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Hemp in the Modern World: The Years-Long Wait for FDA Action

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Chairwoman McClain, Ranking Member Porter, Chairman Comer, Ranking Member Raskin, and members of the Subcommittee on Health Care and Financial Services:

Thank you for inviting me to participate in today's hearing. I appreciate the opportunity to talk about our research and its utility in an overall evaluation of cannabidiol (CBD) to support its safe use as a dietary ingredient. I am a toxicologist and Senior Managing Scientist in the Foods & Consumer Products Practice at ToxStrategies LLC, a multidisciplinary scientific consulting firm that provides scientific, technical, and regulatory support for clients in the public and private sectors across a broad range of industries, including consumer goods. I have experience in the safety assessment of ingredients that are often used in food, dietary supplements, and/or animal feed. Since the implementation of the 2018 Farm Bill, I have been actively involved in the safety assessment of CBD and other hemp-derived products, including managing toxicology testing programs.

I am here today to present our recently published preclinical safety studies that provide key information needed to conduct robust science-based assessments and evaluate the safety of CBD as a dietary ingredient. CBD is generally the most prevalent non-psychoactive cannabinoid to be associated with oral consumer product use. In addition, while many consumer products are hemp extracts containing a mixture of cannabinoids and terpenes, CBD typically makes up a large fraction of the ingested material. As companies continue to innovate and manufacture hemp-derived products for oral use, understanding the safety of CBD in dietary supplements, foods, and/or beverages is critical to ensuring consumer protection.

The need for the studies presented to you today was determined based on standard practice typically employed by regulators and other risk assessors in the safety evaluation of dietary ingredients. All studies were performed according to the highest standards available and involved the collaboration and oversight of scientists from multiple disciplines, including those from various research organizations and within our firm. In addition, three manuscripts summarizing these studies have undergone a rigorous independent peer-review process and are now publicly available in widely disseminated scientific journals.

First, CBD did not cause DNA or chromosomal damage in our testing program. This is critical, because a genotoxic finding would have precluded its use as a dietary ingredient. Next, our studies demonstrated that CBD was well tolerated following repeated consumption in animal models up to the highest dose tested of 140 milligram per kilogram bodyweight per day (mg/kg bw/day). In our reproductive study, exposure up to 100 mg/kg bw/day of CBD did not cause adverse effects on fertility or reproduction in female animals, nor did it produce developmental effects in offspring. When converted to mg/day based on body weight, this value would equate to a safety margin of 100-fold for dietary supplement products containing 70 mg of CBD. In addition, no adverse effects on male reproductive parameters were observed up to 300 mg/kg bw/day, the highest amount of CBD tested. This suite of studies conducted on a hemp-derived CBD isolate provides the body of baseline safety data that is typically required to evaluate use of a dietary ingredient, such as would be expected in the assessment of a New Dietary Ingredient (NDI) in dietary supplements or a Generally Recognized as Safe (GRAS) substance for use in foods and/or beverages.

Science-based evaluations and an aligned consensus on the safety of CBD are in the best interest of public health and the consumer. The studies presented to you today provide an important contribution to help inform the existing database of safety studies on CBD. Taken together, the existing data from human clinical trials and preclinical studies in animal models provide a sufficient basis from which to determine safe levels of CBD for oral consumption by consumers. The process for conducting such an assessment would follow the same principles that we, as risk assessors, apply when evaluating any ingredient for dietary use. The resulting conclusions and recommended consumption levels for safe use could be refined as necessary when new data become available to reflect the evolving science on CBD.

Establishing the Safety of Dietary Ingredients

To ensure consumer protection, dietary ingredients must meet the relevant prescribed safety standards for their intended use, such as those established for NDIs.[1] Evaluations for a specific product include information on the identity, manufacturing process, specifica-

[1] Guidance Document. Draft Guidance for Industry: New Dietary Ingredient Notifications and Related Issues (August 2016). Last updated 4 October 2016. FDA website. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-new-dietary-ingredient-notifications-and-related-issues.

tions, and stability. Any history of human consumption of the ingredient, as well as clinical and preclinical (i.e., toxicology) studies, provide necessary data to support a conclusion of safety.

The first step in any safety assessment is typically a comprehensive literature search and review to evaluate any data already available on key safety endpoints and to determine whether additional studies may be needed. The typical suite of preclinical toxicology studies used to demonstrate safe use of an ingredient includes a battery of genotoxicity studies (typically two *in vitro* [in cells] and one *in vivo* [in laboratory animals]) and a repeated oral toxicity study (e.g., a 90-day subchronic study), as well as data on the toxicokinetics of the substance (e.g., absorption and metabolism). In addition, depending on the intended use and target population of the proposed substance (e.g., including use in children or pregnant individuals) and/or if existing data indicated a potential adverse effect, a developmental and reproductive toxicity study may also be necessary. Consideration of other data may also be warranted, such as from chronic toxicity/carcinogenicity, allergenicity, human clinical, and drug interaction studies. Studies should be assessed for relevance, reliability, and quality, and studies considered key to the safety determination must be deemed scientifically robust by qualified experts.

A fundamental concept in any safety assessment is that the finding of a potential adverse effect does not automatically mean there is a risk of that effect occurring. Substances considered to be beneficial and even necessary for health, can be toxic if consumed in large enough amounts. Understanding at what levels such harmful effects might occur provides the information necessary to assess risk and determine levels of consumption that are unlikely to be associated with such effects. With that in mind, preclinical toxicology studies are intentionally designed to be conducted at high enough exposure levels to enable identification of adverse effects. This is an important distinction, because exposure levels associated with human consumption may be very different from (and are typically much lower than) exposure levels tested in toxicology studies.

When sufficient data are available for an ingredient, a risk assessment can be performed that culminates in safety calculations based on information from safety studies and actual exposure levels (consumption) in the consumer. Typically, the dose levels associated with the most sensitive and relevant adverse effects identified in the most relevant toxicology studies are adjusted by what are known as safety factors to derive an adjusted value. This adjusted value can then be compared to human exposure levels as part of the risk assessment to determine whether a sufficiently protective margin of safety exists.

Ultimately, the objective of a safety assessment for a proposed dietary ingredient such as CBD is to aid in determining a safe level of CBD intake for consumers. Providing the science to accomplish this goal is an integral first step to ensuring protection of the consumer.

Large Existing Database of Clinical and Preclinical Studies on CBD

A large database of safety-related studies is already available for CBD and includes studies in humans, laboratory animals, and cell cultures. The most comprehensive data package available is that of Epidiolex®, a CBD-based drug which has been approved by the US Food and Drug Administration (FDA) for the treatment of patients 2 years of age and older.[2] The clinical and preclinical studies submitted to and reviewed by FDA as part of the data packages for Epidiolex provide important information that can be incorporated into an assessment of CBD consumer safety. More recently, FDA also indicated that they had reviewed preclinical safety-related studies commissioned and conducted by the agency; however, to my knowledge, these studies are not yet available to the public.[3] In addition to studies on Epidiolex, human clinical trials conducted with CBD for other indications are available in the public domain; while most involve patients of various disease states, more recent studies also include healthy populations. Some preclinical toxicology studies have also been conducted and are published in peer-reviewed journals, while others that may have been conducted by different stakeholders are not yet publicly available.

While all available studies should be considered as part of a safety assessment for CBD, it is important to note that the studies I am presenting to you today were the first guideline-compliant toxicology studies to be published on CBD isolate and were only made available to the public earlier this year.

New Toxicology Testing Program to Support the Safety Evaluation of CBD Isolate

Beginning in 2019, our group has conducted various reviews of the safety data available on CBD relevant to oral consumer use. One of the goals of these reviews was to recommend a sufficient approach for additional toxicology testing that would be in line with existing regulatory expectations for food and dietary supplements. This was a proactive approach to generate data that could be used by regulators if and when a federal pathway were to become available for oral CBD products. To this end, we reviewed all data available at the time of the assessments and made recommendations based on our experience with other proposed ingredients for use as NDIs or GRAS substances. Our approach for these assessments was consistent with standard professional practice for safety assessment of food and/or dietary supplement ingredients. Specifically, we evaluated the need for additional data to adequately characterize potential adverse effects in the body following oral consumption of CBD.

To specifically address data needs identified for one CBD isolate product, we embarked on the safety testing program that I am here to present today. Our program was designed to

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[2] https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210365Orig1s000TOC.cfm

[3] https://www.fda.gov/news-events/press-announcements/fda-concludes-existing-regulatory-frameworks-foods-and-supplements-are-not-appropriate-cannabidiol

address the lack of studies in the peer-reviewed literature conducted according to regulatory test guidelines for evaluating the genotoxicity, repeated oral toxicity, and reproductive and developmental toxicity of CBD. Of note, the need for such studies was later confirmed in a separate systematic mapping study that we published on the availability of safety-related data on CBD.[4] The outcome of these new studies would also provide data to address some of the concerns previously highlighted by different global regulatory agencies, such as the FDA.[5,6] In addition, it was critical that these studies be published and widely disseminated in peer-reviewed publications, in order to meet the expectations of GRAS for pivotal data to be "generally accepted" and "generally available," should this pathway ever become an option for the proposed CBD product.

As part of this program, teams of scientific experts collaborated to design, conduct, monitor, and interpret the following six pre-clinical toxicity studies on a hemp-derived CBD isolate (>99% pure).[7] This included experts in different aspects of toxicology within our firm, within contract research laboratories, and other experts, such as pathologists and biostatisticians. All studies were conducted according to the global gold standard of testing guidelines provided by the Organization for Economic Co-operation and Development (OECD). With the exception of the one dose-range-finding study, all studies adhered to Good Laboratory Practice (GLP) guidelines.

These studies were subsequently summarized in three manuscripts, of which I am the lead author. All manuscripts underwent a formal independent peer-review process by the journal reviewers and were published in widely disseminated scientific journals earlier in 2023. Importantly, to our knowledge, these were the first of such guideline-compliant studies to be published on a CBD isolate, thus changing the landscape of publicly available data on this substance. Brief summaries of these studies are provided below, and a copy of each manuscript is appended to this written testimony (Appendix A).

Genotoxicity Studies[8]

Three studies were conducted to evaluate the ability of CBD isolate to interact with DNA or cause chromosomal damage. Multiple assays were conducted to adequately evaluate the different types of such effects that might occur with exposure to a substance. The testing battery included an *in vivo* mammalian micronucleus test (OECD Test Guideline No. 474),

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4 Henderson RG, Franke KS, Payne LE, Franzen A. 2023. Cannabidiol safety data: A systematic mapping study. Cannabis Cannabinoid Res 8 (1):34–40.

5 https://www.fda.gov/media/168778/download

6 https://www.fda.gov/consumers/consumer-updates/what-you-need-know-and-what-were-working-find-out-about-products-containing-cannabis-or-cannabis

7 ToxStrategies was not involved in the 14- and 90-day studies until after the original study designs were completed. However, we conducted a full review of the protocols to ensure compliance with testing guidelines.

8 Henderson RG, Welsh BT, Trexler KR, Bonn-Miller MO, Lefever TW. 2023. Genotoxicity evaluation of cannabidiol. Regul Toxicol Pharmacol 142:105425; doi: 10.1016/j.yrtph.2023.105425.

an *in vitro* mammalian micronucleus test (OCED Guideline No. 487), and an *in vitro* bacterial review mutation test (OECD Test Guideline No. 471). [9,10,11] The results of all three studies confirmed that CBD was not associated with genotoxic effects under the study conditions. This is critical, because a genotoxic finding would have precluded its use as a dietary ingredient.

Oral Toxicity Studies[12]

Studies were conducted to investigate the potential for toxicity following repeated exposure to oral CBD in male and female rodents. First, a 14-day study was performed to look for signals of potential adverse effects, in order to ascertain what dose levels should be used in the follow-up 90-day (subchronic) oral toxicity study. These studies were conducted following the *FDA Toxicological Principles for the Safety Assessment of Food Ingredients* and OECD Test Guideline Nos. 407 and 408.[13,14,15]

CBD was well tolerated at all dose levels following up to 90 days of treatment. Microscopic liver and adrenal gland changes observed in the 90-day study resolved after a 28-day recovery period and were determined by the experts in the testing laboratory, as well as our team, to be non-adverse. Therefore, CBD dose levels up to 150 and 140 mg/kg-bw/d were determined to be without adverse effects in the 14- and 90-day toxicity studies, respectively. The results of these studies are comparable to findings reported in unpublished studies conducted with other CBD isolates. Because the dose levels in the 90-day study did not identify specific adverse effects, additional studies testing higher doses of CBD will help to further elucidate any potential toxicity associated with repeated consumer ingestion.

Reproductive and Developmental Toxicity Study[12]

This study was designed to investigate the effects of oral CBD exposure on male and female reproductive performance and offspring development in rodents. OECD Test

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[9] OECD (Organisation for Economic Co-operation and Development). 2020. OECD guideline for the testing of chemicals, Section 4. Test No. 471: Bacterial Reverse Mutation Test.

[10] OECD. 2016. OECD Guideline for the Testing of Chemicals, Section 4. Test No. 474 Mammalian Erythrocyte Micronucleus Test.

[11] OECD. 2016. OECD Guideline for the Testing of Chemicals, Section 4. Test No. 487 in Vitro Mammalian Cell Micronucleus Test.

[12] Henderson RG, Lefever TW, Heintz MM, Trexler KR, Borghoff SJ, Bonn-Miller MO. 2023. Oral toxicity evaluation of cannabidiol. Food Chem Toxicol 176:113778; https://doi.org/10.1016/j.fct.2023.113786.

[13] FDA (U.S. Food and Drug Administration). 2007. Guidance for industry and other stakeholders; Toxicological principles for the safety assessment of food ingredients. In: Redbook 2000. Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition.

[14] OECD. 2008. Test No. 407: Repeated dose 28-day oral toxicity study in rodents.

[15] OECD. 2018. Repeated dose 90-day oral toxicity study in rodents (OECD TG 408).

Guideline No. 421 was modified to include extended postnatal dosing through postnatal day (PND) 42, as well as additional hormone analysis.[16] In this study, parental males and females were dosed with CBD starting prior to mating and through weaning of offspring for females; offspring were then dosed directly through PND 42.

The main reproductive finding in this study was lower body weights in male and female offspring, primarily after birth and only at the highest dose of CBD tested (300 mg/kg bw/day). Based on this study, a dose of 100 mg/kg bw/day was determined not to cause reproductive toxicity in females or neonatal toxicity in offspring. When converted to mg/day based on body weight, this value would equate to a safety margin of 100-fold for dietary supplement products containing 70 mg of CBD. Finally, while the highest dose tested in this study (300 mg/kg bw/day of CBD) did not cause male reproductive toxicity under the study conditions, it is important to note that effects on sperm could not be fully assessed in this protocol.

Available Data Support Derivation of a Safe Level for Oral Consumer Use of CBD

As noted earlier in my testimony, the goal of conducting these types of studies in our professional practice is to understand whether a substance is safe for consumption and, if so, at what exposure levels. To reach such a conclusion, the potential adverse effects associated with a substance need to be identified. Subsequently, information on consumption levels can be incorporated into a risk assessment analysis to reach conclusions and make recommendations regarding safe consumer use. Importantly, standard risk assessment practice is designed to account for uncertainties in data sets, such as extrapolation of data from laboratory animal species to humans.

Understanding the toxicological profile of CBD in dietary supplements, foods, and/or beverages is critical to ensuring consumer safety. When conducting hazard and risk assessments for CBD, the data presented to you today should be considered together with all other available safety-related data on CBD. Importantly, studies should be evaluated for quality and reliability according to established criteria and should be relevant to the assessment and intended use of the ingredient as a consumer product.

Taken together, the existing data from human clinical trials and preclinical studies in animal models provide a sufficient basis from which to determine safe levels of hemp-derived CBD for oral consumer use. The process for conducting such an assessment would follow the same principles that we, as risk assessors, apply when evaluating any ingredient for dietary use. As new data continue to emerge, conclusions and recommendations for safe use can be revisited to reflect the evolving science on CBD.

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[16] OECD (Organisation for Economic Co-operation and Development). 2016. Test No. 421: Reproduction/Developmental Toxicity Screening Test.

Other Hemp-Derived Ingredients Require Substance-Specific Assessments

While the studies I presented to you today were conducted on a CBD isolate, it is important to consider that many of the hemp-derived products available to consumers consist of hemp extracts with variable composition and/or other cannabinoid isolates. Extracts are typically mixtures that can include multiple cannabinoids and/or terpenes with differing relative concentrations, depending on many factors related to the raw materials and manufacturing processes. These differences in chemical composition may result in differences in their respective safety profiles. Therefore, safety-related studies on the specific material (e.g., extract or mixture) should be prioritized, and the toxicological profile of individual constituents should be considered in the overall safety assessment. For example, baseline toxicology studies have been conducted on at least four hemp extracts and are available in peer-reviewed scientific journals.[17,18,19,20]

Toxicity data on CBD isolates, such as those I have presented to you today, may be valuable in the safety assessment of products that are mixtures containing CBD and should be evaluated on an individual basis. As with CBD, evaluating the safe use of other hemp-derived products can be accomplished using the same approaches already in place for other dietary ingredients.

Closing Remarks

Thank you again for the opportunity to present these recently published studies on hemp-derived CBD isolate. Providing the science to assess the safety and risk of dietary ingredients is expressly aligned with the objective of protecting the health and well-being of the American consumer.

We have conducted these core safety studies that add to the already extensive body of science for CBD. Based on my experience performing similar evaluations, the data available are sufficient for conducting a safety assessment of CBD following the same principles that we would apply for any ingredient proposed for use in foods or supplements. Recommendations for safe use, including levels of consumption, can be derived based on the available data—what these levels are will depend on number of factors, including the safety

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[17] Marx TK, Reddeman R, Clewell AE, Endres JR, Béres E, Vértesi A, Glávits R, Hirka G, Szakonyiné IP. 2018. An assessment of the genotoxicity and subchronic toxicity of a supercritical fluid extract of the aerial parts of hemp. J Toxicol 7:2018:8143582; doi: 10.1155/2018/8143582.

[18] Dziwenka M, Dolan L, Mitchell J. 2021. Toxicological safety of VOHO Hemp Oil; a supercritical fluid extract from the aerial parts of hemp. PLoS One 16(12):e0261900; doi: 10.1371/journal.pone.0261900.

[19] Dziwenka M, Coppock R, Alexander M, Palumbo E, Ramirez C, Lermer S. Safety 2020. Assessment of a hemp extract using genotoxicity and oral repeat-dose toxicity studies in Sprague-Dawley rats. Toxicol Rep 7:376–385; doi: 10.1016/j.toxrep.2020.02.014.

[20] Dziwenka M, Coppock R, Davidson MH, Weder MA. 2023. Toxicological safety assessment of HempChoice® hemp oil extract; A proprietary extract consisting of a high concentration of cannabidiol (CBD) in addition to other phytocannabinoids and terpenes derived from *Cannabissativa*L. Heliyon 9(6):e16913; doi: 10.1016/j.heliyon.2023.e16913.

data available to those conducting the assessment and the population intended to use the product. As new data continue to emerge on the safety of CBD, any conclusions can be refined, when relevant.

I thank the Subcommittee and its members for your interest, and I look forward to answering any questions you may have.

ATTACHMENT A

Published Preclinical Toxicology Studies on CBD Isolate

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Genotoxicity evaluation of cannabidiol

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ABSTRACT

Consumer use of cannabidiol (CBD) for personal wellness purposes has garnered much public interest. However, safety-related data on CBD in the public domain are limited, including a lack of quality studies evaluating its genotoxic potential. The quality of available studies is limited due to the test material used (e.g., low CBD purity) and/or study design, leading some global regulatory agencies to highlight genotoxicity as an important data gap for CBD. To address this gap, the genotoxic potential of a pure CBD isolate was investigated in a battery of three genotoxicity assays conducted according to OECD testing guidelines. In an *in vitro* microbial reverse mutation assay, CBD up to 5000 μg/plate was negative in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* strain WP2 *uvrA*, with and without metabolic activation. Testing in an *in vitro* micronucleus assay was negative in human TK6 cells up to 10–11 μg/mL, with and without metabolic activation. Finally, an *in vivo* micronucleus assay conducted in male and female rats was negative for genotoxicity up to 1000 mg/kg-bw/d. Bioanalysis of CBD and its primary metabolite, 7-carboxy CBD, confirmed a dose-related increase in plasma exposure. Together, these assays indicate that CBD is unlikely to pose a genotoxic hazard.

1. Introduction

Cannabidiol (CBD) is a naturally occurring cannabinoid, and the dominant cannabinoid found in industrial hemp (*Cannabis sativa* containing <0.3% tetrahydrocannabinol [THC] w/w) (Mechoulam et al., 2007; Pertwee, 2014; VanDolah et al., 2019). Although *C. sativa* plants and preparations thereof have been used for industrial, medicinal, and recreational purposes for thousands of years, the public and medical communities have recently become particularly interested in CBD for its therapeutic potential, following the Hemp Farming Act in the United States (US), part of the Agricultural Improvement Act of 2018 (aka, "2018 Farm Bill") (Rupasinghe et al., 2020). CBD is proposed to have anticonvulsive, analgesic, anti-anxiety, neuroprotective, antioxidant, and antimicrobial properties (Small and Marcus, 2002; Pertwee, 2004; Billakota et al., 2019; Devinsky et al., 2018). Epidiolex® (active ingredient CBD isolate) has been approved by the US Food and Drug Administration (FDA) for the treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome in pediatric patients (Jazz Pharmaceuticals, 2023). In addition, Sativex® (CBD and THC combination), is approved in other countries for the treatment of moderate to severe spasticity due to multiple sclerosis (Jazz Pharmaceuticals, 2023).

