NTP
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NTP Technical Report on the Toxicology and Carcinogenesis Studies of Black Cohosh Root Extract (CASRN 84776-26-1) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and Female B6C3F1/N Mice

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Technical Report 603

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to NTP. The studies described in the NTP Technical Report series are designed and conducted to characterize and evaluate the toxicological potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in NTP Technical Reports are derived solely from the results of these NTP studies, and extrapolation of the results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's carcinogenic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and the Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

NTP Technical Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Explanation of Levels of Evidence of Carcinogenic Activity

The National Toxicology Program (NTP) describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, in as much as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major flaws **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical-related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been

adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as "were also related" to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as "may have been" related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

Peer Review

The National Toxicology Program (NTP) conducted a peer review of the draft *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Black Cohosh Root Extract (CASRN 84776-26-1) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and Female B6C3F1/N Mice* by letter in January 2023 by the experts listed below. Reviewer selection and document review followed established NTP practices. The reviewers were charged to:

(1) Peer review the draft *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Black Cohosh Root Extract (CASRN 84776-26-1) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and Female B6C3F1/N Mice*

(2) Comment on NTP's interpretations of the data.

NTP carefully considered reviewer comments in finalizing this report.

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Abstract

Black cohosh (*Actaea racemose* L.) is widely used as a botanical dietary supplement to alleviate female gynecological symptoms, such as premenstrual syndrome and changes associated with menopause, and to stimulate labor. Despite its popularity, limited data are available on the long-term safety of black cohosh products. To address this knowledge gap, 2-year National Toxicology Program (NTP) carcinogenicity studies were conducted in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats and B6C3F1/N mice. To emulate a potential human exposure scenario in which a woman might use black cohosh throughout pregnancy and lactation, perinatal exposure was included for the rat study.

Time-mated female rats were administered 0, 75, 250, or 750 mg/kg body weight/day (mg/kg/day) of black cohosh root extract (BCE) in 0.5% methylcellulose by gavage starting on gestation day 6 and continuing through lactation. After weaning, female offspring were administered the same doses as their respective dam for 2 years; male offspring were not administered BCE or the methylcellulose vehicle but were maintained for the remainder of the study. Female mice, approximately 5–6 weeks of age, were administered 0, 30, 100, 300, or 1,000 mg/kg/day BCE for 2 years. Interim evaluations of micronucleated peripheral blood erythrocytes, hematology, and bone marrow cytology in these mice were conducted at 3 and 12 months to evaluate the persistence of hematological effects observed in previous studies of BCE.

Two-year Study in Rats

Mean body weights of BCE-dosed dams were lower (within 10%) than that of vehicle control animals throughout gestation and lactation. Total and live litter sizes were significantly decreased on postnatal day (PND) 1 and PND 4 in the highest dosed group, 750 mg/kg/day, but there was no difference in pup survival throughout the lactation period. Pup mean body weights exhibited a negative trend with increasing dose and remained within 8%–12% of vehicle control mean body weights throughout lactation; this pattern continued into the postweaning phase.

After 2 years, dosed females and perinatally only-exposed males had mean body weights approximately 13% and 11% less, respectively, than vehicle control animals, and survival did not differ significantly among groups. There was a marginal increase in the incidence of rare uterine squamous cell papillomas in the 250 mg/kg/day female rat group. The incidence of squamous metaplasia of the uterus was significantly increased in the 750 mg/kg/day female group. There also were dose-related increases in the incidences of dilation, hemorrhage, thrombus, and ulcers in the uterus. Additionally, ovarian atrophy was considered a dose-related nonneoplastic lesion.

Two-year Study in Mice

Female mice dosed with BCE for 2 years had survival rates similar to that of the vehicle control group. Mean body weights were lower with increasing BCE dose, indicative of lower body weight gains. Terminal mean body weights of the 30, 100, 300, and 1,000 mg/kg/day groups were approximately 9%, 18%, 25%, and 40% less, respectively, than that of the vehicle control group. No clinical observations were considered related to BCE administration at any point during the study.

At 3 and 12 months, changes in the erythron were observed; these consisted of a decrease in erythrocyte count and hemoglobin concentration (12 months only) and an increase in mean cell volume with increasing dose. In addition, abnormal metarubricytes were observed in the bone

marrow at both 3 and 12 months. These hematological changes indicated ineffective erythropoiesis and were consistent with a condition known as megaloblastic anemia. Significant, dose-related increases in peripheral blood micronucleated erythrocytes were observed with no significant changes in the percentage of immature erythrocytes at both 3 and 12 months. These effects were consistent with previously reported NTP studies.

No neoplasms in mice were considered related to BCE administration. Dose-related nonneoplastic lesions included necrosis in the liver and follicle dilation in the thyroid gland.

Genetic Toxicology

In addition to interim micronuclei evaluations of peripheral erythrocytes in female mice, two different lots of BCE were tested in two independent bacterial mutagenicity assays in multiple strains of bacteria, with and without induced rodent liver S9 mix. Results were negative for the first BCE lot tested, when both hamster and rat liver S9 was used in seven strains of *Salmonella typhimurium*; the lot of BCE used in the NTP in vivo rodent studies was negative in two bacterial strains (*S. typhimurium* TA100 and *Escherichia coli* WP2 *uvrA* [pKM101]) but was judged to be equivocal in *S. typhimurium* TA98 in the presence of induced rat liver S9.

Conclusions

Under the conditions of these 2-year gavage studies, there was *equivocal evidence of carcinogenic activity* of black cohosh root extract (BCE) in female Hsd:Sprague Dawley® SD® rats based on marginal increases in the incidence of uterine squamous cell papillomas. There was *no evidence of carcinogenic activity* of perinatal BCE exposure in male Hsd:Sprague Dawley® SD® rats at maternal doses of 75, 250, or 750 mg/kg/day.

There was *no evidence of carcinogenic activity* of BCE in female B6C3F1/N mice at doses of 30, 100, 300, or 1,000 mg/kg/day.

Dose-related nonneoplastic lesions were observed in the uterus and ovary in rats and in the liver and thyroid gland in female mice. A significant decrease in litter size was observed in rats.

Interim hematological evaluations and micronucleus assays in female mice showed disruption of normal erythropoiesis and increased frequency of micronucleated erythrocytes at 3 months; at 12 months, the same effects were observed with similar severity and frequency.

Synonyms: *Actaea racemosa* L., *Cimicifuga racemosa* L., black snakeroot, macrotys, rattleweed, rattleroot, snake root, wanzenkraut, bugbane, baneberry

Summary of the Perinatal and Two-year Carcinogenesis and Genetic Toxicology Studies of Black Cohosh Root Extract

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Female B6C3F1/N Mice
Doses in Aqueous Methylcellulose	0, 75, 250, or 750 mg/kg/day (perinatal exposure only)	0, 75, 250, or 750 mg/kg/day (perinatal exposure and postweaning dosing)	0, 30, 100, 300, or 1,000 mg/kg/day (postweaning dosing only)
Survival Rates	15/50, 15/50, 21/50, 16/50	28/50, 24/50, 29/50, 20/50	40/50, 39/50, 44/50, 39/50, 38/50
Body Weights	F_1 generation: *Lactation*: ↓ *Study termination*: ↓ (750 mg/kg/day group 11.3% less than the vehicle control group)	F_0 generation: ↓ F_1 generation: *Lactation*: ↓ *Study termination*: ↓ (750 mg/kg/day group 13.3% less than the vehicle control group)	100 mg/kg/day group 18.5% less than the vehicle control group; 300 mg/kg/day group 25.2% less than the vehicle control group; 1,000 mg/kg/day group 40.0% less than the vehicle control group
Nonneoplastic Effects	–	Uterus: squamous metaplasia (21/50, 25/50, 30/50, 38/49); dilation (2/50, 3/50, 9/50, 22/49); hemorrhage (1/50, 0/50, 0/50, 8/49); thrombus (1/50, 0/50, 5/50, 5/49); ulcers (0/50, 1/50, 3/50, 9/49) Ovary: atrophy (32/50, 39/50, 38/50, 47/49)	2-year evaluation: *Liver*: necrosis (2/50, 0/50, 1/50, 1/50, 8/50) *Thyroid gland*: follicle, dilation (0/50, 1/50, 1/49, 1/49, 5/50)
Hematology	–	–	3- and 12-month interim evaluations: ↓ Erythrocytes ↑ Mean cell volume ↑ Mean cell hemoglobin 3-month interim evaluation: ↓ White blood cells ↓ Neutrophils ↓ Lymphocytes ↓ Monocytes 12-month interim evaluation: ↓ Hematocrit ↓ Hemoglobin

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Female B6C3F1/N Mice
Bone Marrow Cell Count and Cytology	–	–	3-month interim evaluation: abnormal metarubricytes (0/10, 0/10, 3/10, 6/10, 9/10) 12-month interim evaluation: abnormal metarubricytes (0/10, 0/10, 2/10, 4/10, 10/10)
Neoplastic Effects	None[a]	None	None
Equivocal Findings	None	Uterus: squamous cell papilloma (1/50, 1/50, 4/50, 0/49)	None
Level of Evidence of Carcinogenic Activity	No evidence	Equivocal evidence	No evidence
Genetic Toxicology			
Bacterial mutagenicity: First study: Negative in *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, with and without hamster and rat liver S9. Second study: Negative in *S. typhimurium* strain TA100 and *Escherichia coli* strain WP2 *uvrA* (pKM101), with and without S9; equivocal in *S. typhimurium* strain TA98 with S9.			
Micronucleated Erythrocytes (In Vivo)			
Mouse peripheral blood: Positive at both the 3-month and 12-month interim evaluations			

[a]None = no toxicologically relevant effects for this endpoint.

Overview

Botanical dietary supplements, sometimes called herbals or herbal dietary supplements, are products made from plants. National Toxicology Program (NTP) research has contributed to the field of botanical product safety evaluation by reporting hazard characterization of many botanical dietary supplements and supporting development of strategies for their efficient and effective evaluation.

Black cohosh, a perennial species of buttercup, is used to make botanical dietary supplements primarily sold to aid female gynecological symptoms. Because of its widespread use and the lack of safety data, the effects of black cohosh have been thoroughly studied as part of a broad NTP research program.[1] Subchronic studies in female rats and mice demonstrated potential genotoxic and developmental effects. The mechanisms behind these findings were investigated in a follow-up mouse study and in vitro genetic toxicology studies.[2] Potential reproductive and developmental toxicity was further evaluated in rats using a Reproductive Assessment by Continuous Breeding (RACB) study. This report details studies of potential chronic toxicity and carcinogenic activity that were conducted in rats and mice due to concern about long-term use and the genotoxicity findings. Of note, the black cohosh root extract test article used in many of these studies was from the same lot and/or supplier, reducing inherent test article variability and allowing for comparisons and extrapolations across studies.

Introduction

Figure 1. Black Cohosh (CASRN 84776-26-1)

Synonyms: *Actaea racemosa* L*, Cimicifuga racemosa* L., black snakeroot, macrotys, rattleweed, rattleroot, snake root, wanzenkraut, bugbane, baneberry.
Image courtesy of King's American Dispensatory.[3]

Chemical and Physical Properties

Black cohosh (*Actaea racemosa* L.) is a perennial plant of the buttercup family native to eastern North America. It is erect and smooth-stemmed and ranges from 4 to 8 feet tall with long, wand-like, white flowers in midsummer.[4] Preparations of black cohosh include the fresh or dried roots and rhizomes of *A. racemosa* L.[5; 6] Its roots are straight, dark brown, and odorless. When cut, the interior of the roots has the appearance of off-white, porous wood. The plant's rhizomes are harvested in autumn and can be used fresh in tinctures or dried in decoctions, liquid extracts, and tinctures.[7; 8]

Only a small proportion (<10%) of the constituents in black cohosh extract is known.[9; 10] Extracts of black cohosh rhizomes contain various triterpene glycosides, phenolic acids (e.g., isoferulic acid and fukinolic acid), flavonoids, alkaloids, volatile oils, and tannins.[11; 12] Although the constituents responsible for the pharmacological or toxicological effects of black cohosh have not been identified, various components have exhibited biological activity. For example, triterpene glycosides were shown to inhibit breast cancer cell proliferation,[13] and phenolic acids

extracted from black cohosh were shown to prevent menadione-induced DNA damage in breast cancer cells.[14]

Production, Use, and Human Exposure

Black cohosh roots and rhizomes have been used for centuries by eastern North American indigenous groups for enhancing female health, treating abnormal or painful menstruation, relieving pregnancy-related pain or distress, and promoting uncomplicated delivery and quick recovery.[4; 15; 16] Black cohosh also has been used to address other maladies, such as kidney problems, rheumatism, colds, aches, and coughs.[8; 17] Black cohosh was introduced in Europe in the late 19th century. In recent years, its popularity for the treatment of menopause has grown in part from a desire to shift away from hormone-replacement therapies that can produce unwanted side effects.[9] Of women who discontinue hormone therapy and use herbal remedies, an estimated 30% choose black cohosh products.[18]

Black cohosh products are widely available and primarily marketed to alleviate symptoms of menopause, premenstrual syndrome (PMS), and menstrual pain. In 2002, the World Health Organization summarized the evidence for medicinal uses of black cohosh, highlighting that clinical data supported its use in the "treatment of climacteric symptoms such as hot flushes, profuse sweating, sleeping disorders and nervous irritability."[19] Although black cohosh typically is used during menopause, it has been recommended by 20%–30% of midwives in the United States to induce labor in pregnant women or for other purposes.[20-23]

In 2019, black cohosh was the 15th top-selling botanical dietary supplement in the United States.[24] Black cohosh dietary supplements are sold as powdered whole herb, liquid extracts, or dried extracts in pill form and can be found alone or in combinations with other botanicals.[25] Extraction and preparation methods may vary by manufacturer and are often proprietary. Commercial pharmaceutical formulations of black cohosh include Remifemin® (sold in Germany since 1956) and Remixin. Black cohosh is typically found in combination products marketed for improving female health, such as general wellness supplements for use during menopause, phytoestrogen supplements, sleep aids for women, and female-specific vitamins.[25]

Black cohosh products generally are standardized to the content of triterpene glycosides, such as 23-epi-26-deoxyactein (27-deoxyactein), actein, or cimicifugosides.[26; 27] Standardizing to attain a triterpene content of 2.5%, or approximately 1 mg of a given triterpene, is common.[28; 29] Remifemin® has been widely studied for efficacy and is standardized to 20 mg of black cohosh root and rhizome per tablet[30]; however, the chemical makeup of black cohosh products varies widely.[31; 32] One study analyzed triterpene glycoside content in 11 black cohosh products and found amounts ranging from 0.36% to 7.55%; 8 of the 11 products had label claims of 2.5% triterpene glycoside content.[31]

Black cohosh doses in clinical trials evaluating efficacy have ranged from 6.5 to 160 mg/day for 1–12 months.[10; 33] The expert committee on herbal remedies established by the German Federal Institute for Drugs and Medical Devices to independently evaluate the safety and efficacy of phytomedicines, Commission E, recommends 40 mg/day of alcohol extracts of black cohosh, not to exceed 6 months of use.[6] The manufacturer of Remifemin® recommends a dose equivalent to approximately 5 mg/day, taken as two tablets containing 2.5 mg black cohosh root/rhizome per day.[34] Botanical supplements that contain a mixture of active ingredients, such as black cohosh,

further complicate the exposure profile and safety evaluation.[35] Data on internal concentrations of chemical constituents or metabolites of black cohosh after oral intake are limited. Black cohosh products are also marketed in combination with other botanicals, such as red clover, soy isoflavones, St. John's wort, chasteberry, and dong quai.[36; 37]

Regulatory Status

In the United States, black cohosh products are sold as dietary supplements and are therefore regulated under the Dietary Supplement Health and Education Act.[38] For dietary supplements on the market before October 15, 1994, there is no requirement for proof of safety to remain on the market. The labeling requirements for supplements allow warnings and dosage recommendations as well as substantiated "structure or function" claims. All claims must prominently note they have not been evaluated by the U.S. Food and Drug Administration and bear the statement, "This product is not intended to diagnose, treat, cure, or prevent any disease."[39] Thus, dietary supplements containing black cohosh cannot claim to treat PMS or menopausal symptoms.

In Europe, there is general support for the use of black cohosh for gynecological symptoms. Commission E recommended black cohosh for treating PMS, menstrual cramps, and menopausal symptoms for a duration of 6 months or less.[6] The European Medicines Agency's Herbal Medicinal Products Committee released a monograph on *Cimicifuga racemosa* (L.) Nutt., rhizoma, indicating black cohosh for relief of menopausal symptoms such as hot flashes and profuse sweating.[40]

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

The literature contains few studies on the metabolism of black cohosh in rodents. Expression of phase I and II enzymes and transporters in the liver, kidney, and small intestine were evaluated in female C57BL/6J wild type mice following gavage administration of up to 500 mg/kg body weight (mg/kg) of an extract of black cohosh for 28 days.[41] Dose- and time-dependent, liver-specific up-regulation of *Cyp3a11* was observed, with a sevenfold increase in the 500 mg/kg group compared to control animals after 14 days. The authors demonstrated that the mouse pregnane X receptor (PXR) played a direct role in the induction of *Cyp3a11* by using a combination of in vivo (*Pxr*-null, PXR-humanized [hPXR], and double transgenic CYP3A4/hPXR mice) and cell-based assays, whereas human PXR was not activated by the extract. Following a single gavage administration of 35.7 mg/kg actein (a constituent of black cohosh) to female Sprague Dawley rats, the maximum serum concentration (C_{max}) was 2.4 µg/mL at 6 hours.[42] Actein was detected in urine at 24 hours with a reported concentration of 777.07 ng/mL.

Humans

The pharmacokinetics of 23-epi-26-deoxyactein were evaluated in menopausal women following single oral administration of black cohosh capsules (32 mg, 64 mg, or 128 mg extracted with 75% ethanol) containing 1.4 mg, 2.8 mg, or 5.6 mg of 23-epi-26-deoxyactein.[43] Absorption was rapid, with C_{max} reached in ≤3 hours. Area under the concentration-time curve (AUC) and C_{max} (2.2–12.4 ng/L) increased proportionally to the dose. There was no dose-dependent difference in

the serum elimination half-life (2.1–3.0 hours). The dose recovered in urine during the first 24 hours after administration was <0.01%. No metabolites were detected in urine or serum despite low concentrations of the parent compound observed in serum and urine. Metabolites also were not detected following incubation of 23-epi-26-deoxyactein with human hepatocytes. Incubation of 23-epi-26-deoxyactein with simulated human gastric fluid showed that it degraded over time, with an estimated half-life of 80.6 minutes. This finding suggests 23-epi-26-deoxyactein likely undergoes degradation in the stomach following oral administration, which leads to low systemic levels, as found in the pharmacokinetic study.

The literature generally shows limited effects of black cohosh on drug metabolism and disposition in humans. In human liver microsomes, seven tested black cohosh supplements (methanolic extracts) produced slight, but insignificant, activation or inhibition of CYP3A4.[44] This observation is consistent with the finding that black cohosh supplement consumption (40 mg twice daily for 14 days) did not have a significant effect on CYP3A activity.[45] Other studies using human liver microsomes have found that black cohosh (methanolic extract containing ≥5% total triterpene glycosides) inhibits UGT1A4, β-D-glucuronide formation, and 3-O-glucuronidation of estradiol.[46; 47]

The pharmacokinetics of digoxin, a P-glycoprotein (P-gp) substrate, were not influenced following administration of 20 mg (twice daily for 14 days) of black cohosh extract (2.5% triterpene glycosides), suggesting that black cohosh constituents do not modulate P-gp activity.[48] Similarly, 40 mg (twice daily for 14 days) of a black cohosh supplement (2.5% triterpene glycosides) did not modulate CYP2D6 in humans when debrisoquine was the substrate.[49] A significant, but not clinically relevant, inhibition of CYP2D6 was reported, however, following administration of a supplement containing 1,090 mg (twice daily for 28 days) of black cohosh (0.2% triterpene glycosides).[50]

Potential herb-drug interactions of black cohosh have been demonstrated in some in vitro studies. Several studies reported inhibition of hepatic cytochrome P450 enzymes[44; 51-54] and UDP-glucuronosyltransferases[46; 47] by black cohosh. Sevior et al.[53] reported inhibition of CYP2B6, CYP2C19, CYP(D)2C19, and CYP2E1, but not CYP2D6 and CYP3A4, enzymes by a methanolic black cohosh extract with median inhibitory concentration (IC_{50}) values of 49.2, 36.3, 23.9, and 11.5 µg/mL, respectively. Studies have also shown inhibition of CYP1A2, CYP2D6, CYP2C9, and CYP3A4.[51; 52] The active inhibitory constituents, triterpene glycosides, fukinolic acid, and cimicifugic acids A and B, were identified by bioassay-directed fractionation. Although the triterpene glycosides (cimiracemoside A, 23-epi-26-deoxyactein, and actein) were weakly active for CYP2C9 and CYP3A4 (IC_{50}: 25–100 µM), fukinolic acid and cimicifugic acids A and B strongly inhibited all CYP isozymes (IC_{50}: 1.8–12.6 µM).

Toxicity

Experimental Animals

In a 13-week repeat-dose study conducted as part of the National Toxicology Program (NTP) research program on black cohosh, female Wistar Han rats and female B6C3F1/N mice were administered up to 1,000 mg/kg body weight/day (mg/kg/day) of an ethanolic extract of black cohosh via gavage.[55] In both species, dosing did not affect mean body weights but did result in higher liver weights in the 500 and 1,000 mg/kg/day groups. Mild liver necrosis was noted in

two rats in the 1,000 mg/kg/day group, but no significant microscopic findings were noted in mouse livers. Differences in kidney, pituitary gland, and ovary weights were reported but did not have microscopic correlates. Mean thymus weights of rats administered ≥500 mg/kg/day were lower than that of control animals and correlated with increased incidences of apoptosis in the thymus; this histological finding was not observed in mice. Notably, increased frequencies of micronucleated peripheral red blood cells were observed in both species. Dose-dependent hematological changes were also found, consistent with a nonregenerative macrocytic decrease in the erythron that presented more severely in mice than in rats. Additionally, rare-to-occasional poikilocytes (including acanthocytes and schistocytes) and basophilic stippling were observed on the mouse peripheral blood smears. This combination of hematological changes was consistent with a condition known as megaloblastic anemia. Further investigation in female B6C3F1/N mice showed these hematological changes were consistent with functional cobalamin (and potentially folate) deficiencies, which are both known causes of megaloblastic anemia.[2]

Other in vivo studies have investigated hepatotoxicity with mixed findings. Marked changes in hepatic mitochondrial morphology were observed in female Wistar rats administered ≥100 mg/kg/day via gavage for 21 days; microvesicular steatosis developed in the 1,000 mg/kg/day group by study termination.[56] On the other hand, male Wistar rats administered 300 mg/kg/day of a black cohosh extract for 30 days via gavage exhibited no changes in serum AST (aspartate transaminase), ALT (alanine transaminase), GGT (gamma-glutamyltransferase), ALP (alkaline phosphatase), or total or direct bilirubin and had normal liver histopathology.[57] Reductions in hepatic GSH (glutathione) were observed.

