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Induction of apoptosis by cannabidiol and its main metabolites in human Leydig cells

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Abstract

Cannabidiol (CBD) is one of the most prevalent and abundant cannabinoids extracted from the plant *Cannabis sativa*. CBD has been reported to induce male reproductive toxicity in animal models. In this study, we examined the effects of CBD and its main metabolites, 7-carboxy-CBD and 7-hydroxy-CBD, on primary human Leydig cells, which play a crucial role in male reproductive health. Our results showed that CBD, at concentrations below the Bayesian benchmark dose $(BMD)_{50}$, inhibited the growth of human Leydig cells by arresting the cell cycle at G1/S transition, disrupting cell cycle regulators, and decreasing DNA synthesis. Concentration-response transcriptomic profiling identified that apoptosis was one of the top biological processes significantly affected by treatment with CBD for 24 h. The occurrence of apoptosis was confirmed by increased activation of caspase 3/7 and an increased proportion of Annexin V and propidium iodide (PI) positive cells. Similar to CBD, both 7-carboxy-CBD and 7-hydroxy-CBD decreased cell viability and induced apoptosis after treatment for 24 h. 7-Hydroxy-CBD and 7-carboxy-CBD showed lower cytotoxicity than CBD, and 7-carboxy-CBD had the lowest cytotoxicity among the three compounds. Our findings revealed that CBD and its main metabolites can cause adverse effects on primary human Leydig cells.

Keywords

Cannabidiol; 7-Carboxy-CBD; 7-Hydroxy-CBD; Male reproductive toxicity; Leydig cells; Apoptosis; Cell cycle arrest; DNA synthesis

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Compliance with ethical standards

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Introduction

Cannabidiol (CBD) is a major chemical component first isolated in 1940 from the plant *Cannabis sativa* (Adams et al. 1940; Ujvary and Hanus 2016). CBD is thought to be non-intoxicating and has been demonstrated to have anti-epileptic effects (Devinsky et al. 2014; Hill et al. 2012). The U.S. Food and Drug Administration (FDA) approved Epidiolex®, a CBD-based oral solution drug, to treat seizures associated with two rare and severe forms of childhood-onset epileptic disorders (Lennox-Gastaut syndrome and Dravet syndrome) and tuberous sclerosis complex. Due to the increasing public interest, sales of CBD products continue to increase annually in the U.S. (Smith et al. 2021). According to a recent survey, more than half of the participants in the United States (n = 30,288) and Canada (n = 15,042) reported using CBD products (Goodman et al. 2022). However, it is important to note that some CBD products in the market claim unproven beneficial effects, and their quality is unknown (FDA 2020).

CBD has been found to have negative impacts on various aspects such as changes in organ wight, developmental and reproductive toxicities, neuronal development and fetal mortality, liver injury, immune system suppression, mutagenicity and genotoxicity, and interference with liver metabolizing enzymes and drug transport proteins (Gingrich et al. 2023). In particular, animal studies have raised concerns about the potential adverse effects of CBD on the male reproductive system. For example, CBD exposure caused a reduction in testes weight and fertilization rate, an inhibition of spermatogenesis, and changes in gonadal hormones in rhesus monkeys, mice, and rats (Carvalho et al. 2020; Carvalho et al. 2022; Carvalho et al. 2018; Dalterio and deRooij 1986; Rosenkrantz and Esber 1980). Additionally, while significant amounts of 7-carboxy-CBD and 7-hydroxy-CBD have been found in animals and humans (Schwotzer et al. 2023; Taylor et al. 2018; Ujvary and Hanus 2016), less is known about their toxicity profiles. Therefore, our study aims to evaluate the potential mechanisms of toxicity of CBD, 7-carboxy-CBD, and 7-hydroxy-CBD on the human male reproductive system.

Leydig and Sertoli cells are two main cell types in the testes that support sperm production and testicular function (Petersen et al. 2015); they could be potential targets for CBD's reproductive toxicity. Sertoli cells play an essential role in supporting testicular development and sperm production while Leydig cells are crucial to sexual determination and development, spermatogenesis, and the maintenance of secondary sexual features (Shima 2019). Using Sertoli cells, our previous studies have demonstrated that in both mouse and human Sertoli cells, CBD and its two metabolites inhibited cell proliferation and DNA synthesis; and CBD activated the p53 signaling pathway, which eventually led to senescence in primary human Sertoli cells (Li et al. 2023b; Li et al. 2022). However, whether CBD has a negative impact on Leydig cells has not been elucidated. It is important to study the cytotoxicity of CBD and its underlying mechanism in Leydig cells because dysfunction or loss of Leydig cells is often associated with spermatogenic dysfunction, leading to male infertility (de Kretser 2004).

CBD has been shown to induce apoptosis and inhibit cell proliferation in various immune cells as well as in multiple human malignant cell lines (Rieder et al. 2010; Shrivastava

et al. 2011; Valenti et al. 2022). While a CBD-induced apoptotic response could be beneficial in cancer cells, apoptotic cell death in non-cancerous cells may disrupt normal physiological functions. For example, apoptosis of immune cells exposed to CBD resulted in immunosuppression, which could be beneficial or detrimental depending on different patient conditions (Rieder et al. 2010).

Apoptosis is one of the most extensively studied programmed cell death pathways that can occur in response to various stimuli, including DNA damage, cellular stress, immune surveillance, developmental signals, infection, and exposure to toxins (Bertheloot et al. 2021; Carneiro and El-Deiry 2020). Apoptosis is triggered through cascade signaling pathways and subsequently leads to the activation of caspase 3/7, the executioners of apoptosis, eventually leading to cell death (Bertheloot et al. 2021). Before committing to apoptosis, cells can undergo cell cycle arrest to allow repair processes to take place. The cell cycle is regulated by a family of cyclin-dependent kinases (CDKs) that are controlled by the cyclins (Serrano et al. 1993). Two of the major cell cycle checkpoints are the G1/S checkpoint and the G2/M checkpoint (Swift and Golsteyn 2016). The G1/S checkpoint ensures cells do not replicate damaged DNA, whereas the G2/M checkpoint ensures the chromosomes are replicated accurately and the replicated DNA is undamaged before cells progress to the mitotic phase (Swift and Golsteyn 2016). Mitogenic signals promote CDK4/6 and cyclin D forming complexes that facilitate cells exiting from G1 phase and entering the S phase via activating downstream S-phase gene expression and activating cyclin E-CDK2 complex (Serrano et al. 1993; Sherr 1995). The cyclin E-CDK2 complexes then contribute to the irreversibility of the G1/S transition (Sherr and Roberts 1999).

In this study, we characterized the effects of CBD and its two main metabolites, 7-hydroxy CBD and 7-carboxy CBD, on primary human Leydig cells *in vitro*. We conducted RNA-sequencing analysis on CBD-treated primary human Leydig cells to facilitate our understanding on the molecular mechanisms of cytotoxicity of CBD on human male reproductive cells. The significance of this study lies in evaluating the potential male reproductive toxicity of CBD, 7-carboxy-CBD, and 7-hydroxy-CBD in human-relevant cell models and delving further into the underlying mechanisms.

Materials and Methods

Test chemicals

CBD (Batch # NQSS1951, purity 100%), 7-carboxy-CBD (Batch # BDG11603, purity 97.4%), and 7-hydroxy-CBD (Batch # BDG11596, purity 97.6%) were purchased from Purisys (Athens, GA). Dimethyl sulfoxide (DMSO, Catalog # D8418) was used as the vehicle control and purchased from MilliporeSigma (St. Louis, MO). We tested vehicle control did not cause any cytotoxic effect in human Leydig cells. All chemicals were dissolved in DMSO and stored as 1,000 × stock solution at −20 °C.