Interest in hemp-derived CBD consumer products, however, has outpaced the development of a legal pathway for CBD use in foods and dietary supplements. While the FDA has not established tolerable daily intake levels associated with consumer use, an overview of the agency's activities related to evaluating the safe use of CBD in food and dietary supplement products can be found on its website (FDA). The United Kingdom (UK) Food Safety Authority (United Kingdom Food Safety Authority, 2022), Health Canada (2022), and the Australian Therapeutic Goods Administration (Therapeutic Goods Administration, 2021) have conducted safety evaluations resulting in recommended established recommended maximum upper intake levels of CBD by healthy adults (except those planning to be or currently pregnant or breastfeeding). However, these agencies continue to highlight safety data gaps. Specifically, in regard to the potential genotoxicity of CBD, the European Food Safety Authority (EFSA) and the UK FSA (2022) have concluded the available data to be insufficient. While CBD is one of the most well-studied phytocannabinoids, there exist limited safety-related data on CBD in the public domain, including a lack of quality studies conducted according to regulatory test guidelines to evaluate its genotoxic

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Abbreviations

2AA	2-aminoanthracene
2NF	2-nitrofluorene
ANOVA	analysis of variance
API	atmospheric pressure ionization
bw	body weight
CBD	cannabidiol
CP	cyclophosphamide monohydrate
CRL	Charles River Laboratories
CRO	Contract Research Organization
DMSO	dimethyl sulfoxide
EFSA	European Food Safety Authority
FDA	US Food and Drug Administration
FSA	UK Food Safety Authority
GLP	Good Laboratory Practice
cGMP	current Good Manufacturing Practice
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICR	ICR-191 acridine
LC-MS/MS	liquid chromatography with tandem mass spectrometry
MHLW	Ministry of Health, Labour and Welfare of Japan
MMC	mitomycin C
MN	micronucleus
MTD	maximum tolerated dose
NAAZ	sodium azide
NCE	normochromatic erythrocyte
NQNO	4-nitroquinoline-N-oxide
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocyte
QC	quality control
SCGE	single cell gel electrophoresis
SD	standard deviation
SOP	standard operating procedure
TE	total erythrocytes
TGA	Therapeutic Goods Administration
THC	tetrahydrocannabinol
UK	United Kingdom
US	United States
VIN	vinblastine sulfate

potential. Publicly available *in vivo* genotoxicity tests also present limitations with respect to the test material and/or study design (Russo et al., 2019; Zimmerman and Raj, 1980; Marx et al., 2018; Dziwenka et al., 2020, 2021; Carvalho et al., 2022). Studies summarized by the FDA as part of its review of the non-clinical safety data package for Epidiolex provide information on the genotoxic potential of CBD, however, no publications or study reports are available for public review (CDER, 2018).

While the data from non-guideline-compliant studies and those studies using test materials with lower CBD contents can provide corroborative evidence for the safe use of CBD, no publicly available studies on CBD isolate conducted according to regulatory test guidelines have been identified that evaluate genotoxicity, repeated oral toxicity, or reproductive and developmental toxicity endpoints. Here we present the findings from three genotoxicity studies (i.e., Ames, *in vitro* micronucleus, and *in vivo* micronucleus assays) that were conducted according to U.S. Food and Drug Administration Good Laboratory Practice (GLP) and OECD guidelines. These studies were conducted as part of a larger program to investigate the safety of CBD isolate (Henderson et al., 2023a, 2023b).

2. Materials and methods

2.1. Test material

Hemp-derived CBD isolate (99.08–101.46%; CAS No. 13956-29-1) provided by Canopy Growth USA (Evergreen, Colorado) was produced by an ethanol extraction method and subsequent crystallization under current Good Manufacturing Practices (CGMP). The test material was stored at Charles River Laboratories (CRL) protected from light with desiccant at room temperature (19 °C–25 °C), and under nitrogen. The test article Certificate of Analysis (Botancor Laboratories, Denver, CO) demonstrated that the test article was 99.62% CBD. Based on the demonstrated purity, a correction factor of 1.004 was used in the preparation of dose formulations.

2.2. Genotoxicity studies

All genotoxicity studies were conducted in accordance with U.S. FDA (21 CFR Part 58): Good Laboratory Practice for Nonclinical Laboratory Studies, and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice) and Japan (MHLW), except for the characterization analyses of the test article, which were conducted to GMP standards.

2.2.1. Microbial reverse mutation assay

The assay design was based on OECD Guideline 471 (OECD, 2020). *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 *uvrA* were originally obtained from Molecular Toxicology, Inc. (Boone, NC).

2.2.1.1. Test article and positive controls preparation. CBD isolate was tested in an initial dose range-finding plate incorporation assay with a limited number of strains, followed by the full mutagenicity assay with all strains, performed under identical conditions (Ames et al., 1975; Maron and Ames, 1983). On the day of the range-finding and mutagenicity assays, CBD isolate was prepared as a formulation in dimethyl sulfoxide (DMSO) at a stock concentration up to 50 mg/mL. On the day of the repeat mutagenicity assay (i.e., TA100, without metabolic activation), CBD isolate was prepared at 1.00 mg/mL. The plate incorporation method was conducted using molten agar (2.0 mL) as the medium for transference of the test and control articles, bacterial culture (0.1 mL), control or test article (0.10 mL), and saline or Aroclor™ 1254-induced rat liver S9 fraction metabolic activation system (0.5 mL; Molecular Toxicology, Inc.), allowing colony growth.

The dose range-finding assay included CBD doses of 1.0, 5.0, 10.0, 50, 100, 500, 1000, or 5000 μg/plate with and without S9 (one plate per dose) using the tester strains TA100 and WP2 *uvrA*. Based on the results, the definitive mutagenicity assay evaluated CBD doses of 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, 2500, or 5000 μg/plate with and without S9. Three test plates per strain per treatment condition were used. Positive controls used in the absence of metabolic activation were as follows: 2-nitrofluorene (2NF; Sigma-Aldrich) at 2.5 μg/plate with TA98, sodium azide (NAAZ; Sigma-Aldrich) at 1.0 μg/plate with TA100 and TA1535, ICR-191 acridine (Sigma-Aldrich) at 0.5 μg/plate with TA1537, and 4-nitroquinoline-N-oxide (Acros Organics) at 2.0 μg/plate with *E. coli* WP2 *uvrA*. With metabolic activation, 2-aminoanthracene (2AA; Sigma-Aldrich) was used as a positive control for all bacterial strains (2.5 μg/plate), and 10 μg/plate for *E. coli* WP2 *uvrA*. The vehicle control used in the assay was dimethyl sulfoxide (DMSO; Sigma-Aldrich).

2.2.1.2. Experimental design. The following procedures were used in both the dose range-finding and the definitive mutagenicity assays.

Study number, tester strain, treatment group, concentration, and the presence or absence of metabolic activation were identified on each test plate. A stock solution of CBD isolate was prepared in DMSO on the day of the assay at a concentration of up to 50 mg/mL and above, while lower concentrations were prepared by serial dilution with DMSO. The dosing volume for all assays was 100 μL per plate.

Briefly, sterile 12 × 75 mm test tubes were placed in heating blocks set to approximately 46 °C, and the relevant items were added stepwise for each concentration of test or control article. After addition of the required components, the mixture was gently mixed and overlaid onto minimal glucose plates and incubated for 2 day at 36–38 °C. All cultures gave acceptable absorbance readings (in the range of 0.2–0.5) prior to each assay. Bacterial background lawn was evaluated macroscopically for test-article precipitate and microscopically for indications of cytotoxicity (i.e., thinning). Evidence of cytotoxicity was scored (by hand or automatic colony counter) relative to the vehicle control plate and recorded along with the revertant counts for all plates at that dose level. Assay acceptance was determined by comparing the vehicle and positive control plates against historical data of revertant count ranges (CRL, 2020). All plates had confluent background lawn; however, cytotoxicity (i.e., reduction in the background lawn and/or mean number of revertant colonies) was observed at ≥ 10 μg/plate in strain TA1537 without metabolic activation, ≥250 μg/plate in strain TA100 with metabolic activation and, ≥1000 μg/plate in strain TA1537 with metabolic activation.

2.2.2. *In vitro micronucleus assay*

The assay design was based on OECD Guideline 487 (OECD, 2016b). Human lymphoblast TK6 cells were originally obtained from Pfizer Global Research (Groton, CT) and subcloned at Charles River (Skokie). All cells used for this assay were free of mycoplasma contamination. The passage number of the cells was 22 for the range-finding assay and 10 for the micronucleus assay.

2.2.2.1. Test article and positive controls preparation. CBD (200 mg/mL in DMSO) isolate was tested in an initial dose range-finding cytotoxicity assay, as a stock solution in DMSO at a target concentration of 200 mg/mL, to determine the highest soluble concentration in the vehicle, followed by the micronucleus assay, which used a CBD isolate concentration of 2.20 mg/mL. The metabolic activation system used was Aroclor™ 1254-induced rat liver S9 fraction (Molecular Toxicology, Inc.). Positive controls were vinblastine sulfate (VIN; Sigma-Aldrich, target dose levels 0.003 and 0.0025 μg/mL) for the 27-h treatments without metabolic activation, cyclophosphamide monohydrate (CP; Sigma-Aldrich, target dose levels 11.9 and 4.7 μg/mL) for the 4-h treatments with metabolic activation, and mitomycin C (MMC, Sigma-Aldrich, target dose levels 0.125 and 0.0625 μg/mL) for the 4-h treatments without metabolic activation. Each culture flask was labeled with the study number, assay date, treatment group, concentration, length of treatment, and the presence or absence of metabolic activation. The vehicle control was dimethyl sulfoxide (DMSO; Sigma-Aldrich).

2.2.2.2. Experimental design. The dose range-finding cytotoxicity assay evaluated target concentrations of CBD isolate: 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000, or 2000 μg/mL; with and without metabolic activation. Cytotoxicity was assessed using cell-count data obtained from Coulter counts and an appropriate calculation of cytotoxicity (i.e., relative population doubling); cultures exhibiting ≥60% cytotoxicity were not processed and analyzed. Based on the results of the range-finding assay, target concentrations of CBD used during the micronucleus assay ranged from 0.100 to 22.0 μg/mL for the 4-h treatments with and without metabolic activation and for the 27-h treatment without metabolic activation, as shown in Table 1.

The test system was treated with the test article, positive control, or vehicle in the presence and absence of metabolic activation for short incubations (4 h) and in the absence of activation for the long incubation (27 h). The metabolic activation mixture was adjusted and added as appropriate, equal to the volume (mL) of cell culture in ICM at the adjusted cell density multiplied by 0.02. A harvest time of approximately 27 h was used for the 27-h exposure without S9 with no recovery period. A harvest time of approximately 44 h was used for the 4-h exposures with and without metabolic activation, with a 40-h recovery period. Cultures were resuspended at harvest, and an aliquot was removed for counting via Coulter counter and for micronucleus evaluation by flow cytometry. Micronucleus frequencies were analyzed from the processed cultures in at least 20,000 nucleated events (approximately 10,000 nucleated events per culture). All test-article concentrations up to the cytotoxic limit, along with the vehicle control and two concentrations of the positive control, were scored for micronuclei in each treatment condition.

Table 1
Treatment conditions and test material concentrations processed for micronuclei in the main *in vitro* micronucleus study.

Group	Treatment Conditions and Concentrations		
	~4 h without S9	~4 h with S9	~27 h without S9
DMSO (%)	1.0	1.0	1.0
Vinblastine (μg/mL)	–	–	0.0025 and 0.003
Cyclophosphamide (μg/mL)	–	4.7 and 11.9	–
Mitomycin C (μg/mL)	0.0625 and 0.125	–	–
CBD isolate (μg/mL)	0.100, 0.250, 0.500, 1.00, 2.00, 4.00, 6.00, 8.00, 9.00, 10.0, and 11.0	0.100, 0.250, 0.500, 1.00, 2.00, 4.00, 6.00, 8.00, 9.00, and 10.0	0.100, 0.250, 0.500, 1.00, 2.00, 4.00, and 6.00

Cultures for micronucleus evaluation were processed according to the manufacturer's instructions for the *in vitro* MicroFlow kit (Litron Labs, Rochester, NY), and the final samples were analyzed after ≥30 min (and up to 24 h) at ambient temperature, protected from light. Alternatively, samples were stored refrigerated for up to 80 h, prior to analysis.

2.2.2.3. Micronuclei analysis. Data acquisition and analysis was conducted using a FACSCanto II (or equivalent) with FACSDiva Software following CRL Standard Operating Procedures (SOPs). The test article was considered positive for micronuclei induction if a significant increase (z'≥0.6) in percentage of multinucleated cells was observed at one or more concentrations (Wojciechowski et al., 2016), and any observed dose-response was defined as a statistically significant Cochran-Armitage test ($p \leq 0.05$). The test article was considered negative for inducing micronuclei if the positive response criteria were not met and results were not comparable to the historical control range of the vehicle control. Cases that did not clearly fit either criteria were judged equivocal.

2.2.3. *In vivo micronucleus assay*

The assay design was based on OECD Guideline 474 (OECD, 2016a) and the International Council for Harmonisation (ICH) Harmonized Tripartite Guideline S2 (R1).

2.2.3.1. Test article and vehicle control preparations. Oral gavage dose formulations were prepared fresh daily by mixing appropriate concentrations of CBD in olive oil (vehicle control; Spectrum, New Brunswick, NJ) and heating at 35 ± 5 °C for 30 min. Preparations were dispensed prior to dosing and stored at controlled room temperature while stirring to maintain homogeneity.

2.2.3.2. Bioanalysis. Bioanalysis was conducted using a validated method (Charles River Testing Facility Study No. 3281–011) to determine the concentrations of CBD and 77-carboxy-CBD (7-COOH-CBD) in 50 μL of standard, quality control (QC), or rat plasma samples using a SCIEX API [atmospheric pressure ionization] 5000 triple quadrupole LC-MS/MS (liquid chromatography with tandem mass spectrometry) system. The calibration range of the assay was 20.0 to 20,000 ng/mL. Certified reference materials from Cerilliant Corporation (Round Rock, TX) were used as internal standards: cannabidiol-D_3 (99.6%) and 7-carboxy cannabidiol-D_3 (99.1%). The biological matrix used was Sprague-Dawley rat plasma with K_2EDTA obtained from BioIVT (Hicksville, New York).

2.2.3.3. Animals. Male and female Sprague Dawley, CD® [Crl:CD® (SD)] rats, approximately 7–7.5 weeks of age, were obtained from CRL (Raleigh, NC, or Stone Ridge, NY) and randomized into test groups. Animals were housed single sex, three per cage, in solid-bottom cages with nonaromatic bedding and environmental enrichment in a room that maintained temperatures of 20–26.1 °C, relative humidity of 30–70%, and a 12-h light/dark cycle. Rats were fed Lab Diet® (Certified Rodent Diet #5002, PMI Nutrition International, Inc.) *ad libitum*. Following a 7-day acclimation period, the animals weighed between 152 and 284 g at initiation of dosing. Animals were cared for according to the published National Research Council guidelines.

2.2.3.4. Experimental design. The following in-life assessments were performed for all animals at least daily: mortality/cageside observations, clinical/post-dose observations, detailed clinical observations, individual body weights, and food consumption.

Doses were selected based on dose range-finding toxicity studies in rats and on limit dose recommendations (ICH, 2012). For the range-finding study, animals (three/sex/group) were administered 500, 1000, or 2000 mg/kg-bw/d CBD by oral gavage once daily for two consecutive days. Based on observed toxicity at the 2000-mg/kg-bw/d dose, 1000 mg/kg-bw/d was selected at the maximum tolerated dose (MTD) in the main study, and subsequent doses were based on 50% of the next-highest dose. In the main study, six animals/sex/group were administered 0 (vehicle control), 250, 500, or 1000 mg/kg-bw/d CBD once daily on two consecutive days. Blood samples (approximately 0.5 mL) were collected from non-fasted, anesthetized animals via cardiac puncture prior to terminal necropsy and processed to plasma for determination of plasma CBD and 7-COOH-CBD concentrations. On Day 3, animals were euthanized, and bone marrow was collected from animals (5/group) for micronucleus evaluation. Slides were prepared and maintained at controlled room temperature and shipped to CRL (Skokie, IL) for analysis. To verify scorer proficiency, positive control reference slides from a historical experiment in which 60 mg/kg cyclophosphamide was administered via oral gavage were used (CRL, 2016).

2.2.3.5. Micronuclei analysis. Coded slides were stained with acridine orange solution prior to analysis. Two separate counts were made for each animal: 1) ≥ 500 total erythrocytes (TE; equals polychromatic erythrocytes [PCEs] + normochromatic erythrocytes [NCEs]) were counted and the PCE:TE ratio was determined; and 2) the number of micronucleated PCEs (MN-PCEs) in a total of 4000 PCEs scored. The % MN PCE and PCE:TE ratio results were compared between the test article and vehicle control groups, and between the positive and vehicle control groups, using analysis of variance (ANOVA). The MN-PCE frequencies were analyzed using a one-tailed test; PCE:TE ratios were analyzed using a two-tailed test. The Cochran-Armitage test was used to evaluate dose-response. Statistical significance was determined at a 95% confidence level ($p \leq 0.05$).

3. Results

3.1. Analytical verification of CBD dose formulation

For the bacterial reverse mutagenicity assay, CBD formulations of 0.5 and 50 mg/mL quantified at 96.2% and 96.6% of the nominal concentration, respectively. For the repeat mutagenicity assay, CBD formulations of 0.05 and 1.00 mg/mL quantified at 99.1% and 105% of the nominal concentration, respectively. These concentrations met the acceptance criterion of ≥90% of nominal. The lowest concentration (0.0025 mg/mL) from the mutagenicity and repeat mutagenicity assays was collected but not reported because this concentration was outside the validated range. For the *in vitro* micronucleus assay, CBD formulations of 0.0100, 1.00, and 2.20 mg/mL quantified at 96.8%, 103% and 103% of the nominal concentration, respectively, meeting the acceptance criterion of ≥90% of nominal.

For the *in vivo* micronucleus assay, CBD formulations of 50, 100, and 200 mg/mL quantified at 99%, 95.7% and 95.9% of the nominal concentration, respectively, meeting the acceptance criterion of ±15% of nominal. CBD was not detected in vehicle control samples from any of these studies.

3.2. Bacterial reverse mutagenicity assay

In the range-finding assay, precipitates were observed in both strains (TA100 and WP2 *urvA*), at ≥ 500 μg/plate without metabolic activation and at ≥ 1000 μg/plate with metabolic activation. Cytotoxicity was observed at ≥ 50 μg/plate in strain TA100 without metabolic activation and ≥500 μg/plate in strain TA100 with metabolic activation.

In the definitive assay, precipitates were observed in the following conditions: ≥250 μg/plate in strain TA1535 without metabolic activation and in strains TA98 and TA100 with metabolic activation; at ≥ 500 μg/plate in strains TA98, TA1537, and WP2 *uvrA* without metabolic activation and in strains TA1535 and TA1537 with metabolic activation; and at ≥ 1000 μg/plate in strain WP2 *uvrA* with metabolic activation. Cytotoxicity was observed at ≥ 10 μg/plate in strain TA1537 without metabolic activation, ≥250 μg/plate in strain TA100 with metabolic activation, and ≥1000 μg/plate in strain TA1537 with metabolic activation. A reduction in the background lawn was observed at 500 μg/plate in strain TA98 without metabolic activation and in strain WP2 *uvrA* with and without metabolic activation, and at 250 μg/plate in strain TA1535 without metabolic activation. However, the concentrations higher and lower than these doses showed mean revertant counts comparable to vehicle control. Therefore, this was not a dose-dependent response and determined not to be biologically relevant. Additionally, the highest concentration evaluated was as per the OECD guidelines and was limited by solubility, indicating that the test article was tested up to the maximum feasible limits in the present assay.

Under the conditions of the study, CBD did not cause an increase in the number of histidine revertants (*Salmonella* strains) or tryptophan revertants (*E. coli*) per plate in the presence or absence of S9 microsomal enzymes (Table 2). Positive controls produced the expected (or greater) increase in mutation frequency and all criteria for a valid study were met.

3.3. In vitro micronucleus assay

Precipitates were observed in the range-finding assay at ≥ 125 μg/mL in the 4-h treatment with metabolic activation and ≥500 μg/mL 27-h treatment without metabolic activation and in the 4-h treatment without metabolic activation at the end of CBD treatment. Excessive cytotoxicity was observed at ≥ 8 μg/mL in the 27-h treatment without metabolic activation; at ≥ 12 μg/mL in the 4-h treatment without metabolic activation; and at ≥ 11 μg/mL in the 4-h treatment with metabolic activation. Changes in the pH were not observed in any treatment at the end of test article treatment.

Table 2
Mean (±SD) revertant colonies per plate in main bacterial reverse mutation assay with CBD.

Treatment Group	μg/plate	TA98		TA100[B]		TA1535		TA1537		WP2 *uvrA*	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
DMSO	100 μL	9 ± 2	11 ± 5	103 ± 6	112 ± 9	7 ± 1	7 ± 5	3 ± 1	6 ± 2	68 ± 10	69 ± 14
CBD	0.25	9 ± 6	16 ± 3	107 ± 20	125 ± 14	6 ± 1	10 ± 2	3 ± 2	4 ± 4	70 ± 11	63 ± 3
	0.5	12 ± 7	16 ± 7	117 ± 9	141 ± 18	8 ± 3	7 ± 1	4 ± 1	5 ± 2	66 ± 15	72 ± 8
	1	14 ± 3	10 ± 3	116 ± 12	204 ± 11	10 ± 3	5 ± 1	2 ± 1	2 ± 1	68 ± 21	70 ± 14
	2.5	9 ± 2	11 ± 3	112 ± 4	125 ± 13	9 ± 1	8 ± 2	2 ± 1	4 ± 1	66 ± 5	68 ± 14
	5	11 ± 3	19 ± 4	104 ± 32	104 ± 11	8 ± 4	6 ± 2	3[S] ± 1	3 ± 1	65 ± 13	56 ± 11
	10	8 ± 3	11 ± 7	89 ± 9	97 ± 10	7 ± 3	6 ± 3	–[R]	3 ± 2	85 ± 10	65 ± 9
	25	9 ± 1	13 ± 6	73[S] ± 9	85[S] ± 4	6 ± 3	7 ± 3	–[R]	3 ± 3	60 ± 9	61 ± 8
	50	9[S] ± 1	17 ± 3	74[S] ± 7	91[S] ± 12	6 ± 3	5 ± 4	–[R]	3 ± 2	49 ± 7	65 ± 18
	100	6[S] ± 2	12 ± 3	–[R]	93[S] ± 13	8 ± 1	7 ± 2	–[R]	6 ± 1	63 ± 2	67 ± 5
	250	6[S] ± 2	14[P] ± 2	NT	–[PR]	–[PR]	8 ± 1	–[R]	2[S] ± 2	62 ± 3	54 ± 18
	500	–[PR]	14[P] ± 3	NT	–[PR]	10[PS] ± 3	7[P] ± 2	–[PR]	3[PS] ± 1	–[PR]	–[R]
	1000	8[P] ± 3	8[P] ± 2	NT	–[PR]	6[P] ± 2	9[P] ± 3	–[PR]	–[PR]	37[P] ± 3	43[P] ± 7
	2500	8[P] ± 3	10[P] ± 3	NT	–[PR]	7[P] ± 3	9[P] ± 3	–[PR]	–[PR]	33[P] ± 9	29[P] ± 2
	5000	13[PS] ± 8	12[P] ± 3	NT	–[PR]	15[P] ± 2	9[P] ± 1	–[PR]	–[PR]	42[P] ± 6	52[P] ± 9
2AA	2.5	NA	2207 ± 1311[C]	NA	1153 ± 156[C]	NA	322 ± 40[C]	NA	134 ± 14[C]	NA	NA
	10.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	335 ± 80[C]
2NF	2.5	1126 ± 296[C]	NA	NA	NA	NA	NA	NA	NA	NA	NA
NAAZ	1.0	NA	NA	417 ± 35[C]	NA	536 ± 139[C]	NA	NA	NA	NA	NA
ICR	0.5	NA	NA	NA	NA	NA	NA	151 ± 15[C]	NA	NA	NA
NQNO	2.0	NA	NA	NA	NA	NA	NA	NA	NA	641 ± 80[C]	NA

2AA – 2-Aminoanthracene; 2NF – 2-Nitrofluorene; CBD – cannabidiol; ICR – ICR-191 acridine; NAAZ – sodium azide; NQNO – 4-nitroquinoline-N-oxide; SD – standard deviation; DMSO – dimethyl sulfoxide; NA – not applicable; NT – not tested.
Note: All plates had confluent background lawn, unless otherwise noted.
[A] Calculated from triplicate plates.
[B] Data from repeat assay for TA100 without activation; data from vehicle control for strain TA100 without metabolic activation in original assay was outside the historical control data.
[C] Protocol criteria for a positive response met.
[P] Precipitates present.
[S] Slightly reduced background lawn.
[R] Cytotoxicity: Reduced background lawn, plates not counted.