Humans

Acute complaints associated with black cohosh consumption have included headaches and gastrointestinal symptoms such as nausea, vomiting, and gastric discomfort (reviewed in Huntley[58]). One case of severe hyponatremia was reported when black cohosh was used to induce labor.[59] Another possible concern is liver toxicity, as suggested by case reports of patients developing hepatitis or fulminant hepatic failure.[60-62] In 2008, an expert committee convened by the United States Pharmacopeia evaluated 30 reports of black cohosh products and liver damage and found insufficient evidence to support a causal relationship.[63] The committee nevertheless recommended including a warning label for hepatotoxicity on black cohosh products. Further analysis of data from 11 published case reports and 58 spontaneous reports did not support black cohosh hepatotoxicity.[64] A meta-analysis of five clinical trials evaluating the efficiency of Remifemin® also did not find black cohosh to be associated with abnormal liver function.[65]

A key problem in evaluating the available data on possible hepatotoxicity is the limited information on the type, amount, and quality of black cohosh products used and incomplete and/or unclear descriptions of the clinical presentation.[64] Multiple sources of variation also exist in the production of botanical dietary supplements,[66] including differences in preparation methods and potential adulteration with other types of cohoshes or toxicants. This variability may help to explain the heterogeneity of clinical reports, both in terms of clinical efficacy and toxicity. Overall, the consensus evidence for black cohosh-induced hepatotoxicity is weak, and more systematic investigation of this adverse effect is warranted.

Reproductive and Developmental Toxicity

Experimental Animals

Despite the predominant use of black cohosh by women, some of child-bearing age, minimal information is available regarding its reproductive and developmental effects. The literature contains no reproductive toxicity studies in animals. Regarding developmental effects, the previously described subchronic NTP studies found delayed onset of puberty in female Wistar Han rats and B6C3F1/N mice.[55]

Given the use of black cohosh for gynecological symptoms, the estrogenic activity of black cohosh and its constituents has been studied extensively.[29; 67] Findings in molecular studies evaluating the ability of components found in black cohosh to bind to estrogen receptors (ERs) are conflicting. Extracts of black cohosh were found to bind to ERs in rat, porcine, and human uterine cytosol preparations[68-70] but not when incubated with recombinant ERα and ERβ receptors.[69; 71; 72] In vivo studies in rodents have reported negative findings in uterotrophic assays,[55; 73; 74] although some found decreased luteinizing hormone concentrations in ovariectomized rats.[70; 75] Findings in ovariectomized Sprague Dawley rats with black cohosh-supplemented diets showed an estrogenic effect in bone (particularly osteoblasts), which reduced serum and urinary biomarkers indicative of bone turnover or loss and preserved bone mineral density.[74; 76] Black cohosh may also exert estrogenic activity in the brain. A proposed mechanism for the regulation of hot flashes is the binding of black cohosh components to serotonin receptors found in the thermoregulatory region of the brain, rather than activation of ERs.[77]

Humans

The literature contains few studies on adverse effects of black cohosh on female reproductive health. The available evidence generally suggests minimal effects.

In vitro, black cohosh extracts did not stimulate proliferation in human breast cancer MCF-7 cells, which would be expected of an ER agonist, but rather inhibited growth through induction of apoptosis.[13; 78; 79] Proliferation of human prostate cancer cells also was inhibited by black cohosh extracts, possibly through activation of aryl hydrocarbon receptors.[80] Exposure to an isopropanolic extract of black cohosh increased osteoprotegerin gene expression and protein secretion in human osteoblastic cells, suggesting an estrogenic effect in the bone.[81]

In line with findings from animal studies, a review of clinical studies did not find indications of estrogenic activity in the mammary gland or the endometrium.[82] There was no increase in endometrial proliferation in a study of postmenopausal women given 40 mg of black cohosh daily for 1 year.[83] No change in vaginal cytology or menstrual cyclicity was observed in women consuming 160 mg/day for up to 1 year.[84] Black cohosh supplementation also did not modify reproductive hormones such as estradiol, follicle stimulating hormone, prolactin, or luteinizing hormone.[28; 84-89] In contrast, some studies have shown a decrease in luteinizing hormone or increased proliferation of the vaginal epithelium among women taking black cohosh products.[70; 90] These discrepancies could result from the complexity of the biological effect and variation in the chemical makeup of black cohosh products.

Immunotoxicity

Immune modulation has been observed with black cohosh and some of its constituents. Studies using extracts of black cohosh found decreased cytokines in human peripheral whole blood[91] and macrophages,[92] inhibition of histamine release in rat peritoneal mast cells,[93] and reduced nitric oxide production in murine macrophages.[94] Immunological evaluations conducted as part of the NTP black cohosh research program showed increased liver weight and decreased spleen cell populations in female B6C3F1/N mice administered 1,000 mg/kg/day of the same black cohosh extract lot used in the present studies for 28 days.[95] In that study, minimal changes in thymus cell populations and bone marrow cellularity were observed. Importantly, apart from an enhanced cytotoxic T lymphocyte response, no changes in the function of innate, cell-mediated, or humoral immunity were detected.[95]

Studies on ferulic and isoferulic acid, constituents found in extracts of black cohosh, suggested they could be responsible for some of the anti-inflammatory effects of black cohosh products. Ferulic acid and/or isoferulic acid decreased cytokine production in murine macrophages and in female ICR mice,[96] inhibited COX-2 expression in lipopolysaccharide-induced macrophages,[97] and reduced inflammation in rats.[98] Other constituents of black cohosh—actein, cimigenol, and cimicifugoside—have been found to be immunosuppressive.[99; 100] In vitro assays of rhizome extracts from other species of the *Cimicifuga* genus found anticomplement activity in human serum[101] and suppression of concanavalin A-stimulated BALB/c mice spleen cell proliferation,[102] lymphocyte proliferation in mixed BALB/c and C57BL/6 mouse spleen cells,[103] and C57BL/6 mouse T cell differentiation.[104]

Carcinogenicity

Experimental Animals

Given that black cohosh might be estrogenic and because of its prevalent use as a supplement among women, much of the available literature evaluates the relationship of black cohosh product use with breast cancer. However, over time, experimental studies using breast and endometrial cancer cell lines have shown that black cohosh is likely not estrogenic and either inhibits or has no effect on cancer cell growth.[40; 58; 67]

In vivo animal studies have shown a similar pattern in which black cohosh either inhibits or does not promote breast or uterine cancer growth. One study reported a decrease in the incidence of mammary fibroadenomas in female Sprague Dawley rats administered up to 35.7 mg/kg of black cohosh (n-butanolic extract) via oral intubation 6 days per week for 40 weeks.[105] Consumption of 40 mg/day of an isopropanolic extract of black cohosh in the diet for up to 14 months did not promote or inhibit growth of mammary tumors in MMTV-neu mice (transgenic mice that spontaneously develop mammary tumors).[106] In this study, however, increased pulmonary metastases were observed in animals consuming black cohosh. Endometrial adenocarcinoma cells implanted in ovariectomized rats did not respond negatively or positively to black cohosh consumption (60 mg of an isopropanolic extract of black cohosh per kg body weight via drinking water prepared daily for 5 weeks).[107]

The role of black cohosh in other cancers is less studied. Extract of black cohosh inhibited tumor development in immunodeficient nu/nu mice inoculated with LNCaP cells.[108] Overall, there is

little evidence that black cohosh promotes cancer growth, but further research is needed to evaluate its role in carcinogenesis in various tissues.

Humans

The available human data do not support an association between black cohosh use and increased risk of breast cancer.[67; 109; 110] The risk of developing other cancers with consumption of black cohosh has not been evaluated in human population studies. In relation to other types of cancer, extracts of black cohosh inhibited proliferation of HepG2 human liver cancer cells,[111] HT29 human colon cancer cells,[112] and LNCaP human prostate cancer cells.[80; 113]

Genetic Toxicity

A published NTP study reported that an ethanolic black cohosh extract (the same lot used in the present studies) produced significant, dose-dependent increases in micronucleated reticulocytes and micronucleated erythrocytes in the peripheral blood of female B6C3F1/N mice at doses ranging from 62.5 to 1,000 mg/kg/day via gavage for 13 weeks.[55] Significant increases in micronucleated reticulocytes also were observed in the peripheral blood of female Wistar Han rats administered doses ranging from 15 to 1,000 mg/kg/day for 13 weeks via gavage.[55] In a subsequent NTP study, a significant increase in micronuclei (reported as Howell-Jolly bodies) was observed in peripheral blood from female B6C3F1/N mice administered 1,000 mg/kg/day for 13 weeks by gavage.[2]

To evaluate whether the genotoxicity of black cohosh was specific to the lot used for the NTP animal studies, the same lot, as well as 14 other black cohosh products (including a standard reference material [XRM, ChromaDex, Inc.] for black cohosh extract), were tested in an in vitro flow-cytometric micronucleus assay using human B-lymphoblastoid TK6 cells.[114] The lot used in the previous and present NTP studies produced significant, dose-dependent increases in micronuclei in TK6 cells when tested over a range of 30–500 µg/mL for 4 hours or 10–125 µg/mL for 24 hours. It did not induce micronuclei in the presence of 10% induced rat liver S9 mix, suggesting the active compound (or compounds) may have been detoxified. Samples from all 15 black cohosh sources induced significant increases in micronuclei in the absence of S9 mix when cells were first conditioned in culture medium containing a more physiologically relevant concentration of folic acid. Furthermore, use of the MultiFlow® DNA Damage Assay indicated that induction of micronuclei in TK6 cells by the lot used in the present studies and the XRM standard reference material was due to an aneugenic (whole chromosome loss) versus a clastogenic (chromosome breakage) mode of action, an observation that was repeated in a subsequent study, also with TK6 cells.[115] These same two samples shared characteristics with chemicals known to destabilize microtubules, as shown in a MultiFlow Aneugen Molecular Mechanism Assay, which uses TK6 cells to categorize chemicals with aneugenic activity as tubulin destabilizers, tubulin stabilizers, or Aurora kinase inhibitors.[115] The observation that black cohosh extract—the same lot that was used for NTP animal studies—induces micronuclei in TK6 cells was repeated in another laboratory.[116]

TK6 cells exposed to 25–250 µg/mL of black cohosh extract from the same lot as that used for NTP animal studies were assessed for DNA damage at 0.5, 1, 2, 4, and 24 hours after exposure using the comet assay.[116] DNA damage (% tail DNA) was significantly increased at the 0.5-, 1-, 2-, and 4-hour time points when cells were exposed to 250 µg/mL of black cohosh extract. The

level of test article-induced DNA damage was highest at the 0.5-hour time point and decreased over time. DNA damage was not detected, however, in human hepatoblastoma HepG2 cells exposed to 50–500 µg/mL of this lot of black cohosh extract over the same time course using the comet assay.[116] In the same study, phosphorylation of H2AX (γH2AX), a biomarker for double-strand DNA breaks, was observed in TK6 cells after 4 hours and 24 hours of exposure, accompanied by phosphorylation of cell cycle checkpoint proteins Chk1 and Chk2 at both time points and increases in p53 and p21 after 24 hours exposure.

The same lot of black cohosh extract was tested in *Salmonella typhimurium* strains TA100 and TA98 and *Escherichia coli* strain WP2 *uvrA* (pKM101) at doses ranging from 100 to 10,000 µg/plate, with or without 10% induced rat liver S9 mix.[114; 117] The result was judged to be equivocal in TA98 in the presence of S9 mix and otherwise negative in these tests. The same three tester strains were used to assay five additional black cohosh products from different lots or sources at doses ranging from 187.5 to 6,000 µg/plate, with or without 10% induced rat liver S9 mix, and judged all results to be negative.

In summary, black cohosh extract was genotoxic in rats and mice, and studies with human cells showed that a variety of extracts from different suppliers were genotoxic, indicating general genotoxic activity. Furthermore, studies with human cells showed that micronuclei were induced by an aneugenic mode of action, likely due to destabilization of microtubules.[114; 115] The same black cohosh lot as used in the present studies might also be capable of directly causing DNA damage, as indicated by the comet assay and activation of the DNA damage response,[116] although clastogenic activity was not identified as a mode of action in TK6 cells exposed to similar black cohosh extract concentrations.[114; 115] Black cohosh does not appear to cause gene mutations, as assessed in bacterial mutagenicity assays.

Study Rationale

In the NTP 3-month repeat-dose studies, black cohosh root extract (BCE) administration increased frequencies of micronucleated peripheral erythrocytes in both female rats and mice.[55] Given the correlation of positive micronucleus assays and carcinogenicity[118] and the lack of publicly available data to evaluate cancer risk, it was determined that a carcinogenicity study on black cohosh was warranted. These studies were conducted using the same lot of BCE used in previous NTP animal studies that showed increases in micronucleated peripheral erythrocytes.[55] Perinatal exposure was included in the rat study to reflect the potential use of black cohosh products by women during pregnancy. Male offspring were maintained during the study, but not administered BCE, to detect potential effects of in utero exposure. Because black cohosh products are used by women of reproductive age, BCE was administered to female rat offspring after weaning and to female mice starting from 5 to 6 weeks of age for 2 years.

Materials and Methods

Procurement and Characterization

Black Cohosh Root Extract

Black cohosh root extract (BCE) was obtained from PlusPharma, Inc. (Vista, CA) in a single lot (3012782). According to the manufacturer's certificate of analysis, lot 3012782 was a 50% aqueous ethanolic extract standardized to 7.8% (w/w) total triterpene glycosides. Identity, purity, and stability analyses were conducted by the study laboratory Battelle (Columbus, OH). Reports on analyses performed in support of the BCE studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

The BCE test lot was a light-brown powder. It was confirmed to be authentic black cohosh by multiple laboratories using high-performance thin-layer chromatography and DNA barcoding. Fourier transform infrared (IR) spectroscopy detected the presence of phenolic compounds as well as plant components such as sugars, terpenes, and glycosides. Proton-induced X-ray emission spectroscopy analysis conducted by Element Analysis Corporation (Lexington, KY) indicated that the BCE was composed almost entirely of carbon (69.5%), hydrogen (11.7%), and oxygen (15.4%). Potassium (2.5%), magnesium (0.3%), and calcium (0.3%) were also detected. Water content in lot 3012782 was determined to be <7.9% via Karl Fisher analysis (Prevalere Life Sciences, Inc., Whitesboro, NY) and weight loss on drying.

Analysis of the extract by liquid chromatography (LC) coupled with quadrupole time-of-flight mass spectrometry (MS) or quadrupole ion trap MS identified multiple constituents, including caffeic acid, ferulic acid, isoferulic acid, actein, 23-epi-26-deoxyactein (27-deoxyactein), cimifugin, allocryptopine, cimicifugoside H-1, cimiracemoside C, 26-deoxycimicifugoside, magnoflorine, and prim-o-glucosylcimifugin. Quantitation of analytes by LC coupled with tandem MS showed that the extract contained (w/w) 0.28% caffeic acid, 0.04% ferulic acid, 0.70% isoferulic acid, 0.47% actein, 2.09% 23-epi-26-deoxyactein (27-deoxyactein), 2.13% cimiracemoside C, 0.08% 26-deoxycimicifugoside, and 0.01% magnoflorine. Cimifugin, prim-o-glucosylcimifugin, allocryptopine, and cimifugoside H-1 were not quantifiable and were estimated to be <0.01% of the extract. The measured constituents accounted for approximately 5.8% of the total extract weight.

An aliquot of the test lot was evaluated for environmental contaminants by Covance Laboratories, Inc. (Madison, WI) (Appendix A). The heavy metals arsenic, cadmium, and lead were present at low levels, below the threshold limits for botanical dietary supplements.[119; 120] Of >300 pesticides screened, only chlorpropham and 2-phenylphenol were detected, and these were present at concentrations below the lowest respective U.S. Environmental Protection Agency residue tolerance levels.[121; 122] Aflatoxins B_1, B_2, G_1, and G_2 and the pesticide imazilil could not be accurately detected due to matrix interferences. All other pesticides tested were determined to be below the limits of quantitation.

A comparison of high-performance liquid chromatography (HPLC)-charged aerosol detection chromatographic profiles of BCE lot 3012782 with root extract reference material and a black cohosh product available to the public in the United States showed that the chemical profiles were similar.

Accelerated stability studies were conducted, using isoferulic acid as the marker, by storing samples of lot 3012782 in amber glass bottles for 14 days at frozen (−20°C), refrigerated (5°C), room (25°C), and elevated (60°C) temperatures. Stability was confirmed for at least 14 days at ≤25°C. Further details identifying the test lot as BCE, as well as a thorough description of its composition, are available in a separate publication by Waidyanatha et al.[123]

Six plastic bags of the BCE test lot were originally received. Each was double bagged and rolled for 5 minutes to homogenize. All bags were then transferred to 4 L plastic bottles. Samples were removed and stored to serve as analytical standards, reference standards, and an archive sample. The remaining bulk test article was sealed and stored at room temperature. Reanalysis of the bulk chemical, using isoferulic acid as the marker, was performed once (mice) or three times (rats) before the start of the study, four times during the study, and one time after study termination using HPLC with ultraviolet (UV) detection. No degradation of the bulk test article was detected by analysis of the marker constituent, comparison of the constituent profile with frozen reference material, or the appearance of the bulk material.

Methylcellulose

Methylcellulose used to make the 0.5% aqueous vehicle for gavage formulations was obtained from Spectrum (Gardena, CA) in two lots (2CB0045 and 2AJ0439). The identity of methylcellulose was confirmed by the study laboratory using IR spectroscopy. Methoxy content of both lots was measured by Galbraith Laboratories, Inc. (Knoxville, TN) to determine purity. Purity reanalysis was conducted at 6-month intervals over the course of the study. Deionized tap water was used to make the 0.5% (w/v) aqueous methylcellulose vehicle for gavage formulations.

Preparation and Analysis of Dose Formulations

Dose formulations were prepared at the study laboratory by mixing the BCE test lot with 0.5% aqueous methylcellulose following the protocols outlined in Appendix A. For the rat study, formulations were prepared at 0, 15, 50, and 150 mg/mL. For the mouse study, formulations were prepared at 0, 3, 10, 30, and 100 mg/mL.

The homogeneity and stability of the dose formulations were determined by the study laboratory. Isoferulic acid was used as the marker for BCE formulation analysis. Homogeneity was evaluated using 3, 20, 100, and 200 mg/mL formulations and was confirmed at these doses. Stability of the 3, 30, and 100 mg/mL formulations was investigated using marker constituents actein, 27-deoxyactein, isoferulic acid, or cimiracemoside C prior to and following study termination. Stability of the 3, 30, and 100 mg/mL dose formulations was confirmed for up to 42 days in sealed, refrigerated, amber glass bottles. In addition, the 3, 30, and 100 mg/mL dose formulations were stable under simulated animal room conditions for at least 3 hours. The dose formulations were stored in sterile, clear glass, straight-sided bottles under inert headspace at 2°C–8°C (refrigerated).

The dose formulations were used within 42 days during the first 11 months of the studies. Before dose administration on April 13, 2013, unidentified particles resembling mold were observed in the 15 mg/mL rat dose formulation and in the 3 and 10 mg/mL mouse dose formulation. To determine whether storing the formulation frozen would prevent mold growth, an experiment was conducted by storing formulations at approximately −20°C in polycarbonate jars for

26 days. The frozen formulations were then thawed and analyzed using HPLC/UV. All dose formulations were within 10% of the target concentrations, and the results indicated no differences between refrigerated and frozen samples. Following this finding, subsequent dose formulations were used within 21 days and included backup formulations that were stored frozen. There were no further reports of mold growth in the dose formulations during the 2-year studies.

Animal Source

Time-mated (F_0) female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN). Male and female B6C3F1/N mice were obtained from the National Toxicology Program (NTP) colony maintained by Taconic Biosciences, Inc. (Germantown, NY).

Animal Welfare

Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by AAALAC International. Studies were approved by the Battelle (Columbus, OH) Animal Care and Use Committee and conducted in accordance with all relevant National Institutes of Health (NIH) and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

Two-year Studies

Dose Selection Rationale

Dose selection was based on findings from the 3-month repeat-dose studies, which observed no effect on survival and marginal effects on body weight (within 10% of control values) in female Wistar Han rats and B6C3F1/N mice.[55] As the primary toxicity observed was mild hematological effects, a similar dose range up to 1,000 mg BCE/kg body weight/day (mg/kg/day) was used for the mouse chronic study. Perinatal exposure was included in the rat study because women use BCE for general female health, which may include use during pregnancy. Males were not dosed postweaning but were maintained in the study to evaluate potential in utero effects. Because preliminary data indicated mortality of pups from dams administered 1,000 mg/kg/day via gavage throughout gestation and lactation, the highest dose administered to rats was 750 mg/kg/day (NTP, unpublished).

Study Design for Rats

F_0 female rats were 12–15 weeks old upon receipt. Evidence of mating is defined as gestation day (GD) 1; F_0 females were received on GD 2 and held for 4 days. F_0 females were randomly assigned to one of four dose groups on GD 5. Randomization was stratified by body weight that produced similar group mean weights using PATH/TOX SYSTEM software (Xybion Medical Systems Corporation, Lawrenceville, NJ).

F_0 females were quarantined for 12 days after receipt. Ten nonmated females received with the time-mated females were designated for disease monitoring 11 days after arrival; samples were collected for serological analyses, and the rats were euthanized, necropsied, and examined for the

presence of disease or parasites. The health of the F_1 rats was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix C). Pinworms (*Syphacia* spp.) were diagnosed in sentinel animals during routine health monitoring evaluations. Infected animals did not display clinical signs, and no pathological lesions were noted in relation to the presence of the pinworms. Following this finding, a successful plan of pinworm containment and eradication without the use of medication was developed and implemented in coordination with the testing laboratory. The testing laboratories were required to actively monitor animals to ensure the continued exclusion of pinworms from all studies going forward. All other test results were negative.

Beginning on GD 6, groups of 38 F_0 time-mated female rats were administered BCE in 0.5% aqueous methylcellulose by gavage throughout gestation and lactation at one of three doses (75, 250, or 750 mg/kg/day) or the vehicle control only (0.5% aqueous methylcellulose). Formulations were administered daily, except for the day of delivery if the dam was in the process of delivering; dosing volumes were 5 mL/kg. Groups of 50 F_1 female rats per dose group continued in the study after weaning and were administered 0.5% aqueous methylcellulose by gavage containing the same respective BCE doses for 2 years. Groups of 50 F_1 male rats were not dosed after weaning but were assigned to groups corresponding to the doses their respective dams received during the perinatal phase.

F_0 female rats were housed individually during gestation and with their respective litters during lactation. Feed and water were available ad libitum. F_0 female body weights were recorded at arrival, on GD 5, and then daily throughout the rest of gestation and lactation. The day of parturition was considered to be lactation day (LD) 0. On apparent GD 25, all time-mated female rats that failed to deliver were euthanized, and the uteri were examined and stained for evidence of implantation. Total litter weight and litter weights by sex were collected on postnatal day (PND) 1. Individual F_1 pup weights were recorded on PNDs 4, 7, 12, 15, 18, 21, 24, and 28. Clinical observations and survival were evaluated throughout lactation.

F_1 litters were standardized on PND 4 to eight pups per litter, with at least two pups of each sex and a preference for four males and four females each. Litters that did not meet the minimum of eight pups (or had fewer than two pups of either sex) were removed from the study. On the day the last litter reached PND 26, pups (two/sex/litter) were randomly selected from 25 litters for each dose group and assigned to the 2-year study. On the day the last litter reached PND 28, dams were removed from the cages, and the pups were weaned. Weaning marked the beginning of the 2-year study.

