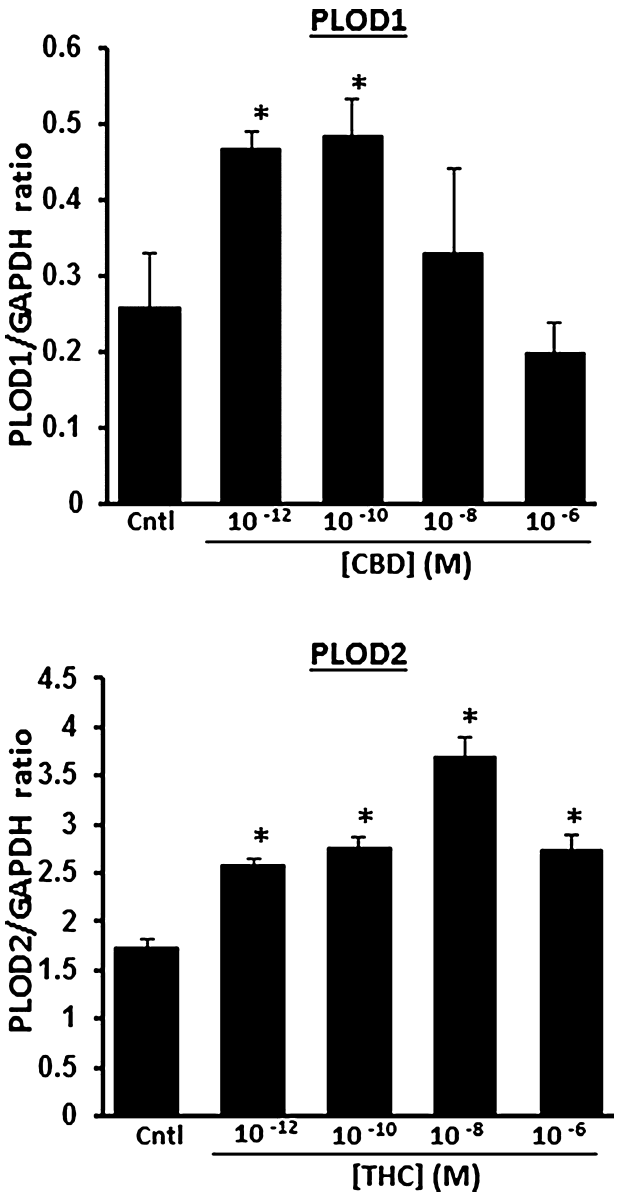

Fig. 6. Cannabis components modulate mRNA expression of lysyl hydroxylases in osteoblasts. Data are mean ± SE obtained in quadruplicate cultures per condition. *$p < 0.05$ versus Cntl. Cntl = control; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol.

environmental, systemic, local, and pharmacological factors. Most relevant to our study is the high fracture incidence among young adults, who are also common drug users, especially of marijuana and hashish. Surprisingly, the amount of experimental and clinical information related to drug abuse and fracture healing is scanty and focuses mainly on the adverse effects of nicotine and alcohol.[21] The combined 3D quantitative μCT analysis and biomechanical testing in this study demonstrates a specific CBD-induced enhancement of the callus strength and toughness, most probably through an effect on osteoblastic bone formation, because this stimulation was apparent only during the late phases of healing, when osteogenesis, rather than chondrogenesis prevails. We further demonstrate the probability that the CBD-induced enhancement is mediated by enzymes catalyzing collagen crosslinking, implicating for the first time a role of these enzymes in fracture healing.

Bone cells express cannabinoid receptors and endocannabinoid metabolizing enzymes.[22,23] Cannabinoid receptors are also expressed by skeletal sympathetic nerve terminals[24] and cannabinoids have an important role in the regulation of skeletal remodeling and mass.[1] With the recent progress in approving cannabis for medical indications and even recreational use,[25] it is important to assess its possible beneficial and adverse effects on skeletal healing. Many fractures heal by a process known as endochondral ossification. In this process initial bridging across the fracture gap is made by a cartilaginous callus that mineralizes, and is subsequently resorbed and replaced by a bony callus. The bony callus is further remodeled to form mature bone that is similar to the prefracture tissue.[26] Interestingly, a single experimental study on the effect of marijuana reported inhibition of the early stages (30 days) of bone healing around endo-osseous implants.[27] By contrast, our results suggest that the CBD-induced stimulation of fracture healing occurs during the later phases of healing (after 6 weeks). Also, peri-implant ossification is intramembranous, the early phases of which, eg, blood clot formation and/or its organization through primary bone formation,[28] may be susceptible to the deleterious effects of cannabis. During fracture healing, our data suggests that the initial cartilaginous phase, absent in intramembranous ossification, protects the process from such effects.