Table 3
Cytotoxicity and micronucleus summary data from *in vitro* micronucleus study with CBD.

		Cytotoxicity (%)	Mean MN (%)	z'	Cytotoxicity (%)	Mean MN (%)	z'	Cytotoxicity (%)	Mean MN (%)	z'
Treatment Group	**μg/mL**	4-Hour Treatment without Metabolic Activation			27-Hour Treatment without Metabolic Activation			4-Hour Treatment with Metabolic Activation		
DMSO	1%	0.00	0.12	NA	0.00	0.21	NA	0.00	0.33	NA
CBD	0.100	0.77	0.21	<0	6.83	0.15	<0	2.59	0.32	<0
	0.250	1.41	0.28	<0	5.30	0.10	<0	4.15	0.31	<0
	0.500	0.40	0.26	<0	3.18	0.13	<0	3.55	0.26	<0
	1.00	1.40	0.21	<0	6.36	0.10	<0	5.62	0.29	<0
	2.00	1.54	0.28	<0	12.76	0.07	<0	5.43	0.30	<0
	4.00	7.01	0.24	<0	23.03	0.14	<0	10.64	0.32	<0
	6.00	11.20	0.34	0.10	57.09	0.55	0.24	17.75	0.28	<0
	8.00	18.79	0.29	<0	NA	NA	NA	35.38	0.47	<0
	9.00	18.08	0.30	<0	NA	NA	NA	48.11	0.47	<0
	10.0	29.97	0.34	0.10	NA	NA	NA	40.86	0.52	<0
	11.0	45.09	0.43	0.31	NA	NA	NA	NA	NA	NA
MMC	0.0625	19.28	1.56	0.77[a]	NA	NA	NA	NA	NA	NA
	0.125	36.58	4.18	0.88[a]	NA	NA	NA	NA	NA	NA
VIN	2.5	NA	NA	NA	54.09	3.09	0.84[a]	NA	NA	NA
	1.0	NA	NA	NA	56.24	3.33	0.85[a]	NA	NA	NA
CP	4.7	NA	NA	NA	NA	NA	NA	26.20	2.55	0.80[a]
	11.9	NA	NA	NA	NA	NA	NA	59.78	7.17	0.90[a]

No data are shown for CBD concentrations that exhibited excessive cytotoxicity (designated as NA), i.e., at ≥ 8 μg/mL in the 27-h treatment without metabolic activation; at ≥ 12 μg/mL in the 4-h treatment without metabolic activation; and at ≥ 11 μg/mL in the 4-h treatment with metabolic activation.
CBD – cannabidiol; CP – Cyclophosphamide monohydrate; DMSO – Dimethyl sulfoxide; MMC – Mitomycin C; NA – Not Applicable; VIN – Vinblastine sulfate.
MN – Micronucleated cells.
[a] z' ≥ 0.6.

In the definitive assay, precipitates were not observed in any treatment with or without activation, up to 22.0 μg/mL. Excessive cytotoxicity was observed at ≥ 8 μg/mL in the 27-h treatment without metabolic activation; at ≥ 12 μg/mL in the 4-h treatment without metabolic activation; and at ≥ 11 μg/mL in the 4-h treatment with metabolic activation. The vehicle and positive control data were comparable to the relevant historical control values.

Cannabidiol was considered negative for inducing micronuclei in TK6 cells in the 27-h treatment without metabolic activation and in the 4-h treatments with and without metabolic activation under the conditions of this test system (Table 3).

3.4. *In vivo* micronucleus assay

3.4.1. *In-life and clinical observations*

In the preliminary dose range-finding experiment, absolute body weights were similar between groups (data not shown). Average food consumption per animal decreased in an apparent dose-proportional manner in males; however, females in the 2000 mg/kg-bw/day group had increased consumption on Day 2 (data not shown). Overall, the lowest dose (500 mg/kg-bw/d CBD) was generally well-tolerated; all animals showed only mild incoordination and three animals decreased activity following dosing. At the mid dose (1000 mg/kg-bw/d CBD), all males and females were observed with pronounced piloerection, partially closed eyes, moderate to severe incoordination, and decreased activity. Similar or more severe signs were noted at the highest dose (2000 mg/kg-bw/d CBD), along with observations of shallow breathing, intermittent tremoring, and cold to touch in all animals and low carriage in all males. A few animals were observed convulsing. Based on these findings and the severity of the toxic effects, all animals in the 2000 mg/kg-bw/d dose group were humanely euthanized late on Day 2 and the functional MTD was considered to be 1000 mg/kg-bw/d CBD.

In the main micronucleus experiment, no difference in absolute body weights was reported in animals administered up to 500 mg/kg-bw/d CBD compared to concurrent control animals. However, at 1000 mg/kg-bw/d CBD, average body weights on Day 3 were statistically significantly decreased in males, along with a non-significant decrease observed in females. Dose-dependent decreases in the average rate of body weight gain relative to concurrent controls was observed in animals of the 500 and 1000 mg/kg-bw/d CBD groups; however, this finding was only statistically significant in males. Average food consumption per animal decreased in a dose proportional manner in males and females. There were no notable observations recorded throughout the study for animals receiving 250 mg/kg-bw/d CBD and animals receiving 500 mg/kg-bw/d CBD were minimally affected, with a single male and female each presenting with wet fur on their ventral surface and a single male presenting with decreased activity. Animals administered 1000 mg/kg-bw/d CBD were more noticeably affected, as they were observed with hunched posture (one male, two females), incoordination (one per sex), and decreased activity (four per sex) after 2 days of dosing. One female at this high dose was also observed with low carriage and abnormal gait.

3.4.2. *Bioanalysis*

Plasma samples (n = 36) from the main experiment were analyzed for CBD and 7-COOH-CBD. Administration of CBD to male and female animals resulted in significant, dose-related exposure to both CBD and 7-COOH-CBD at all dose levels (Table 4).

3.4.3. *Micronucleus analysis*

There was no statistically significant or dose-dependent increase in the %MN PCEs in male or female rats at any CBD dose level as compared to the vehicle control group (Table 5). No evidence of bone marrow cytotoxicity (decreases in PCE:TE ratio) was found in any animal at any CBD dose level. Group mean values for %MN-PCEs and PCE:TE ratios for the vehicle and positive controls were within 95% of the historical control intervals obtained by CRL (Skokie, IL), demonstrating the acceptability of the assay (Table 5) (CRL, 2016). Therefore, CBD was negative for clastogenic activity and/or disruption of the mitotic apparatus under the conditions of this assay.

Table 4
Average CBD and 7-COOH-CBD rat plasma concentrations on Day 3 following CBD administration (*in vivo* micronucleus assay).

CBD Dose Group (mg/kg-bw/d)	Gender	CBD Plasma Concentration (ng/mL) ± SD	7-COOH-CBD Plasma Concentration (ng/mL) ± SD
250	M	677 ± 338	2025 ± 1589
	F	911 ± 724	3736 ± 4734
500	M	4969 ± 3192	16305 ± 10888
	F	4800 ± 4138	12288 ± 8943
1000	M	26250 ± 15642	43333 ± 8815
	F	16800 ± 2990	39483 ± 11788

CBD – cannabidiol; M – male; F – female; SD – Standard deviation.

Table 5
Summary of micronucleus assay data for Sprague Dawley rats administered CBD for two consecutive days.

Treatment Group (mg/kg-bw/d)	Sex	% MN-PCEs ± SD	PCE:TE Ratio ± SD
CBD: 0 (vehicle control)	M	0.06 ± 0.05	0.58 ± 0.06
	F	0.09 ± 0.03	0.54 ± 0.10
CBD: 250	M	0.10 ± 0.02	0.53 ± 0.07
	F	0.05 ± 0.04	0.53 ± 0.08
CBD: 500	M	0.10 ± 0.04	0.60 ± 0.04
	F	0.05 ± 0.04	0.55 ± 0.06
CBD: 1000	M	0.09 ± 0.03	0.55 ± 0.07
	F	0.07 ± 0.05	0.49 ± 0.10
CP: 60 (positive control)	M	1.63 ± 0.46*	0.45 ± 0.14
	F	0.97 ± 0.13*	0.28 ± 0.05*

CBD – pure cannabidiol isolate; CP – cyclophosphamide monohydrate; MN – micronucleated; PCE – polychromatic erythrocyte; SD – standard deviation; vehicle control – olive oil; *statistically different from vehicle control $p \leq 0.01$.

4. Discussion

The commercial availability of hemp-derived products in the US has increased dramatically since the passage of the Hemp Farming Act, part of the 2018 Farm Bill. Despite the increase in consumer use of hemp-derived CBD in the US and in other countries globally, few high-quality, guideline-based genotoxicity studies have been conducted or published on CBD itself. In addition, EFSA and UK FSA (2022) have highlighted this endpoint as a data gap, concluding the currently available studies to be insufficient for reaching a conclusion regarding genotoxic potential. Interpretations of the results of previously published genotoxicity assays using CBD and CBD-containing mixtures have been inconsistent and complicated by issues of purity and potency of the test article and/or limitations in the study design. For example, an early investigation of CBD's genotoxic potential found evidence of micronucleus induction in bone marrow cells following intraperitoneal injection of 10 mg CBD/kg-bw in (C57BL x C3H)F1 mice for 5 consecutive days, resulting in structural and numerical chromosomal aberrations (Zimmerman and Raj, 1980). However, no abnormal effects on sperm morphology were observed with CBD exposure for 5 days followed by a 35-day recovery as reported (Zimmerman et al., 1979). More recently, the Epidiolex non-clinical safety review (CDER, 2018) describes negative results from an *in vivo* micronucleus study with a pure CBD test material. However, the public summary of this report does not describe the justification for dose selection or why the doses used were limited to a top dose of 500 mg/kg, nor does it provide any indication that CBD was confirmed to reach the bone marrow compartment. Results from *in vitro*

comet assays have been inconsistent. The CDER (2018) review summarizes a study in which CBD did not induce DNA damage in the liver of rats at doses up to 500 mg/kg-bw/day in the alkaline comet assay. Carvalho et al. (2022) reported significantly increased DNA damage in sperm, but not leukocytes in comet assays. Whereas, Russo et al. (2019) reported CBD-induced DNA damage in single cell gel electrophoresis (SCGE) experiments in a human liver cell line (HepG2) and in buccal-derived cells (TR146) In addition, results of an *in vitro* micronucleus assay using HepG2 cells, found CBD to be positive for induction of micronuclei (Russo et al., 2019).

To date, three publications have explored the genotoxic potential of CBD-containing hemp extracts following OECD guidelines. Marx et al. (2018) conducted a guideline-compliant study using a battery of genotoxicity assays (i.e., an *in vitro* reverse mutation Ames assay [OECD 471], an *in vitro* micronucleus assay [OECD 473], and *in vivo* mouse micronucleus assay) [OECD 474] on a hemp extract (~25% CBD). Although genotoxicity results were negative from all assays, extrapolation of results to pure CBD is difficult. Slight reduction in polychromatic erythrocytes was observed *in vivo* (evidence of bone marrow toxicity), but was not considered biologically significant (Marx et al., 2018). Results from this assay cannot be considered definitively negative, due to a failure to demonstrate the presence of CBD in the blood or toxicity to bone marrow. Dziwenka et al. (2020, 2021) have also conducted OECD guideline studies on hemp extracts (~7% CBD), although negative results were obtained, the low CBD content in the test articles decreases the utility of the data for understanding the genotoxicity of pure CBD. Given the absence of high-quality, guideline-compliant genotoxicity assessments on pure CBD, additional research to fully assess the safety of this compound is warranted.

In the present study, CBD isolate (>99%) did not produce an increase in the number of revertants in the presence or absence of S9 microsomal enzymes in the Ames assay. This negative result aligns with previously conducted Ames assays using hemp extracts containing ~7–25% CBD (Marx et al., 2018; Dziwenka et al., 2020, 2021), as well as unpublished data reviewed by CDER (2018) in which CBD was negative in an Ames assay up to 5000 μg/plate, with and without metabolic activation. Results from the *in vitro* micronucleus assay indicate that CBD was negative for inducing micronuclei in TK6 cells in both the 27-h treatment without metabolic activation and the 4-h treatments with and without metabolic activation. Notably, this finding contrasts with other published work that used a pure CBD test material; however, our study was conducted in the human TK6 cell line, which is considered by the OECD 487 guideline to be validated more extensively for this assay than the HepG2 cell line previously reported (Zhang et al., 1995; OECD, 2016b; Russo et al., 2019). Finally, the *in vivo* micronucleus study provides additional support for a lack of genotoxicity of CBD. There was no significant or dose-dependent increase in the %MN PCEs in male or female Sprague Dawley rats for any CBD dose level tested, up to 1000 mg/kg-bw/d. This result is similar to the negative findings obtained at up to 2000 mg/kg-bw/d in a mouse micronucleus study using a hemp extract containing approximately 25% CBD, as well as up to 500 mg/kg-bw/d in rats using CBD isolate in the Epidiolex non-clinical review package (Marx et al., 2018; CDER, 2018).

Finally, the conclusions from the studies presented here are supported by the results of a 2-year cancer bioassay reviewed by the FDA (CDER, 2018) in which a CBD Botanical Drug Substance (containing 57.5–67.2% CBD) was administered in the diet up to 50 mg/kg-bw/d and demonstrated no treatment-related increase in tumor incidence. However, test-article impurities and the dietary route of exposure were concerns highlighted by the FDA for this study, which limit its relevance to the present assessment.

In conclusion, three GLP- and OECD guideline-compliant mutagenicity and genotoxicity studies were performed to test the ability of CBD to induce mutation or cause chromosomal damage. The results from this testing battery indicate that pure CBD isolate was nonmutagenic, nonclastogenic, and nongenotoxic under the study conditions. These studies are the first to be published using guideline-compliant methods on a pure CBD isolate, and together, they provide information critical to assessing the safe consumer use of CBD in food and dietary supplements.

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CRediT authorship contribution statement

Rayetta G. Henderson: Conceptualization, Writing – original draft, Writing – review & editing. **Brian T. Welsh:** Study monitoring, Writing – original draft, Writing – review & editing. **Kristen R. Trexler:** Conceptualization, Writing – review & editing. **Marcel O. Bonn-Miller:** Conceptualization, Supervision. **Timothy W. Lefever:** Conceptualization, Writing – review & editing.

Declaration of competing interest

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Data availability

Data will be made available on request.

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Oral toxicity evaluation of cannabidiol

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ABSTRACT

Use of cannabidiol (CBD) in humans has increased considerably in recent years. While currently available studies suggest that CBD is relatively safe for human consumption, data from publicly available studies on CBD conducted according to modern testing guidelines are lacking. In the current study, the potential for toxicity following repeated oral exposure to hemp-derived CBD isolate was evaluated in male and female Sprague Dawley rats. No adverse treatment-related effects were observed following administration of CBD via oral gavage for 14 and 90 days at concentrations up to 150 and 140 mg/kg-bw/d, respectively. Microscopic liver and adrenal gland changes observed in the 90-day study were determined to be resolved after a 28-day recovery period. CBD was well tolerated at these dose levels, and the results of this study are comparable to findings reported in unpublished studies conducted with other CBD isolates. The current studies were conducted as part of a broader research program to examine the safety of CBD.

1. Introduction

Cannabis sativa L. and cannabis-derived products in various forms have been used widely throughout the world for thousands of years for medicinal and recreational purposes (Bergamaschi et al., 2011; Rupasinghe et al., 2020). While delta-9-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, has historically been the primary focus of much research, attention has also turned to other phytocannabinoids and terpenes. In particular, cannabidiol (CBD), a non-intoxicating phytocannabinoid, has received much recent attention from both the general public and the scientific community for its purported anticonvulsive, analgesic, anti-anxiety, neuroprotective, antioxidant, and antimicrobial properties (Small and Marcus, 2002; Pertwee, 2004; Billakota et al., 2019). Epidiolex® (active ingredient CBD isolate) has been approved by the United States (US) Food and Drug Administration (FDA) for the treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome in patients 2 years of age and older (Jazz Pharmaceuticals, 2023). In addition, Sativex®, a combination of CBD and THC, is approved in other countries for the treatment of moderate to severe spasticity due to multiple sclerosis (Jazz Pharmaceuticals, 2023).

Cannabidiolic acid (CBDa), typically the most common phytocannabinoid in fiber (hemp) plants, is converted to CBD through decarboxylation (Formato et al., 2020; Rupasinghe et al., 2020). CBD and its metabolites identified in human plasma have been shown to possess low affinity and lack appreciable functional activity at classical cannabinoid receptors 1 and 2 (CB1 and CB2; CDER, 2018a). A substantial body of data exists that describes the different pharmacodynamic properties of CBD and its modulation of targets unrelated to the endocannabinoid system (ECS), such as serotonin 1a (5HT1a) (Russo et al., 2005; Gomes et al., 2011). CBD has the ability to interact with multiple 7-transmembrane receptor systems, ion channels, transporters, and enzymes (Small and Marcus, 2002; Pertwee, 2004). Although a number of other targets have been identified *in vitro*, their potential physiological implications are currently theoretical.

Following implementation of the Hemp Farming Act, part of the Agricultural Improvement Act of 2018 (aka, "2018 Farm Bill"), interest in hemp-derived products, especially CBD, has outpaced the development of a legal pathway for their use in foods and dietary supplements in the US. Data submitted to FDA as part of the nonclinical and clinical packages for Epidiolex® (CDER, 2018a, b) are key to understanding CBD consumer safety; however, only summaries of such studies are available to the public. While the FDA has not established tolerable daily intake

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Abbreviations

ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
BUN	blood urea nitrogen
Ca	calcium
CB1, CB2	cannabinoid receptors 1 and 2
CBD	cannabidiol
CHOL	total cholesterol
Cl	chloride
ECS	endocannabinoid receptor system
FDA	US Food and Drug Administration
FOB	functional observational battery
FSA	UK Food Safety Authority
GLOB	globulin
GLP	Good Laboratory Practice
GLU	fasting glucose
H&E	hematoxylin and eosin
HDL	high-density lipoprotein
K	potassium
LDL	low-density lipoprotein
Na	sodium
NOAEL	no-observed-adverse-effect level
OECD	Organization for Economic Co-operation and Development
PHOS	inorganic phosphorus
SD	standard deviation
SDH	sorbitol dehydrogenase
TAG	triglycerides
TBIL	total bilirubin
TGA	Therapeutic Goods Administration
THC	delta-9-tetrahydrocannabinol
TP	total serum protein
TSH	thyroid stimulation hormone
UK	United Kingdom
US	United States

levels associated with consumer use, an overview of the agency's activities related to evaluating the safe use of CBD in food and dietary supplement products can be found on its website (FDA, 2023). In addition, the United Kingdom (UK) Food Safety Authority (UK FSA, 2022), Health Canada (2022), and the Australian Therapeutic Goods Administration (TGA, 2021) have established recommended maximum upper intake levels of CBD by healthy adults, except those planning to be or currently pregnant or breastfeeding. While some limited safety-related data on CBD are available in the public domain, these regulatory agencies continue to highlight gaps in available toxicology and other related data. In addition, recent literature reviews, including a systematic mapping study, have been published summarizing the available CBD toxicity data and knowledge gaps (Henderson et al., 2023a; Li et al., 2021). Specifically, no publicly available studies on CBD conducted according to regulatory test guidelines are identified to evaluate genotoxicity, repeated oral toxicity, or reproductive and developmental toxicity endpoints.

Given that consumer use of CBD has increased drastically in recent years, it is essential to continue to generate data on which to evaluate its safety. Additional research is needed to fill the aforementioned data gaps and, subsequently, to enable calculation of a margin of safety/exposure. The present study was conducted to investigate the potential for toxicity following repeated exposure to oral CBD in male and female Sprague Dawley rats. The current studies were conducted as part of a larger program to investigate the safety and potential for toxicity of CBD isolates (Henderson et al., 2023b).

2. Materials and methods

2.1. Test material

Hemp-derived CBD isolate (99.08–101.46%; CAS No. 13956-29-1) was provided by Canopy Growth USA (Evergreen, Colorado). The test substance was stored under ambient conditions and remained stable through the duration of the study, as demonstrated by analysis on samples of the test substance (neat) collected at the beginning, middle, and end of the in-life phase (data not shown).

2.2. Animals

Seven or eight-week-old CRL Sprague Dawley CD® IGS rats (20/sex) were obtained from Charles River Laboratories (Raleigh, North Carolina). The animals were housed individually in single polycarbonate cages in temperature-controlled and humidity-monitored rooms with a 12-h light/dark cycle. Test animals were provided filtered tap water and 2016 Certified Envigo Teklad Global Rodent Diet® *ad libitum* throughout the study. Animals were cared for according to the published National Research Council guidelines. The testing laboratory is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited.

2.3. 14-day oral toxicity study in rats

2.3.1. Dose selection and test article preparation

Dose levels of 0 (vehicle control; olive oil), 30, 70, or 150 mg/kg-bw/d of the CBD were selected. The high dose was selected based on available data from studies submitted as part of the Epidiolex non-clinical data package reviewed by FDA, including a 26-week study in rats (CDER, 2018a; Study number GWTX1412). The low- and mid-dose levels were selected to derive a dose-response for observed effects. Fresh preparations containing 20, 46.7, and 100 mg/mL of the test substance mixed in olive oil (w/v) were prepared daily using a dosing volume of 1.5 ml/kg. Individual doses were calculated weekly and adjusted based on current body weights. Samples from each dose were collected and tested to verify homogeneity and concentration. Analytical chemistry results can be found in Supplementary Table 1A.

2.3.2. Experimental design

The design was conducted following the principles of FDA Toxicological Principles for the Safety Assessment of Food Ingredients (FDA, 2007) and Organization for Economic Co-operation and Development Test Guideline 407 (OECD, 2008). All animals were acclimated for 5 days prior to testing. Rats were distributed into four groups (one vehicle control and three test substance groups; n = 5/sex). Dose levels of 0 (vehicle control), 30, 70, or 150 mg/kg-bw/d of CBD were administered once daily via oral gavage for 14 days. Throughout the study, animals were observed daily for signs of gross toxicity and behavioral changes, and weekly for a battery of detailed observations. Body weight and food consumption were recorded weekly. Animals were fasted overnight prior to sacrifice on day 16. Necropsies were performed on all study animals, and any gross observations, including lesions, were recorded. Wet weights of the liver, kidneys (combined), and adrenal glands (combined) from each animal were recorded, and tissues were fixed in 10% neutral buffered formalin for histopathological examination.