After weaning, F_1 rats were housed up to two (males) or up to five (females) per cage. Feed and water were available ad libitum. Cages were changed at least once weekly through PND 4, then changed at least twice weekly. Racks were changed and rotated at least every 2 weeks. Further details of animal maintenance are given in Table 1.

Two diets were utilized in the rat studies: (1) NIH-07 during the perinatal phase and (2) NTP-2000 during the postnatal phase. The NIH-07 diet is a higher protein diet that supports reproduction and lactation in rodents, whereas the NTP-2000 diet is a lower protein diet that decreases the incidence of chronic nephropathy in adult rats. Information on feed composition and contaminants for both diets is provided in Appendix B.

Study Design for Mice

Female B6C3F1/N mice were approximately 3–4 weeks old upon receipt and were quarantined for 11 days before study start. Mice were randomly assigned to one of five dose groups (n = 70 female mice per dose group). Randomization was stratified by body weight that produced similar group mean weights using PATH/TOX SYSTEM software (Xybion Medical Systems Corporation, Lawrenceville, NJ).

Twenty male and 20 female mice were randomly selected for parasite evaluation and gross observation of disease. The health of the mice was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix C). All test results were negative.

Mice were administered BCE in 0.5% aqueous methylcellulose by gavage at one of four doses (30, 100, 300, or 1,000 mg/kg/day) or the vehicle control (0.5% aqueous methylcellulose). Formulations were administered daily for 2 years; dosing volumes were 10 mL/kg. Ten female mice per dose group were removed for each interim evaluation at 3 and 12 months.

Mice were housed up to two (cyclicity males) or up to four (females) per cage. Up to four male mice were maintained per rack to ensure cyclicity in female mice, but these male mice were not evaluated in the study. Feed and water were available ad libitum. Cages were changed at least once weekly (males) or twice weekly (females) and rotated every 2 weeks. Racks were changed and rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is given in Appendix B.

Clinical Examinations and Pathology

In the 2-year studies in rats and mice, animals were observed twice daily for signs of morbidity and moribundity and were weighed initially, weekly for the next 3 months, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks and at study termination.

At the 3-month and 12-month mouse interim evaluations, blood was collected from the retroorbital sinus for hematology and micronuclei determinations. Mice were anesthetized with a carbon dioxide/oxygen mixture and bled in a random order. Blood was collected into tubes containing K_3 EDTA (tripotassium ethylenediaminetetraacetic acid). Hematology parameters were analyzed using an Advia® 120 system (Bayer Diagnostics Division, Tarrytown, NY). The parameters measured are listed in Table 1. Samples for erythrocyte micronuclei determination were stored at 2°C–8°C immediately after collection and shipped to Integrated Laboratory Systems, LLC (ILS, Durham, NC) for analysis. Full necropsy was not performed on these animals. Spleen weights were collected, and histopathology was performed on the spleen and left femur only. Bone marrow was collected from the right femur for determination of total cell count and from the sternum for cytological evaluation (myeloid:erythoid [M:E] ratio and morphological assessment). Bone marrow total cell counts were quantified per femur using an Advia 120 system (Bayer Diagnostics Division, Tarrytown, NY). Three slides were prepared from the sternum bone marrow sample using the paint brush technique. A 500-cell differential cell count was performed on the sternum bone marrow slides stained with Wright's Giemsa stain to calculate the M:E ratio. Bone marrow cytological evaluation was performed by the study clinical pathologist.

Complete necropsies and microscopic examinations were performed on all F_1 male and female rats and female mice that continued in the 2-year studies. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, except for eyes, testes (rats), vaginal tunics (rats), and epididymides (rats), which were first fixed in Davidson's solution or modified Davidson's solution. Tissues were processed and trimmed, embedded in paraffin, sectioned to a thickness of 4–6 μm, and stained with hematoxylin and eosin (H&E) for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. The uterine horns were bisected at their midpoint, and one transverse section was taken from the midpoint of each horn for histopathology evaluation; the two free portions of each uterine horn, the uterine body, including the cervix, and the vagina were also examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by a quality assessment (QA) pathologist at a pathology laboratory independent of the study laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a QA pathologist evaluated slides from all neoplasms and all potential target organs, which included the bone marrow, kidney, thymus, and thyroid gland of male and female rats; the mammary gland, ovary, pituitary gland, spleen, uterus, and vagina of female rats; and the liver of male rats. For mice, the target organs included the spleen of female mice in the 3-month and 12-month interim evaluations and the adrenal gland, bone marrow, liver, ovary, skin, thymus, thyroid gland, uterus, and vagina of female mice in the 2-year study.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to a Pathology Working Group (PWG) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologists were resolved by the Division of Translational Toxicology (DTT) pathology peer-review process. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, DTT pathologist, QA pathologist(s), and the PWG coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman[124] and Boorman et al.[125] For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined generally was based on the guidelines of Brix et al.[126]

In addition to the routine pathology review, a step section analysis of the kidneys from the perinatal and 2-year rat study was performed. Paraffin-embedded kidneys from male and female rats were sectioned at 1 mm intervals to obtain three to four additional sections per kidney to allow for observation of additional renal tumors. The evaluation of these additional kidney slides was conducted by the QA pathologist. A PWG, separate from the PWG for the perinatal and 2-year rat study, evaluated representative slides from the kidney step section review. Final diagnosis of the kidney step section review represents a consensus between the QA pathologist, the DTT pathologist, and the kidney step PWG.

Table 1. Experimental Design and Materials and Methods in the Perinatal and Two-year Gavage Studies of Black Cohosh Root Extract

Rats	Mice
Study Laboratory	
Battelle (Columbus, OH)	Same as rats
Strain and Species	
Sprague Dawley (Hsd:Sprague Dawley® SD®)	B6C3F1/N
Animal Source	
Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN)	Taconic Biosciences, Inc. (Germantown, NY)
Time Held Before Studies	
F_0 females: 4 days	11 days
Average Age When Studies Began	
F_0 females: 12–15 weeks	5–6 weeks
Date of First Dose	
F_0 females: May 18, 2012	April 9, 2012
F_1 females: July 2, 2012	
F_1 males: July 3, 2012 (not dosed postweaning, perinatal exposure only)	
Duration of Dose	
F_0 females: GD 6 through LD 28	Interim evaluations: 3 and 12 months
F_1: Perinatal only (males) or perinatal plus 2 years (females)	2-year study: 2 years
Date of Last Dose	
F_0 females: July 2, 2012	3-month interim evaluation: July 8, 2012
F_1: June 30, 2014 (females)	12-month interim evaluation: April 7, 2013
	2-year study: April 10, 2014
Necropsy Dates	
F_1: June 30–July 3, 2014	3-month interim evaluation: July 9, 2012
	12-month interim evaluation: April 8, 2013
	2-year study: April 7–11, 2014
Average Age at Necropsy	
F_1: 2 years	3-month interim evaluation: 3 months
	12-month interim evaluation: 12 months
	2-year study: 2 years
Size of Study Groups	
F_0 females: 38	3- and 12-month interim evaluations: 10 females each
F_1 (2-year study): 50/sex	2-year study: 50 females

Rats	Mice
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights	Same as rats
Animals per Cage	
F_0 females: 1 (with litter)	Cyclicity males: Up to 2
F_1: Up to 2 (males) or up to 5 (females)	Females: Up to 4
Method of Animal Identification	
F_0 females: Cage card and tail marking with permanent pen	Cage card and tail tattoo
F_1 (pups): Limb tattoo	
F_1 (2-year study): Cage card and tail tattoo	
Diet	
Irradiated NIH-07 wafer feed (perinatal phase) or irradiated NTP-2000 wafer feed (2-year study) (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed twice weekly	Irradiated NTP-2000 wafer feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed once weekly
Water	
Tap water (Columbus, OH municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as rats
Cages	
Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly through PND 4, then twice weekly, rotated every 2 weeks	Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (males) or twice weekly (females), rotated every 2 weeks
Bedding	
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed with cage changes	Same as rats
Rack Filters	
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks	Same as rats
Racks	
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks	Same as rats
Animal Room Environment	
Temperature: 71°F–74°F Relative humidity: 23%–78% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 69°F–80°F Relative humidity: 27%–69% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Doses	
F_0 and F_1 females: 0, 75, 250, and 750 mg/kg/day in 0.5% aqueous methylcellulose; 5 mL/kg dosing volume	0, 30, 100, 300, and 1,000 mg/kg/day in 0.5% aqueous methylcellulose; 10 mL/kg dosing volume

Rats	Mice
Type and Frequency of Observation	
F_0 females: Observed twice daily. Weighed on GD 5 and then daily throughout the rest of gestation and lactation.	Observed twice daily. Weighed at randomization, day 1, weekly for the first 3 months, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks and at study termination.
F_1 rats: Observed twice daily. Litter data (litter count by sex, litter weights by sex, and litter observations) were recorded on PND 1. Pups were weighed on PNDs 4, 7, 12, 15, 18, 21, 24, and 28, weekly for the next 3 months, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks, beginning on day 29, and at study termination.	
Method of Euthanasia	
Carbon dioxide	Same as rats
Necropsy	
Necropsies were performed on all F_1 rats.	Necropsies were performed on all female mice in the 2-year study. Spleen weights were collected at the 3-month and 12-month interim evaluations.
Histopathology	
Complete histopathology was performed on all F_1 rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (olfactory bulbs, fronto-parietal cortex including basal ganglia, mid-parietal cortex and thalamus, mid-brain with substantia nigra and red nucleus, posterior colliculi, mid-cerebellum including cranial nerve VIII, and posterior medulla), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage, and articular surface]), Harderian glands, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver (two sections including left lateral lobe and median lobe) lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland with adjacent (inguinal) skin, nasal cavity and nasal turbinates, ovaries, pancreas, parathyroid glands, pituitary gland, preputial glands, prostate gland, salivary glands, seminal vesicles, spleen, stomach (forestomach and glandular), testes with epididymides, thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina with cervix. If neurological signs were present, the spinal cord and nerves (sciatic, tibial, and trigeminal with ganglion) were examined microscopically. If neuromuscular signs were present, the thigh muscle was examined microscopically.	3- and 12-month interim evaluations: Histopathology was performed on the spleen and left femur (including bone marrow) only. 2-year study: Complete histopathology was performed on all female mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (olfactory bulbs, fronto-parietal cortex including basal ganglia, mid-parietal cortex and thalamus, mid-brain with substantia nigra and red nucleus, posterior colliculi, mid-cerebellum including cranial nerve VIII, and posterior medulla), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage, and articular surface]), gallbladder, Harderian glands, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and median lobe), kidney, liver, lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland with adjacent (inguinal) skin, nasal cavity and nasal turbinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, salivary glands, spleen, stomach (forestomach and glandular), thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina with cervix. If neurological signs were present, the spinal cord and nerves (sciatic and trigeminal with ganglion) were examined

Rats	Mice
	microscopically. If neuromuscular signs were present, the thigh muscle was examined microscopically.
Hematology	
None	Following 3 months and 12 months of dose administration, blood was collected from the retroorbital sinus for hematology and micronuclei determinations.
	Hematology: erythrocyte count, hemoglobin, manual hematocrit, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, leukocyte count and differential, reticulocyte count, platelet count, and qualitative evaluation of morphological features in all cellular components.
Bone Marrow Cell Count and Cytology	
None	Following 3 months and 12 months of dose administration, bone marrow from the right femur was collected for total cell count per femur. Bone marrow from the sternum was collected for cytological evaluation (M:E ratio, morphology).

GD = gestation day; LD = lactation day; PND = postnatal day; M:E = myeloid:erythoid.

Statistical Methods

For all analyses, p values ≤0.05 were considered statistically significant. Statistical significance is one component of the "weight of evidence" approach to evaluate carcinogenicity (described in the Explanation of Levels of Evidence of Carcinogenic Activity section).

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier[127] and is presented graphically. Animals surviving to the end of the observation period are treated as censored observations, as are animals dying from unnatural causes within the observation period. Animals dying from natural causes are included in analyses and are treated as uncensored observations. For the 2-year mouse study, dose-related trends are identified with Tarone's life-table test,[128] and pairwise dose-related effects are assessed using Cox's log-rank method.[129] For the rat perinatal and 2-year study, dose-related trends and pairwise dose-related effects on survival are assessed using a Cox proportional hazards model[129] with a random litter effect. All reported p values for the survival analyses are two-sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented as the numbers of animals bearing such lesions at a specific anatomical site. For calculation of incidence rates, the denominator for most neoplasms and all nonneoplastic lesions is the number of animals for which the site was examined microscopically. When macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, and Zymbal's gland) before microscopic evaluation, however, the denominator

consists of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominator consists of the number of animals on which a necropsy was performed. Additional study data also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal euthanasia.

Analysis of Neoplasm and Nonneoplastic Lesion Incidence

Statistical analyses of neoplasm and nonneoplastic lesion incidence considered two features of the data. Some animals did not survive the entire 2 years of the study, so survival differences between groups had to be considered. In addition, for the rat perinatal and 2-year study, up to two animals per sex were randomly selected from each litter to participate in the study. The statistical analysis of lesion incidence used the Poly-3 test to account for survival differences, with a Rao-Scott adjustment for litter effects, as described below.

The Poly-k test[130-132] was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to account for survival differences. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is 1 if the animal had a lesion at that site or if it survived until terminal euthanasia; if the animal died before terminal euthanasia and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only on the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time.[130] Unless otherwise specified, a value of k = 3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier[130] after an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control Fischer 344 rats and B6C3F1 mice.[133] Bailer and Portier[130] showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams.[134] Poly-3 tests used the continuity correction described by Nam.[135]

Littermates tend to be more like each other than like fetuses/pups in other litters. Failure to account for correlation within litters leads to underestimates of variance in statistical tests, resulting in higher probabilities of Type I errors ("false positives"). Because up to two pups/sex/litter were present in the rat perinatal and 2-year study, the Poly-3 test was modified to accommodate litter effects using the Rao-Scott approach.[136] The Rao-Scott approach accounts for litter effects by estimating the ratio of the variance in the presence of litter effects to the variance in the absence of litter effects. This ratio is then used to adjust the sample size downward to yield the estimated variance in the presence of litter effects. The Rao-Scott approach was implemented in the Poly-3 test as recommended by Fung et al.[137] formula $\overline{T}_{RS2}$.

Tests of significance included pairwise comparisons of each dosed group with control groups and a test for an overall dose-related trend. Continuity-corrected Rao-Scott-adjusted Poly-3 tests were used in the analysis of rat lesion incidence and reported p values are one sided. The significance of a lower incidence or negative trend in lesions is approximated as 1−p with the letter N added (e.g., p = 0.99 is presented as p = 0.01N). For the mouse study in which neoplasms and nonneoplastic lesions were observed without litter structure, Poly-3 tests that included the continuity correction, but without adjustment for potential litter effects, were used for trend and pairwise comparisons to the control group.

To evaluate incidence rates by litter in the rat perinatal and 2-year study, the proportions of litters affected by each lesion type were tested among groups. Cochran-Armitage trend tests and Fisher's exact test[138] were used to test for trends and pairwise differences from the control group, respectively.

Analysis of Continuous Variables

Before statistical analysis, outliers identified using the Dixon and Massey test[139] for small samples ($n < 20$) and Tukey's outer fences method[140] for large samples ($n \geq 20$) were examined by DTT personnel, and biologically implausible values (likely due to experimental error) were eliminated from the analysis. Organ and body weight measurements, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[141] and Williams.[142; 143] Litter sizes, pup survival, gestation lengths, hematology endpoints, and proportions of male pups per litter for all studies were analyzed using the nonparametric multiple comparison methods of Shirley[144] [as modified by Williams[145]] and Dunn,[146] given that these endpoints typically have skewed distributions. For all quantitative endpoints unaffected by litter structure, the Jonckheere test[147] was used to assess the significance of the dose-related trends and to determine at the 0.01 level of significance, whether a trend-sensitive test (the Williams or Shirley test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (the Dunnett or Dunn test).

Postweaning body weights were measured on two pups/sex/litter in the rat perinatal and 2-year study; more than two pups/sex/litter were possible in preweaning body weight measurements. The analyses of pup body weights and pup body weights adjusted for litter size (described below) took litter effects into account using a mixed model with litter as a random effect. To adjust for multiple comparisons, a Dunnett-Hsu adjustment was used.[148] Dam body weights during gestation and lactation were analyzed with the parametric multiple comparison procedures of Dunnett[141] or Williams,[142; 143] depending on whether the Jonckheere test indicated the use of a trend-sensitive test. P values for these analyses are two-sided.

Analysis of Gestational and Fertility Indices

Cochran-Armitage trend tests were used to test the significance of trends in gestational and fertility indices across dose groups in the rat perinatal and 2-year study. Fisher's exact test was used to conduct pairwise comparisons of each dosed group with the control group. P values for these analyses are two-sided.

Body Weight Adjustments

Preweaning pup body weights in the rat perinatal and 2-year study were adjusted for live litter size as follows. A linear model was fit to body weights as a function of dose and litter size. The estimated coefficient of litter size was then used to adjust each pup body weight according to the difference between its litter size and the mean litter size. Prestandardization PND 4 body weights were adjusted for PND 1 litter size, and body weights measured between PND 4 poststandardization and PND 28 were adjusted for PND 4 poststandardization litter size. After adjustment, body weights were analyzed with a linear mixed model with a random litter effect.

Historical Control Data

The concurrent control group is the most valid comparison to the dosed groups and is the only control group analyzed statistically in NTP bioassays. Historical control data are often helpful in interpreting potential dose-related effects, however, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical control data must be generally similar. Significant factors affecting the background incidence of neoplasms at a variety of sites are diet, sex, strain/stock, and route of exposure. The NTP historical control database contains all 2-year studies for each species, sex, and strain/stock with histopathology findings in control animals completed within the most recent 5-year period,[149-151] including the concurrent control for comparison across multiple technical reports. In general, the historical control data for a given study includes studies using the same route of administration, and the overall incidence of neoplasms in controls for all routes of administration is included for comparison, including the current study. In the historical control data set, only one comparator for same mode of administration (*trans*-resveratrol) was available for methylcellulose gavage in rats.

Quality Assurance Methods

The 2-year studies were conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice Regulations.[152] In addition, the 2-year study reports were audited retrospectively by an independent QA contractor against study records submitted to the NTP Archives. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by DTT staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

Genetic Toxicology

The genetic toxicity of BCE was assessed by testing whether the chemical induces mutations in various strains of *Salmonella typhimurium* and *Escherichia coli* or increases the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocol for these studies and the results are given in Appendix D.

The genetic toxicity studies have evolved from an earlier effort to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the relationship between the molecular structure of the chemical and its observed effects in short-term in vitro and in vivo genetic

toxicity tests (structure-activity relationships). The short-term tests were developed originally to clarify proposed mechanisms of chemical-induced DNA damage, given the relationship between electrophilicity and mutagenicity,[153] and the somatic mutation theory of cancer.[154; 155] Not all cancers, however, arise through genotoxic mechanisms.

Bacterial Mutagenicity

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites.[156] A positive response in the *Salmonella* test was shown to be the most predictive in vitro indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens).[157; 158] Additionally, no battery of tests that included the *Salmonella* test improved predictivity over the *Salmonella* test alone. Other tests, however, can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

Peripheral Blood Micronucleus Test

Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division.[159; 160] Acute in vivo bone marrow chromosome aberration and micronucleus tests appear to be less predictive of carcinogenicity than the *Salmonella* test.[161; 162] However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a weak response in one sex only or negative results in both sexes in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies.[163] Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, determination of in vivo genetic effects is important to overall understanding of risks associated with exposure to a particular chemical.

Results

Data Availability

All study data were evaluated. Data relevant for evaluating toxicological findings are presented here. All study data are available in the National Toxicology Program (NTP) Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-TR-603.[164]

Rats

Two-year Study (Perinatal Phase)

No changes in dam survival, pregnancy status, number of dams littered, or gestation length were related to administration of black cohosh root extract (BCE) (Table 2).

Table 2. Summary of the Disposition of F_0 Female Rats during Perinatal Exposure in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
Reproductive Performance				
Time-mated Females (GD 6)	38	38	38	38
Females Pregnant (%)[a]	33 (86.8)	32 (84.2)	34 (89.5)	36 (94.7)
Females Not Pregnant (%)	5 (13.2)	6 (15.8)	4 (10.5)	2 (5.3)
Dams Not Delivering with Evidence of Pregnancy (%)	2 (6.1)	0 (0)	2 (5.9)	1 (2.8)
Dams with Litters on LD 0 (%)[a]	31 (93.9)	32 (100.0)	32 (94.1)	35 (97.2)
Gestation Length (Days)[b,c]	22.3 ± 0.1 (31)	22.2 ± 0.1 (32)	22.2 ± 0.1 (32)	22.3 ± 0.1 (35)
Litters Poststandardization (PND 4)[d]	30	30	30	30
Weaned Males/Females	121/116	122/117	117/115	122/112

GD = gestation day; LD = lactation day; PND = postnatal day.
[a]Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests. No statistically significant findings were noted at $p \leq 0.05$.
[b]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests. No statistically significant findings were noted at $p \leq 0.05$.
[c]Gestation length calculated for sperm-positive females that delivered a litter. Data are presented as mean ± standard error (number of dams).
[d]Standardization to eight pups/litter (four pups/sex).

Mean body weights of dams administered BCE were lower than that of vehicle control animals throughout gestation and lactation; changes in body weight were within 10% of vehicle control animals (Table 3). Beginning around gestation day (GD) 9, mean body weights of animals administered 250 or 750 mg BCE/kg body weight/day (mg/kg/day) were significantly decreased (up to 8% less in the 750 mg/kg/day group) compared to that of vehicle control animals. There was a negative trend in mean body weights with increasing dose at most time points during gestation and lactation. During lactation, the differences were consistently significant in the 750 mg/kg/day group from lactation day (LD) 1 through LD 25 and in the 250 mg/kg/day group from LD 1 through LD 14 (Table 3). Dam body weight changes were significantly decreased in all dosed groups compared to the vehicle control group for gestation interval GD 6–21 but tended to be higher than that in the vehicle control group for lactation interval LD 1–28.