Here, either THC or CBD reduced the callus size 4 weeks after fracture. This decrease similarly affected the unmineralized and the mineralized constituents of the callus and may have resulted from transient enhancement of the cartilaginous callus resorption followed by uninterrupted bone formation. Indeed, at 6 and 8 weeks, when most of the callus is bony, neither CBD nor THC affects its size. CBD does not target CB1 or CB2, the classical cannabinoid receptors. Hence, we were surprised that CBD but not THC markedly enhanced the callus maximum force and work-to-failure, or toughness, specifically 8 weeks after fracturing, a time point representing primarily bony bridging of the fracture gap. CBD has been suggested as a moderator of many of the effects historically assigned to THC.[29–32] Hence, CBD and THC may counteract each other. Indeed, adding to the preparation an equal amount of THC considerably modified the effect of CBD, significantly increasing the stiffness at 8 weeks, increasing the toughness at 6 weeks, and attenuating it at 8 weeks. This effect of THC in combination with CBD may be clinically meaningful in terms of the healing rate.

The enhanced callus mechanical properties at the 6-week and 8-week time points, together with the absence of structural differences between the cannabinoid-treated and VEH-treated animals at these time points, suggests that CBD and/or THC affects the material properties of the newly formed bone bridge. Bone strength is proportional to its mineral density.[19] However, our results, although showing a temporal increase in the callus mineral density, do not demonstrate treatment-related differences.

Another factor that may affect the bone mechanical properties is the quality of the collagenous matrix. Although previously unexplored in the context of fracture healing, one factor that may affect the matrix is the degree of the collagen intramolecular and intermolecular crosslinking,[33] which is in turn regulated through expression of the enzymes catalyzing this process, namely, 3 lysyl hydroxylases, 5 lysyl oxidases, and prolyl hydroxylase. The lysyl hydroxylases act intracellularly,[34–36] followed by extracellular oxidation of lysine and