2.3.3. Serum chemistry

Blood samples were collected from the inferior vena cava in all animals at terminal sacrifice. Serum from each sample was separated via refrigerated centrifugation, transferred to a fresh tube, and stored at −80 °C. Clinical chemistry parameters evaluated on a Cobas C 311 Analyzer (Roche Diagnostics) included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine, total cholesterol (CHOL), triglycerides (TAG), fasting glucose (GLU), total serum protein (TP), albumin (ALB), globulin (GLOB), calcium (Ca), inorganic phosphorus (PHOS), sodium (Na), potassium (K), and chloride (Cl).

2.3.4. Histopathology

During necropsy, selected organs (liver, kidneys, and adrenal glands) from the control and high-dose animals were placed in 10% formalin. Fixed tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). Slides were prepared and evaluated by a board-certified veterinary pathologist at Histo-Scientific Research Laboratories (HSRL).

2.4. 90-day oral toxicity study in rats

2.4.1. Dose selection and test article preparation

Dose levels of 0 (vehicle control; olive oil), 50, 80, 120, or 140 mg/kg-bw/d of CBD were selected for this study. As with the previous 14-day oral toxicity study, the high dose was selected based on available data from studies submitted as part of the Epidiolex non-clinical data package reviewed by FDA (CDER, 2018a). One 26-week study in rats demonstrated no toxicologically significant effects of pure CBD at doses up to 150 mg/kg-bw/day (Study number GWTX1412); however, other studies reviewed by CDER (2018a) reported adverse effects following similar exposure levels to mixtures containing high concentrations of CBD (e.g., 50–65.6% CBD; Study numbers GWTX10124 and JJG0001). As such, the doses in the current study were selected to confirm the findings of the 26-week study with pure CBD. Fresh preparations containing 50, 80, 120, and 140 mg/mL of the test substance mixed in olive oil (w/v) were prepared daily using a dosing volume of 1 ml/kg. Individual doses were calculated weekly and adjusted based on current body weights. Samples from each dose were collected and tested to verify homogeneity and concentration at the beginning, middle, and end of the study. Analytical chemistry results can be found in Supplementary Table 1B.

2.4.2. Experimental design

The study was conducted in compliance with FDA (21 CFR Part 58) and the OECD Principles of Good Laboratory Practice (GLP) ENV/MC/CHEM (98)17. The study design followed FDA Toxicological Principles for the Safety Assessment of Food Ingredients (FDA, 2007) and OECD Test Guideline 408 (OECD, 2018). All animals were acclimated for 5–6 days prior to testing. Rats were distributed into five main groups (one vehicle control and four treatment groups; n = 10/sex). An additional five recovery groups (n = 5/sex) also received the same dose levels as the main test group for 90 days, followed by a 28-day recovery period. CBD was administered daily via oral gavage 92 days (males) and 93 days (females). Ophthalmologic evaluations were conducted once during the acclimation period and again on dosing Day 87 for all study animals. Animals were observed twice daily for viability, signs of gross toxicity, and behavioral changes, in addition to weekly detailed clinical observations. Body weight and food consumption were recorded weekly. All rats were fasted overnight prior to terminal sacrifice. Necropsies were performed on all study animals, and any gross observations, including lesions, were recorded. Wet weights of the liver, kidneys (combined), adrenal glands (combined), brain, heart, spleen, thymus, epididymides (combined), testes (combined), uterus, and ovaries with oviducts (combined) were recorded for all animals.

2.4.3. Functional observational battery

During week 12 of the study, a functional observational battery (FOB) was performed on all main-test animals using a validated protocol (Product Safety Labs Standard Operating Procedure, issue date 04/05/18). Each rat was evaluated for the following: excitability, autonomic function, gait and sensorimotor coordination, reactivity and sensitivity, and other abnormal clinical signs. The observer was blind to treatment groups, and all animals were observed in random order. In addition, duplicate measurements of foot splay and triplicate measurements of grip strength of forelimb and hindlimb (Dillon GS Series Digital Force Gage, Fairmont, Minnesota) were recorded for each animal, and the corresponding mean was calculated.

2.4.4. Motor activity

During week 12 of the study, motor activity was evaluated on all main-test animals. Activity was monitored using an automated Photobeam Activity System®, San Diego Instruments, Inc. Each rat was placed into a polycarbonate solid-bottom cage and evaluated for 1 h in a quiet, darkened room. Photobeam counts accumulated over six 10-min intervals.

2.4.5. Clinical pathology

Blood samples for hematology (except those for coagulation analyses) and thyroid hormone assessment were collected following an overnight fast from main-test animals on days 93 (male) and 94 (female) and from recovery animals on Day 122. The day prior, animals were placed in metabolism cages, and urine was collected from all animals after at least 15 h of fasting. At terminal sacrifice, blood was sampled for clinical chemistry, as well as determination of prothrombin time and activated partial thromboplastin time. Additional selected hematology analyses were determined with an ADVIA 120 Hematology System (Siemens Healthineers) and included white blood cell count (WBC) and differential leukocyte count, red blood cell count (RBC), red cell distribution width, hematocrit (Hct), hemoglobin concentration (Hgb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC; calculated), reticulocyte count, and platelet count. Coagulation times were determined on a Siemens Sysmex CA620 (Siemens Healthineers) automated system. Thyroid hormone analysis was done only for main-test animals; ELISA was used to measure triiodothyronine (T3), thyroxine (T4), and thyroid stimulation hormone (TSH). Clinical chemistry parameters determined on a COBAS C311 (Roche Diagnostics) analyzer included: AST, ALT, ALP, SDH, TBIL, BUN, creatinine, CHOL, high-density lipoprotein (HDL), low-density lipoprotein (LDL), TAG, GLU, TP, ALB, GLOB, Ca, PHOS, Na, K, and Cl. Urinalysis (CLINITEK Advantus urinalysis analyzer, Siemens Healthineers) included quality, color, clarity, volume, pH, glucose, specific gravity, total protein, ketone, bilirubin, blood, urobilinogen, and microscopic urine sediment.

2.4.6. Histopathology

Tissues and organs were collected and stored in 10% buffered formalin and included prostate and seminal vesicles, adrenals, ileum with Peyer's patches, rectum, jejunum, salivary glands, kidneys, larynx, aorta, liver, skeletal muscle, bone (femur), lungs, skin, bone marrow (femur and sternum), mandibular and mesenteric lymph nodes, spinal cord (cervical, mid-thoracic, and lumbar), brain (medulla/pons, cerebellar, and cerebral cortex), mammary gland, nasal turbinates, spleen, nose, sternum, cecum, ovaries, stomach, cervix, oviducts, thymus, colon, pancreas, thyroid, duodenum, parathyroid, trachea, esophagus, peripheral nerve (sciatic), urinary bladder, Harderian gland, pharynx, uterus, heart, pituitary gland, vagina, and all gross lesions. Eyes, epididymides, optic nerve, and testes samples from the main-test group were preserved in Davidson's fixative and stored in ethanol.

Histological examination was performed on all samples from the control and high-dose groups. In addition, samples of adrenal gland and liver from all main-test animals in the 50-, 80-, and 120¬mg/kg-bw/

d groups were processed. Fixed tissues were paraffin embedded, sectioned, and stained with H&E. Slides were prepared and assessed by a board-certified veterinary pathologist.

2.5. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9 software (San Diego, CA).

Mean and standard deviations (SDs) were calculated for all quantitative data. In both studies, in-life data from treatment and control groups were compared using a two-way analysis of variance (ANOVA; Motulsky, 2014) and tested for time effect, group effect, and time/group interaction effect. Repeated measures were accounted for in one independent variable (i.e., time). Dunnett's test (Dunnett, 1964, 1980) was used as the post hoc multiple comparisons test to compare individual treatment groups to the control group within each time variable. Endpoints with single measurements of continuous data within groups (e.g., organ weight, clinical pathology) were evaluated for homogeneity of variances (Bartlett, 1937) and normality (Shapiro and Wilk, 1965). One-way ANOVA was subsequently used between treatment and control groups where homogeneous variances and normal distribution were observed. If one-way ANOVA was significant, treated groups were compared to controls using a multiple comparisons test (e.g., Dunnett's test). If variances were considered significantly different, groups were compared using a non-parametric method (e.g., Kruskal-Wallis non-parametric ANOVA; Kruskal and Wallis, 1952). If non-parametric ANOVA was significant, treated groups were compared to control using Dunn's test (Dunn, 1964).

For clinical pathology data (90-day study), when variances were considered significantly different, data were log transformed to achieve variance homogeneity and normality. If log transformation failed, a non-parametric method (e.g., Kruskal-Wallis non-parametric ANOVA) was used. When variance was significant, a comparison of treated groups to control was performed (e.g., Dunn's test). One outlier value was identified in the control group of the main 90-day study in males for the hematology parameter reticulocytes. This outlier was identified using the ROUT test method, a method combining regression and outlier removal, with a cutoff Q value set to 0.1% (Motulsky and Brown, 2006). This value was removed prior to performing statistical analysis.

For histopathology of terminal sacrifice animals, Fishers exact test was used to compare the incidence of each microscopic finding between control and the high-dose group animals, and between each group and controls, where specific findings were noted. The extended Mantel-Haenszel (MH) test was also used. Statistical analysis of microscopic findings in recovery animals was not performed due to a lack of sample size and associated power.

3. Results

3.1. 14-Day oral toxicity study

No treatment-related deaths or clinical signs were observed throughout the study. Mean body weights (Table 1; Suppl. Table 2) and food consumption (Suppl. Table 3) of male and female rats administered CBD for 14 days were similar to that of control groups. Mean relative liver weights increased ($p < 0.05$) in the males of the high-CDB-dose group (150 mg/kg-bw/d), while mean absolute and relative liver weights were increased ($p < 0.001$–0.05) in females in the two highest CBD dose groups (70 and 150 mg/kg-bw/d) compared to control groups. Of note, all mean absolute liver weights were within the laboratory's historical control range for this parameter (Product Safety Labs, 2022). No other changes in weights of organs evaluated were found (Table 1). In general, significant differences in serum chemistry parameters were observed in a non-dose-dependent manner and were within range of biological variation and/or lab historical ranges, and therefore, were considered to be not toxicologically relevant (Suppl. Table 4; Product Safety Labs, 2022). The only treatment-related adverse change observed in serum parameters was elevated total cholesterol levels in female rats administered 150 mg/kg-bw/d CBD; however, the mean value was well within the laboratory's historical control range for this parameter (Product Safety Labs, 2022). No treatment-related macroscopic observations were observed. However, liver histopathology (Table 2) revealed a dose-dependent increase in the mean severity of centrilobular hepatocellular hypertrophy, with increased cytoplasmic volume in the hypertrophic cells in both male and female rats, correlating with an increase in liver weights in the higher dose groups. Under the conditions of this 14-day study and the endpoints evaluated, Sprague Dawley rats tolerated an oral dose of 150 mg/kg-bw/d CBD.

Table 1
Absolute and relative organ weights of male (A) and female (B) rats administered 0, 30, 70, or 150 mg/kg-bw/day CBD isolate for 14 days.

A				
Terminal	Treatment group (mg/kg-bw/day)			
Weights	0	30	70	150
Mean organ weights (g)				
Body Weight	314.80 ± 19.52	320.20 ± 22.80	315.20 ± 19.06	315.40 ± 16.85
Liver	10.72 ± 1.87	10.92 ± 1.10	12.01 ± 1.61	13.24 ± 1.48
Adrenal Glands	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Kidneys	2.56 ± 0.30	2.66 ± 0.30	2.67 ± 0.18	2.70 ± 0.27
Mean organ-to-body weight (g/kg)				
Liver	33.90 ± 4.10	34.13 ± 2.46	38.18 ± 5.37	42.04 ± 4.84*
Adrenal Glands	0.22 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.19 ± 0.04
Kidneys	8.15 ± 0.88	8.32 ± 1.05	8.49 ± 0.52	8.54 ± 0.58
B				
Terminal	Treatment group (mg/kg-bw/day)			
Weights	0	30	70	150
Mean organ weights (g)				
Body Weight	196.80 ± 12.56	190.20 ± 13.03	202.20 ± 10.76	201.20 ± 11.12
Liver	7.96 ± 1.51	9.01 ± 0.76	10.06 ± 0.68*	11.82 ± 1.14***
Adrenal Glands	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01*
Kidneys	1.65 ± 0.15	1.71 ± 0.10	1.75 ± 0.08	1.84 ± 0.15
Mean organ-to-body weight (g/kg)				
Liver	40.41 ± 7.01	47.35 ± 1.79	49.78 ± 3.21*	58.73 ± 3.83***
Adrenal Glands	0.33 ± 0.04	0.38 ± 0.05	0.38 ± 0.06	0.41 ± 0.05
Kidneys	8.40 ± 0.66	9.03 ± 0.58	8.67 ± 0.10	9.13 ± 0.49

Data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's test (n = 5). * indicates a p-value <0.05, ** indicates a p-value <0.01, and *** indicates a p-value <0.001 compared to control group.

3.2. 90-Day oral toxicity study

3.2.1. Survival and clinical observations

No treatment-related mortality was observed throughout the study. One female control rat was euthanized for humane reasons on Day 22 due to moderate visible swelling in the chest, later confirmed during necropsy to be caused by a dosing error. No clinical observations throughout the study were attributed to administration of the CBD. In male rats, incidental clinical signs included unilateral red ocular discharge, slight hypersalivation, slight to moderate alopecia on forelimb/hindlimb or head, superficial eschar on the head or tail, and slight to moderate visible swelling on the right ear. Incidental findings for females included slight to extreme hypersalivation, slight alopecia on the head or forelimb, slight moist rales, abnormal gait, a damaged left hindlimb, eschar on the head, and slight swelling in the right ear. These

Table 2
Histopathology results for male (A) and female (B) rats administered 0, 30, 70, or 150 mg/kg-bw/day CBD isolate for 14 days.

A

Treatment group (mg/kg-bw/day)	Liver: Hypertrophy	Adrenal Glands	Kidneys: Chronic Progressive Nephropathy	Kidneys: Dilation
0	No remarkable findings	No remarkable findings	1 ≥ 4	No remarkable findings
30	1 ≥ 2 2 ≥ 1 3 ≥ 2	–	–	–
70	3 ≥ 3 4 ≥ 2	–	–	–
150	3 ≥ 2 4 ≥ 3	No remarkable findings	1 ≥ 3	1 ≥ 1

B

Treatment group (mg/kg-bw/day)	Liver: Hypertrophy	Adrenal Glands	Kidneys: Chronic Progressive Nephropathy
0	No remarkable findings	No remarkable findings	1 ≥ 1
30	1 ≥ 2 2 ≥ 3	–	–
70	3 ≥ 3 4 ≥ 2	–	–
150	3 ≥ 1 4 ≥ 4	No remarkable findings	No remarkable findings

– = no data; histopathology severity scores: 1 ≥ Minimal, 2 ≥ Mild, 3 ≥ Moderate, 4 ≥ Marked, 5 ≥ Severe; (n = 5).

findings were sporadic among controls and treatment groups, and therefore, were considered unrelated to the CBD test material.

3.2.2. Body weight and food consumption

Body weight and body weight gain for all treatment groups were comparable to that of the control group through the 28-day recovery period (Fig. 1; Suppl. Table 5). Additionally, there were no significant changes in food consumption or food efficiency in any of the treatment groups during both the main toxicity test and recovery period (Suppl. Table 6).

3.2.3. Ophthalmologic examinations, functional observation battery, and motor activity assessment

Ophthalmologic examinations revealed no abnormalities in any of the treatment or control groups at either time point. Similarly, functional observations showed no treatment-related findings. Mean quantitative measurements for forelimb/hindlimb grip strength and hindlimb foot splay were comparable between animals in the control and CBD-treated groups (Suppl. Table 7). Overall, motor activity measurements (i.e., mean total movements) for CBD-treated groups were considered comparable to those of the control group. Mean total movements were statistically significantly higher in males in the rats administered 120 mg/kg-bw/d (time intervals 2 and 3 only) and 140 mg/kg-bw/d (time intervals 1 and 3 only), compared to concurrent controls, with mean total movements similar to controls for the remainder of the time intervals. For females, all groups exhibited a similar level of movement over all intervals, with the single exception of females in the 80 mg/kg-bw/d group during the sixth time interval (Suppl. Table 8).

3.2.4. Clinical chemistry and pathology

CBD-treated females in some groups exhibited a significant increase in total cholesterol (140 mg/kg-bw/d), HDL (≥120 mg/kg-bw/d), and LDL (≥120 mg/kg-bw/d) compared to the female control group after the 90-day study (Table 3). However, these observed increases in CHOL,

Table 3
Serum clinical chemistry parameters for male (A) and female (B) rats administered 0, 50, 80, 120, or 140 mg/kg-bw/day CBD isolate for 90 days.

A

Serum Parameters	Treatment Group (mg/kg-bw/day) 0	50	80	120	140
AST (U/L)	91.90 ± 48.14	76.60 ± 18.14	75.80 ± 11.41	82.50 ± 17.60	79.80 ± 24.42
ALT (U/L)	37.90 ± 17.63	31.50 ± 6.62	28.70 ± 5.56	30.20 ± 7.07	38.70 ± 23.92
ALKP (U/L)	77.50 ± 17.67	76.60 ± 10.95	78.90 ± 21.95	79.90 ± 13.53	83.60 ± 16.52
BUN (mg/dL)	15.30 ± 2.21	15.90 ± 1.79	15.70 ± 2.00	16.90 ± 2.33	15.10 ± 2.23
Ca (mg/dL)	11.42 ± 0.42	11.15 ± 0.77	11.11 ± 0.88	11.31 ± 0.43	11.31 ± 0.75
Cl (mmol/L)	100.70 ± 2.73	101.30 ± 3.08	101.30 ± 2.08	101.80 ± 2.54	100.60 ± 3.36
Na (mmol/L)	143.80 ± 4.29	143.90 ± 4.33	144.10 ± 4.33	145.30 ± 4.03	144.90 ± 4.56
K (mmol/L)	8.25 ± 1.46	7.60 ± 1.26	7.34 ± 1.54	7.61 ± 0.68	7.50 ± 1.73
CHOL (mg/dL)	63.50 ± 16.98	58.40 ± 7.76	56.60 ± 7.34	64.30 ± 15.87	66.10 ± 12.85
LDL (mmol/L)	0.26 ± 0.12	0.27 ± 0.07	0.18 ± 0.07	0.28 ± 0.08	0.27 ± 0.08
HDL (mmol/L)	1.03 ± 0.25	0.91 ± 0.16	0.92 ± 0.11	1.02 ± 0.18	1.02 ± 0.21
GLU (mg/dL)	258.90 ± 59.10	248.00 ± 69.58	226.60 ± 41.74	233.60 ± 51.41	210.80 ± 47.15
CREAT (mg/dL)	0.31 ± 0.05	0.29 ± 0.05	0.29 ± 0.04	0.33 ± 0.05	0.31 ± 0.03
PHOS (mg/dL)	9.25 ± 0.70	8.48 ± 1.04	8.43 ± 1.03	8.60 ± 0.54	8.82 ± 0.88
TBIL (mg/dL)	0.07 ± 0.02	0.05 ± 0.01*	0.04 ± 0.02**	0.04 ± 0.02**	0.04 ± 0.02*
TAG (mg/dL)	69.10 ± 26.92	72.50 ± 20.91	115.30 ± 56.83	65.00 ± 27.00	68.50 ± 34.78
SDH (U/L)	20.01 ± 5.91	22.22 ± 11.54	20.43 ± 6.12	26.39 ± 13.23	28.08 ± 13.04
TP (g/dL)	6.33 ± 0.25	6.31 ± 0.39	6.42 ± 0.47	6.55 ± 0.33	6.61 ± 0.39
ALB (g/dL)	4.07 ± 0.13	3.98 ± 0.25	4.01 ± 0.23	4.12 ± 0.20	4.19 ± 0.28
GLOB (g/dL)	2.26 ± 0.28	2.33 ± 0.23	2.41 ± 0.31	2.43 ± 0.28	2.42 ± 0.25

B

Serum Parameters	Treatment Group (mg/kg-bw/day) 0	50	80	120	140
AST (U/L)	69.33 ± 8.78	72.80 ± 18.12	94.40 ± 80.44	64.70 ± 13.12	81.50 ± 38.59
ALT (U/L)	25.78 ± 3.99	28.30 ± 11.70	28.70 ± 18.75	25.20 ± 3.58	36.20 ± 16.80
ALP (U/L)	43.44 ± 13.47	32.70 ± 9.88	32.40 ± 6.50	37.70 ± 14.23	36.90 ± 10.74
BUN (mg/dL)	18.67 ± 4.47	20.50 ± 2.27	18.80 ± 3.52	17.80 ± 2.53	17.60 ± 4.06
Ca (mg/dL)	11.92 ± 0.47	11.66 ± 0.70	11.65 ± 1.35	11.50 ± 082	11.92 ± 0.86
Cl (mmol/L)	101.80 ± 2.54	99.15 ± 1.89*	100.80 ± 1.14	100.40 ± 1.82	99.89 ± 1.62
Na (mmol/L)	144.80 ± 3.31	140.90 ± 2.96	143.00 ± 2.63	143.00 ± 2.83	143.30 ± 2.79
K (mmol/L)	7.39 ± 1.54	7.69 ± 0.62	6.97 ± 0.85	7.18 ± 2.11	6.45 ± 0.94
CHOL (mg/dL)	94.44 ± 12.76	92.70 ± 17.16	100.50 ± 29.90	120.90 ± 16.35	141.40 ± 45.81*
LDL (mmol/L)	0.24 ± 0.07	0.24 ± 0.06	0.29 ± 0.13	0.40 ± 0.13*	0.44 ± 0.18**
HDL (mmol/L)	1.83 ± 0.21	1.88 ± 0.29	1.93 ± 0.43	2.27 ± 0.27*	2.62 ± 0.58***
GLU (mg/dL)	246.00 ± 66.35	214.70 ± 78.20	227.10 ± 68.64	218.10 ± 58.76	227.80 ± 42.93
CREAT (mg/dL)	0.43 ± 0.09	0.36 ± 0.05	0.42 ± 0.07	0.40 ± 0.06	0.37 ± 0.08

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Table 3 (*continued*)

PHOS (mg/dL)	8.50 ± 1.10	9.17 ± 1.59	8.88 ± 1.43	8.46 ± 1.50	8.17 ± 1.35
TBIL (mg/dL)	0.07 ± 0.03	0.04 ± 0.02**	0.05 ± 0.02	0.04 ± 0.01*	0.04 ± 0.02*
TAG (mg/dL)	80.44 ± 19.07	67.90 ± 41.30	67.50 ± 29.07	66.10 ± 16.78	68.40 ± 39.91
SDH (U/L)	13.36 ± 1.85	15.59 ± 4.82	20.68 ± 15.03	14.72 ± 2.54	24.43 ± 20.07
TP (g/dL)	7.21 ± 0.40	7.38 ± 0.74	7.35 ± 0.58	7.45 ± 0.52	7.77 ± 0.73
ALB (g/dL)	5.29 ± 0.33	5.14 ± 0.69	5.05 ± 0.54	5.20 ± 0.51	5.38 ± 0.46
GLOB (g/dL)	1.92 ± 0.21	2.24 ± 0.32	2.30 ± 0.28*	2.25 ± 0.31	2.39 ± 0.41**

Data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's test or Dunn's test if data failed tests for normality or homogeneity (n = 10). * indicates a p-value <0.05, ** indicates a p-value <0.01 and *** indicates a p-value <0.001 compared to control group.