Cell culture and chemical treatments

Primary human Leydig cells (Catalog # 4510), isolated from a 25-year-old male donor, were purchased from ScienCell Research Laboratories (Carlsbad, CA). The identification of the

primary human Leydig cells was performed by the manufacturer using immunofluorescence staining for GATA-4 and CK-18, which are markers for Leydig cells. The cells were cultured in vessels pre-coated with poly-L-lysine (2 μg/cm^2, Catalog # P1274, MilliporeSigma) in Leydig cell medium (Catalog # 4511) supplemented with 5% FBS (Catalog # 0025), 1% Leydig cell growth supplement (Catalog # 4562), and 1% penicillin/streptomycin solution (Catalog # 0503) (all from ScienCell Research Laboratories). After thawing, the human Leydig cells were seeded at a density of 1.0 × 10^6 cells/dish (5,000 cells/cm^2) in 100 mm tissue culture dishes (Catalog # 430617, Corning, Glendale, AZ) and sub-cultured every three to four days at 95% confluency using 0.05% trypsin-EDTA solution (Catalog # 0183, ScienCell Research Laboratories) and trypsin neutralization solution (Catalog # 0113, ScienCell Research Laboratories) for up to 5 passages. The cells were cultured at 37°C with a sustained 5% CO_2 supply in a humidified incubator. Before treatment with the chemicals or vehicle (0.1% DMSO), the cells were seeded at a density of 1.7 × 10^4 cells/cm^2 and allowed to attach to the surface of the dishes or multi-well plates for 24 h.

Measurement of cell viability and cytotoxicity

Human Leydig cells were seeded at a density of 1.7 × 10^5 cells/well in 6-well plates (Catalog # 3506, Corning) and treated with 0.1% DMSO, 10–30 μM CBD, 60–140 μM 7-carboxy-CBD, or 10–30 μM 7-hydroxy-CBD for 24 h. Viable cell numbers were determined after staining with 0.4% Trypan blue (Catalog # 15250061, ThermoFisher Scientific, Waltham, MA) and counting with the Countess 3 Automated Cell Counter (Catalog # AMQAX2000, ThermoFisher Scientific). The relative cell viability was calculated by normalizing the viable cell numbers to those of DMSO controls. The Bayesian benchmark dose (BMD) method employs a set of eight continuous models to fit dose-response curves, representing a scientifically more advanced approach than traditional methods such as no-observable-adverse-effect-level (NOAEL) or lowest-observable-adverse-effect-level (LOAEL). BMD utilizes a wider range of dose-response data and enables a more comprehensive quantification of uncertainties associated with the data. We used BMD_{50} to determine the concentration of chemicals that induced a 50% relative cytotoxicity (Shao and Shapiro 2018). Model averaging was used to estimate BMD values to address model uncertainty. The lactate dehydrogenase (LDH) assay was used to assess cytotoxicity exactly as describe previously (Chen et al. 2018).

Cell cycle analysis

Human Leydig cells were seeded at a density of 1.7 × 10^5 cells/well in 6-well plates (Catalog # 3506, Corning). After incubating with 10–20 μM CBD or 0.1% DMSO for 24 h, cells were collected by trypsinization and centrifugation. Cell numbers for each sample were adjusted to 1.0 × 10^6 cells and cells were re-suspended in 300 μL of ice-cold 1 × DPBS buffer (Catalog # 0303, ScienCell Research Laboratories). To fix the samples, 700 μL of ice-cold 70% ethanol (Catalog # BP2818500, ThermoFisher Scientific) was added and the cells were incubated on ice for 1 h. After washing the cells with 1 × DPBS buffer, cells were re-suspended in 250 μL of 1 × DPBS containing RNase A (200 μg/mL, Qiagen, Valencia, CA) to digest RNA at 37°C for 1 h. Finally, 250 μL of 1 × DPBS containing 10 μg/mL propidium iodide (PI, Catalog # P1304MP, ThermoFisher Scientific) was added and the

samples were incubated in darkness overnight at 4°C. Data were acquired on a FACSCanto™ flow cytometer using FACSDiva™ software (BD Biosciences, San Jose, CA) and analyzed by FlowJo® software (Ashland, OR).

EdU proliferation assay

DNA synthesis activity is a commonly used parameter to evaluate cell proliferation. 5-Ethynyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine, can be incorporated into DNA and reflect the DNA synthesis activity (Buck et al. 2008). Human Leydig cells were seeded at a density of 1.7×10^5 cells/well in 6-well plates (Catalog # 3506, Corning) and exposed to 0.1% DMSO, 15 μM CBD, 80 μM 7-carboxy-CBD, or 15 μM 7-hydroxy-CBD for 24 h. After treatment, cells were harvested, and the number of cells was adjusted to 1×10^6 cells/sample. EdU staining and flow cytometric analysis were conducted as described previously (Li et al. 2022).

Western blot analysis

Human Leydig cells were seeded at a density of 1.0×10^6 cells/dish in 100 mm dishes (Catalog # 430617, Corning) and exposed to 10–15 μM CBD or 0.1% DMSO vehicle for 24 h. After treatment, cells were harvested by trypsinization and centrifugation. The cell pellets were lysed with ice-cold RIPA buffer (Catalog # 89901, ThermoFisher Scientific) containing 1 × Halt™ protease inhibitor cocktail (Catalog # 1861279, ThermoFisher Scientific). The protein concentrations of the cell lysates were quantified by the Bradford protein assay (Catalog # 5000006, Bio-Rad laboratories, Hercules, CA). Standard SDS-PAGE and transfer procedures were performed to analyze protein profiles as described previously (Chen et al. 2020). Primary antibodies from Cell Signaling Technology (Danvers, MA) were used to target cyclin D1 (# 2978), cyclin D3 (# 2936), CDK2 (# 2546), CDK4 (# 12790), CDK6 (# 3136), and GAPDH (# 5174). Secondary horseradish peroxidase (HRP)-conjugated antibodies, anti-rabbit IgG-HRP (sc-2357) and anti-mouse IgG-HRP (sc-516102), were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein band images were captured using FluorChem E imaging system (ProteinSimple, San Jose, CA), and the data were analyzed by AlphaView software (San Jose, CA).

Immunofluorescence staining

An 18-well chambered coverslip (Catalog # 81817, Ibidi, Martinsried, Planegg, Germany) was used to culture human Leydig cells at a density of 5,000 cells/well. After a 24 h CBD (15 μM) or DMSO (0.1%) treatment, the medium was removed, and cells were washed with 1 × DPBS. The cells were then fixed with 4% formaldehyde (Catalog # F8775, MilliporeSigma) for 10 min at room temperature. Fixed cells were permeabilized with 0.5% Triton ™ X-100 (Catalog # T9284, MilliporeSigma) in 1 × DPBS and blocked with 1 × Blocker™ BSA (Catalog # 37525, ThermoFisher Scientific) for 30 min at room temperature. F-actin was stained with DyLight 488-Phalloidin (Catalog # A12379, ThermoFisher Scientific) in 1 × Blocker™ BSA at a dilution of 1:300 for 30 min at room temperature. Cell nuclei were stained with Hoechst 33342 (Catalog # 62249, ThermoFisher Scientific) at a concentration of 1 μg/mL for 10 min. Using a Cytation 5 Cell Imaging Reader (Agilent BioTek, Santa Clara, CA), images were captured from samples obtained

from three independent repeats. Random fields were selected under the microscope and at least three images were captured for each sample.