Table 3. Summary of Mean Body Weights and Body Weight Gains of F_0 Female Rats during Gestation and Lactation in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

Parameter[a,b]	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
Gestation Body Weight				
Gestation Day				
6	234.5 ± 1.5 (33)	237.5 ± 2.2 (32)	233.4 ± 2.4 (34)	234.8 ± 2.3 (36)
9	253.9 ± 1.4** (33)	248.8 ± 3.0 (32)	244.1 ± 2.0** (34)	241.2 ± 2.2** (36)
12	269.0 ± 1.4** (33)	264.1 ± 2.0 (32)	260.7 ± 2.1** (34)	257.0 ± 2.3** (36)
15	286.7 ± 1.9** (33)	281.0 ± 2.7 (32)	276.4 ± 2.4** (34)	271.6 ± 2.2** (36)
18	331.0 ± 2.8** (33)	325.6 ± 2.9 (32)	319.0 ± 3.7** (34)	309.0 ± 2.6** (36)
21	378.0 ± 4.2** (33)	365.9 ± 4.8 (32)	363.1 ± 5.6* (34)	347.4 ± 3.4** (36)
Gestation Weight Change				
Gestation Day Interval				
6–9	19.3 ± 1.2** (33)	11.2 ± 1.1** (32)	10.7 ± 1.5** (34)	6.4 ± 1.1** (36)
9–12	15.2 ± 1.1 (33)	15.3 ± 1.5 (32)	16.6 ± 1.0 (34)	15.8 ± 1.5 (36)
12–15	17.7 ± 1.2* (33)	16.9 ± 1.0 (32)	15.7 ± 1.0 (34)	14.6 ± 1.5 (36)
15–18	44.3 ± 1.6** (33)	44.6 ± 1.3 (32)	42.6 ± 1.7 (34)	37.4 ± 1.2** (36)
18–21	46.9 ± 1.7** (33)	40.3 ± 3.1 (32)	44.0 ± 2.2 (34)	38.4 ± 1.9** (36)
6–21	143.4 ± 4.1** (33)	128.4 ± 4.5* (32)	129.7 ± 5.1* (34)	112.6 ± 3.2** (36)
Lactation Body Weight				
Lactation Day				
1	279.6 ± 2.5** (31)	274.0 ± 2.3 (32)	271.8 ± 2.6* (32)	266.9 ± 2.4** (35)
4	293.4 ± 2.1** (31)	285.5 ± 2.6* (32)	285.2 ± 2.7* (32)	277.5 ± 2.2** (35)
7[c]	301.9 ± 2.5** (30)	293.4 ± 2.3* (30)	291.9 ± 3.1** (30)	285.8 ± 2.1** (30)
14	321.9 ± 2.5** (30)	312.7 ± 2.7* (30)	313.1 ± 2.9* (29)[c]	308.6 ± 2.6** (30)
21	305.8 ± 2.8** (30)	299.6 ± 3.0 (30)	299.7 ± 2.5 (29)	288.4 ± 2.7** (30)
25	295.1 ± 2.0 (30)	289.8 ± 2.8 (30)	292.5 ± 2.6 (29)	285.2 ± 3.2* (30)
28	289.5 ± 2.5 (30)	284.3 ± 3.2 (30)	286.1 ± 2.8 (29)	285.5 ± 2.2 (29)[c]
Lactation Weight Change				
Lactation Day Interval				
1–4	13.7 ± 1.9 (31)	11.5 ± 1.3 (32)	13.3 ± 1.7 (32)	10.7 ± 1.4 (35)
4–7[c]	8.0 ± 1.9 (30)	6.2 ± 1.6 (30)	7.2 ± 2.8 (30)	9.6 ± 1.3 (30)
7–10	8.7 ± 1.9 (30)	10.6 ± 1.7 (30)	8.9 ± 1.8 (29)[c]	12.7 ± 1.2 (30)
10–14	11.3 ± 1.8 (30)	8.6 ± 2.3 (30)	11.5 ± 1.1 (29)	10.1 ± 1.7 (30)
14–18	−9.7 ± 1.7 (30)	−6.7 ± 1.8 (30)	−4.6 ± 1.2 (29)	−6.1 ± 1.7 (30)
18–21	−6.3 ± 2.0** (30)	−6.3 ± 1.9 (30)	−8.8 ± 2.2 (29)	−14.1 ± 1.8** (30)
21–25	−10.7 ± 2.2** (30)	−9.9 ± 1.3 (30)	−7.2 ± 1.8 (29)	−3.3 ± 3.5* (30)
25–28	−5.5 ± 1.4 (30)	−5.4 ± 1.3 (30)	−6.4 ± 0.9 (29)	0.4 ± 2.8* (29)[c]
1–28	9.9 ± 2.5** (30)	8.9 ± 2.9 (30)	14.9 ± 2.1 (29)	20.9 ± 2.2** (29)

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard error (number of dams). Body weight data are presented in grams.
[b]Each dosed group was compared to the vehicle control group with the Williams test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunnett test when no trend was present.
[c]Changes in n are the result of standardization on lactation day (LD) 4 and removing litters with no surviving pups by LD 8 (one litter in the 250 mg/kg/day group) and LD 28 (one litter in the 750 mg/kg/day group).

Total and live litter sizes were significantly decreased in the 750 mg/kg/day group relative to the vehicle control group on postnatal day (PND) 1 and PND 4 (combined male and female prestandardization) only (Table 4).

Table 4. Summary of Mean Litter Size and Survival Ratio of F_1 Male and Female Rats during Lactation in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

Parameter	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
PND 1				
Total[a,b]	13.10 ± 0.45* (31)	12.52 ± 0.61 (31)	13.09 ± 0.41 (32)	11.69 ± 0.47* (35)
Live[b]	13.23 ± 0.40** (30)	12.45 ± 0.60 (31)	12.97 ± 0.42 (32)	11.51 ± 0.46** (35)
% Male/Litter[a,b,c]	50.5 ± 2.1 (28)	49.4 ± 3.5 (23)	50.0 ± 2.8 (24)	52.8 ± 3.0 (34)
% Male[c,d,e]	50.1 (369)	51.8 (280)	50.5 (307)	52.4 (389)
Male[a,b]				
PND 1[c]	6.61 ± 0.30 (28)	6.30 ± 0.54 (23)	6.46 ± 0.47 (24)	6.00 ± 0.40 (34)
PND 4 Prestandardization[c]	6.61 ± 0.30 (28)	6.26 ± 0.53 (23)	6.38 ± 0.47 (24)	6.09 ± 0.40 (33)
PND 4 Poststandardization	4.07 ± 0.07 (30)	4.10 ± 0.10 (30)	4.03 ± 0.06 (30)	4.17 ± 0.14 (30)
Female[a,b]				
PND 1[c]	6.57 ± 0.38 (28)	5.87 ± 0.49 (23)	6.33 ± 0.42 (24)	5.44 ± 0.37 (34)
PND 4 Prestandardization[c]	6.57 ± 0.38 (28)	5.83 ± 0.51 (23)	6.29 ± 0.42 (24)	5.52 ± 0.35 (33)
PND 4 Poststandardization	3.93 ± 0.07 (30)	3.90 ± 0.10 (30)	3.97 ± 0.06 (30)	3.83 ± 0.14 (30)
Male and Female[a,b]				
PND 4 Prestandardization	13.23 ± 0.40** (30)	12.39 ± 0.60 (31)	12.84 ± 0.42 (32)	11.34 ± 0.51** (35)
PND 4 Poststandardization	8.00 ± 0.00 (30)	8.00 ± 0.00 (30)	8.00 ± 0.00 (30)	8.00 ± 0.00 (30)
PND 28	7.90 ± 0.06 (30)	7.93 ± 0.05 (30)	7.73 ± 0.27 (30)	7.53 ± 0.27 (30)
Survival per Litter				
Total Dead: PND 1–4[e,f]	9 (3)	4 (4)	8 (7)	12 (9)
Total Dead: PND 4–28[e,f]	3 (3)	2 (2)	8 (1)	14 (6)
Dead/Litter: PND 1–4[a,b]	0.29 ± 0.18 (31)	0.13 ± 0.06 (31)	0.25 ± 0.09 (32)	0.34 ± 0.12 (35)
Dead/Litter: PND 4–28[a,b]	0.10 ± 0.06 (30)	0.07 ± 0.05 (30)	0.27 ± 0.27 (30)	0.47 ± 0.27 (30)
Survival Ratio: PND 1–4[a,b,g]	1.000 ± 0.000 (30)	0.995 ± 0.004 (31)	0.991 ± 0.006 (32)	0.965 ± 0.029 (35)
Survival Ratio: PND 4–28[a,b,h]	0.988 ± 0.007 (30)	0.992 ± 0.006 (30)	0.967 ± 0.033 (30)	0.942 ± 0.034 (30)

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
PND = postnatal day.
[a]Each dosed group was compared to the vehicle control group with the Shirley test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunn test when no trend was present.
[b]Data are presented as mean ± standard error (number of litters).
[c]Litters in which the male/female pup counts were inconsistent between PND 1 and PND 4 were excluded from the male/female-specific endpoints.
[d][100 × (number of live males in dosed group)/(number of live males and females in dosed group)](number of pups).
[e]No statistical analysis performed on this endpoint.
[f]Total number of dead pups in dosed group (number of litters contributing dead pups).
[g]Survival/litter: Number of pups prestandardization on PND 4/number of live pups on PND 1.
[h]Survival/litter: Number of live pups on PND 28/number of live pups poststandardization on PND 4.

There was a marginal decrease in pup survival ratio from PND 1 to PND 4, but no significant differences in pup survival were observed during lactation (Table 4). Mean body weights of BCE-exposed pups were within 12% of the vehicle control groups (Table 5). Pups tended to gain less weight with increasing exposure, resulting in significantly different mean body weights relative to the vehicle control pups at all time points from PND 1 until weaning on PND 28. Male and female pup mean body weights of the 750 mg/kg/day groups were significantly different from those of the vehicle control groups throughout the lactation period; the difference ranged from 8% to 11% (male pups) or from 10% to 12% (female pups). In the 250 mg/kg/day group, female pups had significantly different mean body weights (5%–8% less than that of the vehicle control group) starting from PND 12 until weaning; this was not observed in male pups.

Table 5. Summary of Preweaning F_1 Male and Female Rat Pup Mean Body Weights Following Perinatal Exposure to Black Cohosh Root Extract

Parameter	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
Male (g)				
PND 1[a,b,c,d]	7.08 ± 0.10** (28)	6.85 ± 0.11 (22)	6.94 ± 0.17 (24)	6.64 ± 0.12* (34)
PND 4[e,f,g,h]	10.24 ± 0.17** (197/30)	9.88 ± 0.18 (206/30)	9.96 ± 0.17 (210/32)	9.17 ± 0.17** (208/34)
PND 7[e,f,i]	15.36 ± 0.35* (122/30)	14.90 ± 0.38 (123/30)	14.84 ± 0.44 (121/30)	14.03 ± 0.34* (124/30)
PND 12[e,f,i]	26.80 ± 0.45** (121/30)	26.14 ± 0.50 (123/30)	25.63 ± 0.58 (117/29)	24.14 ± 0.54** (122/30)
PND 15[e,f,i]	34.20 ± 0.50** (121/30)	33.47 ± 0.58 (122/30)	32.64 ± 0.60 (117/29)	31.44 ± 0.65** (122/30)
PND 18[e,f,i]	41.51 ± 0.70** (121/30)	40.71 ± 0.69 (122/30)	40.01 ± 0.74 (117/29)	38.00 ± 0.78** (122/30)
PND 21[e,f,i]	53.66 ± 0.81** (121/30)	52.41 ± 0.89 (122/30)	50.67 ± 1.06 (117/29)	47.96 ± 1.00** (122/30)
PND 24[e,f,i]	67.94 ± 1.09** (121/30)	66.24 ± 1.10 (122/30)	63.88 ± 1.31* (117/29)	60.30 ± 1.24** (122/30)
PND 28[e,f,i]	88.35 ± 1.31** (121/30)	86.75 ± 1.24 (122/30)	83.85 ± 1.60 (117/29)	79.26 ± 1.56** (122/30)
Female (g)				
PND 1[a,b,c,d]	6.79 ± 0.11* (28)	6.51 ± 0.08 (23)	6.59 ± 0.16 (24)	6.38 ± 0.12* (33)
PND 4[e,f,g,h]	9.75 ± 0.18** (200/30)	9.28 ± 0.18 (177/31)	9.29 ± 0.16 (201/32)	8.70 ± 0.17** (189/34)
PND 7[e,f,i]	14.75 ± 0.38** (118/30)	13.91 ± 0.35 (117/30)	13.73 ± 0.41 (119/30)	13.18 ± 0.34** (115/30)
PND 12[e,f,i]	26.00 ± 0.53** (117/30)	24.52 ± 0.52 (117/30)	24.14 ± 0.55* (115/29)	22.89 ± 0.53** (112/30)
PND 15[e,f,i]	33.24 ± 0.57** (117/30)	31.31 ± 0.57 (117/30)	30.91 ± 0.58* (115/29)	29.96 ± 0.65** (112/30)
PND 18[e,f,i]	40.39 ± 0.76** (117/30)	38.11 ± 0.68 (117/30)	37.83 ± 0.73* (115/29)	36.45 ± 0.76** (112/30)
PND 21[e,f,i]	51.29 ± 0.85** (117/30)	48.43 ± 0.85 (117/30)	47.29 ± 0.92** (115/29)	45.40 ± 0.99** (112/30)
PND 24[e,f,i]	63.70 ± 1.13** (117/30)	60.34 ± 1.01 (117/30)	59.03 ± 1.14* (115/29)	56.64 ± 1.18** (112/30)
PND 28[e,f,i]	80.26 ± 1.17** (116/30)	77.06 ± 1.09 (117/30)	75.21 ± 1.34* (115/29)	72.22 ± 1.39** (112/30)
Male and Female (g)				
PND 1[a,b,c]	6.95 ± 0.10** (30)	6.65 ± 0.07 (31)	6.68 ± 0.12 (32)	6.48 ± 0.11** (35)
PND 4[e,f,g,h]	10.00 ± 0.17** (397/30)	9.57 ± 0.18 (383/31)	9.64 ± 0.16 (411/32)	8.94 ± 0.16** (397/34)
PND 7[e,f,i]	15.05 ± 0.35** (240/30)	14.42 ± 0.36 (240/30)	14.29 ± 0.41 (240/30)	13.64 ± 0.31* (239/30)
PND 12[e,f,i]	26.41 ± 0.47** (238/30)	25.37 ± 0.50 (240/30)	24.90 ± 0.55 (232/29)	23.57 ± 0.50** (234/30)
PND 15[e,f,i]	33.73 ± 0.50** (238/30)	32.42 ± 0.56 (239/30)	31.79 ± 0.57* (232/29)	30.76 ± 0.61** (234/30)
PND 18[e,f,i]	40.96 ± 0.70** (238/30)	39.45 ± 0.67 (239/30)	38.93 ± 0.71 (232/29)	37.25 ± 0.74** (234/30)
PND 21[e,f,i]	52.49 ± 0.79** (238/30)	50.46 ± 0.86 (239/30)	49.01 ± 0.97* (232/29)	46.79 ± 0.94** (234/30)
PND 24[e,f,i]	65.86 ± 1.07** (238/30)	63.34 ± 1.03 (239/30)	61.49 ± 1.19* (232/29)	58.58 ± 1.14** (234/30)
PND 28[e,f,i]	84.41 ± 1.19** (237/30)	82.00 ± 1.13 (239/30)	79.58 ± 1.43* (232/29)	75.94 ± 1.38** (234/30)

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
PND = postnatal day.
[a]Data are presented as mean ± standard error (number of litters).
[b]Each dosed group was compared to the vehicle control group with the Williams test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunnett test when no trend was present.
[c]Total litter weight at PND 1 divided by number of live pups in litter at PND 1.
[d]Litters in which the male/female pup counts were inconsistent between PND 1 and PND 4 were excluded from the male/female-specific endpoints.
[e]Statistical analysis performed using linear mixed models with random litter effect for both trend and pairwise tests, using the Dunnett-Hsu adjustment for multiple comparisons.
[f]Data are presented as the mean of litter means ± standard error (number of pups/number of litters).
[g]PND 4 prestandardization.
[h]Individual pup weights first adjusted for live litter size on PND 1. Litters with differing pup counts between PND 1 and PND 4 were excluded.
[i]Individual pup weights first adjusted for live litter size on PND 4 poststandardization.

Two-year Study (Postweaning Phase)

After weaning, female rats were administered BCE via daily gavage at the same dose as their respective dams. Male rats were not administered BCE or the vehicle during the postweaning phase. Consequently, "exposed male rats" hereafter refers to animals that experienced perinatal BCE exposure only; "exposure concentration of male rats" refers to the dose their respective dams received.

Estimates of 2-year survival probabilities for male and female rats are shown in Table 6 and in the Kaplan-Meier survival curves (Figure 2). Survival of all groups of exposed male rats was not significantly different from the vehicle control group. There was a slightly lower survival rate ($p = 0.055$) of female rats in the 750 mg/kg/day group.

Table 6. Summary of Survival of Male and Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
Male				
Animals Initially in Study	50	50	50	50
Moribund	16	14	11	8
Natural Deaths	19	21	18	26
Animals Surviving to Study Termination	15	15	21	16
Percent Probability of Survival at Study Termination[a]	30.0	30.0	42.0	32.0
Mean Survival (Days)[b]	655.8 ± 15.3	647.3 ± 18.0	658.5 ± 15.4	650.7 ± 13.7
Survival Analysis[c]	p = 0.903	p = 0.541	p = 0.596N	p = 0.747
Female				
Animals Initially in Study	50	50	50	50
Moribund	13	14	15	14
Natural Deaths	9	12	6	16
Animals Surviving to Study Termination	28	24	29	20
Percent Probability of Survival at Study Termination	56.0	48.0	58.0	40.0
Mean Survival (Days)	679.2 ± 9.3	655.6 ± 15.4	684.7 ± 12.5	632.3 ± 15.9
Survival Analysis	p = 0.056	p = 0.398	p = 0.784N	p = 0.055

[a]Kaplan-Meier determinations.
[b]Mean of litter means of all deaths (uncensored, censored, and study termination) ± standard error.
[c]The result of the Cox proportional hazards trend test is in the vehicle control group column, and the results of the Cox proportional hazards pairwise comparisons to the vehicle control group are in the dosed group columns. A negative trend or lower mortality in a dosed group is indicated by N.

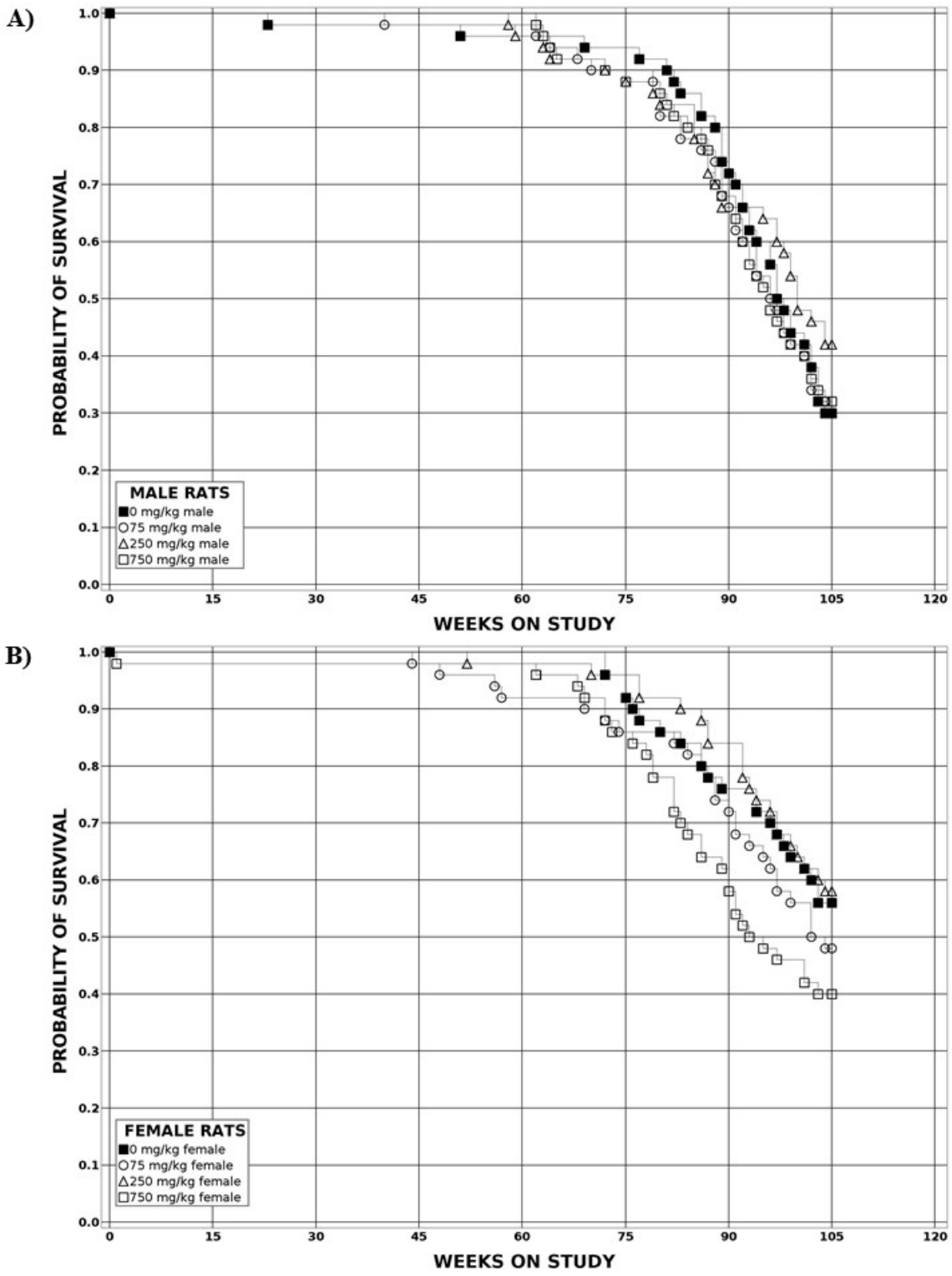

Figure 2. Kaplan-Meier Survival Curves for Male Rats Exposed Perinatally and Female Rats Exposed Perinatally and Administered Black Cohosh Root Extract by Gavage for Two Years

Survival curves are shown for (A) males and (B) females.

Throughout the study, groups of BCE-exposed male rats had mean body weights within 12% of that of the vehicle control males (Table 7; Figure 3). Mean body weights of the female 75 and 250 mg/kg/day groups were within 10% of that of the vehicle control group throughout the study (Table 8; Figure 3). The mean body weight of the 750 mg/kg/day female group was generally within 10% of that of the vehicle control group until study day 309 and thereafter ranged from 12% to 18% less than that of the vehicle control group until study termination.

No clinical observations in groups of exposed male or dosed female rats were considered related to BCE (Appendix E).