HDL, and LDL were not associated with lesions that reflect alterations in lipid metabolism in the liver (Section 3.2.6); in addition, all CHOL and HDL levels were within the laboratory's historical control values (Product Safety Labs, 2022). All levels were back to control levels at the end of the 28-day recovery period (Suppl. Table 10). Total bilirubin was significantly higher in males (all doses) and females (50, 120, and 140 mg/kg-bw/d); this finding was considered test substance related but not adverse, as it was not dose dependent, returned to control levels after the recovery period, and correlated with adaptative changes in the liver (Hall et al., 2012). All other differences in clinical chemistry parameters between treatment groups and controls were minimal and random and were determined to occur as a result of biological variation among rats (Table 3; Suppl. Table 10).

The serum levels of thyroid hormones T3 and T4 did not change with treatment. TSH levels increased significantly in male and female rats administered 80–140 mg/kg-bw/d CBD when compared to their respective control groups following the 90-day exposure (Suppl. Table 11).

Absolute reticulocyte levels were significantly reduced in males in the 50, 120, and 140 mg/kg-bw/d groups compared to control animals; this finding was considered nonadverse, as it was not dose dependent, was within the historical control range for this parameter (Product Safety Labs, 2022), and returned to control levels following the recovery time period. In addition, this finding was not accompanied by changes in red blood cells or lesion in the bone marrow (Suppl. Tables 12 and 13). The few other observed differences in hematology parameters were considered a result of biological variation among rats and appeared to have occurred sporadically (Suppl. Tables 12 and 13). Additionally, no treatment-related changes in coagulation or urinalysis parameters were observed (Suppl. Tables 14 and 15).

3.2.5. Organ weights and gross pathology

At terminal sacrifice on Day 93/94 and recovery sacrifice on Day 122, all gross findings were determined to be incidental or commonly found in Sprague Dawley rats; findings were of similar incidence in both control and treatment groups and were therefore not related to CBD administration.

In comparison to control groups at terminal sacrifice, mean absolute and relative liver weights were increased significantly in male rats administered 80–140 mg/kg-bw/d CBD, as well as in female rats administered 120 and 140 mg/kg-bw/d CBD. Mean relative kidney weights were increased significantly in male rats at the highest dose, with female rats in the two highest dose groups (120 and 140 mg/kg-bw/d) having significantly increased absolute and kidney weights compared to control rats. Weights of adrenal glands were increased significantly in male rats treated with 120 mg/kg-bw/d CBD (relative only), and in female rats that received 80–140 mg/kg-bw/d CBD

Table 4
Absolute and relative organ weights of male (A) and female (B) rats administered 0, 50, 80, 120, or 140 mg/kg-bw/day CBD isolate for 90 days.

A

Terminal Weights	Treatment Group (mg/kg-bw/day) 0	50	80	120	140
Mean organ weights (g)					
Body Weight	573.20 ± 49.53	524.70 ± 58.36	590.20 ± 51.05	543.60 ± 51.39	546.20 ± 45.02
Adrenal Glands	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Brain	2.33 ± 0.15	2.29 ± 0.08	2.34 ± 0.15	2.36 ± 0.13	2.29 ± 0.06
Epididymis	1.52 ± 0.17	1.41 ± 0.16	1.51 ± 0.28	1.49 ± 0.20	1.44 ± 0.16
Heart	1.56 ± 0.11	1.45 ± 0.18	1.59 ± 0.26	1.45 ± 0.18	1.48 ± 0.14
Kidneys	3.53 ± 0.37	3.44 ± 0.42	3.67 ± 0.32	3.61 ± 0.45	3.74 ± 0.24
Liver	15.08 ± 1.90	14.72 ± 1.61	18.16 ± 2.22**	18.18 ± 1.96**	18.69 ± 2.57**
Spleen	0.97 ± 0.09	0.85 ± 0.17*	0.91 ± 0.12	0.82 ± 0.12*	0.78 ± 0.08**
Testes	3.92 ± 0.36	3.74 ± 0.42	3.43 ± 0.91	3.67 ± 0.31	3.85 ± 0.26
Thymus	0.28 ± 0.07	0.33 ± 0.06	0.27 ± 0.12	0.22 ± 0.05	0.23 ± 0.07
Mean organ-to-body weight (g/kg)					
Adrenal Glands	0.11 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.14 ± 0.02*	0.13 ± 0.02
Brain	4.09 ± 0.49	4.43 ± 0.57	4.00 ± 0.46	4.36 ± 0.31	4.22 ± 0.33
Epididymis	2.65 ± 0.16	2.75 ± 0.27	2.59 ± 0.57	2.76 ± 0.39	2.65 ± 0.26
Heart	2.72 ± 0.19	2.79 ± 0.32	2.69 ± 0.34	2.67 ± 0.13	2.71 ± 0.29
Kidneys	6.16 ± 0.38	6.67 ± 0.71	6.24 ± 0.57	6.64 ± 0.62	6.88 ± 0.54*
Liver	26.24 ± 1.37	28.16 ± 1.67	30.69 ± 1.68***	33.45 ± 1.93***	34.14 ± 3.02***
Spleen	1.69 ± 0.16	1.63 ± 0.29	1.54 ± 0.19	1.51 ± 0.16	1.43 ± 0.14*
Testes	6.86 ± 0.67	7.25 ± 0.65	5.89 ± 1.67	6.79 ± 0.73	7.11 ± 0.96
Thymus	0.49 ± 0.10	0.61 ± 0.13	0.46 ± 0.21	0.41 ± 0.10	0.42 ± 0.14

B

Terminal Weights	Treatment Group (mg/kg-bw/day) 0	50	80	120	140
Mean organ weights (g)					
Body Weight	300.33 ± 29.43	285.90 ± 22.12	315.70 ± 31.57	299.80 ± 25.53	289.80 ± 18.21
Adrenal Glands	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01*	0.09 ± 0.01***	0.10 ± 0.02***
Brain	2.07 ± 0.07	2.09 ± 0.09	2.07 ± 0.06	2.09 ± 0.10	2.05 ± 0.11
Heart	1.01 ± 0.11	0.93 ± 0.10	0.98 ± 0.07	0.94 ± 0.07	0.99 ± 0.08
Kidneys	1.86 ± 0.10	1.93 ± 0.15	2.05 ± 0.13	2.15 ± 0.31*	2.23 ± 0.21**
Liver	8.52 ± 0.84	8.71 ± 1.13	10.64 ± 1.22	12.13 ± 1.16 ***	12.98 ± 2.05 ***
Spleen	0.51 ± 0.08	0.51 ± 0.06	0.60 ± 0.07	0.53 ± 0.09	0.53 ± 0.08
Ovaries w/ Oviducts	0.13 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.03	0.12 ± 0.01
Thymus	0.24 ± 0.07	0.23 ± 0.04	0.25 ± 0.04	0.23 ± 0.09	0.21 ± 0.05
Uterus	0.70 ± 0.21	0.64 ± 0.22	0.88 ± 0.70	0.83 ± 0.32	0.71 ± 0.06

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Table 4 (*continued*)

Mean organ-to-body weight (g/kg)					
Adrenal Glands	0.23 ± 0.04	0.25 ± 0.03	0.27 ± 0.04	0.31 ± 0.03***	0.33 ± 0.06***
Brain	6.94 ± 0.61	7.37 ± 0.67	6.63 ± 0.72	7.01 ± 0.59	7.10 ± 0.52
Heart	3.37 ± 0.23	3.24 ± 0.19	3.11 ± 0.22	3.15 ± 0.34	3.42 ± 0.24
Kidneys	6.24 ± 0.46	6.76 ± 0.38	6.54 ± 0.62	7.17 ± 0.84**	7.70 ± 0.47***
Liver	28.40 ± 1.15	30.44 ± 2.91	33.91 ± 4.33	40.51 ± 2.87***	44.68 ± 5.12***
Spleen	1.72 ± 0.30	1.80 ± 0.24	1.90 ± 0.30	1.77 ± 0.26	1.81 ± 0.23
Ovaries w/ Oviducts	0.43 ± 0.05	0.45 ± 0.06	0.42 ± 0.05	0.44 ± 0.07	0.41 ± 0.05
Thymus	0.81 ± 0.25	0.81 ± 0.13	0.78 ± 0.09	0.77 ± 0.32	0.72 ± 0.20
Uterus	2.35 ± 0.76	2.25 ± 0.81	2.91 ± 2.67	2.78 ± 1.12	2.44 ± 0.25

Data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's test or Dunn's test if data failed tests for normality or homogeneity (n = 10). * indicates a p-value <0.05, ** indicates a p-value <0.01, and *** indicates a p-value <0.001 compared to control group.

(absolute: 80–140 mg/kg-bw/d, relative: 120–140 mg/kg-bw/d). Furthermore, spleen weights decreased significantly only in males treated with 50 and 120 mg/kg-bw/d (absolute) or 140 mg/kg-bw/d CBD (absolute and relative) compared to the concurrent control group. All other organ weights for male and female treatment groups were similar to controls after the 90-day exposure (Table 4).

Fewer differences in organ weights were observed following 28 days of recovery in the group of animals sacrificed on Day 122. Mean relative kidney weights were increased significantly in males receiving 120 mg/kg-bw/d, compared to controls. Mean absolute liver weights were significantly higher in females treated with 80 mg/kg-bw/d. Mean absolute and relative ovaries (with oviducts) weights were significantly higher in females treated with 140 mg/kg-bw/d CBD, when compared to the female control group. All other reported organ weights for male and female rats in the recovery group were similar across groups, including spleen weights (Suppl. Table 9).

3.2.6. *Histopathology*

CBD-related histopathological changes were found in the livers of male and female rats, as well as in the adrenal glands of males, following the 90-day exposure (Table 5). Increases in incidence of liver hypertrophy observed in both sexes were found to be statistically significant starting at 80 mg/kg-bw/d; however, hepatocellular hypertrophy fully resolved in both male and female rats following the 28-day recovery period (Table 5). Of note, the incidence and severity of hepatocellular hypertrophy correlated with the dose-dependent increase in liver weights (Table 4). Increased adrenal gland vacuolation observed in males was found to be statistically significant in the two highest dose groups at the end of dosing (Fig. 2). The vacuolation of male adrenal glands decreased after 28 days of recovery, with only minimal (<1) histopathology severity scores in one animal (0, 50, and 120 mg/kg-bw/d), two animals (140 mg/kg-bw/d), or no animals (80 mg/kg-bw/d). Notably, two male rats from the control group also received a minimal severity score for adrenal cortical vacuolation after either Day 90 or Day 122 of the study (Table 5). No other tissues showed remarkable changes due to CBD administration on histopathologic examination, including in the spleens of animals in all dose groups.

Under the conditions of this 90-day study oral toxicity study (followed by 28-day recovery period) and the toxicological endpoints evaluated, the NOAEL for the oral CBD administration was determined to be 140 mg/kg-bw/d for male and female Sprague Dawley rats.

4. Discussion

Consumer interest in and use of foods and dietary supplements

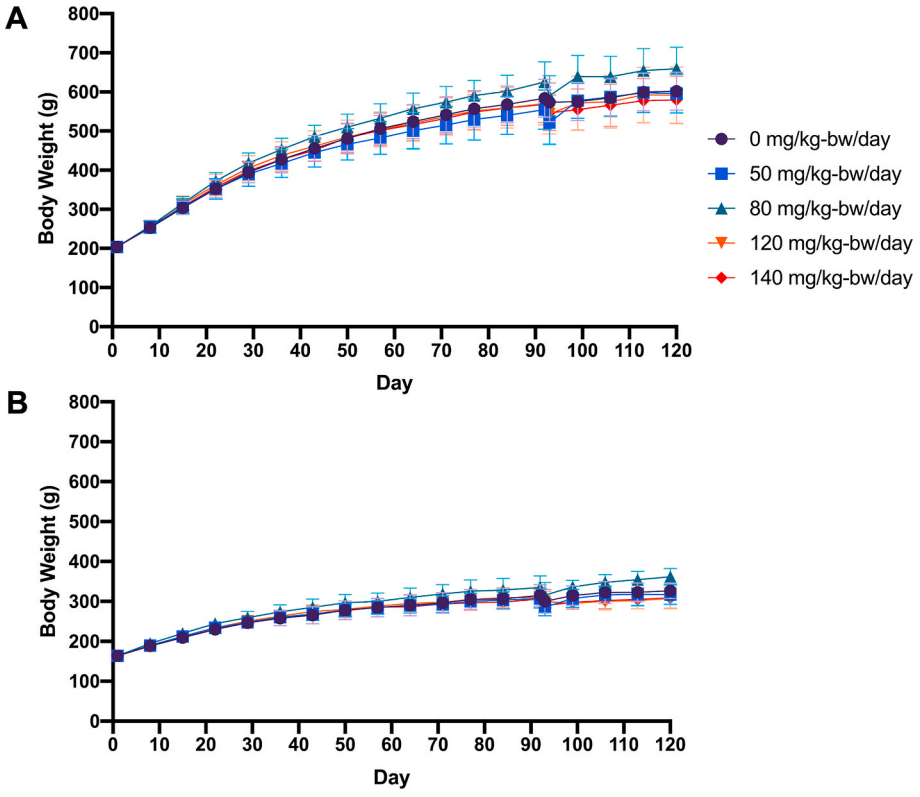

Fig. 1. Mean body-weight data for male (1A) and female (1B) rats administered CBD isolate by oral gavage for 90 days.

Table 5
Histopathology results for male (A) and female (B) rats administered 0, 50, 80, 120, or 140 mg/kg-bw/day CBD isolate for 90 days followed by a 28-day recovery period.

A

Treatment group (mg/kg-bw/day)	90-day Toxicity Study (Day 93/94)		28-day Recovery Period (Day 122)	
	Liver: Hypertrophy	Adrenal Glands: Vacuolation	Liver: Hypertrophy	Adrenal Glands: Vacuolation
0	No remarkable findings	1 ≥ 1	No remarkable findings	1 ≥ 1
50	1 ≥ 3	1 ≥ 4	No remarkable findings	1 ≥ 2
80	1 ≥ 8* 2 ≥ 1	1 ≥ 5	No remarkable findings	No remarkable findings
120	1 ≥ 7* 2 ≥ 3	1 ≥ 3* 2 ≥ 6	No remarkable findings	1 ≥ 1
140	1 ≥ 3* 2 ≥ 7	1 ≥ 3* 2 ≥ 6	No remarkable findings	1 ≥ 2

B

Treatment group (mg/kg-bw/day)	90-day Toxicity Study (Day 93/94)		28-day Recovery Period (Day 122)	
	Liver: Hypertrophy	Adrenal Glands: Vacuolation	Liver: Hypertrophy	Adrenal Glands: Vacuolation
0	No remarkable findings	No remarkable findings	No remarkable findings	No remarkable findings
50	No remarkable findings	No remarkable findings	No remarkable findings	No remarkable findings
80	1 ≥ 8*	No remarkable findings	No remarkable findings	No remarkable findings
120	1 ≥ 2* 2 ≥ 7	No remarkable findings	No remarkable findings	No remarkable findings
140	1 ≥ 1* 2 ≥ 8	No remarkable findings	No remarkable findings	No remarkable findings

* indicates a p-value <0.05 for dose group.
Histopathology severity scores: 1 ≥ Minimal, 2 ≥ Mild, 3 ≥ Moderate, 4 ≥ Severe; (n = 5¬10).

containing hemp-derived CBD is increasing. As a result, it is critical that CBD safety be demonstrated using validated, guideline-compliant methods, and that data supporting the derivation of safe levels be widely distributed in peer-reviewed publications. These are the first guideline-compliant repeat-dose toxicity studies on a hemp-derived CBD isolate to be made available in a scientific journal. In 14-day subacute and 90-day subchronic toxicity studies, administration of CBD at concentrations up to 150 and 140 mg/kg-bw/d, respectively, by oral gavage did not produce any significant toxic effects. CBD was well tolerated at these dose levels, as evidenced by the absence of major treatment-related changes in the general condition and appearance of the rats, as well as growth, feed and water intake, ophthalmoscopic examinations, routine hematology and clinical chemistry parameters, urinalysis, necropsy, and histopathological findings.

The NOAEL in this 90-day study was determined to be the highest dose tested—140 mg/kg-bw/d—in male and female Sprague Dawley rats. The results of this study are comparable to findings reported in studies used for safety support that were included as part of the Epidiolex Non-Clinical data package reviewed by FDA (CEER, 2018a,b), but not published in the peer-reviewed literature. In one such study, rats were administered CBD for 26 weeks followed by a 28-day recovery period (Study number GWTX1412). In a second study, dogs were administered CBD for 39 weeks followed by a 28-day recovery period (Study number GWTX1413). In each study, the NOAEL was identified as the highest dose tested of 150 and 100 mg/kg bw/day, respectively; the main findings of liver effects were determined by CDER (2018a) not to be toxicologically significant based on reversibility.

The transient changes in motor activity observed in males of the 90-day study measured at this single timepoint were determined to be non-adverse; changes were not dose dependent, habituation was similar to that of controls, and no changes in functional observations were seen. In addition, hyperactivity is inconsistent with other repeat dose studies administering oral CBD, in which motor activity was either unchanged or decreased (CDER, 2018a).

With regard to histopathological findings, microscopic liver and adrenal gland changes observed in the 90-day study were resolved after a 28-day recovery period, and the incidences of microscopic changes were deemed comparable to controls at this time point. The observed hepatocellular hypertrophy fully resolved in both male and female rats following the recovery period. The treatment-related effects on liver weights and histopathology in this study are concluded to be non-adverse as they indicate induction of both phase 1 and phase 2 metabolic enzymes; with phase 2 enzymes critical in the elimination of thyroid hormones (Papineni et al., 2015; Noyes et al., 2019). Similar changes were noted in a recent reproductive toxicity study conducted with CBD in male and female rats (reported in our companion paper (Henderson et al., 2023b). Hepatocellular hypertrophy without other changes in histopathology or clinical chemistry measures indicative of liver toxicity, as is the case with CBD both in this study, and in the aforementioned reproductive toxicity study, is considered adaptive and non-adverse, as described in a review by Hall et al. (2012).

There are several pathways by which chemicals can produce anti-thyroid effects by perturbing thyroid-pituitary homeostasis, e.g., reduction of circulating thyroid hormones (T3 and T4) with increase TSH levels resulting in thyroid hyperplasia/hypertrophy (Hurley et al., 1998; Zabke et al., 2011; Noyes et al., 2019; Huisinga et al., 2020). One pathway involves chemical induction of liver enzymes that conjugate glucuronic acid to T3 and T4 via uridine diphosphate glucuronosyltransferase (UDPGT), which leads to increased T3 and T4 elimination and decreased serum concentrations of these hormones that trigger an increased synthesis of TSH (Papineni et al., 2015; Noyes et al., 2019). Serum levels of TSH were significantly increased in male and female rats at 80 mg/kg-bw/d CBD treatment and above compared to concurrent controls, without any change in T3 and T4 levels. The small change in serum TSH was determined not to be test article specific as no dose response was observed, values were within the laboratory's historical control range (Product Safety Labs, 2022), and the change did not coincide with a change in thyroid weight or histopathologic changes in the thyroid glands of rats in the high-dose groups. In addition, the small change observed in TSH levels across dose groups may also represent a lack of specificity of the immunoassay due to cross-reactivity of antibodies to other molecules (Li et al., 2019). Although the pattern of liver changes observed in these CBD studies may reflect hepatic microsomal enzyme induction, including UDPGT activity, the thyroid pathway was not perturbed in this study. Also of critical importance is that this liver induction is adaptive; this is reflected in the resolution of the liver lesions when CBD exposure ends, as demonstrated in the current study.

CBD administration resulted in an increase in the incidence and severity of cytoplasmic vacuolation of cells within the adrenal zona fasciculata in male rats of the two highest dose groups (120 and 140 mg/kg-bw/d). However, these lesions were resolved at the recovery time point, and the increased incidence of vacuolation in male adrenal glands in treated groups (0–2 per group) were not considered meaningfully different from the incidence in controls (1 per group). In addition, in the present 14- and 90-day studies, clinical pathology changes did not support the histopathology changes in the adrenal gland of male rats with changes in cholesterol (14- and 90-day study) and HDL (90-day

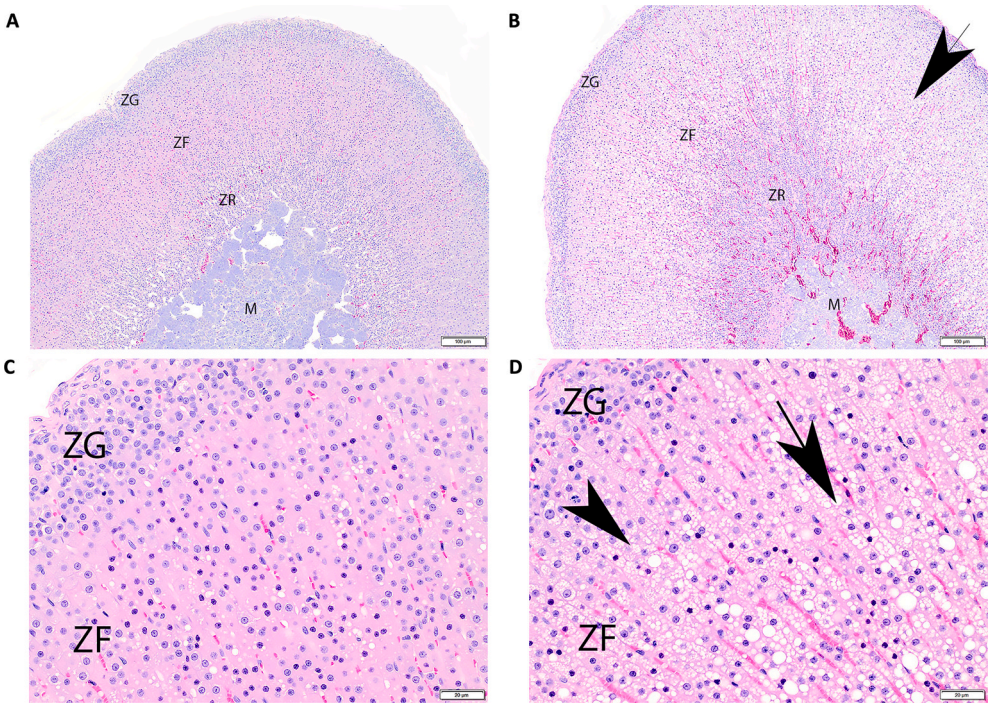

Fig. 2. Adrenal gland sections (H&E stained) from male Sprague-Dawley rats following oral gavage with daily doses of CBD Isolate for 90 days. A. Adrenal gland from a vehicle control male rat (Group 1 Animal 7006×, 40× magnification) with little cytoplasmic vacuolization noted in the cells of the zona fasciculata (ZF), below the minimum threshold for recording as a microscopic abnormality in this animal. B. Adrenal gland from a male rat administered 140 mg/kg-bw/d (Group 5 Animal 7122; 40× magnification) with cells of the ZF expanded by clear cytoplasmic vacuoles (arrow). This degree of cytoplasmic vacuolization was considered mild (Grade 2 severity). C. Adrenal gland from a vehicle control male rat (Group 1 Animal 7006×, 200× magnification) with little cytoplasmic vacuolization was noted in the cells of the ZF. D. Adrenal gland from a male rat administered 140 mg/kg-bw/d (Group 5 Animal 7122; 200× magnification) showing cytoplasmic microvacuolization (arrowhead) and macrovacuolization (arrow) that is appreciable in the cells of the ZF. ZG = zona glomerulosa; ZR = zona reticularis; M = medulla.

study) occurring only in females. The adrenal gland is a common target organ for chemical toxicity, and at the same time, it is not uncommon to observe non-specific cytotoxic effects in the adrenal cortex following administration of high dose levels of various test substances (Rosol et al., 2001). In addition, adrenal cortical vacuolization is considered a background lesion in laboratory rats (Laast et al., 2014) and is proposed to represent the accumulation of cholesterol and other steroid precursors. This vacuolization has been noted to be increased by xenobiotics that interfere with steroid synthesis (Brändli-Baliocco et al., 2018) and has been reported previously in laboratory rats administered cannabinoids (Dziwenka et al., 2020). For this reason, the fact that only male rats had an increase in the adrenal lesion, which was resolved when exposure stopped, combined with the knowledge that this is typically a background lesion in rats, support the conclusion that this lesion does not represent an adverse effect associated with administration of CBD.