RNA isolation and mRNA sequencing

Human Leydig cells were seeded at a density of 1.7×10^5 cells/well in 6-well plates (Catalog # 3506, Corning) and treated with 10–20 μM CBD or 0.1% DMSO for 24 h. Total RNA was extracted from the cells using a RNeasy Mini kit (Catalog # 74106, Qiagen). The quality and purity of RNA were measured using a NanoDrop™ 8000 (ThermoFisher Scientific), RNA 6000 Nano kits (Agilent Technologies), and an Agilent 2100 Bioanalyzer (Agilent Technologies). Library construction was performed according to Illumina's TruSeq-stranded-mRNA sample preparation protocol with poly-A pull down and paired-ended sequencing was performed using Illumina's NovaSeq 6000 sequencing system (Illumina, San Diego, CA). The resulting mRNA expression profiles were generated using perl scripts, Cutadapt, HISAT2, and StringTie. Concentration-dependency analyses were performed as previously described (Li et al. 2019). Briefly, the raw gene count matrix was filtered to remove genes with low counts (mean count less than or equal to 1), and the resulting counts were normalized by DESeq2 (Anders and Huber 2010) before being imported into BMDExpress2 for Williams' trend tests (2-fold cutoff and $P < 0.05$) (Phillips et al. 2019). Specifically, 27,238 genes from human Leydig cells were inputted into BMDExpress2 and annotated by genome ID hg38 (*Homo sapiens*). Among these genes, 8,927 genes were found to be altered in a concentration-dependent manner by CBD. Subsequently, the DAVID Functional Annotation tool (https://david.ncifcrf.gov/home.jsp) was used to analyze these genes for pathway enrichment (Sherman et al. 2022), with a pathway considered significantly enriched if the Bonferroni adjusted *P*-value was < 0.05. 7,287 genes from *Homo sapiens* were identified in the DAVID gene list manager, with 5,610 (77.0%) of these genes from human Leydig cells being enriched in the Gene Ontology (GO) biological processes (direct) database using the DAVID tool.

Detection of apoptosis using Caspase-Glo® 3/7 assay and Annexin V/PI staining

Human Leydig cells were seeded at a density of 5,000 cells/well in 96-well white wall/clear bottom plates (Catalog # 3903, Corning). Caspase-Glo® 3/7 assay (Catalog # G8092, Promega, Madison, WI) was used to detect caspase 3/7 activities as previously described (Chen et al. 2020). Following treatment with 0.1% DMSO, 10–30 μM CBD, 10–30 μM 7-hydroxy-CBD, or 60–140 μM 7-carboxy-CBD for 24 h, the supernatant was removed and replaced with 50 μL of a mixture of Caspase-Glo® 3/7 reagents and serum-free Leydig cell media according to manufactures' protocol. The luminescence signals were measured in a Cytation 5 reader after incubation at room temperature for 30 min. The caspase 3/7 activity was normalized to the control group.

The apoptotic response was quantified using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Catalog # V13245, Invitrogen™, ThermoFisher scientific) and analyzed by flow cytometry. Annexin V binds to phosphatidylserine, which translocates to the outer leaflet of the plasma membrane during apoptosis (van Engeland et al. 1998). PI, which can bind to nucleic acid but cannot enter live cells, was used to differentiate viable cells. Viable cells were double negative for Annexin V and PI. Early apoptotic cells were Annexin V

positive and PI negative, while late apoptotic cells were both Annexin V and PI positive; the sum percentage of early and late apoptotic cells were identified as the percentage of apoptotic cells. Necrotic cells with disrupted membranes stained positively with PI. Human Leydig cells were seeded at a density of 1.7×10^5/well in 6-well plates (Catalog # 3506, Corning) and treated with 20–30 μM CBD, 15–30 μM 7-hydroxy-CBD, or 120–140 μM 7-carboxy-CBD for 24 h. At the designated time points, cells were harvested, washed with ice-cold 1 × DPBS buffer, and re-suspended in 1 × annexin V binding buffer. The cells were then stained with Alexa Fluor® 488 annexin V and 1 μg/mL PI for 15 min at room temperature in darkness. The samples were immediately analyzed using a FACSCanto™ flow cytometer with FACSDiva™ software (BD Biosciences, San Jose, CA) and analyzed with FlowJo® software. The distribution of cells was plotted in two-dimensional dot plots.

Measurement of cellular growth

Cellular growth was monitored by measuring population doubling levels (PDL) as describe previously (Li et al. 2023b). Briefly, human Leydig cells were seeded at a density of 3.4×10^4/well in 6-well plates (Catalog # 3506, Corning); day 0 was defined as the start of CBD treatment. Cell numbers were counted every other day by trypsinization dissociation to calculate population doubling levels using the formula PDL = log(N2/N1)/log2, where N1 represents the cell number at the earlier time point, and N2 represents the cell number at the subsequent time point. PDL represents the total number of times of cell division occurrences. Specifically, DMSO-treated cells were counted on days 2, 4, and 5 before reaching 100% confluency and reseeded into new cell culture vessels with fresh media containing 0.1% DMSO. During a pilot study, we observed that the cells number of CBD-treated cells did not exceed the initial seeded number (3.4×10^4) on day 2 and was insufficient for reseeding. Therefore, the CBD-treated cells were kept in their initial plates, and media containing 12.5 or 15 μM CBD was changed on day 2 and 4. The CBD-treated cells were counted on day 2, 4, and 5. Additionally, CBD-treated human Leydig cells detached on day 5, and thus monitoring was stopped on day 5.

Statistical analyses

All data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical analyses were conducted using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, as appropriate, using GraphPad Prism 9 (San Diego, CA). Concentration-related trends were assessed by linear regression analyses. A $p < 0.05$ was considered statistically significant.

Results

CBD caused cytotoxicity in human Leydig cells

We first investigated whether CBD induced cytotoxicity in primary human Leydig cells by measuring cell viability and LDH release. Cell viability was determined by counting the viable cells in the control and treatment groups. At 24 h, CBD treatment at concentrations of 15 μM or higher significantly decreased the cell viability in a concentration-dependent manner compared to the DMSO control (Figure 1A). The BMD_{50} of CBD, measured by cell number counting at 24 h, was 21.6 μM (Table 1). Furthermore, significant LDH release was

observed at 25 μM CBD treatment. An increase of 27.8% in LDH release occurred at 30 μM CBD treatment, indicating that more profound cellular damage occurred at the higher concentrations (Figure 1B).

Sub-lethal CBD concentrations disrupted cell cycle progression and inhibited DNA synthesis

We previously reported that CBD induced cell cycle disruption in primary human Sertoli cells (Li et al. 2022). To investigate whether CBD alters the cell cycle in human Leydig cells, we performed PI staining at sub-lethal concentrations of CBD. CBD at the concentrations of 12.5 – 20 μM for 24 h induced a significant increase in the percentage of cells in G1 phase and a decrease in the percentage of cells in S and G2/M phases (Figures 1C and 1D), indicating that CBD arrested cell cycle in G1 phase. Specifically, 20 μM of CBD increased the percentage of cells in G1 phase from 49.8% to 66.5% with simultaneous decreases in S phase from 21.2% to 6.7% and G2/M phases from 24.5% to 16.9% (Figure 1D). Additionally, the proportion of sub G1 phase (fractional DNA content) increased from 1.0% to 7.5% at the 20 μM CBD treatment, indicating the occurrence of apoptosis.

To understand the molecular mechanism of cell cycle disruption, we examined the protein levels of cell cycle regulators. After a 24 h CBD treatment, the protein levels of CDK2, CDK4, CDK6, cyclin D1, cyclin D3, and cyclin E2 were all decreased (Figures 2A and 2B). Down-regulation of these proteins may contribute to the cell cycle arrest in the G1 phase and is consistent with our previous observation that the percentage of cells in the G1 phase increased and cells in the S phase decreased. Therefore, CBD disturbed the cell cycle progression by down-regulating proteins that regulate the G1/S phase transition in human Leydig cells.