Table 7. Summary of Survival and Mean Body Weights of Male Rats Exposed Perinatally in the Perinatal and Two-year Study of Black Cohosh Root Extract

Study Day[a]	0 mg/kg/day		75 mg/kg/day			250 mg/kg/day			750 mg/kg/day		
	Av. Wt. (g)[b]	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters
1	97.4	25	97.7	100.3	25	93.8	96.3	25	88.5	90.8	25
8	141.0	25	141.9	100.7	25	137.5	97.5	25	126.1	89.4	25
15	192.4	25	192.3	100.0	25	184.5	95.9	25	178.8	93.0	25
22	243.6	25	243.2	99.8	25	235.2	96.5	25	226.8	93.1	25
29	288.2	25	287.6	99.8	25	277.9	96.4	25	267.5	92.8	25
36	320.7	25	311.9	97.3	25	309.3	96.5	25	292.0	91.0	25
43	347.2	25	345.5	99.5	25	337.1	97.1	25	318.0	91.6	25
50	365.7	25	366.5	100.2	25	357.2	97.7	25	335.5	91.7	25
57	377.7	25	385.4	102.0	25	372.5	98.6	25	353.1	93.5	25
64	394.9	25	397.6	100.7	25	387.5	98.1	25	367.1	93.0	25
71	406.6	25	409.8	100.8	25	397.7	97.8	25	376.5	92.6	25
78	417.8	25	418.2	100.1	25	404.0	96.7	25	385.9	92.4	25
85	428.8	25	426.8	99.5	25	413.3	96.4	25	394.3	92.0	25
113	460.7	25	459.1	99.6	25	446.4	96.9	25	423.3	91.9	25
141	478.8	25	483.4	101.0	25	471.2	98.4	25	445.0	92.9	25
169	498.0	25	505.1	101.4	25	486.4	97.7	25	462.4	92.8	25
197	511.2	25	517.5	101.2	25	501.8	98.1	25	483.9	94.7	25
225	535.2	25	534.0	99.8	25	519.1	97.0	25	499.1	93.3	25
253	551.6	25	558.1	101.2	25	541.5	98.2	25	513.1	93.0	25
281	560.6	25	573.9	102.4	25	552.4	98.5	25	529.7	94.5	25
309	580.2	25	586.9	101.2	25	564.4	97.3	25	539.5	93.0	25
337	592.5	25	597.5	100.8	25	579.6	97.8	25	549.4	92.7	25
365	603.1	25	611.1	101.3	25	590.2	97.9	25	563.7	93.5	25
393	612.3	25	618.9	101.1	25	598.2	97.7	25	565.4	92.3	25
421	624.6	25	622.9	99.7	25	604.6	96.8	25	572.6	91.7	25
449	630.7	25	624.8	99.1	24	621.5	98.5	24	585.1	92.8	25
477	628.2	25	624.1	99.4	24	621.0	98.9	24	589.1	93.8	25
505	621.1	25	613.3	98.7	24	613.3	98.7	24	582.5	93.8	25
533	622.3	25	614.5	98.7	24	619.7	99.6	24	581.4	93.4	24
561	630.8	25	626.9	99.4	22	622.2	98.6	24	578.6	91.7	24
589	627.2	24	613.7	97.8	22	620.8	99.0	24	589.8	94.0	23
617	621.8	24	604.2	97.2	21	618.6	99.5	22	586.4	94.3	23
645	622.4	23	610.1	98.0	18	617.4	99.2	21	570.5	91.7	21
673	594.2	20	595.2	100.2	16	603.7	101.6	21	561.0	94.4	17
701	607.9	18	600.4	98.8	15	613.2	100.9	17	563.3	92.7	15
EOS	**628.4**	**13**	**566.7**	**90.2**	**11**	**606.8**	**96.6**	**16**	**557.3**	**88.7**	**11**

No trend or pairwise statistical tests were performed on these data.
No. of litters = number of litters represented in weight average; EOS = end of study.
[a]Study day 1 is the day animals were placed on study after pups were weaned.
[b]Average weights shown are means of litter means.

Table 8. Summary of Survival and Mean Body Weights of Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

Study Day[a]	0 mg/kg/day		75 mg/kg/day			250 mg/kg/day			750 mg/kg/day		
	Av. Wt. (g)[b]	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters
1	85.1	25	80.8	95.0	25	78.6	92.4	25	74.7	87.9	25
8	115.6	25	109.6	94.8	25	106.8	92.4	25	99.9	86.4	25
15	146.4	25	142.3	97.2	25	140.1	95.7	25	134.8	92.1	25
22	171.8	25	169.9	98.9	25	169.1	98.4	25	163.2	95.0	25
29	193.3	25	190.6	98.6	25	189.8	98.2	25	184.2	95.3	25
36	210.0	25	205.1	97.7	25	204.6	97.4	25	196.5	93.6	25
43	225.5	25	221.0	98.0	25	219.5	97.3	25	212.5	94.2	25
50	237.6	25	230.7	97.1	25	230.5	97.0	25	222.5	93.7	25
57	241.8	25	238.4	98.6	25	239.9	99.2	25	230.2	95.2	25
64	255.1	25	248.9	97.6	25	249.9	97.9	25	234.5	91.9	25
71	259.5	25	253.7	97.8	25	254.4	98.0	25	238.6	92.0	25
78	264.4	25	257.2	97.3	25	257.5	97.4	25	249.8	94.5	25
85	266.6	25	262.1	98.3	25	262.5	98.5	25	255.2	95.7	25
113	279.8	25	275.7	98.6	25	277.2	99.1	25	265.3	94.8	25
141	292.8	25	288.5	98.5	25	286.3	97.8	25	275.7	94.2	25
169	298.9	25	293.0	98.0	25	289.9	97.0	25	276.1	92.4	25
197	304.9	25	302.8	99.3	25	302.9	99.4	25	288.1	94.5	25
225	315.1	25	311.5	98.9	25	309.6	98.3	25	292.3	92.8	25
253	324.7	25	319.1	98.3	25	317.0	97.6	25	297.0	91.4	25
281	331.4	25	331.1	99.9	25	322.9	97.4	25	298.3	90.0	25
309	339.1	25	332.1	97.9	25	331.7	97.8	25	301.0	88.8	25
337	346.8	25	336.7	97.1	25	327.3	94.4	25	305.0	88.0	25
365	354.4	25	345.9	97.6	25	336.1	94.8	25	307.4	86.7	25
393	362.7	25	353.6	97.5	25	349.3	96.3	25	314.9	86.8	25
421	369.9	25	361.2	97.6	25	359.6	97.2	25	319.9	86.5	25
449	383.8	25	372.3	97.0	25	370.3	96.5	25	322.8	84.1	25
477	389.8	25	379.4	97.3	25	376.4	96.6	25	323.7	83.0	25
505	391.2	25	377.0	96.4	25	365.5	93.4	24	327.4	83.7	25
533	388.6	25	388.8	100.0	25	377.4	97.1	24	333.0	85.7	25
561	398.7	25	398.1	99.8	25	386.4	96.9	24	344.0	86.3	25
589	417.1	25	409.3	98.1	25	394.9	94.7	24	344.5	82.6	23
617	419.5	25	414.4	98.8	22	405.4	96.7	24	357.2	85.2	23
645	416.4	25	414.0	99.4	21	405.3	97.3	24	351.0	84.3	21
673	417.7	23	426.8	102.2	20	401.8	96.2	24	357.4	85.6	19
701	426.8	23	420.0	98.4	20	403.5	94.5	24	352.8	82.6	18
EOS	**417.1**	**22**	**418.7**	**100.4**	**19**	**388.8**	**93.2**	**22**	**361.6**	**86.7**	**16**

No trend or pairwise statistical tests were performed on these data.
No. of litters = number of litters represented in weight average; EOS = end of study.
[a]Study day 1 is the day animals were placed on study after pups were weaned.
[b]Average weights shown are means of litter means.

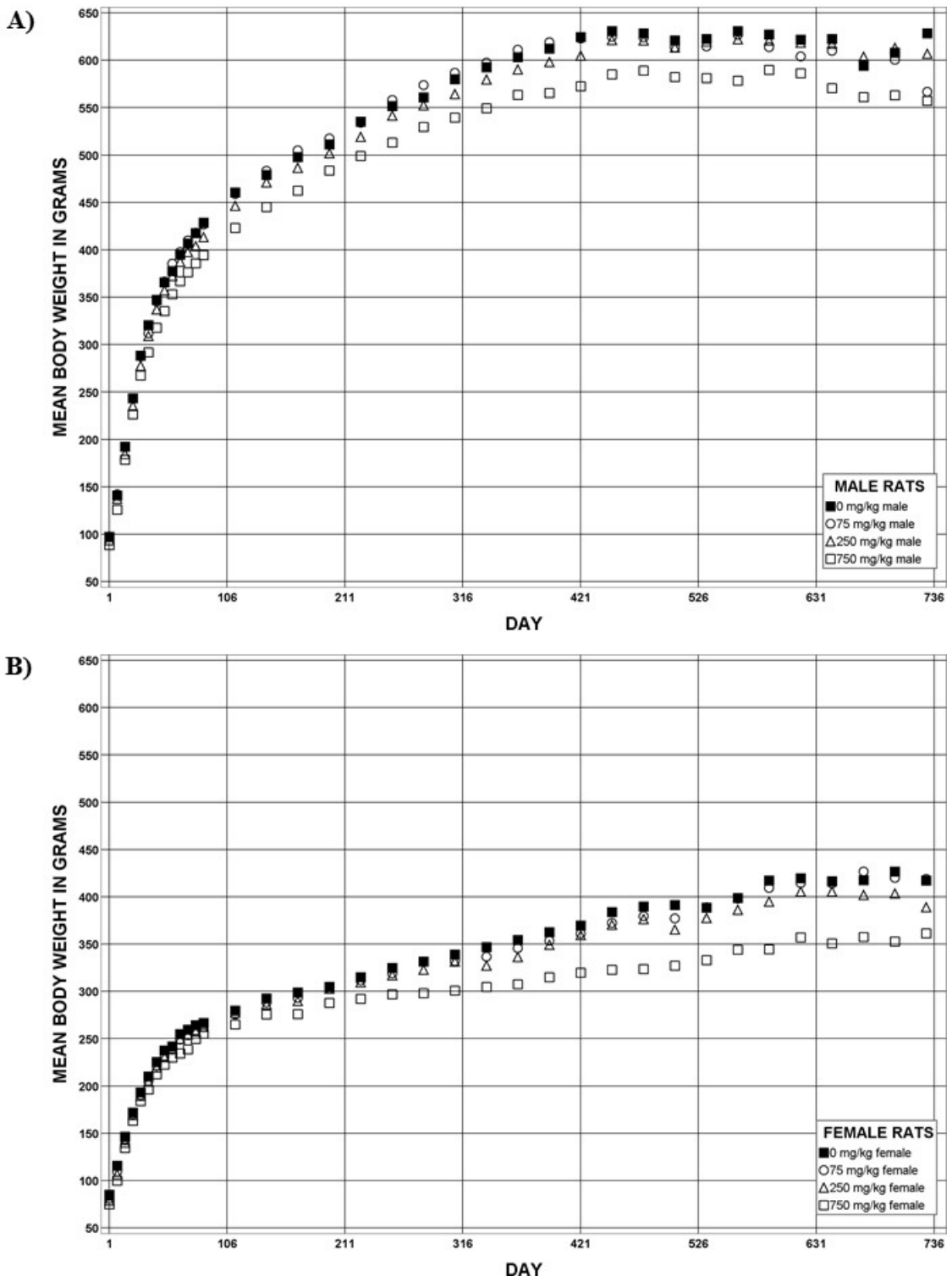

Figure 3. Growth Curves for Male Rats Exposed Perinatally and Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

Growth curves are shown for (A) males and (B) females.

Histopathology

This section describes the statistically significant or biologically noteworthy changes in the incidence of neoplasms and nonneoplastic lesions of the uterus and ovary.

Uterus: Squamous cell papillomas and squamous cell carcinomas of the uterus were present in higher numbers in the 250 mg/kg/day group than in the vehicle control group, but there was not a significant difference between the incidences (Table 9). Squamous cell papillomas were characterized by well-circumscribed masses composed of papillary fronds of squamous epithelium with abundant overlying keratin. Squamous cell papillomas projected into the uterine lumen (Figure 4) and did not invade the underlying myometrium. In contrast, squamous cell carcinomas were poorly demarcated neoplasms arising from the endometrium, which invaded the underlying myometrium and often filled the uterine lumen with keratin. They were composed of variably differentiated squamous epithelial cells in nests and clusters that occasionally contained central keratin pearls. Neoplastic cells were typically polygonal, with abundant eosinophilic cytoplasm and round nuclei; intercellular bridges were visible between some of the cells.

Nonneoplastic lesions observed in the uterus included squamous metaplasia, dilation, hemorrhage, thrombus, and ulcers. There was a positive trend for the incidence of squamous metaplasia, and the incidence was significantly increased in the 750 mg/kg/day group compared to the vehicle control group (Table 9). Squamous metaplasia involved either the endometrial glands, the luminal epithelium, or both, and was characterized by a replacement of the normal epithelium with squamous epithelium (Figure 5).

Significantly increased incidences of uterine dilation occurred in the 250 and 750 mg/kg/day groups compared to the vehicle control group (Table 9). Most of the occurrences of dilation were marked in severity, causing thinning of the uterine wall (Figure 6) and loss of endometrial glands. The remaining uterine parenchyma frequently contained mixed inflammatory cells, hemorrhage, necrotic cell debris, and fibrin.

The incidence of hemorrhage was significantly increased in the 750 mg/kg/day group compared to the vehicle control group (Table 9). Most of the occurrences of uterine hemorrhage were moderate or marked and were characterized by large numbers of erythrocytes filling the lumen of the uterus (Figure 7A); these animals usually had dilation of the uterus as well. Less commonly, hemorrhage was characterized by extravasated erythrocytes within the parenchyma of the uterine wall.

Higher incidences of thrombus were present in the 250 and 750 mg/kg/day groups compared to the vehicle control group (Table 9). Histologically, thrombi were characterized by large vessels between the myometrium and perimetrium that were partially to fully occluded by abundant, homogeneous to lamellated, eosinophilic material consistent with fibrin (Figure 7B, Figure 7C); some vessels had undergone recanalization. Variable numbers of lymphocytes and macrophages were present in the vessel walls, and occasionally, basophilic mineral was observed within the thrombi. Vessels in regions with thrombi were frequently ectatic; affected uteri often had hemorrhage and dilation as concurrent changes.

Compared to the vehicle control group, a significantly increased incidence of ulcers was present in the 750 mg/kg/day group (Table 9). Ulcers were characterized by a focal to focally extensive

loss of the endometrial epithelium (Figure 8). Ulcers were associated with neutrophilic inflammation, necrotic debris, and fibrin.

Table 9. Incidences of Neoplastic and Nonneoplastic Lesions of the Uterus in Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
n[a]	50	50	50	49
Squamous Metaplasia[b]	21** (1.8)[c]	25 (1.4)	30 (2.1)	38** (2.3)
Dilation	2** (4.0)	3 (3.3)	9* (3.4)	22** (3.8)
Hemorrhage	1** (3.0)	0	0	8* (3.1)
Thrombus	1*	0	5	5
Ulcers	0**	1 (1.0)	3 (1.7)	9** (2.7)
Squamous Cell Papilloma[d]				
Overall rate[e]	1/50 (2%)	1/50 (2%)	4/50 (8%)	0/49 (0%)
Rate/litters[f]	1/25 (4%)	1/25 (4%)	4/25 (16%)	0/25 (0%)
Adjusted rate[g]	2.4%	2.5%	9.2%	0%
Rao-Scott-adjusted Poly-3 test[h]	p = 0.397N	p = 0.733	p = 0.232	p = 0.527N
Squamous Cell Carcinoma[i]	1	0	2	1
Squamous Cell Papilloma or Squamous Cell Carcinoma (Combined)[j]				
Overall rate	2/50 (4%)	1/50 (2%)	4/50 (8%)	1/49 (2%)
Rate/litters	2/25 (8%)	1/25 (4%)	4/25 (16%)	1/25 (4%)
Adjusted rate	4.8%	2.5%	9.2%	2.9%
Rao-Scott-adjusted Poly-3 test	p = 0.511N	p = 0.507N	p = 0.363	p = 0.534N

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant ($p \leq 0.05$) from the vehicle control group by the Rao-Scott-adjusted Poly-3 test; **$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[d]Historical control incidence for 2-year methylcellulose gavage study of *trans*-resveratrol: 0/50; all routes (mean ± standard deviation): 1/500 (0.2% ± 0.63%); range: 0% to 2%.
[e]Number of animals with neoplasm/number of animals necropsied.
[f]Number of litters with neoplasm-bearing animals/number of litters examined at site.
[g]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[h]Beneath the control incidence is the p value associated with the trend test. Beneath the dosed group incidence is the p value corresponding to pairwise comparisons between the vehicle control group and that dosed group. The Rao-Scott test adjusts the Poly-3 test (which accounts for differential mortality in animals that do not reach study termination) for within-litter correlation. A negative trend or a lower incidence in a dosed group is indicated by N.
[i]Historical control incidence for 2-year methylcellulose gavage study of *trans*-resveratrol: 0/50; all routes: 4/500 (0.8% ± 1.4%); range: 0% to 4%.
[j]Historical control incidence for 2-year methylcellulose gavage study of *trans*-resveratrol: 0/50; all routes: 5/500 (1% ± 1.7%); range: 0% to 4%.

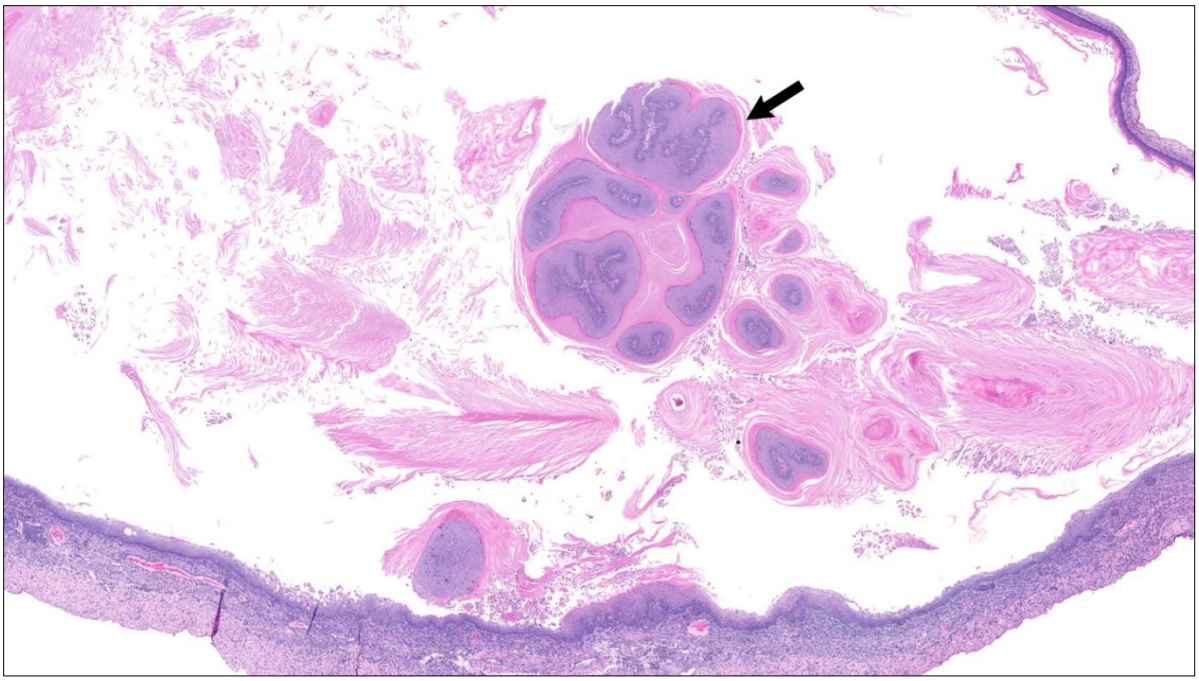

Figure 4. Representative Image of a Squamous Cell Papilloma in the Uterus of a Female Rat in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

A squamous cell papilloma (arrow) is present within the lumen of the uterus of a 250 mg/kg/day rat (2.6×). Because of sectioning, the stalk attaching the papilloma to the wall of the uterine lumen is not present in this section. Squamous metaplasia is also present. H&E = hematoxylin and eosin stain.

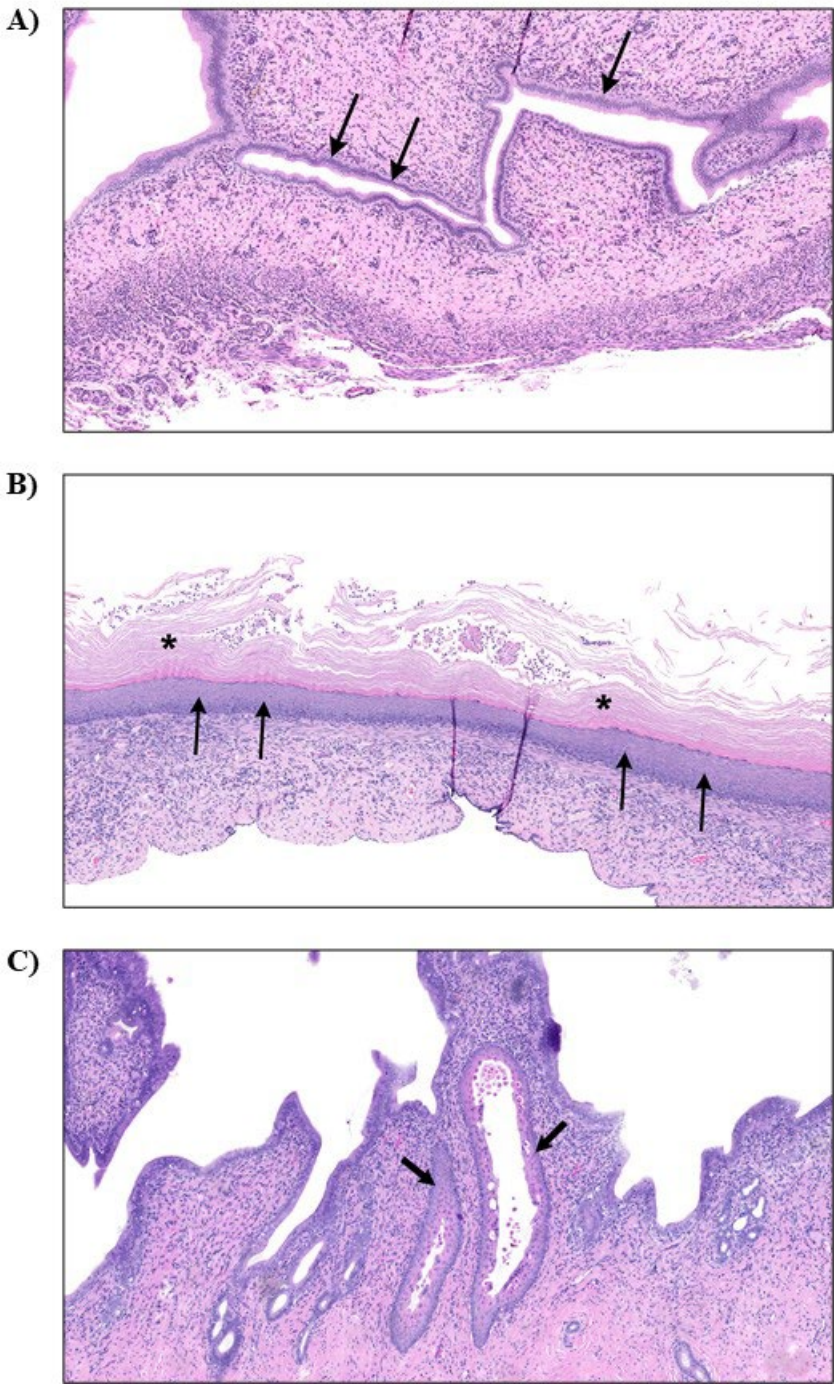

Figure 5. Representative Images of a Normal Uterus and Squamous Metaplasia of the Uterus in Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

(A) Normal lining of the uterus from a vehicle control rat is shown (8×). The luminal epithelium is low cuboidal to columnar (arrows). (B) Squamous metaplasia of the luminal epithelium in the uterus of a 250 mg/kg/day female rat is shown (8×). The lining of the endometrium is replaced by squamous epithelium (arrows) instead of the regular cuboidal or columnar epithelium. An associated orthokeratotic hyperkeratosis (asterisks) is shown, which is commonly seen with squamous metaplasia of the epithelium. (C) Squamous metaplasia of endometrial glands in the uterus of a 250 mg/kg/day female rat is shown (8×). Squamous metaplasia can also involve the glands in the endometrium (arrows). H&E = hematoxylin and eosin stain.