5. Conclusion

No adverse treatment-related effects were observed following up to 90 days of treatment with a pure hemp-derived CBD isolate at any dose level tested. The oral NOAEL was therefore determined to be 150 and 140 mg/kg-bw/d in 14- and 90-day toxicity studies, respectively. These findings fill an important research gap in publicly available data on the safety profile of CBD, thus providing key data to support its safe use in foods and dietary supplements. Future studies testing higher doses of CBD will help to further elucidate any potential toxicity associated with repeat consumer ingestion.

CRediT authorship contribution statement

Rayetta G. Henderson: Conceptualization, Investigation, Writing – original draft. **Timothy W. Lefever:** Conceptualization, Writing – review & editing. **Melissa M. Heintz:** Writing – original draft. **Kristen R. Trexler:** Writing – review & editing. **Susan J. Borghoff:** Writing – review & editing. **Marcel O. Bonn-Miller:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

This work was funded by Canopy Growth Corporation. Authors KRT, TWL, and MOB-M were employees of Canopy Growth Corporation during the conduct and drafting of this study; during their employment, they received stock options. ToxStrategies, a private consulting firm providing services on toxicology and risk assessment issues, received funds for conducting this work. Authors RGH, SJB, and MMH are employees of ToxStrategies.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.113778.

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Reproductive and developmental toxicity evaluation of cannabidiol

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ABSTRACT

An important data gap in determining a safe level of cannabidiol (CBD) intake for consumer use is determination of CBD's potential to cause reproductive or developmental toxicity. We conducted an OECD Test Guideline 421 GLP-compliant study in rats, with extended postnatal dosing and hormone analysis, where hemp-derived CBD isolate (0, 30, 100, or 300 mg/kg-bw/d) was administered orally. Treatment-related mortality, moribundity, and decreased body weight and food consumption were observed in high-dose F_0 adult animals, consistent with severe maternal toxicity. No effects were observed on testosterone concentrations, F_0 reproductive performance, or reproductive organs. Hepatocellular hypertrophy in the 100- and 300 mg/kg-bw/day groups correlated with hypertrophy/hyperplasia in the thyroid gland and changes in mean thyroid hormone concentrations in F_0 animals. Mean gestation length was unaffected; however, total litter loss for two females and dystocia for two additional females in the high-dose group occurred. Other developmental effects were limited to lower mean pup weights in the 300 mg/kg-bw/d group compared to those of concurrent controls. The following NOAELs were identified for CBD isolate based on this study: 100 mg/kg-bw/d for F_0 systemic toxicity and female reproductive toxicity, 300 mg/kg-bw/d for F_0 male reproductive toxicity, and 100 mg/kg-bw/d for F_1 neonatal and F_1 generation toxicity.

1. Introduction

The implementation of the Hemp Farming Act—part of the Agricultural Improvement Act of 2018 (aka, "2018 Farm Bill")—has led to greater market availability and public interest in consumer products containing hemp-derived cannabidiol (CBD) in the United States (US). While various forms of cannabis have been used globally for medicinal and recreational purposes for thousands of years, only recently has a CBD drug (Epidiolex®) been approved by the US Food and Drug Administration (FDA) for the treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome in patients 2 years of age and older (Jazz Pharmaceuticals, 2023). In addition, Sativex®, a combination of CBD and delta-9 tetrahydrocannabinol (THC), is approved in other countries for the treatment of moderate to severe spasticity due to multiple sclerosis (Jazz Pharmaceuticals, 2023). CBD is also proposed to have analgesic, anxiolytic, neuroprotective, antioxidant, and antimicrobial properties (Small and Marcus, 2002; Pertwee, 2004; Billakota et al., 2019; Devinsky et al., 2018).

The FDA has not identified a suitable regulatory pathway for use of CBD in food or dietary supplements, nor has the agency established tolerable daily intake levels associated with consumer use. An overview of the FDA's activities related to evaluating the safe use of CBD in food and dietary supplement products can be found on its website (FDA, 2023). However, based on recent evaluations of the available safety data, the United Kingdom (UK) Food Safety Authority (FSA, 2022), Health Canada (2022), and the Australian Therapeutic Goods Administration (TGA, 2021) have established recommended maximum upper intake levels of CBD by healthy adults, except those planning to be or currently pregnant or breastfeeding. In addition, recent literature reviews, including a systematic mapping study, have been published summarizing the available CBD toxicity data and knowledge gaps (Henderson et al., 2023a; Li et al., 2021). While limited safety-related data on CBD are available in the public domain, regulatory agencies continue to highlight data gaps in the understanding of CBD toxicology. Specifically, no publicly available non-clinical studies on CBD isolate have been conducted according to regulatory test guidelines to evaluate genotoxicity, repeated oral toxicity, or reproductive and developmental

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Abbreviations

AGD	anogenital distance
ANOVA	analysis of variance
BSA	bovine serum albumin
CASA	computer-aided sperm analysis
CBD	cannabidiol
CDER	Center for Drug Evaluation and Research
CFR	US Code of Federal Regulations
FDA	US Food and Drug Administration
FSA	UK Food Safety Authority
GD	gestational day
GLP	Good Laboratory Practice
HPLC	high-performance liquid chromatography
IACUC	institutional animal care and use committee
LD	lactation day
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PND	postnatal day
POD	point of departure
SD	standard deviation
T3	triiodothyronine
T4	total thyroxine
TGA	Therapeutic Goods Administration
THC	delta-9-tetrahydrocannabinol
TSH	thyroid stimulating hormone
UDPGT	uridine diphosphate glucuronosyltransferase
UK	United Kingdom
US	United States of America

toxicity endpoints. Understanding potential effects of CBD on reproduction and/or offspring development is critical in determining a safe CBD intake level for consumer use (e.g., in dietary supplements, foods, and/or beverages).

Using studies reviewed by CDER (2018a), as well as other, published studies, to review CBD safety, Li et al. (2021) summarized the reproductive and developmental toxicity findings in rats, mice, and rabbits: "A full battery of assessments was conducted including litter size, body weight, physical and functional development, sexual milestones, auditory startle, motor activity, and learning and memory. Adverse effects of CBD treatment have been observed primarily in the dose groups of 150 or 250 mg/kg-bw/day including decreased pup body weights, delays in achieving developmental landmarks (eye opening, pupillary reflex, and sexual maturation in male and female), neurobehavioral changes (decreased locomotor activity), and adverse effects on reproductive system structure (small testis) and possibly function." Studies reviewed by the FDA as part of the Epidiolex non-clinical package provide data that can be incorporated into an overall assessment of the potential reproductive and developmental toxicity of CBD (CDER, 2018a). However, none of these studies conducted on CBD isolate included dosing in both sexes starting prior to mating and continuing through weaning, and thus have been deemed insufficient by some regulatory agencies for evaluation of CBD for consumer use. Similarly, published *in vitro* and *in vivo* studies evaluating the developmental and reproductive toxicity of CBD are diverse and include acute and repeated dosing, different species (from mammals to invertebrates), and various dose levels and routes of exposure but do not address the key data gaps identified by regulatory agencies needed to evaluate safety for use in food and dietary supplements (e.g., Carvalho et al., 2018a,b and 2022; Rosenkrantz et al., 1981; Rosenkrantz and Esber, 1980; Dalterio et al., 1982, 1984a,b; Patra and Wadsworth, 1991). Other investigators have hypothesized mechanisms of action for some of the reproductive effects observed with CBD. For example, a recent review article by Carvalho et al. (2020) provides an extensive overview of the available data regarding the potential effects of CBD on the male reproductive system. While these studies contribute to the overall information on CBD safety, none provide sufficient data from which to derive a point of departure (POD) for human health risk assessment. Furthermore, some potential adverse reproductive effects have been observed inconsistently across studies, such as effects on testosterone concentrations and sperm parameters in males (Carvalho et al., 2018a; Dalterio et al., 1982; Marx et al., 2018).

In response to this need for developmental and reproductive toxicity data on CBD, the present study evaluated the effects of repeat oral dosing of pure (>99%) hemp-derived CBD on male and female reproductive performance and offspring development in rats. Testing was performed according to the Organization for Economic Co-operation and Development (OECD) Test Guideline No. 421 (OECD, 2016) with extended offspring evaluation through postnatal day (PND) 42. This study was conducted as part of a larger program to investigate the safety of CBD isolate (Henderson et al., 2023b)

2. Materials and methods

2.1. Test material and vehicle

Hemp-derived CBD isolate (99.08–101.46%; CAS No. 13956-29-1) was provided by Canopy Growth USA (Evergreen, Colorado). CBD was stored, protected from light and with desiccant, at room temperature (19 °C–25 °C) under nitrogen. Third-party analysis (Botanacor Laboratories, Denver, CO) by high-performance liquid chromatography (HPLC) with UV absorbance detection, certified the isolate to be 99.62% CBD and 0.16% cannabidivarin; all other cannabinoids tested were below the limit of quantification (Botanacor, Denver, CO).

The CBD was mixed into an olive oil vehicle (Spectrum, New Brunswick, NJ), which was also used for dosing the control animals. Based on the measured purity, a correction factor of 1.004 was used for dose formulations. Dose formulations for oral gavage were prepared approximately weekly, and all preparations were dispensed into daily aliquots, stored at controlled room temperature (18–24 °C) and protected from light until use. CBD formulations were confirmed to be stable when stored refrigerated (5 °C) and at room temperature for 8 days. On the day of dosing, preparations were heated to 35 ± 5 °C for at least 30 min, followed by continuous stirring at room temperature while dosing to maintain homogeneity. Concentration analyses of the first and last dose preparations confirmed that the dosing formulations contained 94.5%–100.4% of the target concentrations and were within the protocol-specified ranges. CBD was not detected in vehicle control formulations.

2.2. Animals

Sprague Dawley, CD® [Crl:CD®] rats were obtained from Charles River Laboratories (Raleigh, North Carolina) at approximately 10–11 weeks of age. Following a 7-day acclimation period, animals were assigned to test groups using a stratified randomization procedure. Females not exhibiting a normal 4- to 5-day estrous cycle were not assigned to groups. Females and males weighed 198–261 g and 274–407 g, respectively, at initiation of dosing. Animals were housed in solid-bottom cages with nonaromatic bedding and environmental enrichment in a room that maintained temperatures of 68–78 °F, relative humidity of 30%–70%, and a 12-h light/dark cycle. During the acclimation and pre-mating period, animals were housed 2–3 per cage (single sex), and then, during the cohabitation period for mating, the females were paired 1:1 with a male in the male's home cage. On

successful mating or at the end of the mating period, all adult males remained individually housed until termination. Following positive signs of mating or the end of the mating period, females were housed individually and remained in their cages with their litters until termination. On PND 4, eight pups per litter (four/sex) were selected where possible, and remaining pups were euthanized by intraperitoneal sodium pentobarbital after collecting blood for thyroid hormone assessment. Standardization of litters was not done for litters of fewer than eight pups. All offspring selected after weaning for the F_1 generation were housed in groups of 2–3 by sex. Rats were provided treats and cage enrichment and had access to municipal tap water treated by reverse osmosis and UV irradiation, and were given food [Lab Diet® (Certified Rodent Diet #5002, PMI Nutrition International, Inc.)] *ad libitum*. Animals were cared for according to the published National Research Council guidelines.

2.3. Reproductive toxicology

The *in vivo* reproductive toxicology study was conducted in accordance with US Code of Federal Regulations (CFR) Title 40, Parts 160 and 792: Good Laboratory Practice (GLP) Standards. The protocol was reviewed and approved by an institutional animal care and use committee (IACUC). The study design was based on the OECD Guideline for the Testing of Chemicals, Guideline 421, Reproduction/Development Toxicity Screening Test, July 2016 (Modified) and is summarized in Table 1.

2.3.1. Experimental design

For the main study, the control group and three CBD dose groups (30, 100, 300 mg/kg-bw/d) each consisted of 10 animals per sex. The oral route was chosen, because it is the most likely route of exposure for humans. As described in the OECD (2016) guidelines, dose levels were selected based on results from existing reproductive toxicity studies conducted with CBD isolate. The high dose of 300 mg/kg-bw/day was not expected to cause death or severe suffering and was selected based on the highest dose tested of 250 mg/kg-bw/day in the most relevant available study, in which rats were exposed to CBD for two weeks prior to mating and until gestation day (GD) 6 (reviewed by CDER, 2018a; study number GW1456[1]). In that study, decreased weight gain was observed in parental males and females and slight decreases in fertility indices were observed in the mid- and high-dose groups. In a separate pre- and postnatal study (GD 6 to postnatal day [PND] 21), some reproductive and developmental effects were also noted at doses up to 250 mg/kg bw/d (reviewed by CDER, 2018a; study number GWTX1532[2]). Based on these two studies reviewed by CDER (2018a), and taking into consideration other available studies reviewed by FDA (CDER, 2018a) and findings from a male reproductive study conducted in monkeys (Rosenkrantz et al., 1981), reproductive effects were expected at the selected high dose of 300 mg/kg bw/d and the low- and mid-dose levels were selected to derive a graded dose-response for any toxicity effects observed.

Animals were dosed via oral gavage once daily at a dosing volume of 5 mL/kg. F_0 males assigned to the main study were dosed for 14 days prior to mating and continuing through one day prior to euthanasia. F_0 females assigned to the main study were dosed for 14 days prior to mating and continuing through lactation day (LD) 20. Offspring selected as the F_1 generation were dosed by oral gavage from weaning on PND 21 through PND 42 (any prior exposure *in utero* or via nursing was not assessed).

Estrous cyclicity was determined in all F_0 females by daily vaginal lavage for 14 days prior to randomization and through the mating period until mating was confirmed. Stage of estrous was determined by microscopic examination of vaginal cells, and cycle length was calculated over the period of observation.

The following in-life assessments were performed at least daily for all F_0 animals: mortality/cage-side observations, detailed clinical observations prior to and approximately 2 h after dosing, and individual body weights. On the day of parturition, females were observed three times per day for completion of delivery or signs of dystocia or other difficulties, and live pups were counted. Food consumption was measured twice weekly until cohabitation and in females on gestation days (GDs) 0, 4, 7, 11, 14, 17, and 20, and on LDs 1, 4, 7, 10, 14, 17, 20, and 21.

The F_0 generation was necropsied with anatomic histopathology (gross lesions [all groups] and microscopic evaluations [high- and low-dose groups only]) and sperm collection for quantitation and morphological evaluation. Blood samples were collected for thyroid hormones and testosterone analyses, as described below.

F_1 litters were observed twice daily for general health, mortality, and morbidity. Detailed clinical observations and body weights were collected twice weekly from PND 1 through PND 21. Pups were sexed individually on PNDs 0, 4, 14, and 21. Anogenital distance of all pups was measured on PND 1, and areola/nipple anlagen retention was evaluated in all male pups on PND 13. One pup/sex/litter (same as those used for thyroid hormone assessment) was terminated on PND 21 and underwent necropsy, tissue collection, and recording of thyroid weight (after fixation). Remaining F1 pups were terminated on PND 43 and underwent necropsy, tissue collection, and recording of organ weights.

2.4. Sampling and quantification of hormones

Blood samples for thyroid hormone analyses were collected from a jugular vein around the same time of day (before noon), to reduce variability due to normal diurnal variation. F_0 males and females were sampled at euthanasia (Study Day 28 for males, LD 21 for females), and F_1 pups were sampled on PND 4 (pooled by litter; at least two per litter) and PND 21 (one per sex per litter). Samples were processed to serum and analyzed for triiodothyronine (T3) and/or total thyroxine (T4) using validated ultra-high performance liquid chromatography with dual mass spectroscopy (UHPLC/MS/MS) assays (Lucarell, 2017).

Blood samples for testosterone analyses were collected from F_0 males on Study Day 28, and from F_1 males on PND 43, and processed to serum. Electrochemiluminescence detection of testosterone was performed using a COBAS E411 system (Roche, Indianapolis, Indiana) using appropriate methods; the upper limit of quantification for the assay was 1501 ng/dL.

2.5. Sperm evaluations

Immediately after euthanasia on Study Day 28, the reproductive tract of each male was exposed, and the right cauda epididymis was excised and weighed. An incision was made in the distal region of the right cauda epididymis, and it was then placed in Dulbecco's phosphate buffered saline with 10 mg/mL bovine serum albumin (BSA) at approximately 37 °C. After a minimum 10-min incubation, a sample of sperm was loaded onto a slide for determination of sperm motility at a constant 37 °C. Analysis of at least 200 motile and nonmotile spermatozoa per animal (if possible) was performed to determine percent motile sperm. The right epididymis was then placed in modified Davidson's solution for microscopic examination. Sperm morphology was evaluated by light microscopy via a modification of the wet-mount evaluation technique (Linder et al., 1992). Abnormal forms (double heads, double tails, microcephalic, or megacephalic, etc.) from a differential count of 200 spermatozoa per animal, if possible, were recorded.

[1] Cited in CDER (2018a) as *Epidiolex (Purified CBD): Oral (Gavage) Study of Fertility and Early Embryonic Development in Male and Female Rats (GW Report No. GWTX1456; dated 30/9/16; conducted by [redacted]; GLP)*.

[2] Cited in CDER (2018a) as *Purified CBD: Oral (Gavage) Study of Pre- and Postnatal Development in the Rat (GW Report #: GWTX1532; conducted by [redacted] report dated 4/21/17; GLP)*.

Table 1
Modified OECD 421 multidose reproductive toxicology study in Sprague Dawley rats.

Species	Study Dosing Duration(daily)	Groups	Oral Gavage Dose		Clinical Pathology Sampling[a]	Reproductive, Developmental, and Specialized Endpoints
			Levels (mg/kg-bw/d)	Total Doses		
SD Rat, F_0 generation	M: 14 days prior to mating and throughout mating for a minimum of 28 days F: 14 days prior to mating and throughout mating, gestation, and lactation	10/sex/group	0, 30, 100, 300	28+ 28	Thyroid Hormone M: T4 at termination F: T3 and T4 on LD 21 (at termination) Testosterone M: At termination F: N/A	Pregnancy, mating, and fertility indices; number of estrous cycles and cycle length; hormone analysis (testosterone and thyroid); sperm assessment (count, motility/viability, morphology)
SD Rat, F_1 generation	PND 21 – PND 42[b]	10/sex/group	0, 30, 100, 300	22	Thyroid Hormone M/F: T3 and T4 on PND 4 (culling); T4 on PND 21 (non-selected pup[c] termination) Testosterone M: PND 43 (termination) F: N/A	Litter observations, continuous (e.g., sex ratio - males, mean litter body weights); live birth, viability, and lactation index; anogenital distance; areolae/nipple anlagen

Abbreviations: F, female; FOB, functional observational battery; GLP, good laboratory practices; LD, lactation day; M, male; N/A, not applicable; PND, postnatal day; SD, Sprague Dawley (CD®).
[a] Serum samples from adult males and from pups terminated on PND 21 were analyzed for serum levels of Total T4; serum samples from adult females and culled pups (PND 4) were analyzed for serum levels of Total T3 and T4.
[b] Potentially also exposed to CBD *in utero* and through nursing during lactation.
[c] "Non-selected pups" were those culled but retained for thyroid hormone analysis.

The left testis and cauda epididymis from each male was weighed and stored frozen. The left cauda epididymis was homogenized and evaluated for sperm numbers using the Hamilton Thorne computer-aided sperm analysis (CASA) system (Beverly, Massachusetts) on a minimum of 200 cells, if possible.

2.6. Statistical analyses

Indices were calculated as follows.

- Female mating index = Number of Females with Evidence of Mating (or no confirmed mating date and pregnant)/Number of Females Paired
- Female fertility index = Number of Pregnant Females/Number of Females with Evidence of Mating (or no confirmed mating date and pregnant)
- Female pregnancy index = Number of Pregnant Females/Number of Females Paired
- Male mating index = Number of Males with Evidence of Mating (or female partner confirmed pregnant)/Number of Males Paired
- Male fertility index = Number of Males Impregnating a Female/Number of Males with Evidence of Mating (or female partner confirmed pregnant)
- Male pregnancy index = Number of Males Impregnating a Female/Number of Males Paired
- Live birth index = (Number of Live Newborn Pups x 100)/Number of Newborn Pups
- Viability index = (Number of Live Pups on Day 4 Postpartum x 100)/Number of Liveborn Pups
- Lactation index = (Number of Live Pups on Day 21 Postpartum x 100)/Number of Live Pups on Day 4 Postpartum
- Post-implantation loss/litter = Number of Implants – Number of Newborn Pups (total).

The litter was the unit of comparison for all F_1 litter data through culling on PND 4. Levene's test was used to assess the homogeneity of group variances. Groups were compared using an overall one-way analysis of variance (ANOVA) F-test if Levene's test was not significant, or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test, respectively. For incidence data, Fisher's exact test was used for pairwise group comparisons.