To investigate further the mechanism of cell cycle disruption, we quantified DNA synthesis activity during S phase by measuring EdU incorporation. As shown in Figures 2C and 2D, treatment with 15 μM CBD for 24 h nearly eliminated S phase cells (0.9%) as compared to 29.1% in the control group. The decrease in the proportion of EdU-positive cells in the CBD-treated group indicated a reduction in DNA synthesis and the number of cells in S phase.

CBD disrupted F-actin cytoskeleton distribution

To characterize the morphological changes induced by CBD, we stained filamentous actin (F-actin) with green-fluorescent phalloidin conjugates. F-actin is one of the major cytoskeleton components that plays an essential role as the contractile apparatus in the maintenance of cell shape, cell attachment, proliferation, and migration (Li et al. 2009; Tojkander et al. 2012). CBD treatment disrupted the normal F-actin organization (Figure 2E). The control group had a uniform F-actin distribution and well-aligned bundles of F-actin filaments, while 15 μM CBD-treated human Leydig cells lost the even distribution and had F-actin aggregation, such as abnormally thick bundles or dots (red arrows) (Figure 2E). It has previously been demonstrated that disruption of F-actin amplifies the apoptosis signaling cascade (Desouza et al. 2012; Morley et al. 2003). Thus, the disruption of F-actin may contribute to CBD-induced decrease of cell proliferation and cellular damage.

Transcriptomic analysis of CBD-treated primary human Leydig cells

To understand the global changes in gene expression induced by CBD, RNA samples were isolated from human Leydig cells treated with 10–20 μM CBD for 24 h. A total of 8,927 genes displayed significant changes in a concentration-dependent manner after performing William's trend tests using BMDExpress2 (Phillips et al. 2019). Of the 8,927 genes, 5,610 genes were mapped to the Gene Ontology (GO) biological processes database (Sherman et al. 2022). Biological processes with an adjusted *P* value (Bonferroni) < 0.05 were considered significantly enriched and are shown in Table 2 and Figure 3A. The pathway analysis indicates that the most enriched pathways are related to DNA replication and cell division, which is consistent with the results presented in Figures 1 and 2. The top 20 genes with the highest absolute fold change in pathways of DNA replication and cell division are summarized in Figures 3B and 3C. Specifically, consistent with our Western blot results, the expression of cyclin E2 (*CCNE2*) was found to decrease after CBD treatment (Figure 3B). CBD also decreased the expression of genes that encode enzymes that directly participate in DNA synthesis during DNA replication, such as *LIG1* (DNA ligase 1), *POLA2* (DNA polymerase alpha 2, accessory subunit), *POLE2* (DNA polymerase epsilon 2, accessory subunit), and *PRIM1* (DNA primase subunit 1) (Burgers and Kunkel 2017). The expression of genes in cell division, such as CDK1 (*CDK1*) and cyclin A2 (*CCNA2*) were found to decrease in a concentration-dependent manner upon CBD treatment (Figure 3C). Furthermore, apoptosis (Table 2) was also identified as one of the top biological processes modified by CBD. The top 20 genes with the highest absolute fold change in apoptosis and commonly studied apoptosis biomarker genes are summarized in Figures 3D and 3E. For example, the decrease of *BCL2* (Bcl-2 apoptosis regulator) gene expression and the increase in *CASP7* (Caspase 7) expression support the progression of apoptosis.

CBD treatment induced apoptosis

The mRNA-seq results indicated alterations in apoptosis marker genes in response to CBD treatment. To determine if apoptosis was induced by CBD after 24 h, we measured caspase-3/7 activities and characterized changes in the plasma membrane using Annexin V/PI staining followed by flow cytometry analysis. As shown in Figure 4A, CBD induced a significant increase in caspase-3/7 activities at 22.5–30 μM after a 24 h of treatment. Furthermore, we confirmed that CBD induced apoptosis at 25 and 30 μM using Annexin V/PI staining. At 30 μM CBD, the percentage of human Leydig cells undergoing apoptosis increased from 5.8% to 38.3%, and the portion of viable cells decreased from 94.2% to 55.8% (Figures 4B and 4C). These results indicate that CBD induced apoptosis in primary human Leydig cells.

7-Carboxy-CBD and 7-hydroxy-CBD induced cytotoxicity

We then evaluated the cytotoxicity of the main CBD metabolites, 7-carboxy-CBD and 7-hydroxy-CBD. Cell viability was measured by counting the viable cells after exposure to these two compounds for 24 h. Both 7-carboxy-CBD and 7-hydroxy-CBD exhibited cytotoxicity, which increased with increasing concentrations (Figures 5A and 5B). The BMD_{50} values of 7-carboxy-CBD and 7-hydroxy-CBD were 127.3 and 30.2 μM,

respectively, at 24 h (Table 1). Compared to the BMD_{50} of CBD, which was 21.6 μM (Table 1), 7-hydroxy-CBD and 7-carboxy-CBD were less toxic than CBD.

To examine whether the two CBD metabolites share a mechanism of cytotoxicity similar to CBD, we assessed DNA synthesis activity using EdU labeling in human Leydig cells treated with 7-carboxy-CBD and 7-hydroxy-CBD for 24 h. We found that at concentrations that did not significantly decrease cell viability, 80 μM 7-carboxy-CBD and 15 μM 7-hydroxy-CBD reduced the percentage of EdU-positive cells from 29.1% (control) to 8.8% and 17.6%, respectively (Figures 5C and 5D). By comparison, 15 μM CBD reduced the percentage of EdU-positive cells to 0.9% (Figure 2D), indicating that CBD was more potent than its metabolites in inhibiting DNA synthesis.

Additionally, treatment with 90–140 μM 7-carboxy-CBD and 25–30 μM 7-hydroxy-CBD induced cell death, as indicated by the release of LDH enzymes to the culture media (Figures 6A and 6C). At the same time, the activities of caspase-3/7 significantly increased with treatment at 100–140 μM 7-carboxy-CBD and at 17.5–30 μM 7-hydroxy-CBD for 24 h (Figure 6B and 6D). Furthermore, the results of annexin V/PI staining indicated that 140 μM 7-carboxy-CBD and 20–30 μM 7-hydroxy-CBD induced apoptosis in human Leydig cells (Figure 7).

Induction of apoptosis by low concentration of CBD after 4 days of incubation

To study the long-term effects of CBD exposure, we monitored the growth of CBD-treated human Leydig cells over an extended period of 5 days. Population doubling levels (PDLs) reflect the total number of times the cell population has doubled at a given time. As shown in Figure 8A, the control group (0 μM) exhibited a steady increase in PDL. However, on day 2, the PDLs of the 12.5 and 15 μM CBD-treated groups were 0.55 and 0.14, respectively, suggesting that growth arrest occurred in CBD-treated cells. In addition, on day 5, the PDLs of the 12.5 and 15 μM CBD-treated groups were −0.33 and −3.64, respectively, indicating that the number of cells dropped below the initial seeding number in CBD-treated cells. Therefore, long-term exposure to CBD significantly reduced the number of cells.

Next, we investigated whether apoptosis or necrosis was contributing to the decrease in cell number induced by CBD treatment for 4 and 5 days. After treating cells with 15 μM CBD, the average percentage of viable cells decreased by 13.1% and 34.6% (as indicated by Annexin V/PI staining), and the average percentage of apoptotic cells increased by 9% and 41.7% on day 4 and 5, respectively (Figures 8B and 8C). Therefore, even a sub-lethal concentration of CBD at 24 h caused apoptotic cell death in human Leydig cells after a longer-term treatment.