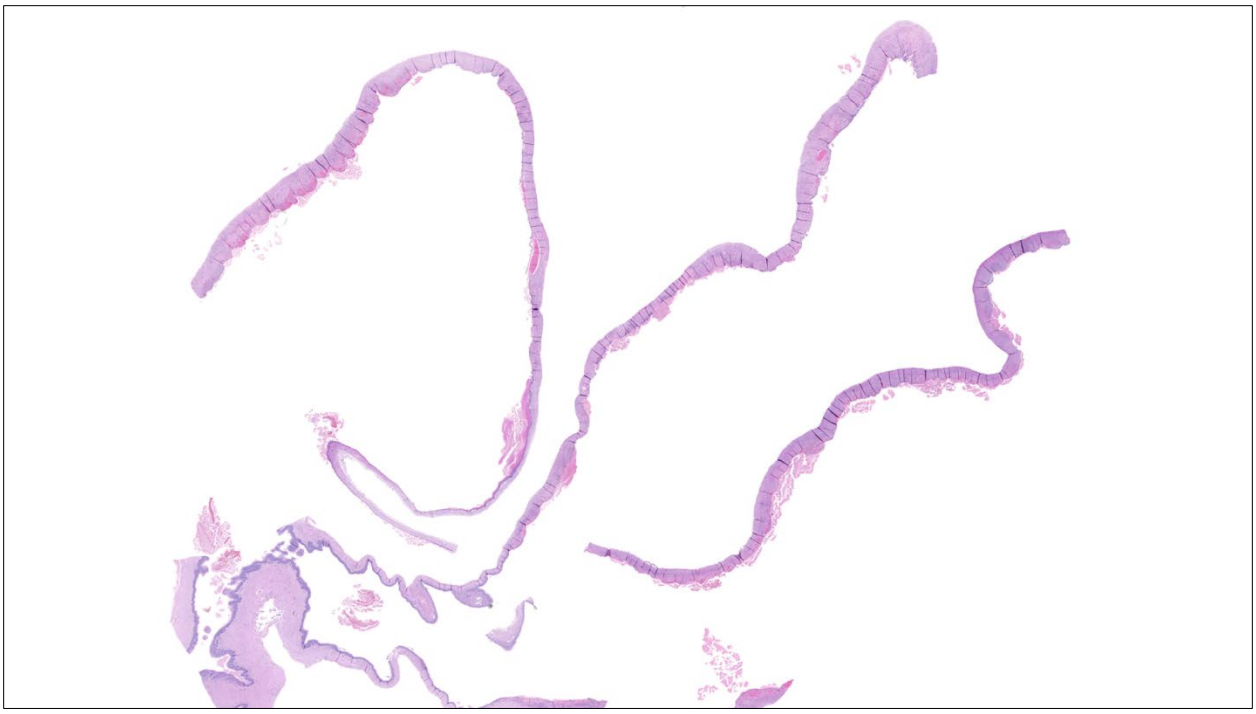

Figure 6. Representative Image of Dilation of the Uterus of a Female Rat in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

Dilation of the uterus from a 750 mg/kg/day rat is shown (0.5×). The lumen of the uterus is markedly enlarged, and the wall of the uterus is thinned. H&E = hematoxylin and eosin stain.

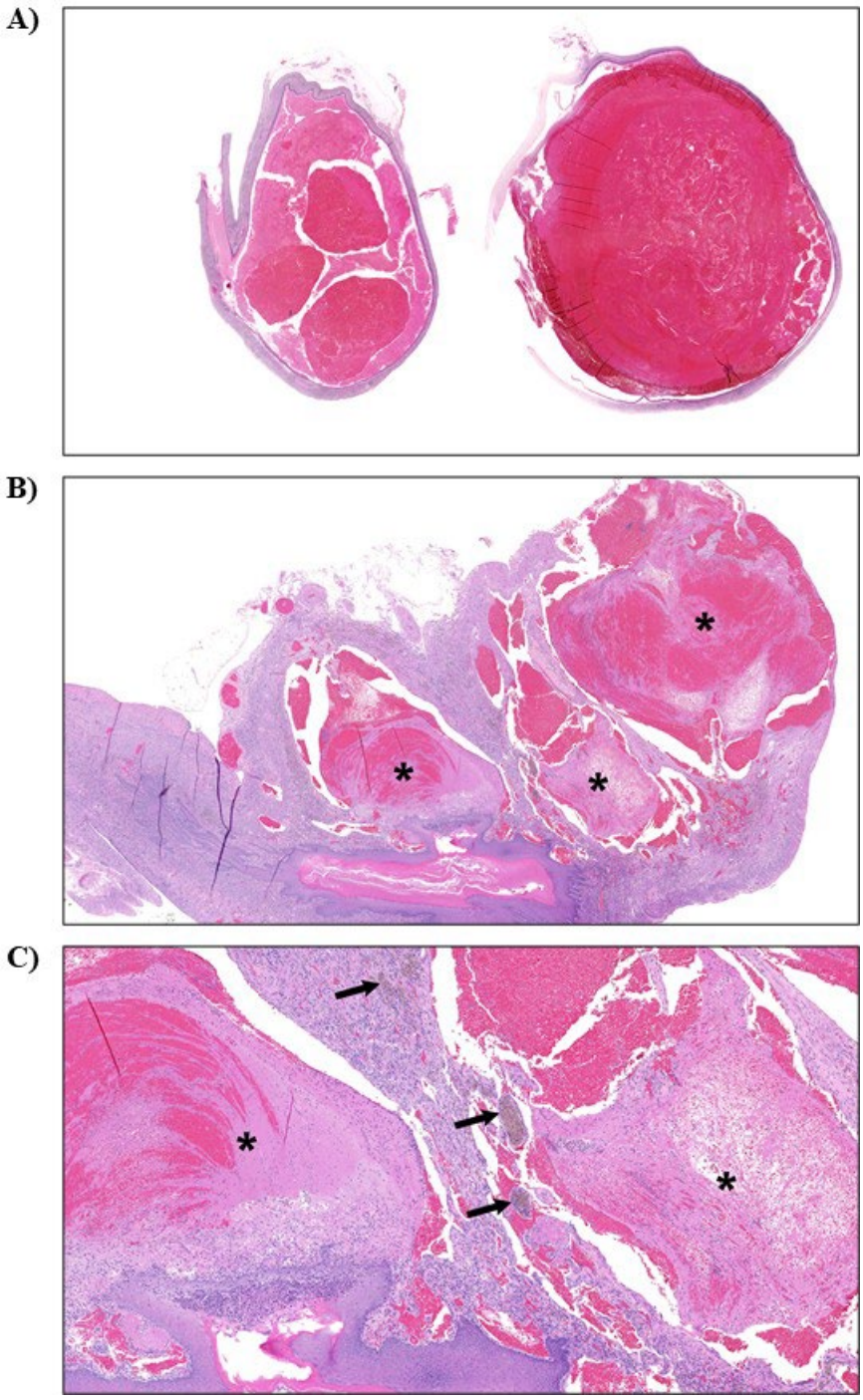

Figure 7. Representative Images of Hemorrhage and Thrombi of the Uterus of a Female Rat in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

(A) Hemorrhage in the uterus of a 750 mg/kg/day rat is shown (0.5×). This cross-sectional cut reveals a blood-filled lumen in both horns of the uterus. (B) Large thrombi (asterisks) within the wall of the uterus from a 750 mg/kg/day rat are shown (1.7×). (C) A higher magnification of panel B shows organized thrombi (asterisks) and pigment (arrows), which indicate hemorrhage occurred prior to death (5×). H&E = hematoxylin and eosin stain.

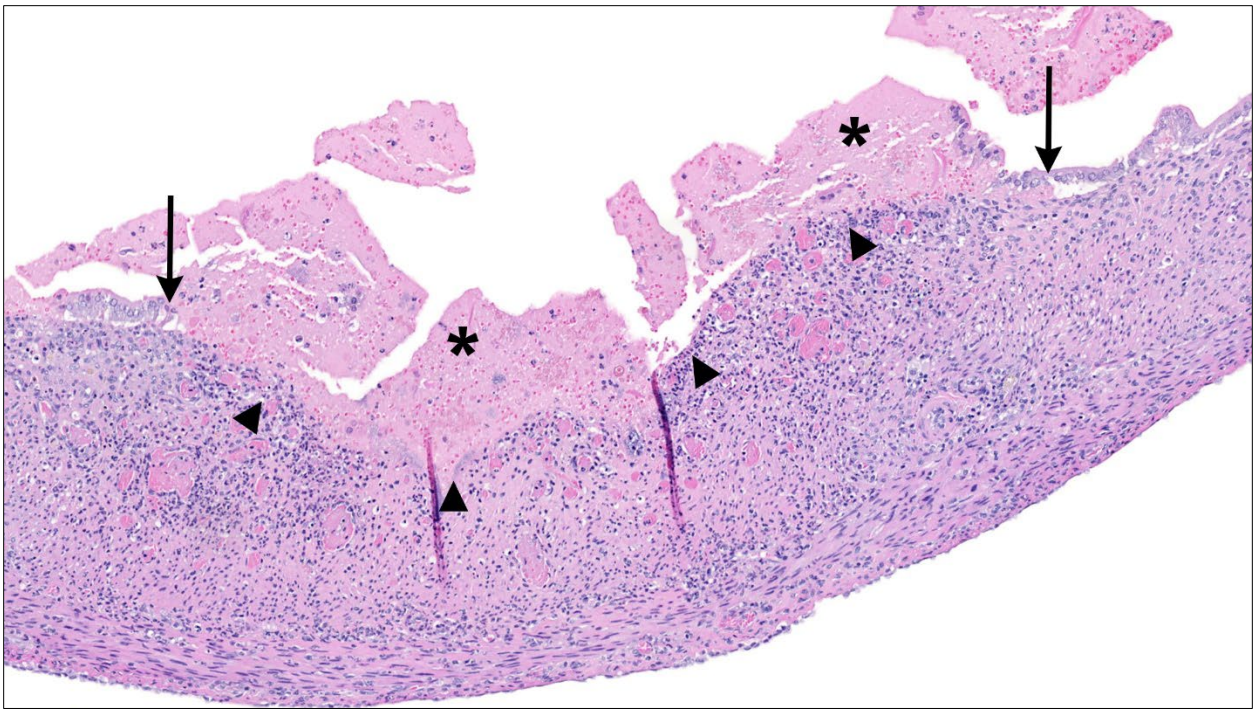

Figure 8. Representative Image of Ulceration of the Uterus of a Female Rat in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

A large area of ulceration (arrowheads) between the remaining epithelium (arrows) of the uterus from a 750 mg/kg/day rat is shown (10×). A layer of necrotic debris (asterisks) covers the area of ulceration. H&E = hematoxylin and eosin stain.

Ovary: In the ovary, the incidence of atrophy was significantly increased in the 750 mg/kg/day group compared to the vehicle control group (Table 10). Most cases across all groups were mild in severity, with little difference in the mean severity scores between the vehicle control and dosed groups. Ovarian atrophy was characterized by ovaries that had decreased numbers of corpora lutea and follicles and were smaller in size than normal (Figure 9). Marked atrophy usually corresponded to a complete lack of corpora lutea and developing follicles.

Other tissues: The incidence of bone marrow hypercellularity was significantly increased in the 750 mg/kg/day females compared to the vehicle control group (Appendix E). In males, the incidence of hemorrhage within the bone marrow was higher compared to the vehicle control group for all exposed groups and was significantly increased in the 75 and 750 mg/kg/day groups. In females, there was a significantly increased incidence of histiocyte cellular infiltrates in the lung at 750 mg/kg/day compared to the vehicle control group. The incidence of granulomatous inflammation was significantly increased in the 250 mg/kg/day females compared to the vehicle control group. In females, the incidences of cardiomyopathy in the heart were higher in all dosed groups compared to the vehicle control group; the increase was significant only in the 250 mg/kg/day group. In perinatally exposed males, the incidences of epithelial hyperplasia of the forestomach were slightly increased in all dose groups compared to the vehicle group, with statistical significance at 750 mg/kg/day. Hyperplasia was characterized by increased layers of squamous epithelium, often associated with inflammation, and in one low-dose animal with an ulcer. None of these lesions, or lesions in other tissues not described here, were considered related to BCE.

There were fewer occurrences of mammary gland hyperplasia in the 250 and 750 mg/kg/day females than in the vehicle control group. The number of mammary gland fibroadenomas and uterine stromal polyps in the 750 mg/kg/day female group was lower than in the concurrent vehicle control group and below the lower end of the range of fibroadenomas in historical controls. Significant decreases in body weight have been associated with a decreased incidence of mammary gland neoplasms in rats.[165] Other contributions to this effect could be the marginally lower survival in this group (not statistically significant) or potential antiestrogenic activity.

Table 10. Incidences of Nonneoplastic Lesions of the Ovary in Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	75 mg/kg/day	250 mg/kg/day	750 mg/kg/day
Ovary	50	50	50	49
Atrophy	32** (2.1)	39 (2.4)	38 (2.2)	47** (2.1)

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant ($p \leq 0.05$) from the vehicle control group by the Rao-Scott-adjusted Poly-3 test; **$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

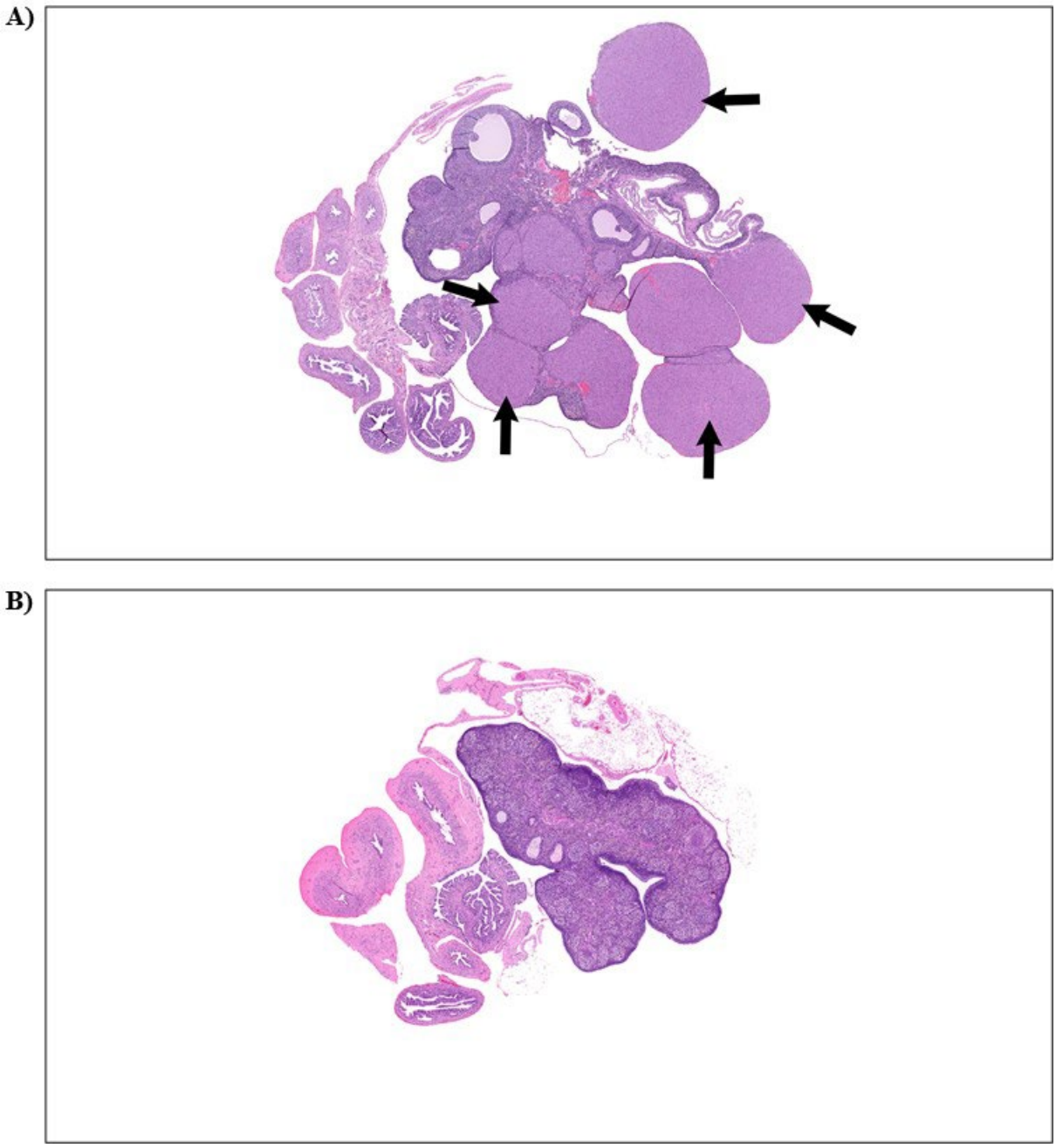

Figure 9. Representative Images of a Normal Ovary and Atrophy of the Ovary in Female Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract (H&E)

(A) A normal ovary from a vehicle control rat is shown (1.2×). Even at this low magnification, numerous corpora lutea (arrows) are visible. (B) An atrophic ovary from a 750 mg/kg/day rat is shown (1.2×). The ovary is grossly smaller than that shown in panel A, and no corpora lutea are present. H&E = hematoxylin and eosin stain.

Mice

Two-year Study Interim Evaluations (3 and 12 Months)

At the 3-month evaluation, absolute spleen weights of all BCE-dosed groups were within 10% of that of the vehicle control group (Table 11). There was a positive trend in absolute and relative spleen weights.

At the 12-month evaluation, absolute spleen weights of all dosed groups were higher than that of the vehicle control group (Table 11). The largest differences were in the 300 and 1,000 mg/kg/day groups, which both had significantly increased absolute mean spleen weights that were approximately 23% higher than that of the vehicle control group. Relative spleen weights increased with increasing dose and were significantly increased for all dosed groups compared to that of the vehicle control group. Mean body weights of dosed groups at the 12-month evaluation decreased with increasing dose, and the spleen weight changes were considered related to BCE administration.

Table 11. Summary of Spleen Weights and Spleen-Weight-to-Body-Weight Ratios for Female Mice in the Three- and Twelve-month Interim Evaluations during the Two-year Gavage Study of Black Cohosh Root Extract[a,b]

	0 mg/kg/day	30 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1,000 mg/kg/day
n[c]	10	10	10	10	10
Three Months					
Terminal Body Wt. (g)	25.7 ± 0.4	25.4 ± 0.5	26.7 ± 0.8	25.5 ± 0.5	25.3 ± 0.5
Spleen					
Absolute (g)	0.080 ± 0.003*	0.073 ± 0.004	0.080 ± 0.002	0.087 ± 0.003	0.086 ± 0.004
Relative (mg/g)[d]	3.11 ± 0.12**	2.87 ± 0.11	3.03 ± 0.11	3.42 ± 0.13	3.40 ± 0.14
Twelve Months					
Terminal Body Wt. (g)	50.3 ± 2.2**	45.2 ± 1.7*	40.8 ± 1.5**	38.7 ± 1.0**	32.3 ± 0.7**
Spleen					
Absolute (g)	0.094 ± 0.003**	0.110 ± 0.005	0.108 ± 0.010	0.116 ± 0.007*	0.116 ± 0.005*
Relative (mg/g)	1.90 ± 0.07**	2.45 ± 0.14*	2.63 ± 0.20**	3.00 ± 0.17**	3.59 ± 0.14**

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed by the Jonckheere (trend) and the Williams or Dunnett (pairwise) tests.
[c]Number of animals examined.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.

The frequency of micronucleated erythrocytes was evaluated in peripheral blood samples obtained at the 3- and 12-month evaluations (Appendix D). At the 3-month evaluation, a significant, dose-related increase in micronucleated erythrocytes was observed (Table 12). To determine whether the responses seen after 3 months of BCE administration increased, decreased, or remained the same after a full year, peripheral blood erythrocytes were evaluated after 12 months of dosing. Again, there was a significant, dose-related increase in the frequencies

of micronucleated erythrocytes in these mice, with a magnitude of response similar to the 3-month evaluation (Table 12). No significant changes were observed in the percentage of immature erythrocytes (% PCE) in the BCE-dosed mice at either evaluation.

Table 12. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Female Mice in the Three- and Twelve-month Interim Evaluations during the Two-year Gavage Study of Black Cohosh Root Extract[a]

	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs (%)[b]	P Value[c]
Three Months							
Dose (mg/kg/day)							
0	5	2.050 ± 0.119		1.056 ± 0.018		1.585 ± 0.101	
30	5	2.080 ± 0.221	1.0000	1.080 ± 0.013	0.3328	1.639 ± 0.117	0.8229
100	5	2.420 ± 0.189	0.8277	1.370 ± 0.045	<0.001	1.826 ± 0.133	0.7443
300	5	3.250 ± 0.099	0.0404	1.908 ± 0.052	<0.001	1.695 ± 0.116	0.7906
1,000	5	5.530 ± 0.485	0.0012	2.890 ± 0.046	<0.001	1.638 ± 0.208	0.8121
Trend[d]		p < 0.001		p < 0.001		p = 0.6474	
Twelve Months							
Dose (mg/kg/day)							
0	5	1.870 ± 0.116		1.002 ± 0.035		1.801 ± 0.167	
30	5	2.100 ± 0.106	0.8271	1.074 ± 0.036	1.0000	1.880 ± 0.230	0.8948
100	5	2.090 ± 0.128	0.8271	1.378 ± 0.118	0.1174	2.081 ± 0.145	0.8261
300	5	3.950 ± 0.695	0.0060	1.892 ± 0.196	0.0070	1.928 ± 0.348	0.8697
1,000	5	5.000 ± 0.359	0.0011	2.641 ± 0.228	<0.001	2.439 ± 0.305	0.2152
Trend		p < 0.001		p < 0.001		p = 0.1440	

PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at Integrated Laboratory Systems, LLC.
[b]Data are presented as mean ± standard error.
[c]Pairwise comparisons with the vehicle control group performed using the Williams or Dunn test ($p \leq 0.025$).
[d]Dose-related trends evaluated by linear regression or the Jonckheere test ($p \leq 0.025$).

A complete blood count, bone marrow cell count, and bone marrow cytological evaluation were performed at the 3- and 12-month evaluations. At 3 months, the mean cell volume (MCV) and mean cell hemoglobin (MCH) were significantly increased in all BCE-dosed groups relative to the vehicle control group (Table 13). The erythrocyte count showed a negative trend with a significant pairwise decrease in the 1,000 mg/kg/day group relative to the vehicle control group. Additionally, the total white blood cell, neutrophil, lymphocyte, and monocyte counts were significantly decreased in various dosed groups. At the 12-month evaluation, the hematocrit and erythrocyte counts were significantly decreased in all dosed groups, and hemoglobin was significantly decreased in the 300 and 1,000 mg/kg/day groups relative to the vehicle control group (Table 13). The MCV and MCH were also significantly increased in most dosed groups

relative to the vehicle control group. The leukogram changes observed at the 3-month evaluation were ameliorated by 12 months.