3. Results

3.1. F_0 generation clinical observations, body weights, and food consumption

3.1.1. Mortality and clinical observations

CBD-related mortality and moribundity were noted in F_0 animals at 300 mg/kg-bw/d. One male exhibited marked body-weight loss (11.5%) from Study Days 21 through 23, salivation, and stained and wet fur, and the animal was found dead on Study Day 24. Also, at the 300 mg/kg-bw/d dose, a total of seven females were euthanized during the study. In general, these animals exhibited erect, stained, and wet fur; skin pallor; and hunched posture, and/or were thin. Pups from these dams were cold to the touch and had no milk band. One female euthanized on Day 25 was nongravid and therefore not included in any further calculations. Two females in the 300 mg/kg-bw/d group exhibited severe maternal toxicity and these females were euthanized *in extremis*, one each on LDs 0 and 2. These dams had severe clinical observations prior to delivery consistent with toxicity observed in other animals, had retained fetuses and/or late resorptions *in utero* at necropsy, and exhibited a lack of maternal care (e.g., not nursing). Based on the pre-existing toxicity, these two litters were excluded from

calculations of Live Birth Index, Live Pups/Litter, and Post Implantation Loss/Litter (Table 4). The other four females euthanized in the 300 mg/kg-bw/d group were included in PND 1 parameters (one *in extremis* due to poor clinical condition (LD 1), two due to total litter losses (LDs 1 and 3), and one with all early resorptions (Day 25)). For parameters calculated starting on PND 4, three total litters were remaining in the 300 mg/kg-bw/d group. A single total litter loss occurred on LD 1 in the control group. The other F_0 animals survived to the scheduled necropsies, except for two females from the 300 mg/kg-bw/d group that were euthanized 25 days after mating—one had all early resorptions,

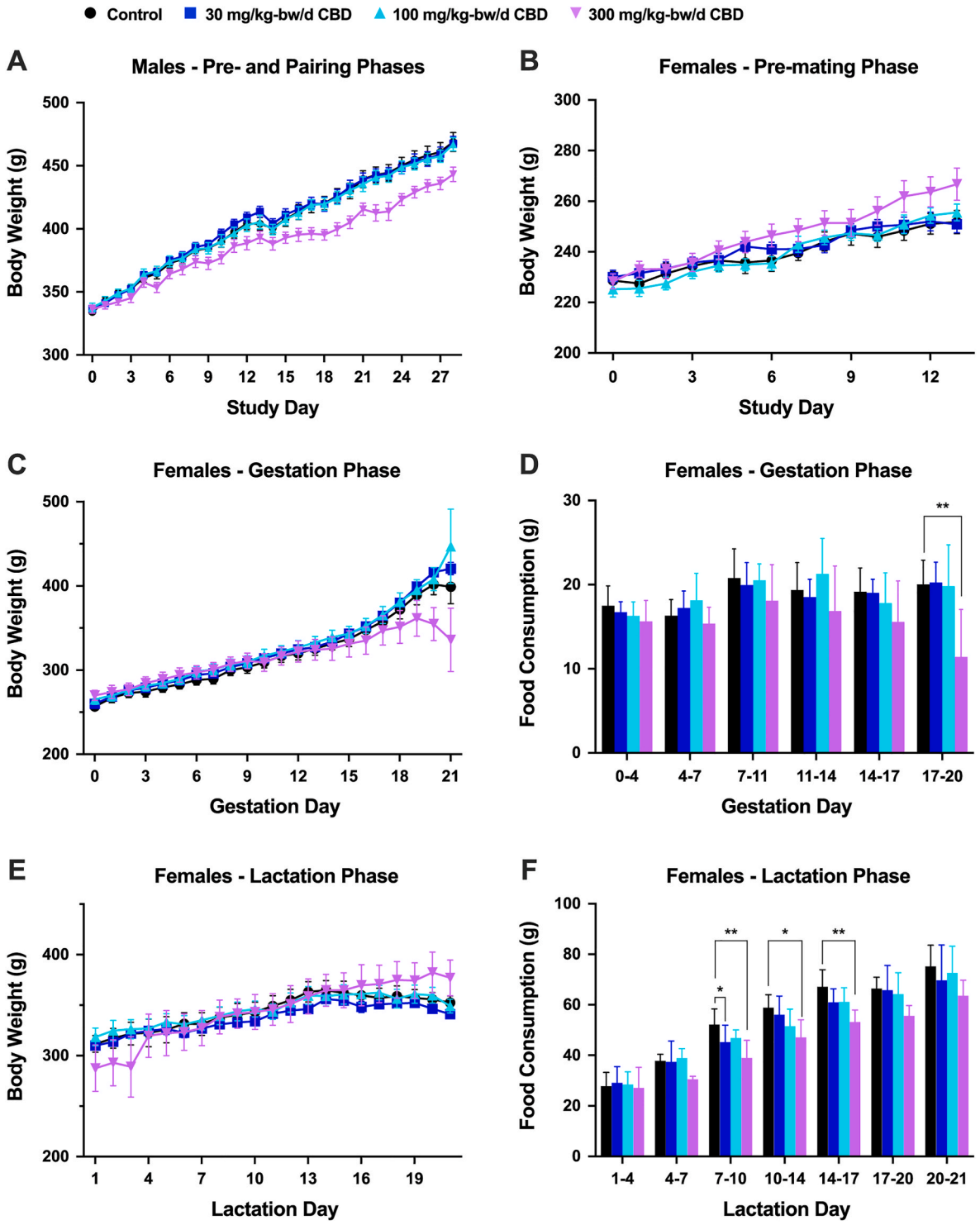

Fig. 1. Body weights and food consumption for F_0 animals. (A) Mean body weights per group for F_0 males; data shown for pre-mating phase through Day 13 and for the pairing/mating phase from Days 14 through 28 (B) Mean body weights per group for F_0 females during the pre-mating phase. (C) Mean body weights per group for F_0 females during the gestation. (D) Mean food consumption per group for F_0 females during the gestation phase (E) Mean body weights per group for F_0 females during the lactation phase. (F) Mean food consumption per group for F_0 females during the lactation phase. All means are shown ±SD. Food consumption is shown as the mean food/animal/d and reported per interval. ANOVA & Dunnett: * = $p \leq 0.05$; ** = $p \leq 0.01$. N = 10/sex/group except for female control group during lactation (n = 9), 300 mg/kg-bw/d males (n = 9), and 300 mg/kg-bw/d females during gestion (n = 9) and lactation (n = 3).

and the other was nongravid. Similar CBD-related adverse clinical observations (erect, stained, and wet fur; skin pallor; hunched posture; and/or thinness) were observed in two females in the 300 mg/kg-bw/d group during late gestation (GDs 12–24) and early lactation (LD 9). Throughout the dosing period, at approximately 2 h following dosing, an increased incidence of salivation and wet fur were noted in the 100- and 300 mg/kg-bw/d group males and females. These observations generally did not persist to the daily examinations and were sporadic at 100 mg/kg-bw/d.

3.1.2. Body weight and food consumption

Prior to pairing and during mating, there was a statistically significant decrease in body weights ($p \leq 0.01$, or 0.05; Fig. 1A) in the 300 mg/kg-bw/d males from Study Days 17–28 compared to those of concurrent contorls; however, these changes were small in magnitude and correlated with a significant decrease in food consumption (Supplementary Table 1A). Body weights and food consumption in males were similar to controls in the 30- and 100 mg/kg-bw/d groups. There was no effect of CBD on female body weights at any dose prior to pairing (Fig. 1B), despite a transient lower mean food consumption at 300 mg/kg-bw/d (Supplementary Table 1B). During gestation, females dosed with 300 mg/kg-bw/d had lower body weights (non-significant; Fig. 1C) and overall, significantly lower food consumption ($p \leq 0.01$; Fig. 1D) than controls from GDs 0–20. Mean body weights and body-weight gains in the 30- and 100 mg/kg-bw/d groups were unaffected by CBD administration during gestation (Fig. 4D; Supplementary Table 2). During lactation (LDs 1–21), there was a nonsignificant increase in mean body weight (Fig. 1E) and significantly lower food consumption (Fig. 1F) in dams at 300 mg/kg-bw/d. However, only three females remained in the highest dosage group after LD 3.

3.2. F_0 reproductive indices, gestation, and parturition

3.2.1. Male and female reproductive indices

No CBD-related effects were observed on precoital interval, estrous cycle length, mating, fertility, or pregnancy indices at any dosage level (Table 2). One mating pair in the 300 mg/kg-bw/d group did not produce a litter.

3.2.2. Gestation length and parturition

Mean gestation lengths in all CBD-treated groups were similar to those in the control group. There were no significant differences in the mean number of implantation sites or proportions of postimplantation loss in the CBD-treated groups compared to controls. As discussed in Section 3.1.1, two females in the 300¬mg/kg-bw/d group that exhibited severe maternal toxicity (with possible dystocia) were euthanized, one each on LDs 0 and 2. There were no effects on parturition or clinical condition of the dams during delivery in the 30- and 100 mg/kg-bw/d groups. Adverse clinical findings were noted for two other females in the 300 mg/kg-bw/d group during early lactation.

Table 2
F_0 male and female reproductive performance parameters.

Parameter	Dose (mg/kg-bw/d)			
	0	30	100	300
Male Mating Index (%)	100.0	100.0	100.0	100.0
Female Mating Index (%)	100.0	100.0	100.0	100.0
Male Fertility Index (%)	100.0	100.0	100.0	90.0
Female Fertility Index (%)	100.0	100.0	100.0	90.0
Male Pregnancy Index (%)	100.0	100.0	100.0	90.0
Female Pregnancy Index (%)	100.0	100.0	100.0	90.0
Estrous Cycle Length (days)	4.00 ± 0.24	4.40 ± 0.52	4.87 ± 1.93	4.38 ± 0.90
Pre-Coital Interval (days)	1.9 ± 1.1	3.0 ± 1.9	2.9 ± 4.0	2.9 ± 2.8

Average parameters are shown as mean ± standard deviation, derived from n = 10 females per group.
See Methods section for detailed description of parameters.

Table 3
F_0 male testosterone and sperm motility, concentration, and morphology.

Parameter	Dose (mg/kg-bw/d)			
	0	30	100	300
Testosterone (ng/dL)	451.9 ± 190.8	326.9 ± 106.8	431.9 ± 175.8	719.6 ± 512.4[a]
Caudal Epididymis, Weight (g)	0.24 ± 0.027	0.22 ± 0.031	0.21 ± 0.038	0.25 ± 0.042
Sperm Concentration (millions/g)	493.8 ± 108.90	464.3 ± 118.16	458.1 ± 144.61	424.3 ± 75.49
Motility (%)	67 ± 20.1	65 ± 16.8	77 ± 10.0	72 ± 8.2
Normal (%)	99.3 ± 0.89	99.6 ± 1.26	99.9 ± 0.17	99.8 ± 0.36
Normally Shaped Head Separated from Flagellum (%)	0.2 ± 0.37	0.4 ± 0.94	0.0 ± 0.00	1.1 ± 0.22
Head Absent with Normal Flagellum (%)	0.6 ± 0.73	0.1 ± 0.32	0.1 ± 0.17	0.1 ± 0.22
Abnormal Head (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Abnormal Flagellum (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Other (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a] The high nominal value (not significantly different from controls) and variability due to two males with values above the upper limit of quantitation. Parameters are shown as mean ± standard deviation, derived from n = 8–10 males per group. All parameters were measured at termination following the end of the mating period and at least 28 days of CBD administration.

3.3. F_0 male testosterone, caudal epididymis weight, and sperm evaluation

There were no statistically significant differences in serum testosterone between control and CBD-treated F_0 males of the low- and mid-dose groups (Table 3). The high nominal value (not significantly different from controls) and variability for testosterone in the 300 mg/kg-bw/d group was due to two males with values above the upper limit of quantitation. Caudal epididymis weight was similar in controls and all CBD-treated groups. All measured sperm parameters were similar between control and CBD-treated males. There were low incidences in all groups of normal sperm heads separated from flagella and normal flagella with heads missing; however, no abnormal sperm heads or sperm flagella were observed in controls or any CBD-treated group.

3.4. F_0 organ weights and histopathology

Mean absolute liver weights (Fig. 2A) and liver weight relative to body or brain weights (Supplementary Tables 1A and 1B) were higher than controls in the 100- and 300 mg/kg-bw/d group F_0 males and females. Mean adrenal gland weight (Fig. 2B) and adrenal gland weight relative to body or brain weight were higher than controls in the 100- and 300 mg/kg-bw/d group males and 300 mg/kg-bw/d group females (Supplementary Tables 1A and 1B). Higher liver weights correlated with noted liver enlargement and microscopic findings of hepatocellular hypertrophy. Higher adrenal gland weights correlated with microscopic findings of adrenal cortical hypertrophy, noted adrenal gland enlargement, and/or pale discoloration. Thyroid plus parathyroid weights (after fixation) were not different between controls and CBD-treated groups (Fig. 2C), although minimal to moderate epithelial hypertrophy/hyperplasia was noted in the 100- and 300 mg/kg-bw/d groups. No other CBD-related organ-weight changes were noted in F_0 animals. Other sporadic organ-weight differences observed were considered incidental and not treatment-related.

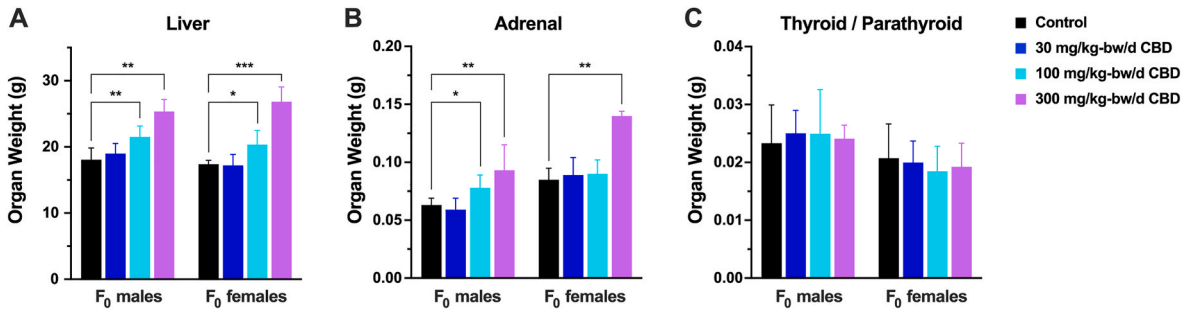

Fig. 2. Selected organ weights for F_0 animals. (A) Mean liver weights by group for F0 males and females. (B) Mean adrenal weights by group for F_0 males and females. (C) Mean thyroid/parathyroid weights by group for F_0 males and females. All means are shown ±SD. Dunnett's test: * = ≤0.05; ** = ≤0.01.

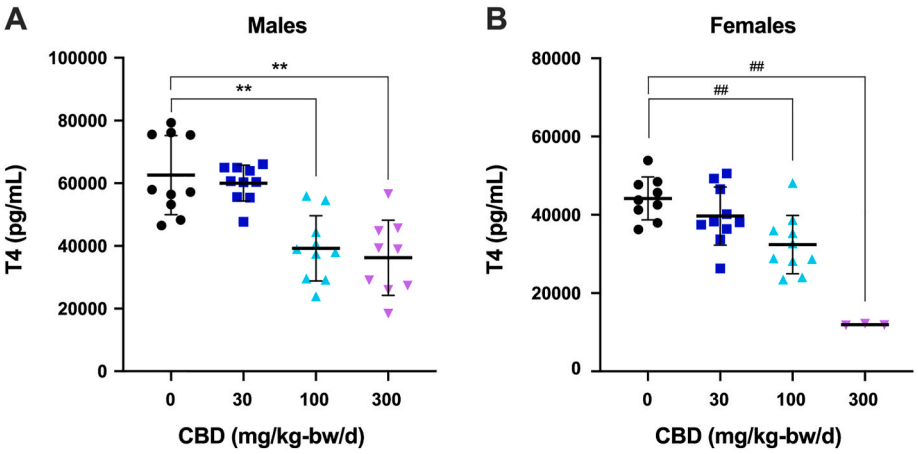

Fig. 3. F_0 Thyroxine (T4) concentrations. (A) Mean T4 concentrations measured in F_0 males on Day 28. (B) Mean T4 concentrations measured in F_0 females on LD 21. All means are shown ±SD. Kruskal-Wallis & Dunn: ** = $p \leq 0.01$; ANOVA & Dunnett: # = $p \leq 0.05$; ## = $p \leq 0.01$.

3.5. F_0 thyroid hormones

Mean T4 concentrations were significantly lower than controls in F_0 males of the 100- and 300¬mg/kg-bw/d groups (Fig. 3A), although these T4 values were within the range of Charles River Ashland (2020) historical control data. Mean T4 concentration in the 30 mg/kg-bw/d group F_0 males was not significantly different from the control group.

In F_0 females, mean T3 concentration on LD 21 was significantly lower than control levels in the 300¬mg/kg-bw/d group (180.3 ± 16.7 pg/mL vs. 316.6 ± 39.3 pg/mL in controls). Mean T4 concentrations on LD 21 in the 100- and 300¬mg/kg-bw/d groups (32,410.0 ± 7460.9 pg/mL and 11,966.7 ± 208.2 pg/mL, respectively) were significantly lower than concurrent control levels (44,200.0 ± 5466.5 pg/mL) (Fig. 3B). The mean T4 concentration in F_0 females at 300 mg/kg-bw/d (11,966.7 ± 208.2 pg/mL), but not at 100 mg/kg-bw/d, was below the minimum mean value in the Charles River Ashland (2020) historical control data (27,770.00 pg/mL). Mean T3 concentrations in the 30- and 100¬mg/kg-bw/d groups F_0 females, and T4 concentrations in the 30 mg/kg-bw/d group F_0 females, were similar to the control group (Supplementary Table 4).

4. F_1 litter data

4.1. PND 0 litter data and postnatal survival

4.1.1. Litter outcomes

There were no significant differences in live birth index, post-implantation loss, or average number of pups (male and/or female) per litter. Mean birth weight was similar across all groups for male pups, while female pups in the 300 mg/kg-bw/d had a significantly lower birth weight than controls (Table 4).

4.1.2. Postnatal survival

Neonatal survival to PND 4 in the in the 300 mg/kg-bw/d group (45.05%) was significantly lower than in the control group (88.89%) (Table 4). These differences were due to two F_0 females in the high-dose group that were euthanized on LDs 0 and 3 following total litter losses, and three females that were euthanized *in extremis* between LD 0 and 2, along with their remaining pups that were pale, cold to the touch, had no milk band, and/or had labored breathing. Survival of the remaining pups from PND 4 to weaning on PND 21 in the 300 mg/kg-bw/d group was comparable to the control group. Postnatal survival to weaning was unaffected by CBD administration in the 30- and 100 mg/kg-bw/d groups. The mean number of pups born and the percentage of males at birth in the 30-, 100-, and 300 mg/kg-bw/d groups were similar to the control-group values. Two pups (from two litters), nine (from four litters), four (from three litters), and sixty (from seven litters) in the control, 30-, 100-, and 300¬mg/kg-bw/d groups, respectively, were found dead or were euthanized *in extremis*. Two pups (from one litter) and one pup each in the 100- and 300¬mg/kg-bw/d groups, respectively, were missing, and five pups (from one litter) in the 300 mg/kg-bw/d group were euthanized due to death of the dam.

4.1.3. Offspring body weights

Male and female pup mean birth weights (PND 1) in the 300 mg/kg-bw/d group were lower (14.39% and 22.06%, respectively) than the control group; the difference was statistically significant for females (Table 4). F_1 male and female pup body-weight gains in this group were lower than the control group throughout the pre-weaning period and mean absolute body weights for males and females were up to 36%

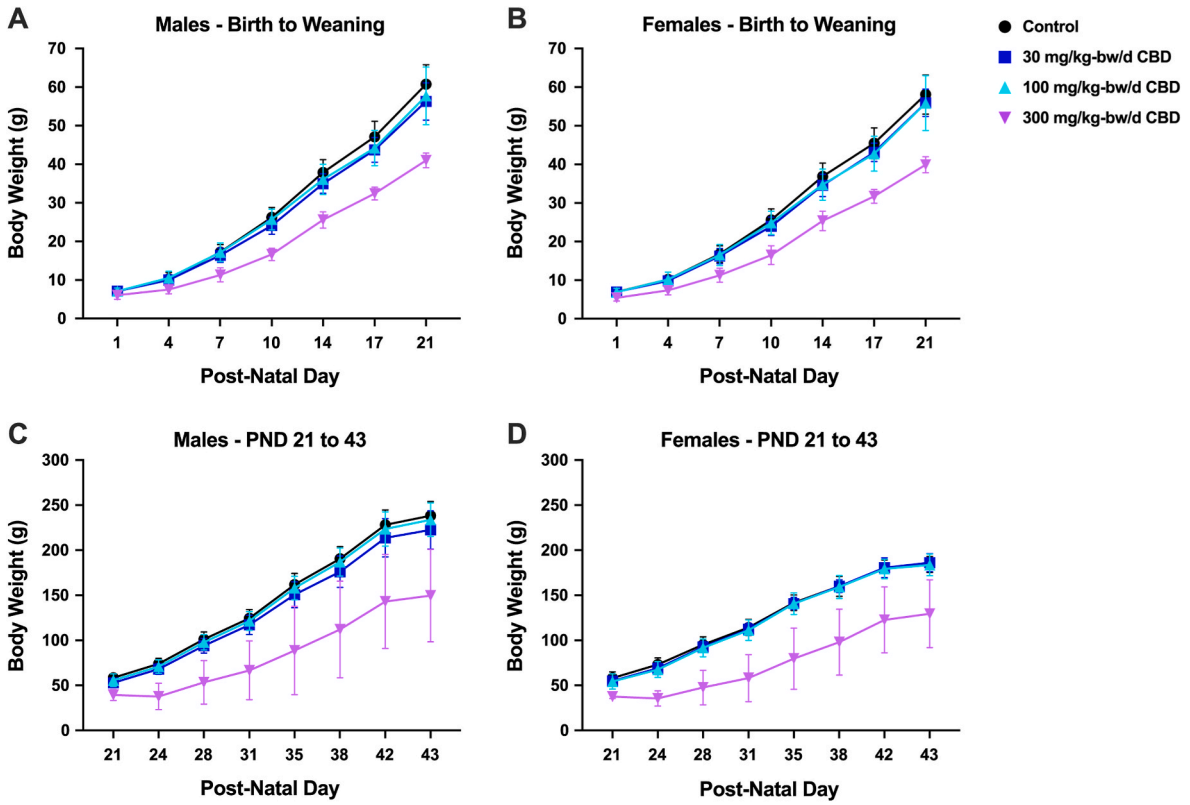

Fig. 4. F_1 Offspring body weight. (A) Mean body weights per group for F_1 males from birth to weaning. (B) Mean body weights per group for F_1 females from birth to weaning. (C) Mean body weights per group for F_1 males post-weaning from post-natal day 21 through 43. (D) Mean body weights per group for F_1 females post-weaning from postnatal day 21 through 43. All means are shown ±SD; statistics calculated using ANOVA and Dunnett test. Starting on PND 21, the number of pups representing a total of 5 litters each were: 9/sex (control), 10 males and 9 female (30 mg/kg-bw/d), and 10/sex (100 mg/kg-bw/d). In the 300 mg/kg-bw/d group, 3 pups/sex represented three litters.