Discussion

It is critical to determine whether male reproductive toxicity of CBD exists in humans, due to the widespread use of CBD. *In vitro* experiments using cultured primary human male reproductive cells are useful models to evaluate the cytotoxicity of CBD and understand its molecular mechanisms. In the current study, we found that the cytotoxicity of CBD in human Leydig cells resulted from cell cycle arrest at G1 phase, inhibition of DNA

synthesis, and induction of apoptosis. Our transcriptomic analysis results aligned well with the observed phenotypes, where we identified subsets of genes whose expression levels were altered by CBD in a concentration-dependent manner and found that these genes were enriched in DNA replication, cell division, and apoptosis. In our previous transcriptomic study, many DNA replication-related genes were also altered with CBD-treated human Sertoli cells (Li et al. 2023b). Changes in DNA replication-related genes in human Leydig cells resembled those in human Sertoli cells, where genes of key replicative proteins, such as DNA polymerase (gene *POLA2*, *POLE*), and genes of proteins control DNA replication initiation, such as MCM complex (gene *MCM4*, *MCM6*, *MCM10*) decreased by CBD in a concentration-dependent manner (Li et al. 2023b).

Unlike human Sertoli cells, which exhibited an absence of apoptosis and underwent cellular senescence (Li et al. 2023b), in this study, we demonstrated that CBD induced apoptosis in human Leydig cells. Leydig cells are the primary source of testosterone biosynthesis, which is essential for male sexual development and function. Thus, the number and quality of Leydig cells influence testosterone production. The number of Leydig cells is determined by the balance between proliferation and cell death. Leydig cells exhibit a low turnover rate and marginal mitotic activity in the testes (Zirkin and Papadopoulos 2018). Thus, apoptosis of Leydig cells seems crucial for their development, maturation, and death. Excessive apoptosis may lead to a decreased number of Leydig cells, resulting in a decline in testosterone production. A correlation between decreased testosterone levels and Leydig cell apoptosis has been reported in many studies (Li et al. 2023a; Liu et al. 2012; Lv et al. 2021; Park et al. 2002; Yang et al. 2021; Zhai et al. 2022). Previous reports indicated that CBD decreased testosterone levels in monkeys and mice (Carvalho et al. 2018; Rosenkrantz and Esber 1980). We attempted to measure testosterone levels from primary human Leydig cell culture supernatants using a Homogeneous Time Resolved Fluorescence assay (testosterone HTFR kit, Cisbio, Waltham, MA, detection limit: 0.1 nM) and an ELISA assay (testosterone ELISA kit, Enzo Life Sciences, Farmingdale, NY, sensitivity: 5.67 pg/mL) but did not detect testosterone with either assay. In addition, hormones such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), and/or human chorionic gonadotropin (hCG) have been reported to stimulate testosterone secretions (Bilinska et al. 2009; Portela et al. 2019). However, we did not detect testosterone after applying these stimulators (data not shown). Previous studies with primary human Leydig cells indicated that testosterone secretion occurs within 3–4 days after isolation (Gaur et al. 2018) and that testosterone production significantly decreases in cryopreserved tissue compared to fresh tissue (Mularoni et al. 2020). In our case, the primary Leydig cells were cryopreserved, so we cannot rule out the possibility that human Leydig cells lost their ability to generate testosterone as a consequence of the cryopreservation, culturing, propagation, and storage processes. We further investigated the expression of key steroidogenic genes in human Leydig cells using RT-PCR assays. We found that *LHCGR* (luteinizing hormone/choriogonadotropin receptor), *HSD3B1* (3β-hydroxysteroid dehydrogenase 1), *CYP17A1* (cytochrome P450 family 17 subfamily A member 1), *HSD17B3* (17β-hydroxysteroid dehydrogenase 3), and *STAR* (steroidogenic acute regulatory protein) genes were not expressed (with Ct values over 35). *CYP11A1* in human Leydig cells was expressed at a low level, with a Ct value around 33.5. At the transcriptomic level based upon RNA-sequencing results, we examined key

steroidogenic genes involving in testosterone biosynthesis pathway and also found low levels of these genes. Therefore, the lack of gene expression supported the notion that the testosterone synthesis pathway was not functional, which is consistent with our observation that testosterone secretion was barely detectable. Thus, cultured primary human Leydig cells may not be a suitable model to study testosterone secretion. Regardless, our findings of CBD-induced apoptosis in Leydig cells may serve as indirect evidence for the negative regulation of testosterone by CBD. However, further investigations using alternative human cell models are necessary to confirm this notion.

Despite differences in activity, 7-hydroxy-CBD and 7-carboxy-CBD shared the same mechanisms of cytotoxicity as CBD. They both inhibited DNA synthesis and induced apoptosis in human Leydig cells. This study suggests that the potential harm from CBD metabolites at the cellular level may resemble that of the parental drug. Therefore, the cytotoxic effects of 7-hydroxy-CBD and 7-carboxy-CBD on male reproductive health should not be overlooked, particularly because 7-carboxy-CBD is the major circulating metabolite of CBD in humans.

Healthy volunteers who received doses of CBD investigated in clinical studies (750 mg twice daily) under fasting conditions for seven consecutive days showed C_{max} values of 2.3 μM, 0.6 μM, and 15.4 μM for CBD, 7-hydroxy-CBD, and 7-carboxy-CBD, respectively, in the afternoon of day 1. The C_{max} of 7-carboxy-CBD reached 28.5 μM on the morning of day 7 (Taylor et al. 2018). The concentrations tested in this study, which ranged from 10–30 μM for CBD and 7-hydroxy-CBD, and 60–140 μM 7-carboxy-CBD, were higher than the reported C_{max}. However, the plasma concentration of CBD, 7-hydroxy-CBD, and 7-carboxy-CBD might be higher in practice due to various factors, such as overdose, long-term usage, high-fat diets (Taylor et al. 2018), hepatic impairment (Taylor et al. 2019), and drug-drug interactions. For instance, following a high-fat breakfast, the C_{max} of CBD, 7-hydroxy-CBD, and 7-carboxy-CBD were enhanced by 5-, 3-, and 2-fold, respectively, compared to the fasted condition (Taylor et al. 2018). Additionally, it is not known whether and to what extent that CBD or its metabolites could accumulate in the testis. Taking into consideration all the factors mentioned above, we believe that the chemical concentrations used in this study could occur in humans who take CBD.

In summary, we demonstrated that CBD inhibited cell growth in primary human Leydig cells by inducing a G1 arrest in cell cycle progression, inhibiting DNA synthesis at the S phase, altering the F-actin organization, and inducing apoptosis. The CBD metabolites 7-carboxy-CBD and 7-hydroxy-CBD also inhibited DNA synthesis and induced apoptosis. The observed *in vitro* toxic effects in both human Leydig and Sertoli cells provide insight into the male reproductive toxicity of CBD.