Table 13. Summary of Select Hematology Data for Female Mice in the Three- and Twelve-month Interim Evaluations during the Two-year Gavage Study of Black Cohosh Root Extract[a,b]

	0 mg/kg/day	30 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1,000 mg/kg/day
Three Months[c]	9	10	10	10	10
Hematocrit (%)	55.6 ± 1.2	56.3 ± 1.0	57.0 ± 1.6	56.5 ± 1.6	53.4 ± 1.0
Hemoglobin (g/dL)	17.1 ± 0.3	17.5 ± 0.3	17.7 ± 0.5	17.7 ± 0.5	16.4 ± 0.3
Erythrocytes (10^6/µL)	11.42 ± 0.21**	11.42 ± 0.21	11.24 ± 0.30	10.76 ± 0.27	9.73 ± 0.19**
Reticulocytes (10^3/µL)	272.8 ± 11.8	275.7 ± 15.9	293.8 ± 14.5	282.2 ± 10.5	255.5 ± 17.6
Mean Cell Volume (fL)	48.7 ± 0.2**	49.4 ± 0.2*	50.7 ± 0.2**	52.5 ± 0.3**	54.9 ± 0.3**
Mean Cell Hemoglobin (pg)	15.0 ± 0.1**	15.3 ± 0.0**	15.7 ± 0.0**	16.5 ± 0.2**	16.9 ± 0.1**
Mean Cell Hemoglobin Concentration (g/dL)	30.8 ± 0.2	31.1 ± 0.1	31.1 ± 0.1	31.4 ± 0.3	30.7 ± 0.1
White Blood Cells (10^3/µL)	4.45 ± 0.45**	3.95 ± 0.31	3.25 ± 0.19	3.61 ± 0.43	2.85 ± 0.36**
Neutrophils (10^3/µL)	0.62 ± 0.07**	0.51 ± 0.06	0.41 ± 0.04*	0.38 ± 0.04*	0.42 ± 0.12**
Lymphocytes (10^3/µL)	3.66 ± 0.39**	3.28 ± 0.25	2.71 ± 0.15	3.14 ± 0.39	2.32 ± 0.25**
Monocytes (10^3/µL)	0.09 ± 0.01**	0.07 ± 0.01	0.08 ± 0.02	0.04 ± 0.01**	0.05 ± 0.01**
Twelve Months	10	10	10	10	10
Hematocrit (%)	52.5 ± 0.6**	51.2 ± 0.5*	51.3 ± 0.6*	49.6 ± 0.6**	50.1 ± 0.4**
Hemoglobin (g/dL)	15.4 ± 0.2**	15.3 ± 0.1	15.2 ± 0.2	14.8 ± 0.2*	14.9 ± 0.1*
Erythrocytes (10^6/µL)	10.34 ± 0.10**	10.02 ± 0.10*	9.77 ± 0.16**	9.22 ± 0.13**	9.00 ± 0.08**
Reticulocytes (10^3/µL)	247.4 ± 16.6	290.0 ± 25.0	276.6 ± 14.9	230.8 ± 15.0	256.9 ± 22.5
Mean Cell Volume (fL)	50.7 ± 0.3**	51.1 ± 0.2	52.6 ± 0.4**	53.8 ± 0.3**	55.7 ± 0.2**
Mean Cell Hemoglobin (pg)	14.9 ± 0.1**	15.2 ± 0.1*	15.6 ± 0.1**	16.0 ± 0.1**	16.6 ± 0.1**
Mean Cell Hemoglobin Concentration (g/dL)	29.4 ± 0.1	29.8 ± 0.2	29.7 ± 0.2	29.7 ± 0.2	29.8 ± 0.1
White Blood Cells (10^3/µL)	4.02 ± 0.35	3.65 ± 0.27	3.39 ± 0.23	3.89 ± 0.28	3.58 ± 0.44
Neutrophils (10^3/µL)	0.80 ± 0.07	0.57 ± 0.09*	0.57 ± 0.04	0.66 ± 0.04	0.71 ± 0.09
Lymphocytes (10^3/µL)	3.05 ± 0.30	2.95 ± 0.19	2.75 ± 0.22	3.07 ± 0.23	2.74 ± 0.35
Monocytes (10^3/µL)	0.10 ± 0.03	0.10 ± 0.03	0.06 ± 0.02	0.11 ± 0.01	0.06 ± 0.01

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed by the Jonckheere (trend) and the Shirley or Dunn (pairwise) tests.
[c]Number of animals examined at each evaluation.

No changes were observed in the bone marrow cell count or bone marrow myeloid:erythroid ratio of the BCE-dosed groups relative to the vehicle control groups (Appendix E). Cytological evaluation of the erythroid, myeloid, and megakaryocyte cell lines revealed mild dysplasia in a

low number of metarubricytes at both the 3- and 12-month evaluations. These abnormal metarubricytes were observed with higher frequency in the 100, 300, and 1,000 mg/kg/day groups relative to the vehicle control group (Table 14; Figure 10). Observed dysplastic features included the following: multilobulated nuclei, micronuclei, nuclear-to-cytoplasmic asynchrony, gigantism, and nuclear fragmentation. In relation to the absolute erythroid precursors, the percentages of abnormal metarubricytes at 3 and 12 months ranged from 0.5 to 10.8, depending on the dose (Table 14). The abnormal metarubricytes were not included in the absolute erythroid precursor counts.

Table 14. Summary of Bone Marrow Cytology Data for Female Mice in the Three- and Twelve-month Interim Evaluations during the Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	30 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1,000 mg/kg/day
n[a]	10	10	10	10	10
Three Months					
Incidence of Abnormal Metarubricytes	0/10	0/10	3/10	6/10	9/10
Min # Abnormal Metarubricytes[b]	0	0	1	1	5
Max # Abnormal Metarubricytes[b]	0	0	3	3	15
Mean # Abnormal Metarubricytes	0	0	1.67	1.83	10.67
Mean # Erythroid Precursors[c]	0	0	179.33	161.67	161.78
Min % Abnormal Metarubricytes[d]	0	0	0.5	0.5	3.7
Max % Abnormal Metarubricytes[d]	0	0	1.7	1.7	8.4
Twelve Months					
Incidence of Abnormal Metarubricytes	0/10	0/10	2/10	4/10	10/10
Min # Abnormal Metarubricytes	0	0	1	1	2
Max # Abnormal Metarubricytes	0	0	3	4	24
Mean # Abnormal Metarubricytes	0	0	2.00	2.25	6.60
Mean # Erythroid Precursors	0	0	173.50	170.50	152.00
Min % Abnormal Metarubricytes	0	0	0.6	0.6	1.4
Max % Abnormal Metarubricytes	0	0	1.7	2.3	10.8

No trend or pairwise statistical tests were performed on these data.
Min = minimum; # = number; max = maximum; % = percent.
[a]Number of animals examined at each evaluation.
[b]Number of abnormal metarubricytes counted in the bone marrow 500-cell differential cell count.
[c]Mean of number of erythroid precursors counted in the bone marrow 500-cell differential cell count from animals with abnormal metarubricytes.
[d]100 × (number of abnormal rubricytes)/(number of erythroid precursors from animals with abnormal metarubricytes).

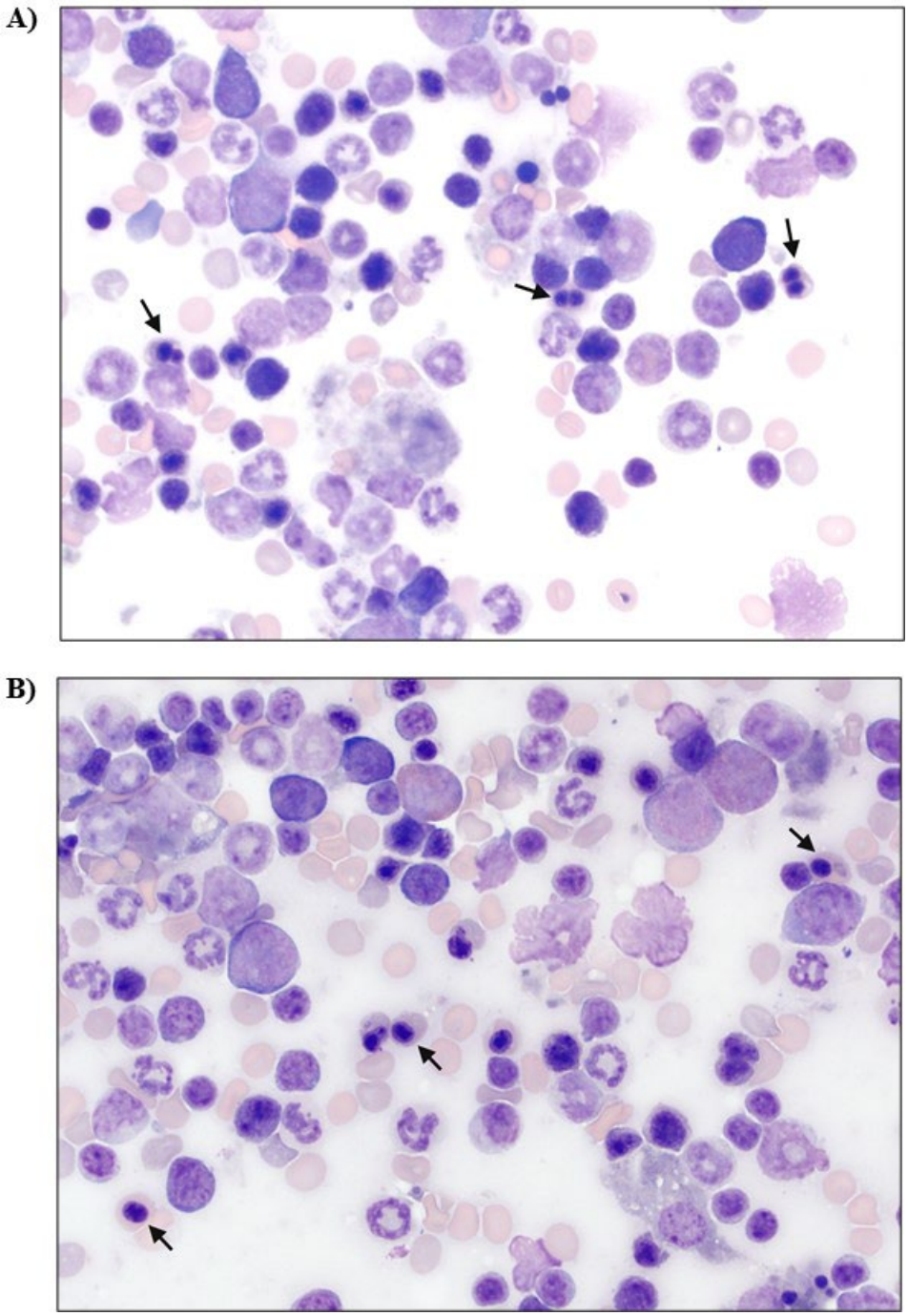

Figure 10. Representative Images of Abnormal Metarubricytes in the Bone Marrow of a Female Mouse in the Two-year Gavage Study of Black Cohosh Root Extract (Wright's Giemsa Stain)

(A) Several binucleated metarubricytes (arrows) in the bone marrow of a 1,000 mg/kg/day mouse is shown (50×). (B) Several metarubricytes displaying nuclear-to-cytoplasmic asynchrony are present (arrows) in the bone marrow of a 1,000 mg/kg/day mouse (50×). Features of nuclear-to-cytoplasmic asynchrony include immature nuclei (more open chromatin) relative to a more mature-looking cytoplasm (pinkish in color due to hemoglobin accumulation).

Histopathology

This section describes the statistically significant or biologically noteworthy changes in the incidence of nonneoplastic lesions of the spleen and bone marrow.

Three-month interim evaluation: There were no significant differences in the incidences of lesions between the vehicle control group and BCE-dosed groups at the 3-month interim evaluation; only the spleen and bone marrow were examined histologically at this evaluation (Appendix E).

Twelve-month interim evaluation: At the 12-month interim evaluation, only the spleen and bone marrow were examined histologically. Compared to the vehicle control group, there was a significantly increased incidence of hematopoietic cell proliferation in the spleens of the 100 mg/kg/day group that was considered sporadic, and not related to BCE administration; no effect was observed at 3 months. There were no significant differences in the incidences of bone marrow lesions between the vehicle control group and BCE-dosed groups (Appendix E).

Two-year Study Final Evaluations

Estimates of 2-year survival probabilities for female mice are shown in Table 15 and in the Kaplan-Meier survival curve (Figure 11). Survival of all BCE-dosed groups was similar to that of the vehicle control group.

Table 15. Summary of Survival of Female Mice in the Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	30 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1,000 mg/kg/day
Animals Initially in Study	50	50	50	50	50
Moribund	4	2	3	3	6
Natural Deaths	6	9	3	7	6
Accidental Deaths	0	0	0	1	0
Animals Surviving to Study Termination	40	39	44	39	38
Percent Probability of Survival at Study Termination[a]	80.0%	80.0%	88.0%	79.6%	76.0%
Mean Survival (Days)[b]	703.2 ± 9.7	714.8 ± 5.9	720.9 ± 3.5	707.0 ± 7.3	692.2 ± 14.4
Survival Analysis[c]	p = 0.389	p = 1.000N	p = 0.393N	p = 1.000	p = 0.785

[a]Kaplan-Meier determinations.
[b]Mean of all deaths (uncensored, censored, and study termination) ± standard error.
[c]The result of the Tarone trend test is in the vehicle control group column, and the results of the Cox log-rank pairwise comparisons to the vehicle control group are in the dosed group columns. A negative trend or lower mortality in a dosed group is indicated by N.

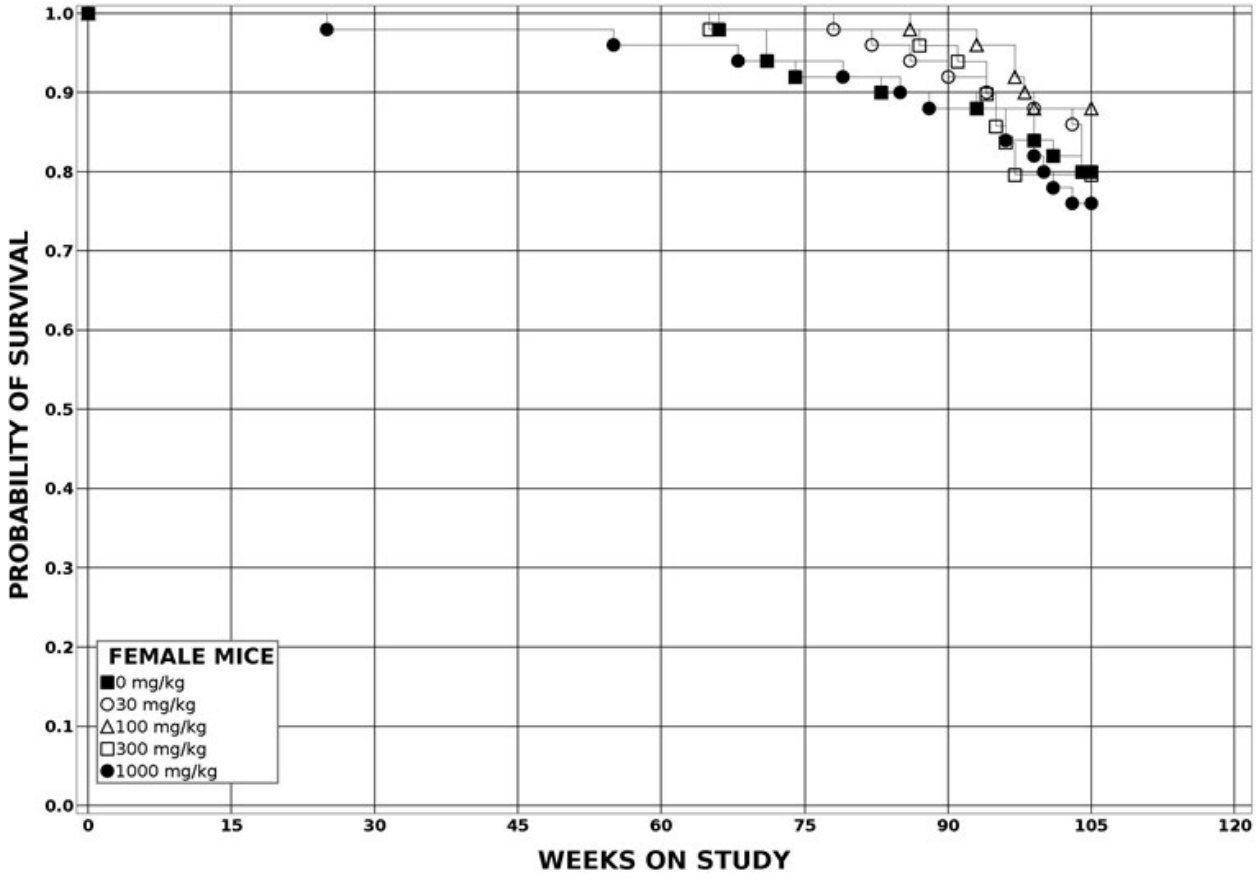

Figure 11. Kaplan-Meier Survival Curves for Female Mice Administered Black Cohosh Root Extract in the Two-year Gavage Study

Mean body weights of the 100, 300, and 1,000 mg/kg/day females were ≤90% of that of the vehicle control group beginning on study day 141 and continued to decrease throughout the remainder of the study (Table 16; Figure 12). Mean body weight of the 30 mg/kg/day females remained within 12% of that of the vehicle control group throughout the study. At study termination, mean body weights of the 30, 100, 300, and 1,000 mg/kg/day groups were 91%, 82%, 75%, and 60%, respectively, of that of the vehicle control group.

No clinical observations in dosed groups of mice were considered related to BCE administration (Appendix E).

Table 16. Summary of Survival and Mean Body Weights of Female Mice in the Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day		30 mg/kg/day			100 mg/kg/day			300 mg/kg/day			1,000 mg/kg/day		
Study Day[a]	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	17.6	50	18.0	102.0	50	17.7	100.4	50	17.8	100.9	50	17.9	101.3	50
8	18.1	50	18.4	101.5	50	18.7	103.3	50	18.7	103.3	50	18.8	103.9	50
15	19.1	50	19.4	101.2	50	19.6	102.5	50	19.8	103.6	50	19.7	103.0	50
22	19.9	50	20.1	100.7	50	20.4	102.2	50	20.6	103.5	50	20.8	104.3	50
29	20.7	50	20.7	100.0	50	20.7	100.2	50	21.3	102.9	50	21.3	103.1	50
36	21.5	50	21.4	99.6	50	21.4	99.7	50	22.0	102.3	50	22.1	102.9	50
43	22.2	50	22.2	100.1	50	22.0	99.1	50	22.7	101.9	50	22.8	102.4	50
50	22.8	50	22.5	98.5	50	22.5	98.6	50	23.3	102.2	50	22.9	100.4	50
57	23.6	50	23.0	97.4	50	23.1	97.6	50	23.7	100.3	50	23.8	100.9	50
64	24.2	50	23.7	98.1	50	23.9	98.7	50	24.2	100.1	50	24.5	101.4	50
71	24.7	50	24.2	98.0	50	24.5	98.9	50	25.5	103.1	50	24.9	100.8	50
78	25.3	50	24.5	96.7	50	24.7	97.4	50	25.3	99.9	50	25.4	100.1	50
85	26.2	50	25.4	96.9	50	25.5	97.2	50	25.9	98.7	50	25.9	98.5	50
113	29.0	50	27.4	94.5	50	27.1	93.4	50	26.4	91.2	50	26.7	92.1	50
141	32.7	50	29.6	90.7	50	29.3	89.6	50	28.6	87.7	50	27.8	85.2	50
169	34.7	50	32.8	94.6	50	31.3	90.1	50	30.5	88.1	50	29.2	84.3	50
197	37.1	50	34.1	92.1	50	31.6	85.3	50	30.9	83.5	50	30.0	80.9	49
225	39.9	50	36.6	91.8	50	34.1	85.4	50	32.9	82.4	50	31.1	77.8	49
253	41.2	50	37.9	92.0	50	35.1	85.2	50	34.2	82.9	50	31.9	77.3	49
281	43.5	50	39.7	91.3	50	36.2	83.4	50	35.5	81.6	50	31.9	73.4	49
309	47.3	50	42.8	90.4	50	38.3	80.9	50	36.9	78.0	50	32.8	69.4	49
337	47.8	50	43.6	91.2	50	38.8	81.1	50	37.5	78.5	50	33.1	69.3	49
365	50.1	50	44.6	89.0	50	40.7	81.2	50	39.1	78.1	50	34.4	68.7	49
393	50.2	50	45.6	90.8	50	41.8	83.3	50	39.1	77.9	50	34.1	68.0	48
421	52.2	50	46.5	89.0	50	42.2	80.9	50	39.4	75.4	50	34.4	65.8	48
449	53.5	50	47.9	89.5	50	44.0	82.2	50	40.6	75.9	50	34.9	65.1	48
477	55.0	49	49.7	90.3	50	45.8	83.2	50	42.2	76.6	49	36.1	65.7	47
505	57.3	47	50.6	88.3	50	46.5	81.1	50	42.6	74.2	49	36.4	63.5	47
533	57.0	46	50.4	88.4	50	46.6	81.7	50	42.4	74.4	49	35.7	62.6	47
561	58.5	46	51.5	88.2	49	47.4	81.1	50	43.4	74.2	49	36.7	62.8	46
589	57.1	45	51.6	90.3	48	46.8	82.0	50	43.5	76.2	48	36.2	63.5	45
617	57.7	45	51.5	89.3	47	46.8	81.1	49	42.6	73.9	47	35.3	61.1	44
645	58.1	45	52.3	89.9	46	47.2	81.2	49	43.4	74.6	46	35.8	61.6	44
673	58.8	44	53.1	90.3	45	47.7	81.1	47	44.3	75.3	41	36.3	61.7	42
701	58.2	42	53.1	91.2	44	47.6	81.7	44	43.6	74.9	39	35.5	61.0	40
EOS	**56.1**	**40**	**51.2**	**91.2**	**39**	**45.7**	**81.5**	**44**	**42.0**	**74.8**	**39**	**33.7**	**60.0**	**38**

No trend or pairwise statistical tests were performed on these data.
EOS = end of study.
[a]Study day 1 is the day animals were placed on study.