Table 4
F_1 litter outcomes and postnatal survival.

Parameter	Dose (mg/kg-bw/d)			
	0	30	100	300[a]
Live Birth Index	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Post-Implantation Loss/Litter	0.8 ± 0.9	0.5 ± 0.7	1.1 ± 1.7	0.2 ± 0.4
Mean Number of Live Pups/Litter (Day 1)				
Males	5.1 ± 3.2	7.9 ± 2.2	6.2 ± 2.7	4.0 ± 3.4
Females	7.1 ± 3.9	7.0 ± 2.1	6.5 ± 2.8	4.8 ± 2.0
Mean Pup Birth Weight (g)				
Males	7.16 ± 0.80	7.21 ± 0.96	7.20 ± 0.84	6.13 ± 1.06
Females	6.94 ± 0.90	6.98 ± 0.88	6.90 ± 1.04	5.41 ± 0.80*
Viability Index (PND 1–4)	88.89 ± 31.43	95.25 ± 7.71	96.21 ± 5.47	45.04 ± 49.91#
Lactation Index (PND 4–21)	100 ± 0	97.5 ± 7.91	100 ± 0	95.83 ± 7.22

Parameters are shown as mean ± standard deviation, derived from n = 9–10 for 0, 30, and 100 mg/kg-bw/d groups. For the 300-mg/kg-bw/d group, n = 6 except for male Mean Pup Birth Weight (n = 5) and Lactation index (n = 3). Lactation Index calculated post-culling. ANOVA and Dunnett: * = $p \leq 0.05$. Kruskal-Wallis and Dunn: # = $p \leq 0.05$.

[a] Two females were euthanized *in extremis* due to severe maternal toxicity on Lactation Day 0 and 2, respectively, with conceptuses retained *in utero*. Therefore, the Total Number Newborn Pups and Number Live Newborn Pups for these females were excluded from the calculations. As a result, the following parameters were not calculated for these litters: Live Birth Index, Live Pups/Litter, and Post-Implantation Loss/Litter. See Section 3.1.1 for additional details.

lower in the 300 mg/kg-bw/d group than in the control group during the pre-weaning period (Fig. 4A and B). Mean body weights for males in the 300 mg/kg/d group were statistically significantly decreased ($p \leq 0.01$, or 0.05) from postnatal day 4 through 21 (Fig. 4A). Mean body weights for females in the 300-mg/kg/d group were statistically significantly decreased ($p \leq 0.01$, or 0.05) on postnatal day 1 and from postnatal day 4 through 21, relative to controls (Fig. 4B). Mean F_1 male and female body weights and body-weight changes in the 30- and 100 mg/kg-bw/d groups during the preweaning period were similar to controls (Fig. 4A and B). Mean body weights for males in the 300¬mg/kg/d group were statistically significantly decreased ($p \leq 0.01$, or 0.05) from postnatal day 21 through 43 (Fig. 4C). Mean body weights for females in the 300-mg/kg/d group were statistically significantly decreased ($p \leq 0.01$, or 0.05) from postnatal day 21 through 43 (Fig. 4D).

4.1.4. Anogenital distance (AGD) and areolae/nipple anlagen retention

The AGD (absolute and relative to the cube root of pup body weight) in the 30-, 100-, and 300¬mg/kg-bw/d groups were similar to the control-group values (Supplementary Tables 5A and 5B). Areola/nipple anlagen in the F_1 male pups were evaluated on PND 13, and no areolae or nipples were noted.

4.1.5. Serum thyroid hormone concentrations on PNDs 4 and 21

Mean T3 and T4 concentrations in F_1 culled pups (pooled by litter) on PND 4 were lower in the 100¬mg/kg-bw/d (166.2 ± 27.1 and 20,377.8 ± 3347 pg/mL, respectively) and 300 mg/kg-bw/d (126.0 ± 9.8 and 16,933.3 ± 4046 pg/mL, respectively) groups compared to the control group (192.1 ± 20.2 and 25,425.0 ± 4422.3 pg/mL, respectively); differences were statistically significant at 300 mg/kg-bw/

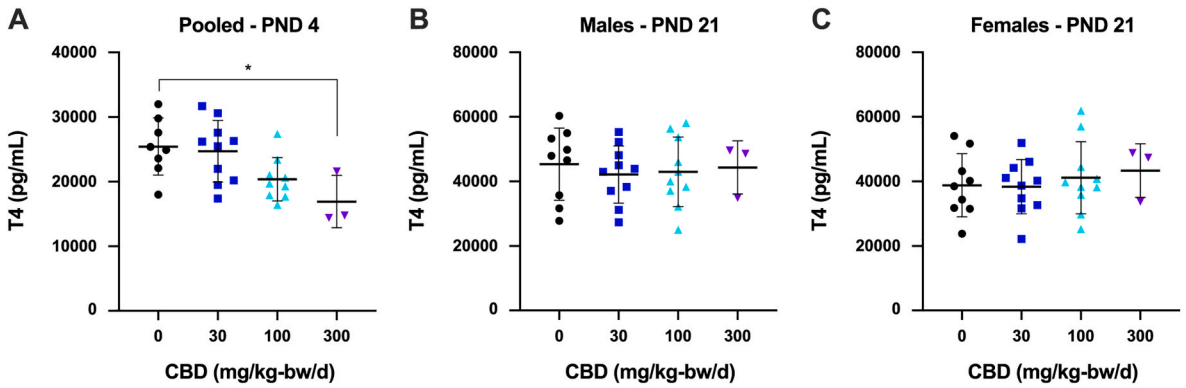

Fig. 5. F_1 Thyroxine (T4) concentrations. (A) T4 concentration of F1 litters pooled from animals culled per litter on PND 4. (B) Mean T4 concentrations of F1 males on PND 21. (C) Mean T4 concentrations of F1 females on PND 21. ANOVA and Dunnett: * = $p \leq 0.05$. On PND 21, the number of pups representing a total of 5 litters each were: 9/sex (control), 10 males and 9 females (30 mg/kg-bw/d), and 10/sex (100 mg/kg-bw/d). In the 300 mg/kg-bw/d group, 3 pups/sex represented three litters.

d (Fig. 5A; Supplementary Table 6). On PND 4, the mean T3 concentration at 300 mg/kg-bw/d, but not 100 mg/kg-bw/d, was below the minimum mean value in the Charles River Ashland (2020) historical control database (158.3 pg/mL). Mean T3 and T4 concentrations in the 30 mg/kg-bw/d group PND 4 culled pups were similar to the control group. There were no CBD-related effects on thyroid hormone concentrations in the F_1 males and females on PND 21 at any maternal dosage level (Fig. 5B and C).

4.1.6. Organ weights

In the 300 mg/kg-bw/d group, significant decreases in absolute epididymides (left and right), testis (right), and ovary/oviduct occurred (data not shown). Of note, only slightly lower mean absolute and higher mean relative (to final body weight) thyroid/parathyroid weights were noted in F_1 males and females in the 300¬mg/kg-bw/d group on PND 21, but these differences were not statistically significant.

4.2. F_1 generation post-weaning

4.2.1. F_1 post-weaning mortality and moribundity

There were no CBD-related effects on mortality or moribundity in the F_1 generation at any post-weaning dosage level; however, due to mortality at 300 mg/kg-bw/d during the preweaning period, only six animals (three/sex, representing three litters) were available for evaluation. In the 300¬mg/kg-bw/d group F_1 males and females, clinical findings similar to those seen in the F_0 generation were noted, including skin pallor, thinness, and partially closed eyes. No CBD-related clinical findings were noted for F_1 generation males and females in the 30-and 100¬mg/kg-bw/d groups.

4.2.2. F_1 post-weaning body weights

After weaning on PND 21, offspring were dosed by oral gavage from PND 21 through 42 at the same dosages administered to the F_0 parental males and females. In the 300 mg/kg-bw/d group, mean body-weight losses or lower mean body-weight gains were noted for F_1 males and females generally throughout the dosing period, resulting in lower mean body-weight gains when the entire post-weaning period (PNDs 21–43) was evaluated (Supplementary Tables 7A and 7B). Mean absolute body weights, but not body-weight gains, for males and females were statistically significantly lower (up to 48% and 50%, respectively), than the control group (Fig. 4C and D). No test-substance-related effects on mean body weight or body-weight gain were noted in F_1 males or females in the 30- and 100 mg/kg-bw/d groups.

4.2.3. F_1 male testosterone analysis

There were no statistically significant differences in testosterone concentrations between controls and CBD-treated F_1 males at any dose level (Supplementary Table 8).

4.2.4. F_1 organ weights

The 300 mg/kg-bw/d group had significantly lower mean absolute epididymides and right testis weights, higher mean relative (to body weight) brain and liver weights, and lower mean relative (to brain weight) epididymides and testis weights. Females in this group had significantly lower mean absolute and relative (to brain weight) ovary/oviduct weights and higher mean (relative to body weight) liver weights compared to controls. There were no CBD-related effects on organ weights in the 30- and 100 mg/kg-bw/d groups (Supplementary Tables 9A and 9B)).

5. Discussion

With increasing availability and public interest in CBD-containing products, it is critical that CBD safety be well investigated, with results widely disseminated in peer-reviewed publications. The present study addresses a critical gap in CBD research–the potential adverse effects on male and female reproduction and offspring development. This research was conducted within the scope of a modified screening study and is the first published standard, guideline-compliant reproductive toxicity study on pure hemp-derived CBD. In this study, OECD Test Guideline No. 421 (OECD, 2016) was modified to include extended postnatal dosing through PND 42 and hormone analysis (testosterone and thyroid hormones). It should be noted that the major circulating metabolite after CBD ingestion in humans is 7-COOH-CBD, whereas CBD is the primary compound measured in rats, followed by 7-COOH-CBD (Deabold et al., 2019; Harvey et al., 1991; CDER, 2018a). Despite these differences, rats appear to be the most appropriate non-primate model for investigating toxicological effects of CBD, as studies in dogs show that 7-COOH-CBD is not a prominant metabolite (CDER, 2018a; Vaughn et al., 2020). Nevertheless, these differences in circulating metabolites should be considered when applying the findings of our study in rats for human health risk assessment purposes.

Dose selection for the current study was based on existing pre- and postnatal toxicity studies conducted with CBD and other CBD-containing test materials. The high dose of 300 mg/kg-bw/d was chosen based on the doses of up to 250–300 mg/kg-bw/d tested in the most relevant available studies (reviewed by CDER, 2018a; study numbers GWTX1456[1] and GWTX1532[2]). Overall, litter parameters and postnatal effects following CBD exposure have only previously been investigated in these and other studies reviewed by FDA (CDER, 2018a), therefore these studies serve as the primary basis for discussion for such effects in our study. Where relevant, data from other study paradigms are also

included for these parameters and are discussed extensively in the context of male reproductive effects.

Treatment-related mortality and moribundity were observed in F_0 animals receiving 300 mg/kg-bw/d, including severe maternal toxicity during pregnancy and lactation. This finding provides important information regarding systemic toxicity in parental animals, in that the previous reproductive toxicity studies reviewed by (CDER, 2018a) that served as the primary basis for dose selection in the current study reported adverse effects, but not severe toxicity, at doses up to 250 mg/kg bw/d.

Treatment-related effects on organ weights and histopathology of the F_0 males and females in this study are concluded to be nonadverse. The constellation of liver changes (e.g., liver enlargement, increased liver weights, and hepatocellular hypertrophy) in F_0 male and female rats at both 100 and 300 mg/kg-bw/d suggests induction of both phase 1 and phase 2 metabolic enzymes involved in thyroid hormone elimination (Papineni et al., 2015; Noyes et al., 2019). Similar changes were noted in a recent 90-day repeat-dose study conducted in male and female rats in which centrilobular hepatocellular hypertrophy was observed and found to be fully resolved following a 28-day recovery period (reported in our companion paper—Henderson et al., 2023b). Hepatocellular hypertrophy without histopathological or other changes indicative of liver toxicity, as is the case with CBD both the present study and in the aforementioned companion paper, is considered adaptive and non-adverse, as described in a review by Hall et al. (2012).

Administration of 100 or 300 mg/kg-bw/d CBD to F_0 male and female rats also resulted in minimum to moderate thyroid hyperplasia/hypertrophy. Although thyroid weights were not changed, these thyroid lesions correlated with significant decreases in serum T4 (male and female at 100 and 300 mg/kg-bw/d) and T3 (females only at 300 mg/kg-bw/d) concentrations; however, only T4 concentrations in females of the high-dose group were below historical control values (Charles River Ashland, 2020). Changes in thyroid hormone levels were considered to be secondary to the adaptive liver changes observed in this study. One possible pathway for the effects of CBD on thyroid hormones may be hepatic microsomal enzyme induction (as evidenced by centrilobular hepatocellular hypertrophy and increased liver weights), with a corresponding increase in thyroid hormone clearance and thyroid follicular cell hypertrophy, a hypothesis previously considered in the review of Epidiolex clinical data (CDER, 2018b). There are several pathways by which chemicals can produce antithyroid effects by perturbing thyroid-pituitary homeostasis and reducing circulating thyroid hormones, increasing thyroid stimulating hormone (TSH) levels, and inducing thyroid hyperplasia/hypertrophy (Hurley et al., 1998; Zabke et al., 2011; Noyes et al., 2019; Huisinga et al., 2020). One pathway involves chemical induction of thyroid hormone conjugation to glucuronic acid by uridine diphosphate glucuronosyltransferase (UDPGT), resulting in increased elimination and decreased serum concentrations of T3 and T4 (Papineni et al., 2015; Noyes et al., 2019). The pattern of liver changes observed in these CBD studies may reflect hepatic microsomal enzyme induction, including UDPGT activity. Also of critical importance is that this liver induction is adaptive; this is reflected in the resolution of the liver lesions when CBD exposure ends, as demonstrated in our 90-day study (Henderson et al., 2023b).

Although T3 and T4 levels in F_1 culled pups (pooled) at PND 4 were lower in the two highest dose groups compared to the controls, differences were only statistically significant at 300 mg/kg-bw/d CBD. These decreased levels observed in the high-dose group may be related to the persistent maternal toxicity and corresponding reduced pup weights observed in this group. In addition, thyroid hormone concentrations were similar across all F_1 groups on PND 21. Changes in thyroid hormone levels on PND 4 were not considered toxicologically significant; such changes have been suggested to indicate slight disturbances of normal homeostasis and therefore may not be biologically significant (Beekhuijzen et al., 2019). Absence of significant developmental neurobehavioral changes in other studies further reduces concern about the limited changes in thyroid hormones. For example, in a pre- and postnatal study conducted in Wistar rats, while thyroid hormone levels were not assessed, doses up to 250 mg/kg-bw/d CBD did not cause any consistent effects on learning or memory on PND 65 (reviewed by CDER, 2018a).

Some previously observed effects were replicated in the current study, such as increased pup mortality and lower pup weight in the high-dose group (reviewed in CDER, 2018a). Lower postnatal survival in the high-dose group was observed, including the two litters with total litter loss and three litters euthanized *in extremis*. Mean pup weights in this group were lower than those of controls, which correlated with decreases in some organ weights. For surviving litters, there were no effects on other developmental parameters, including anogenital distance and areola/nipple retention. However, many findings reported elsewhere, including decreased testis weight, changes in preimplantation loss, and developmental delays (e.g., as reviewed by CDER, 2018a; Dalterio et al., 1984b; Rosenkrantz et al., 1981), were not observed in the present study, even at the high dose of 300 mg/kg-bw/d. Studies reporting these effects did not follow standard guidelines, and in some cases are more than 40 years old; as such, limitations in study design may account for inconsistencies in results. In the current OECD guideline compliant study, no CBD treatment-related effects were observed on F_0 male or female reproductive performance at any dose, and mean gestation lengths were similar between control and CBD-treated groups.

A limitation of this study is that it was designed as a screening study and not a generational reproductive toxicity study (e.g., two-generation or extended one-generation). However, as described by Beekhuijzen et al. (2014), key differences between the current OECD 421 (2016) screening study protocol and generational studies are primarily a lack of a second generation and a limited postnatal period. These authors concluded that only 3% (4 of 134) reproductive toxicity screening studies failed to provide definitive results. The shorter postnatal period is partially addressed in the current study, which extended postnatal dosing out to PND 42. Moreover, Piersma et al. (2011) found that second-generation mating and F_2 offspring data rarely provide additional critical information. In this retrospective analysis of 498 multi-generational studies, no critical differences in sensitivities between generations were found, supporting reliance on the one-generation study. Guidance Document 117 on the Current Implementation of Internal Triggers in Test Guideline 443 for an Extended One Generation Reproductive Toxicity Study, in the United States and Canada (OECD, 2011), presents trigger criteria for needing a second generation, including effects on adults (fertility and estrous cycle) and offspring (litter parameters, developmental landmarks, survival, malformations, live birth index, and body weight). According to these criteria, none of the findings in the current study would have triggered a second generation, because the affected endpoints were driven by severe maternal toxicity. F_1 visceral and skeletal malformations were not analyzed in this study; however, studies reviewed as part of the Epidiolex submission inconsistently found increased fetal variations across gestational exposure studies with CBD at doses up to 250 mg/kg-bw/d (CDER, 2018a).

The male reproductive NOAEL of 300 mg/kg-bw/d under the conditions of this study is an important finding, given that much of the extant research has focused on the male as being critical to understanding the reproductive toxicity of CBD. In a recent narrative review published by Carvalho et al. (2020), the authors concluded that CBD caused male reproductive toxicity, including impaired sexual behavior, reduced testosterone levels, testicular cell degeneration, and decreased fertilization rates. However, the authors acknowledged that data are "still limited, and additional research is required to fully elucidate the mechanisms of action, as well as the reversibility of CBD effects on the reproductive system." In addition to these data gaps, understanding the exposure levels associated with adverse effects is critical to determining a safe level of CBD exposure in consumers. Decreased testosterone in males has been reported in various studies, most involving short-term

exposures (e.g., single day; Dalterio et al., 1984a) and none being standard toxicology assessments. Testosterone levels were decreased in mice receiving oral CBD for 34 days at 30 mg/kg-bw/d but not at 15 mg/kg-bw/d in one study published by Carvalho et al. (2018a) but not in a more recent study published by the same laboratory using the same dosing regimen (Carvalho et al., 2022). In addition, no changes in testosterone concentrations were observed in mice given 50 mg/kg-bw/d CBD orally for 5 weeks (Dalterio et al., 1982). Conversely, testosterone levels were decreased in monkeys receiving oral CBD for 90 days at 300 mg/kg-bw/d but not at 30 or 100 mg/kg-bw/d (Rosenkrantz and Esber, 1980). Carvalho et al. (2018b) reported that exposure of male mice to 15 mg/kg-bw/d CBD for 34 days impaired sexual performance, but exposure to 30 mg/kg-bw/d improved sexual performance. This contrasts with the study by Dalterio et al. (1982), in which 50 mg/kg-bw/d CBD for 50 days in males was associated with reduced impregnation of females.

In the current guideline study, no treatment-related effects on testosterone levels or the testes were seen in F_0 or F_1 males. This finding agrees with available repeat-dose studies in mice, in which CBD doses up to 30–50 mg/kg-bw/d did not affect testes weights (Dalterio et al., 1982; Patra and Wadsworth, 1991; Carvalho et al., 2018b, 2022), whereas other studies reported a decrease in testis weight (Rosenkrantz et al., 1981; Dalterio et al., 1984b). In the current study, sperm analysis was added to further investigate and compare against the findings of Rosenkrantz et al. (1981). Changes in sperm quality and spermatogenesis were reported previously in mice treated with CBD up to 30 and 50 mg/kg-bw/d for 34–35 days (Patra and Wadsworth, 1991; Carvalho et al., 2018a, 2022). In a recent OECD guideline compliant study, Marx et al. (2018) performed a series of studies on the effects of an orally dosed, supercritical fluid extract of the aerial parts of *Cannabis sativa* (26% phytocannabinoids [96% CBD, <1% THC]) in rats. Total sperm count, sperm morphology, and percentage of motile and immotile sperm were found to be similar between control and high-dose males. The findings of Marx et al. (2018) are similar to those reported here, including no changes in sperm motility, viability, morphology, or enumeration in rats dosed with up to 300 mg/kg-bw/d for up to 42 days in the F_0 generation. While no effects on spermatogenesis were observed in the current study, the duration of our study did not encompass a full spermatogenic cycle. As such, and per the OECD (2016) guidelines, these data do "not provide evidence for definite claims of no effects" on sperm parameters. No impact of up to 300 mg/kg-bw/d CBD was observed on reproductive performance in rats, including fertility, in the current study.

6. Conclusion

To aid in the determination of a safe level of CBD intake for consumers, we investigated the potential adverse effects of CBD on male and female reproduction and offspring development in a modified screening study. Exposure to 300 mg/kg-bw/d CBD resulted in treatment-related mortality and decreased body weight in the parental generation. Hepatocellular hypertrophy in the F_0 100 and 300 mg/kg-bw/d groups correlated with thyroid hypertrophy/hyperplasia, as well as hormone changes at the high dose. Body weights were also decreased in F_1 pups in this group; however, no other developmental parameters were adversely affected by CBD administration. While maternal toxicity was associated with adverse reproductive measures in the high-dose group, no effects on male reproductive toxicity were found. However, definitive conclusions regarding effects on sperm parameters could not be made due to limitations in study design. Based on the endpoints evaluated in this study, the following NOAELs were identified for CBD isolate: 100 mg/kg-bw/d for F_0 male and female systemic toxicity and female reproductive toxicity, 300 mg/kg-bw/d for F_0 male reproductive toxicity, and 100 mg/kg-bw/d for F_1 neonatal and F_1 generation toxicity.

CRediT authorship contribution statement

Rayetta G. Henderson: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Brian T. Welsh:** Methodology, and monitoring, Writing – original draft, Writing – review & editing. **John M. Rogers:** Writing – original draft, Writing – review & editing. **Susan J. Borghoff:** Methodology, Writing – review & editing. **Kristen R. Trexler:** Conceptualization, Writing – review & editing. **Marcel O. Bonn-Miller:** Conceptualization, Supervision. **Timothy W. Lefever:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was funded by Canopy Growth Corporation. Authors KRT, TWL, and MOB-M were employees of Canopy Growth Corporation during the conduct and drafting of this study; during their employment, they received stock options. ToxStrategies, a private consulting firm providing services on toxicology and risk assessment issues, received funds for conducting this work. Authors RGH, JMR, and SJB are employees of ToxStrategies; and author BTW was an employee of ToxStrategies during the conduct and drafting of this study.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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