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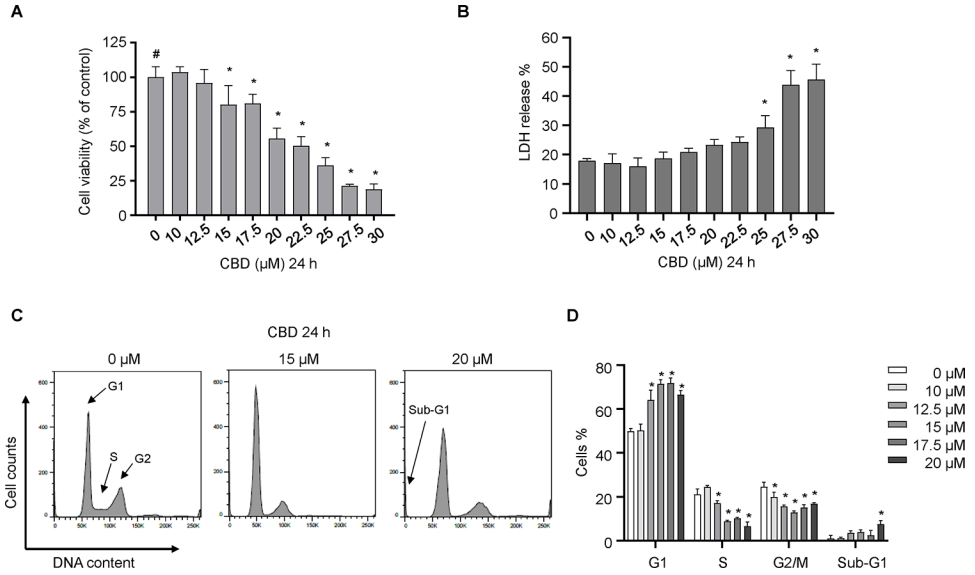

Figure 1. CBD exhibits cytotoxicity in primary human Leydig cells.

Primary human Leydig cells were exposed to CBD at concentrations of 10 – 30 μM for 24 h, and the control group was treated with DMSO (0 μM). (A) The number of viable cells was counted to determine cell viability. (B) LDH assays were performed to measure the percentage of LDH released to supernatants. (C) Flow cytometry was used to analyze DNA content. Representative histograms show DNA content analyses. (D) Bar graphs represent the mean percentage of each cell cycle phase ± SD (n = 3). #, significant concentration-dependent linear trend. *, $p < 0.05$ and significantly different from the control group.

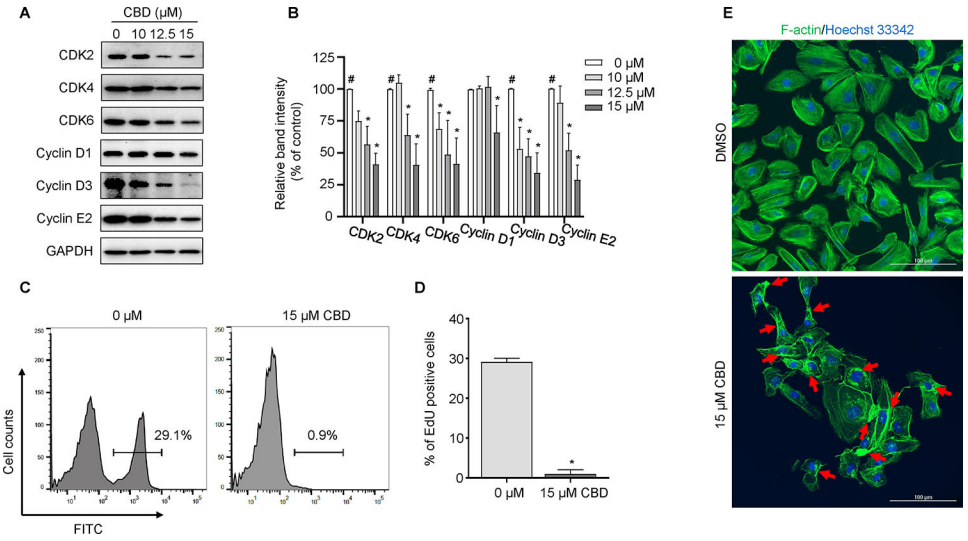

Figure 2. CBD down-regulates cell cycle-related proteins, inhibits DNA synthesis, and induces F-actin changes in primary human Leydig cells.

Total cellular proteins were extracted from cells that were treated with designated concentrations of CBD or DMSO for 24 h. The levels of CDK2, CDK4, CDK6, Cyclin D1, Cyclin D3, and Cyclin E2 were determined by Western blotting, using GAPDH as an internal control. The intensity of each protein band was normalized to its corresponding GAPDH band. Representative images are shown in (A), and quantification is shown in (B). Bar graphs represent means ± SD (n = 3). (C-E) Primary human Leydig cells were treated with either DMSO or 15 μM CBD for 24 h. The cells were stained for EdU to assess DNA synthesis; representative histograms of EdU staining are shown in (C). The bar graph (D) represents the mean percentage of EdU positive cells ± SD (n = 3). #, significant concentration-related linear trend. *, $p < 0.05$ and significantly different from the DMSO control. (E) Cells were stained for F-actin using DyLight 488-phalloidin (green) and nuclei using Hoechst 33342 (blue); immunofluorescent images are representative of three independent experiments. F-actin aggregation is indicated by red arrows. The scale bar indicates a length of 100 μm.

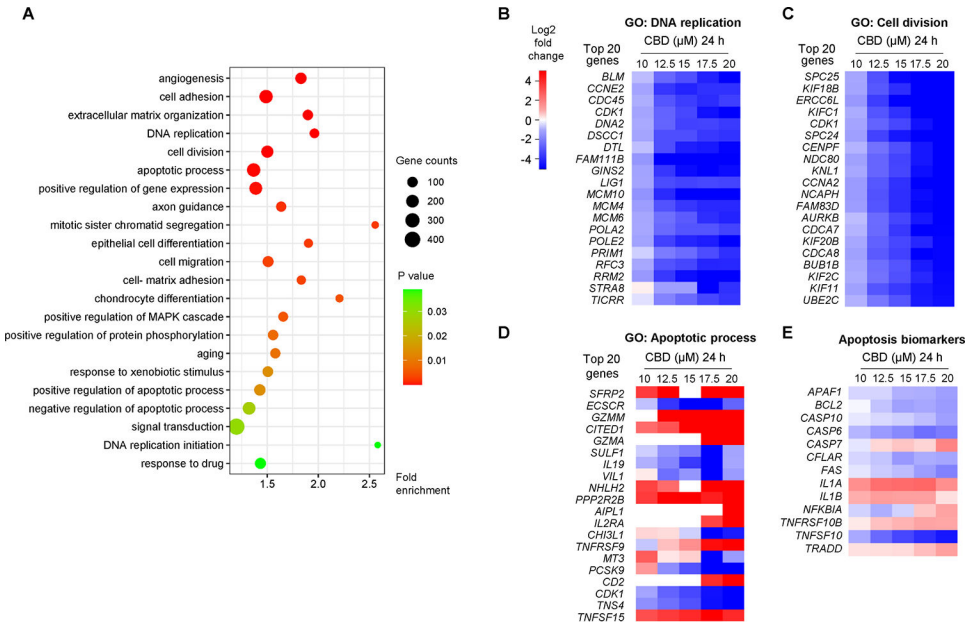

Figure 3. Transcriptomic profiling of 10 – 20 μM CBD treated primary human Leydig cells at 24 h.

Total cellular RNAs were isolated from primary human Leydig cells after 24 h of treatment with either DMSO or 10 – 20 μM CBD. The gene expression profiles were analyzed by mRNA-seq (n = 4/group). (A) The bubble plot displays the results of Gene Ontology (GO) biological processes enrichment analysis. (B-E) Heatmaps illustrate the top 20 genes with the largest absolute fold change from DMSO control at the highest CBD concentration from DNA replication (B), cell division (C), and apoptotic process (D), and the marker genes in apoptosis (E). Color tones represents log2 fold change of gene expression. Blue represents down-regulation and red means up-regulation of gene expression compared to the DMSO control group.