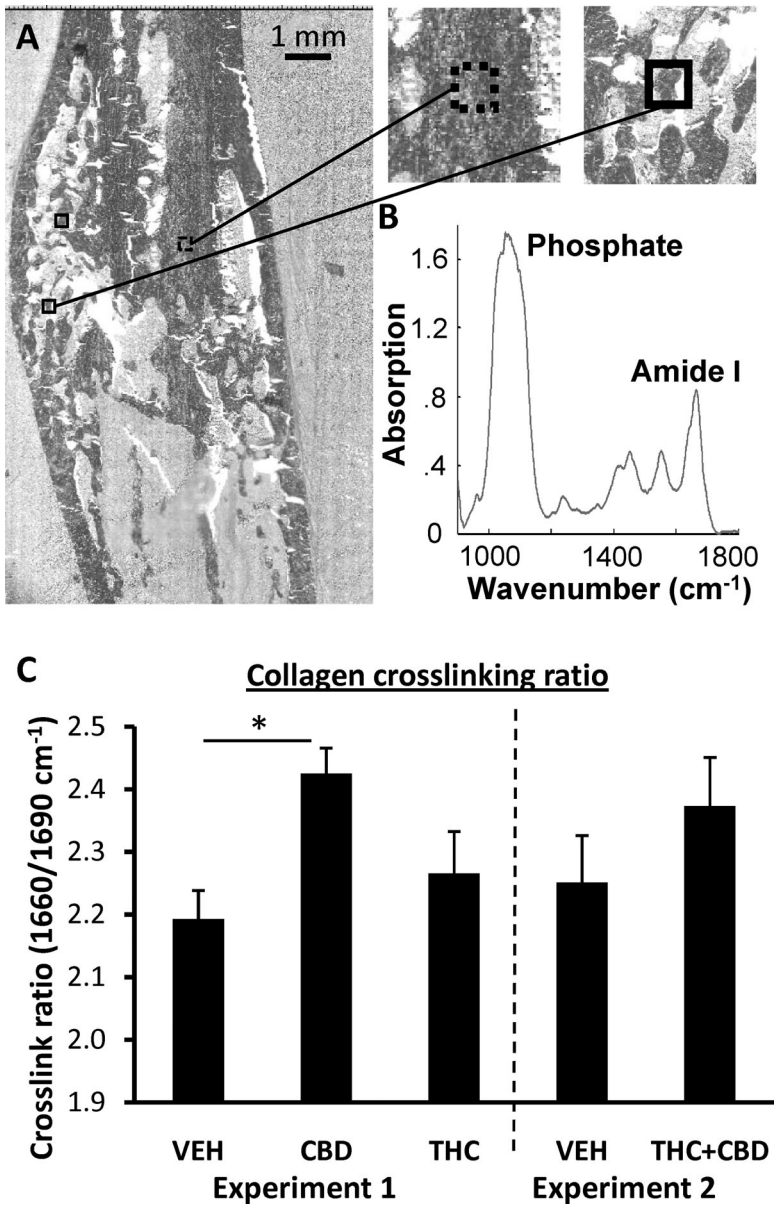

Fig. 7. Cannabis components modulate collagen crosslinking in the fracture callus. (*A*) Longitudinal section for FTIR spectroscopy indicating the two measurement regions of callus tissue (solid lines) and one area of cortex tissue (dashed lines). One region per tissue type is shown at higher magnification (insets). (*B*) Representative bone spectra from the callus tissue showing typical measurement of acid phosphate substitution into the hydroxyapatite. (*C*) Effect of cannabis components on the collagen cross-linking ratio determined by FTIR spectroscopy. Data are mean ± SE obtained in 6 to 8 rats per group. $^*p < 0.05$ between the indicated groups. FTIR = Fourier transform infrared spectroscopy; VEH = vehicle; CBD = cannabidiol; THC = Δ^9-tetrahydrocannabinol.

hydroxylysine residues by lysyl oxydases.[20] Indeed, we show enhancement by CBD of the osteoblastic expression of lysyl hydroxylase 1 (PLOD1), one of the few collagen crosslinking enzymes reported to be associated with bone quality.[37,38] It is likely that this stimulation is specific, because CBD did not affect the mRNA levels of any of the other collagen-crosslinking enzymes. This increased expression of PLOD1 is in line with the increased collagen crosslink ratio in the CBD group measured by FTIR. The collagen crosslink ratio is an estimate of collagen maturity,[16] which is important to bone quality and tissue mechanical properties[39–41]; this may therefore explain the enhanced mechanical properties found in the CDB group. By comparison, mRNA transcripts for PLOD1 were somewhat decreased by THC. This inhibition may offer some explanation for the milder stimulation of both the mechanical strength and collagen crosslink ratio in the THC+CBD as compared to the CBD groups. The only biologically significant effect of THC was the stimulation of PLOD2 mRNA expression. This effect peaked at 1×10^{-8} M concentration, representing 100-fold to 1000-fold lower efficacy and a narrower effective dose window compared to CBD. Furthermore, although PLOD1, targeted by CBD, is reportedly relevant to bone biomechanics, we found no

evidence related to a role of PLOD2 in determining bone quality. Indeed, we found increased APS values in the THC group, which indicates a generally slower tissue maturation.[15]

Implicating PLOD1 in the mechanism of action of CBD may have far-reaching significances, beyond the improvement of fracture healing, in instances such as Ehlers-Danlos syndrome,[42–44] bronchopulmonary dysplasia,[45] bicuspid aortic wall–associated aneurisms,[46] and cancer metastases.[47] Despite of a handful of studies, the mechanisms involved in the CBD actions are not well understood.[48] These results suggest a novel mechanism involving the collagenous extracellular matrix, which may have therapeutic uses both in bone and in extraskeletal tissues.

Modeling cannabis as a mixture of equal amounts of THC and CBD has gained considerable clinical attention as a therapy to relieve neuropathic pain.[49] THC appears to be the main active ingredient in this mixture, with CBD acting to diminish its psychotropic adverse effects.[32] Here we face a different scenario, in which CBD alone is sufficiently effective in enhancing fracture healing, and the combined preparation is not advantageous. Multiple experimental and clinical trials have portrayed CBD as a safe agent,[50] suggesting further studies in humans to assess its usefulness for improving fracture healing. Perhaps more importantly, given the paucity of reports dealing with collagen crosslinking enzymes as therapeutic targets for improving bone mechanical properties, our results suggest further studies on the role of collagen crosslinking in fracture healing and quality of the postfracture bone.

Disclosures

The authors state that they have no conflicts of interest.

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Authors' roles: Study design: IB, YG, NMK, and EM. Study conduct: IB, NMK, SFB, and EM. Data collection: NMK, AB, EW, BR, AB, KSS, RS, AVVE, MAN, SFB, and NM. Data analysis: AB, YG, NMK, BR, KSS, RS, AVVE, NM, and HI. Data interpretation: IB, YG, NMK, RMe, RMu, EM, KSS, and HI. Drafting manuscript: IB and NMK. Revising manuscript content: YG, KSS, RMe, RMu, EM, and HI. Approving final version of manuscript: NMK, AB, EW, BR, AB, KSS, AVVE, RS, MAN, SFB, NM, HI, RMe, RMu, EM, YG, and IB. IB takes responsibility for the integrity of the data analysis.

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