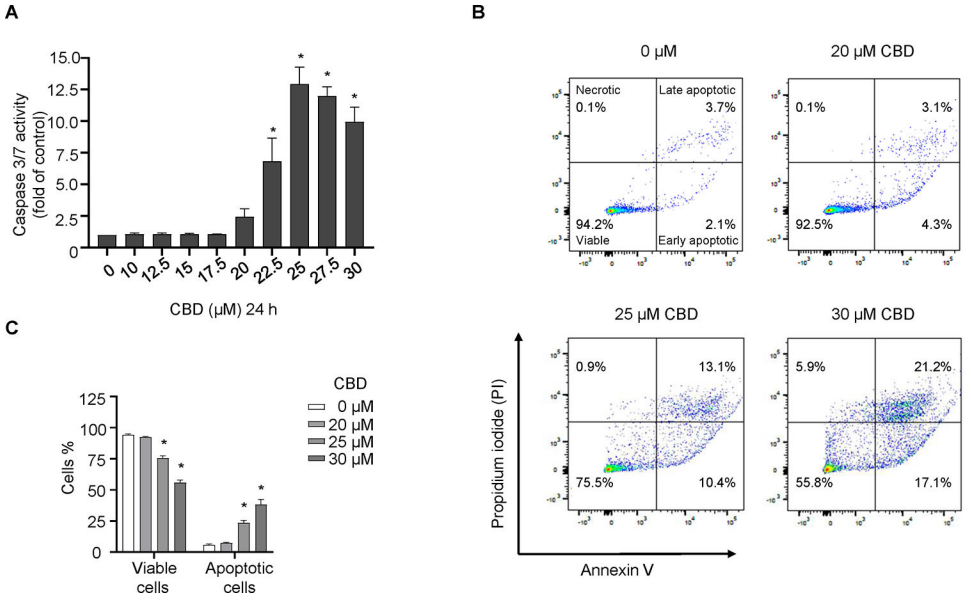

Figure 4. CBD induces apoptosis.

(A) Primary human Leydig cells were exposed to DMSO (0 μM) or 10 – 30 μM CBD for 24 h. Caspase-3/7 activity was measured as an indicator of apoptosis. (B) Detection of apoptotic human Leydig cells by Annexin V/PI staining. Representative scatter plots of Annexin V (X-axis) vs. PI (Y-axis) show the distribution of cells. (C) Quantification of results from (B); the bar graph shows the mean percentage of viable or apoptotic cells ± SD (n = 3). *, $p < 0.05$ and significantly different from the control group.

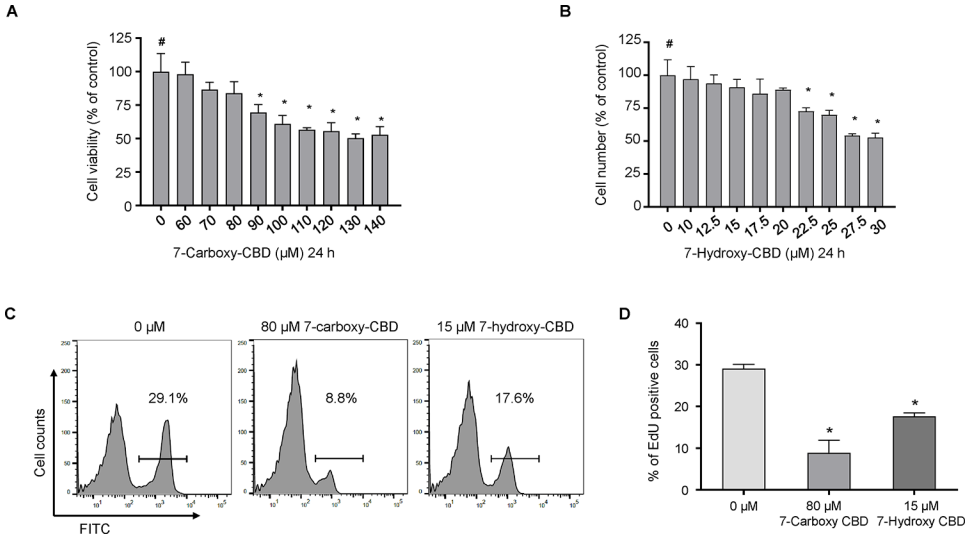

Figure 5. 7-Carboxy-CBD and 7-hydroxy-CBD cause cytotoxicity to primary human Leydig cells and inhibit DNA synthesis at 24 h.

(A and B) Primary human Leydig cells were treated with different concentrations of 7-carboxy-CBD (A) or 7-hydroxy-CBD (B) for 24 h, and cell viability was measured by counting viable cell numbers. (C and D) Primary human Leydig cells were treated with DMSO (0 μM), 80 μM 7-carboxy-CBD, or 15 μM 7-hydroxy-CBD for 24 h, and DNA synthesis was assessed by EdU staining. Representative histograms show EdU staining for DNA synthesis (C); the bar graphs represent the mean percentage of EdU-positive cells ± SD (n = 3) (D). *, $p < 0.05$ and significantly different from the DMSO control. #, significant concentration-related linear trend.

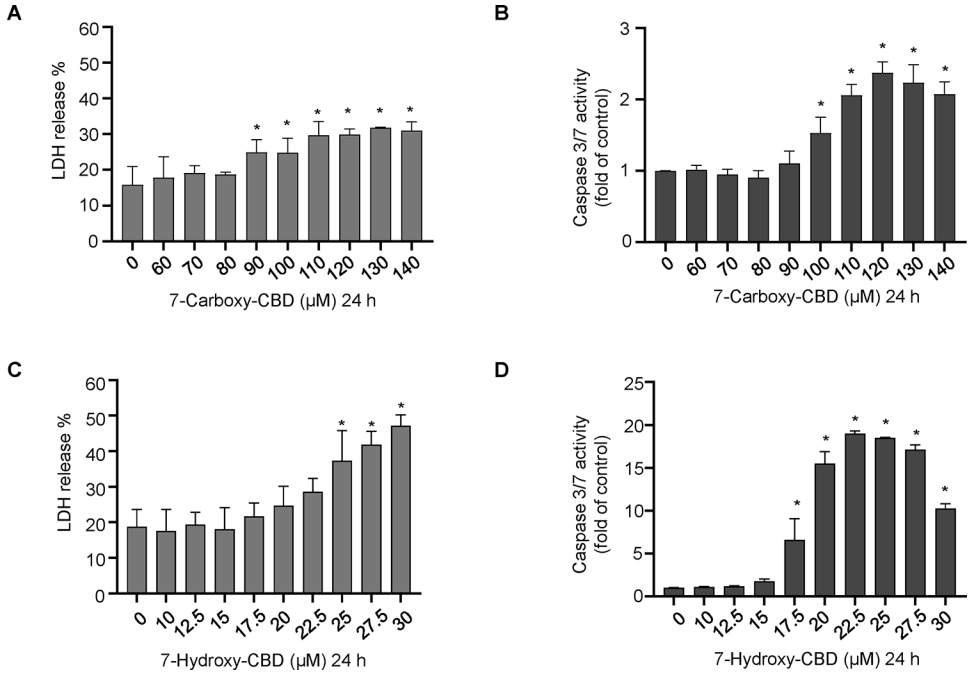

Figure 6. 7-Carboxy-CBD or 7-hydroxy-CBD induces cell death at 24 h.

Primary human Leydig cells were treated with DMSO (0 μM), 60 – 140 μM of 7-carboxy-CBD, or 10 – 30 μM of 7-hydroxy-CBD for 24 h. The percentage of LDH release was measured using LDH assays (A and C). The enzymatic activities of caspase-3/7 were determined using a Caspase-Glo® 3/7 assay (B and D). The results are presented as mean ± SD (n = 3). *, $p < 0.05$ and significantly different from the DMSO control.

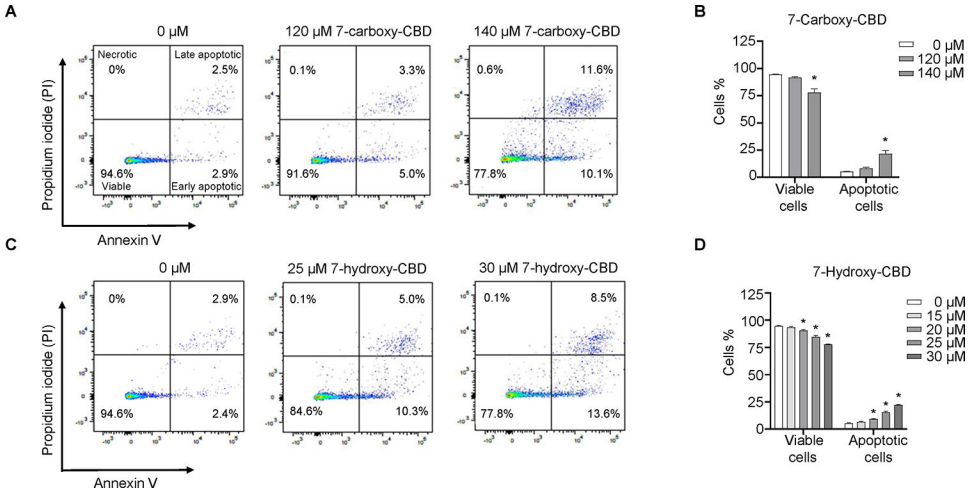

Figure 7. Annexin V/PI staining of primary human Leydig cells following treatment with 7-carboxy-CBD or 7-hydroxy-CBD at 24 h.

Primary human Leydig cells were treated with DMSO (0 μM), 120 and 140 μM 7-carboxy-CBD, or 15 – 30 μM 7-hydroxy-CBD for 24 h. (A and C) Representative scatter plots show the flow cytometric results for Annexin V/PI staining. (B and D) Bar graphs represent the mean percentage of viable or apoptotic cells ± SD (n = 3) at various concentrations. *, $p < 0.05$ and significantly different from the control group.

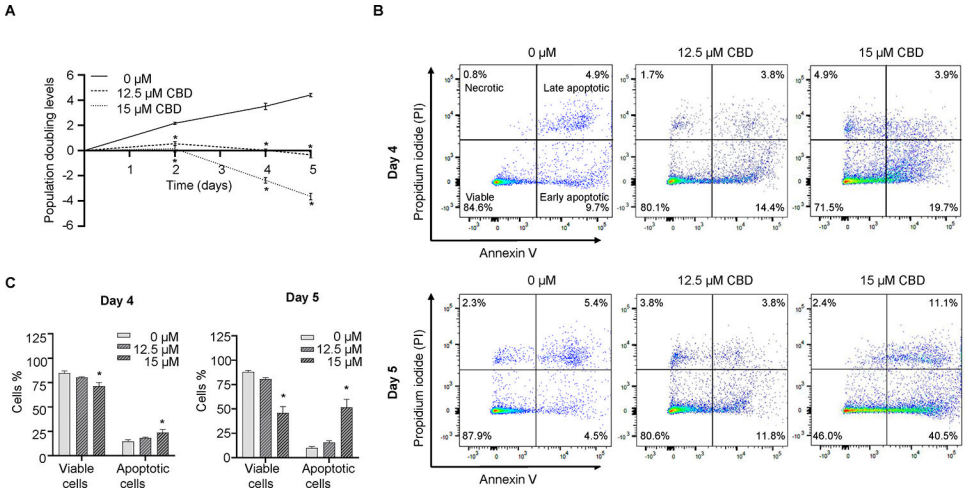

Figure 8. CBD at a low concentration induces apoptosis after long-term incubation.

Primary human Leydig cells were treated with DMSO (0), 12.5, or 15 μM CBD for 5 days. (A) The line graph shows the population doubling levels (n = 3). *, $p < 0.05$ and significantly different from the DMSO control. (B) Representative scatter plots show the flow cytometric results for Annexin V/PI staining on days 4 and 5. (C) Bar graphs show the mean percentage of viable cells or apoptotic cells ± SD (n = 3). *, $p < 0.05$ and significantly different from the DMSO control.

Table 1.

BMD_{50} estimates of CBD, 7-hydroxy-CBD, and 7-carboxy-CBD.[a]

Cell types	Primary human Leydig cells		
Chemicals	**24 h BMD_{50} (μM)**	**BMDL (5^{th} percentile, μM)**	**BMDU (95^{th} percentile, μM)**
CBD	21.6	20.0	22.8
7-Hydroxy-CBD	30.2 *	29.1	31.9
7-Carboxy-CBD	127.3 *,#	113.6	141.8

[a]Primary human Leydig cells were incubated with various concentrations of CBD, 7-hydroxy-CBD, or 7-carboxy-CBD for 24 h. The BMD_{50} estimates, which indicate a 50% relative cytotoxicity compared to controls, were obtained from the cell counting data using Bayesian BMD analysis. The data are presented as the median BMD_{50}, as well as the 5^{th} and 95^{th} percentiles of the estimates.

*, $p < 0.05$ and significantly different from the cells treated with CBD

#significantly different from the cells treated with 7-hydroxy-CBD.

Table 2.

Significantly altered biological processes identified using Gene Ontology (GO) enrichment analysis on concentration-dependent changed genes in response to CBD treatment

No.	GO terms (biological processes)	Adjusted P value (Bonferroni)	Numbers of differentially expressed genes	Percentage of genes in the pathway (%)	Fold enrichment
1	GO:0001525~angiogenesis	1.7E-11	134	1.84	1.83
2	GO:0007155~cell adhesion	6.8E-09	240	3.30	1.49
3	GO:0030198~extracellular matrix organization	5.3E-08	91	1.25	1.90
4	GO:0006260~DNA replication	3.5E-06	69	0.95	1.96
5	GO:0051301~cell division	1.2E-05	167	2.29	1.50
6	GO:0006915~apoptotic process	1.1E-04	241	3.31	1.37
7	GO:0010628~positive regulation of gene expression	3.0E-04	208	2.86	1.39
8	GO:0007411~axon guidance	1.8E-03	87	1.19	1.64
9	GO:0000070~mitotic sister chromatid segregation	2.4E-03	26	0.36	2.56
10	GO:0030855~epithelial cell differentiation	2.5E-03	52	0.71	1.90
11	GO:0016477~cell migration	3.1E-03	117	1.61	1.51
12	GO:0007160~cell-matrix adhesion	3.2E-03	57	0.78	1.83
13	GO:0002062~chondrocyte differentiation	4.8E-03	34	0.47	2.21
14	GO:0043410~positive regulation of MAPK cascade	5.4E-03	77	1.06	1.66
15	GO:0001934~positive regulation of protein phosphorylation	8.0E-03	95	1.30	1.56
16	GO:0007568~aging	8.9E-03	89	1.22	1.58
17	GO:0009410~response to xenobiotic stimulus	1.4E-02	105	1.44	1.51
18	GO:0043065~positive regulation of apoptotic process	1.4E-02	136	1.87	1.43
19	GO:0043066ñegative regulation of apoptotic process	2.7E-02	202	2.77	1.32
20	GO:0007165~signal transduction	3.0E-02	441	6.06	1.20
21	GO:0006270~DNA replication initiation	3.9E-02	21	0.29	2.58
22	GO:0042493~response to drug	3.9E-02	122	1.68	1.43