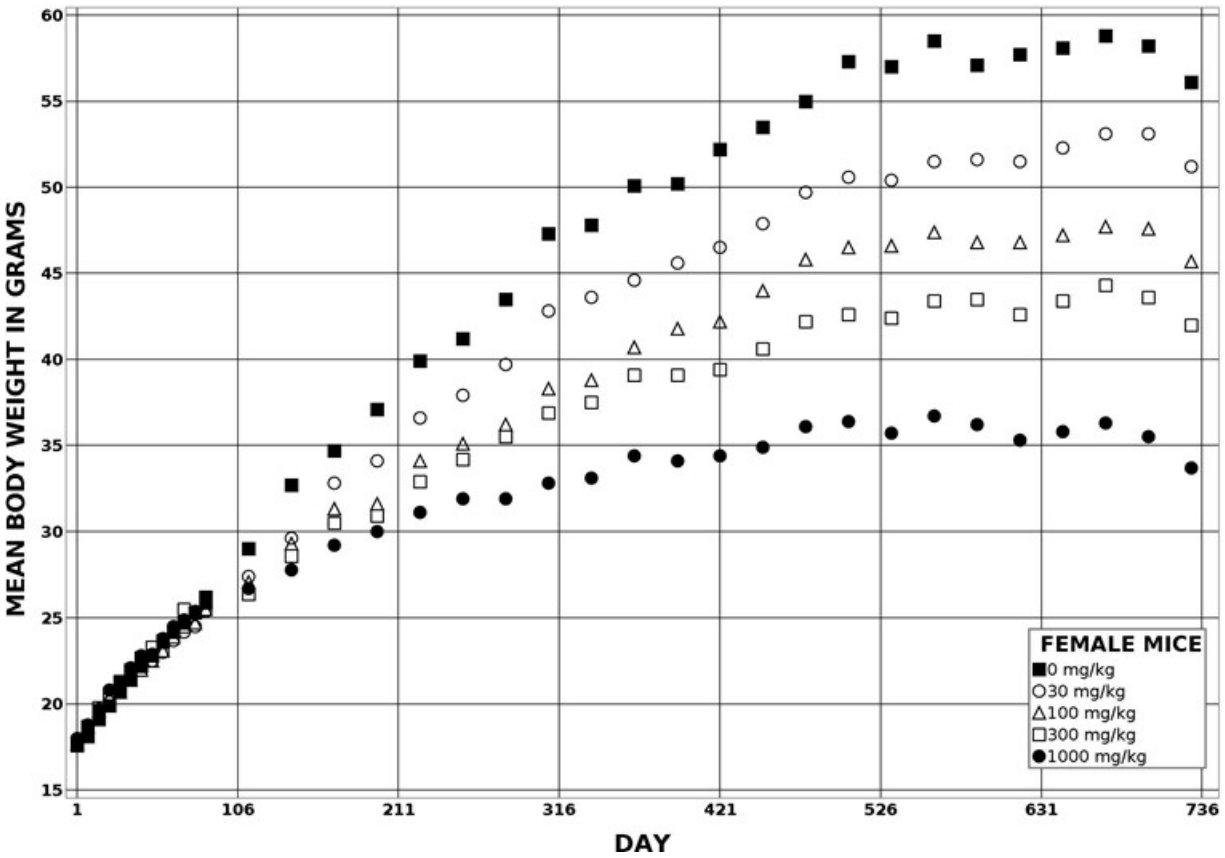

Figure 12. Growth Curves for Female Mice in the Two-year Gavage Study of Black Cohosh Root Extract

Histopathology

This section describes the statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions of the liver and thyroid gland in female mice. No neoplasms were considered related to the administration of BCE in female mice.

Liver: The incidence of necrosis in the liver was significantly increased in the 1,000 mg/kg/day group relative to the vehicle control group (Table 17). Histologically, liver necrosis (Figure 13A) did not have a distinctive lobular pattern; instead, it consisted of randomly scattered focal areas of coagulative necrosis characterized by brightly eosinophilic cytoplasm and shrunken, pyknotic nuclei (Figure 13B). Less commonly, individual necrotic cells were scattered within the hepatic parenchyma. Areas of necrosis, as well as individual necrotic cells (Figure 13C), were often associated with inflammatory cells. Necrosis that was considered secondary to a neoplasm was not recorded separately.

Thyroid gland: The incidence of follicle dilation in the thyroid gland was significantly increased in the 1,000 mg/kg/day group relative to the vehicle control group (Table 17). This change was focal, not diffuse, and was characterized by one or more follicles that were several times larger than normal follicles and caused compression of the adjacent follicles (Figure 14). The epithelium lining the follicles was typically flattened, and there was no evidence of papillary projections or hyperplasia. This change is often seen in the thyroid gland of older mice and is associated with changes in thyroid gland function as the animals age.[166]

Other tissues: There was a significant increase in the incidence of lymphoid hyperplasia in the mandibular lymph node in the 100 and 1,000 mg/kg/day groups relative to the vehicle control group (Appendix E), but this was not considered related to the administration of BCE because of the lack of a consistent response and its inherent variability. Lymphoid hyperplasia was characterized by increased numbers of mature small lymphocytes in the paracortical areas or an increased size and/or number of follicles compared with vehicle control mice.

The incidences of acute inflammation in the uterus and vagina were greater in the vehicle control group than in most dosed groups. Acute inflammation consisted primarily of neutrophils, although occasionally lymphocytes or macrophages were observed. Inflammation can normally be found in the vagina of mice during estrus. In animals in which inflammation was diagnosed, however, there were unusually large numbers of neutrophils, clusters of neutrophils forming pustule-like lesions in the epithelium, or large aggregates of neutrophils in the lumen—changes that are not characteristic of the normal presence of neutrophils found in the vagina during estrus. Inflammation in the vagina of B6C3F1 mice is a common finding, with one article listing the incidence at approximately 40% at 24 months of age.[167]

There was a significant increase in the incidence of squamous hyperplasia of the vaginal epithelium in the 100 mg/kg/day group relative to the vehicle control group. This finding was considered sporadic and not related to BCE administration.

Table 17. Incidences of Nonneoplastic Lesions of Select Organs in Female Mice in the Two-year Gavage Study of Black Cohosh Root Extract

	0 mg/kg/day	30 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1,000 mg/kg/day
Liver[a]	50	50	50	50	50
Necrosis[b]	2** (1.0)[c]	0	1 (1.0)	1 (1.0)	8* (1.1)
Thyroid Gland	50	50	49	49	50
Follicle, dilation	0**	1 (2.0)	1 (1.0)	1 (1.0)	5* (1.4)

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$ by the Poly-3 test; **$p \leq 0.01$.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

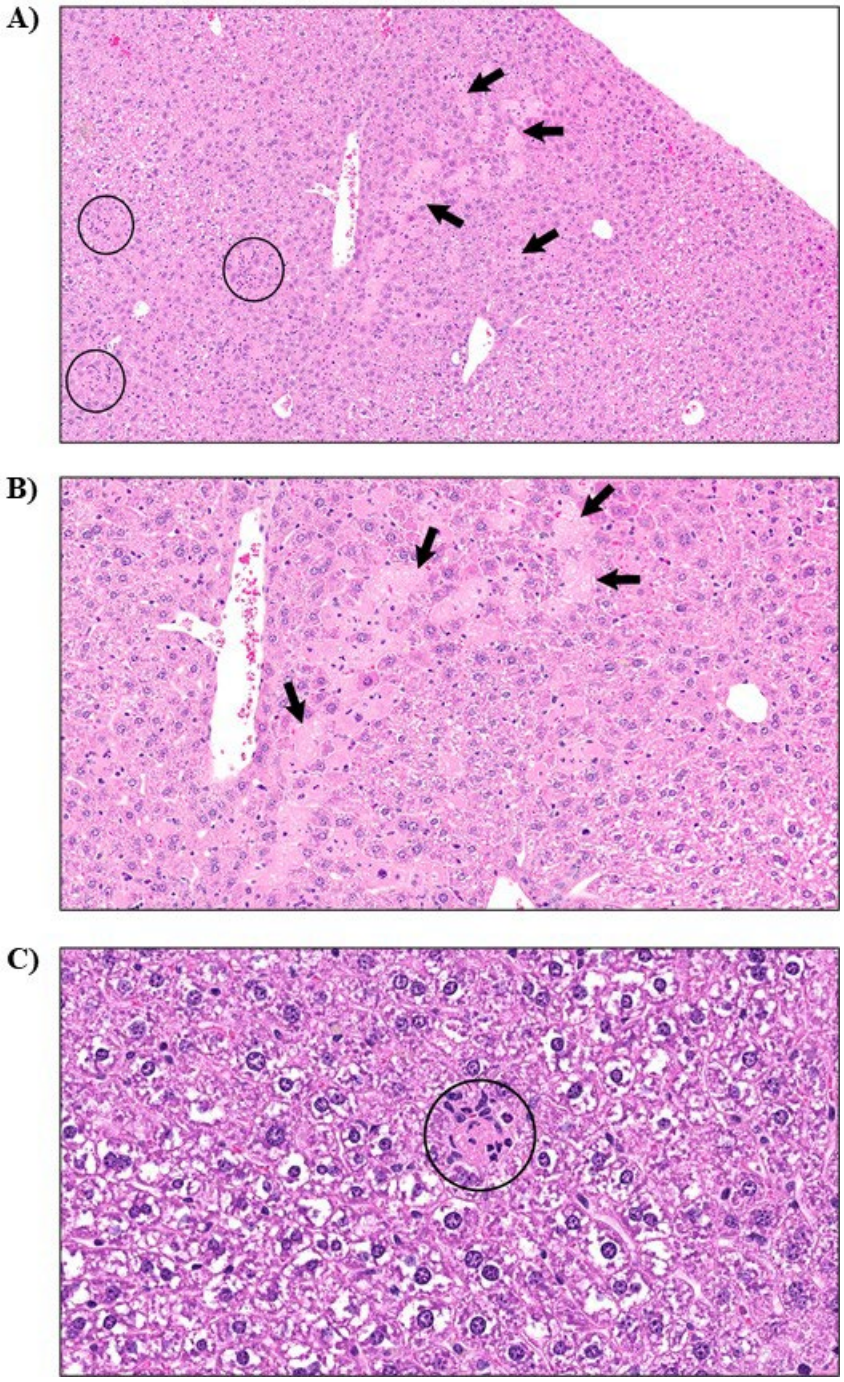

Figure 13. Representative Images of Necrosis in the Liver of a Female Mouse in the Two-year Gavage Study of Black Cohosh Root Extract (H&E)

(A) Necrosis in the liver of a 1,000 mg/kg/day mouse is shown (10×). Several small areas of hepatocellular necrosis (arrows), as well as scattered individual necrotic cells (circles), are indicated. (B) A higher magnification of the liver from panel A is shown (20×). Within the necrotic tissue, the outlines of swollen cells containing pale eosinophilic flocculant material (arrows) are apparent. These necrotic cells lack normal cytoplasmic detail, and some are devoid of a nucleus, while others contain only a small pyknotic nucleus. (C) A higher magnification of a circled area from panel A shows an individual necrotic cell with associated inflammatory cells (circle; 40×). H&E = hematoxylin and eosin stain.

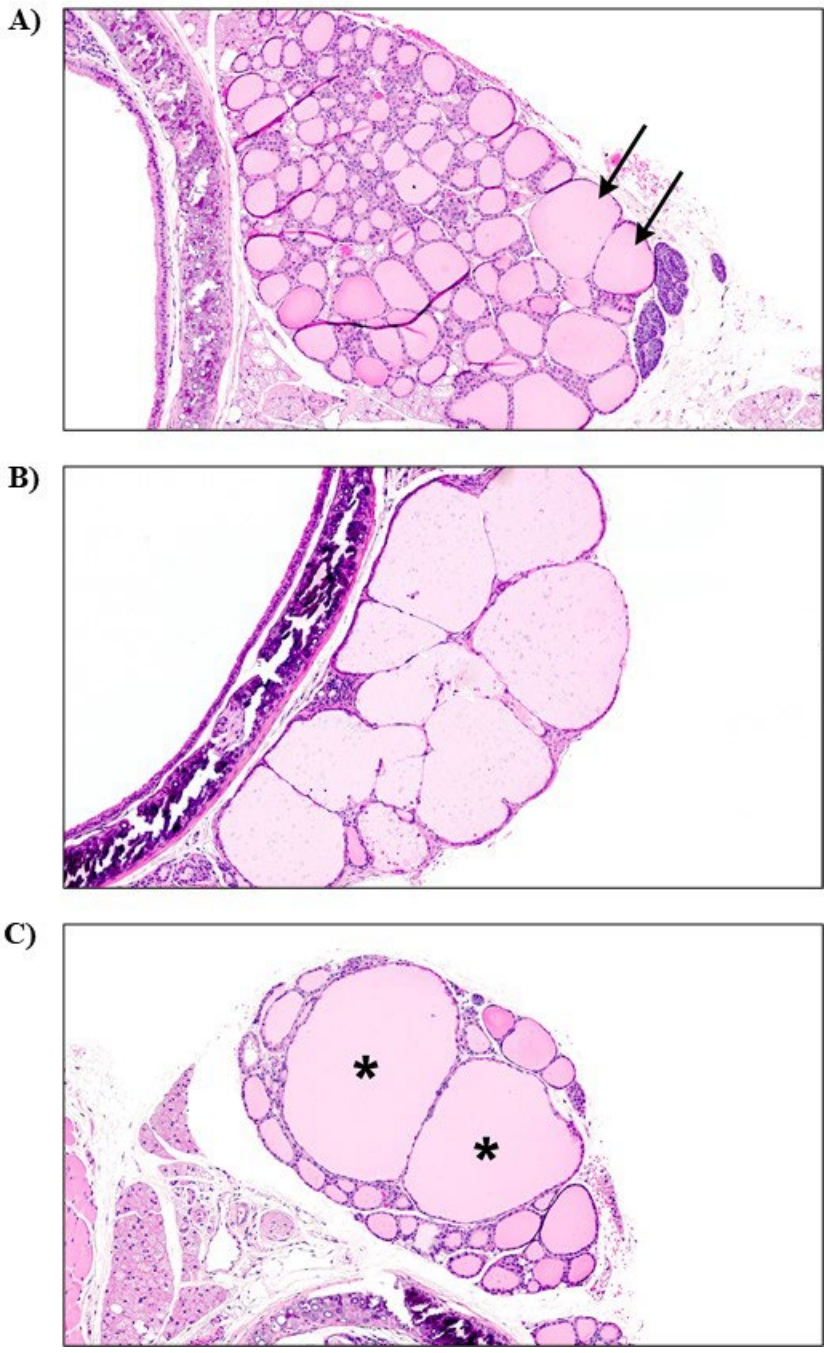

Figure 14. Representative Images of Follicular Dilation in the Thyroid of Female Mice in the Two-year Gavage Study of Black Cohosh Root Extract (H&E)

Dilated follicles are not uncommon in the thyroid glands of mice; dilation of the follicle was not recorded unless the follicles were much larger than normal. (A) In the thyroid gland of a vehicle control mouse, some follicles at the periphery of the gland (arrows) are larger than those toward the center of the gland (10×). Follicle dilation was not recorded. (B) Thyroid follicle dilation in a 1,000 mg/kg/day mouse is shown (10×). There are fewer follicles than in panel A, and those that are present are large and lined by flattened epithelial cells. (C) Another example of thyroid follicle dilation appears in a 1,000 mg/kg/day mouse (10×). Two very large follicles are present within the gland (asterisks). H&E = hematoxylin and eosin stain.

Genetic Toxicology

In addition to interim micronuclei evaluations of peripheral erythrocytes in female mice (Table 12), two different lots of BCE were tested in two independent bacterial mutagenicity assays in multiple strains of bacteria, with and without induced rodent liver S9 mixes. Results were negative in the initial assay that used both hamster and rat liver S9 over a dose range of 100–10,000 µg/plate in six strains of *Salmonella typhimurium* or a dose range of 3.3–10,000 µg/plate in *S. typhimurium* TA97 (Appendix D). In the second study, the BCE lot used in the NTP in vivo rodent studies was tested at doses up to 6,000 µg/plate in three strains of bacteria, with and without induced rat liver S9; this sample was not mutagenic in two strains of bacteria, *S. typhimurium* TA100 and *Escherichia coli* WP2 *uvrA* (pKM101), but it was judged to be equivocal in *S. typhimurium* TA98 in the presence of induced rat liver S9 (Appendix D).[168]

Data from all NTP genetic toxicity tests with BCE are available in the NTP Chemical Effects in Biological Systems database: https://doi.org/10.22427/NTP-DATA-TR-603.[164]

Discussion

Black cohosh herbal supplements, typically made from extracts of roots and rhizomes, are used by women during many stages of life: for treating premenstrual syndrome, for alleviating pain and promoting cervical ripening during pregnancy, and, most commonly, for the management of menopausal symptoms.

Given the widespread use of black cohosh and the lack of safety data regarding long-term use of black cohosh products, a 2-year NTP carcinogenicity study was conducted in rats and mice, administering black cohosh root extract (BCE) via gavage. The dosing paradigms were designed to emulate periods when humans (primarily women) might consume black cohosh products. The human equivalent doses of the BCE doses administered in the present studies, calculated using body surface area conversion factors,[169] were 12.1, 40.3, and 121 mg BCE/kg body weight/day (mg/kg/day; 726, 2,419, and 7,258 mg/day for a 60-kg person) for the rat doses of 75, 250, and 750 mg/kg/day; they were 2.4, 8.1, 24.4, and 81.3 mg/kg/day (146, 488, 1,463, and 4,878 mg/day for a 60-kg person) for the mouse doses of 30, 100, 300, and 1,000 mg/kg/day. As noted previously, clinical trials have generally evaluated lower doses of 6.5–160 mg/day for 1–12 months in women, although the formulations tested have varied.[10; 33] As black cohosh products are sometimes used during pregnancy, the rat study incorporated perinatal exposure. Female rat offspring continued to be administered BCE during the postweaning period, but male rat offspring were not. Female mice were administered BCE for 2 years starting at 5–6 weeks of age to emulate consumption of black cohosh products at an early age. A combination of nontargeted and targeted chemical analyses was used to confirm the authenticity of the test material, absence of contaminants and adulteration from other cohosh species, and similarity to a popular black cohosh product used by consumers.[123]

In rats, administration of BCE during gestation and lactation led to significant effects around the parturition period, including significant decreases in postnatal day (PND) 1 and PND 4 litter size and decreases in mean body weights of BCE-dosed dams during late gestation and early lactation. Pups appeared to gain less weight with increasing dose, and mean body weights remained lower in the highest exposed or dosed groups compared to the vehicle control groups throughout the postweaning period of the study. The difference in mean body weights between exposed or dosed animals and vehicle control animals did not change greatly during the postweaning period, indicating that body weight gain was likely consistent across groups and that the lower terminal body weight was due to lower pup body weight postweaning. No studies evaluating reproductive toxicological effects in rodents are available for comparison, including any with prenatal exposure to BCE.

The only neoplasms in rats that might have resulted from BCE exposure were the uterine squamous cell papillomas. There was a marginally higher incidence of these rare neoplasms in the mid-dose females at 250 mg/kg/day. These findings were not statistically significant, but historical control data indicate that these uterine neoplasms are rare in rats (1/500). There was a supporting finding for the nonneoplastic lesion of uterine squamous metaplasia, wherein the incidence was significantly increased in the highest dosed group of 750 mg/kg/day compared to the vehicle control group. Squamous metaplasia is a common background finding in the uterus of Sprague Dawley rats.[170] A robust dose-response relationship was not observed, however, with the uterine neoplasms in the present studies; the incidences in dosed groups compared to vehicle

control animals lacked significant pairwise differences or trends. Thus, the marginal increase in the incidence of rare uterine squamous cell papillomas was considered to be equivocal evidence of carcinogenicity in female rats.

In female mice, mean body weights were lower with increasing dose; most dosed groups exceeded a 10% difference from the vehicle control group starting around study day 141. The lack of body weight differences in BCE-dosed animals during the first few months of the study is consistent with the lack of body weight differences observed in a previous 3-month NTP study in B6C3F1/N female mice.[55] Subsequent differences in mean body weights relative to the vehicle control group suggest lower body weight gains over the course of the study period.

Complete blood count (CBC) results in female mice at 3 months revealed changes in the erythron that were consistent with previous studies[2; 55]; these changes persisted at 12 months. Specifically, with increasing dose, there was a decrease in the erythrocyte count and an increase in mean cell volume (MCV; macrocytosis) and mean cell hemoglobin with no change in the reticulocyte count (nonregenerative). In previous NTP BCE studies, increased micronuclei (Howell-Jolly bodies) and rare-to-occasional erythrocytic basophilic stippling were observed in the peripheral blood smears of female mice.[2; 55] Basophilic stippling represents ribosomal aggregates within the cytoplasm of erythrocytes and is associated with disruptions in erythropoiesis. In the current study, mild dysplasia of bone marrow metarubricytes (late-stage erythropoietic precursor cells) was observed at both 3 and 12 months and included multilobulated nuclei, micronuclei, and nuclear-to-cytoplasmic asynchrony. The abnormal morphological findings in both the peripheral blood and bone marrow smears paired with the CBC findings indicate a disruption of normal erythropoiesis. In addition, the white blood cell count and differential showed moderate decreases with increasing dose at 3 months; these decreases ameliorated by 12 months.

As previously mentioned, these combinations of hematological changes are consistent with a condition known as megaloblastic anemia, which, despite its name, is a condition that can affect all three hematopoietic cell lines. Causes of megaloblastic anemia include inherited (e.g., orotic aciduria, transcobalamin II deficiency) or drug-induced (e.g., pyrimidine antagonists, antivirals) disorders or either a dietary or functional deficiency in folate or cobalamin (vitamin B_{12}).[171; 172] In a prior NTP 3-month mouse study, circulating levels of homocysteine and methyl-malonic acid (MMA) were increased in female mice exposed to 1,000 mg/kg/day BCE.[2] Homocysteine and MMA are two biomarkers used for the diagnosis or confirmation of folate (homocysteine only) or cobalamin deficiencies (homocysteine and MMA).[171; 173] Taken in its entirety, the results from previous NTP mouse studies and the current study are consistent with a BCE-induced disruption in cobalamin metabolism (functional cobalamin deficiency); however, the exact mechanism by which BCE disrupts cobalamin metabolism is not known at this time.

Megaloblastic anemia and its characteristic hematological changes result from deranged DNA synthesis.[171] Folate and cobalamin are both involved in several interrelated metabolic cycles related to DNA and RNA synthesis, including those required for purine and thymidylate synthesis, as well as for the synthesis of methionine from homocysteine (methionine cycle).[171; 174] Thus, with folate or cobalamin deficiency, DNA synthesis is ultimately impaired. Within hematopoietic tissues, this impairment results in increased death of immature erythroid cells leading to a decrease in the erythron or anemia.[175] Surviving erythroid cells are delayed in their maturation, which leads to a larger cell size (macrocytosis) and is reflected in an increase in the

MCV. In addition, and depending on the cause, severity, and species, megaloblastic anemia will manifest with decreases in white blood cells or platelets with or without dysplastic changes (e.g., giant metamyelocytes, hypersegmented neutrophils).

Increased frequencies of micronucleated peripheral blood erythrocytes were observed in female mice administered ≥100 mg/kg/day for 3 months. This finding is consistent with a previous 3-month NTP study, which showed a significant increase in micronucleated erythrocytes in female B6C3F1/N mice starting at doses of 250 mg/kg/day.[55] Interestingly, the magnitude of the BCE effect in the micronucleus assay remained similar at the 12-month evaluation, suggesting no alteration in the genotoxicity response associated with increasing duration of BCE administration. A variety of genotoxicity studies has been conducted using the same lot of BCE as was used in this study, including bacterial mutation assays, in vitro chromosomal damage (micronucleus) assays, in vitro DNA damage (comet) assays, in vivo micronucleus assays in female rats and mice, and specialized in vitro assays that provide information on the mechanism of action. All but the bacterial mutation assays have shown significant effects.[55; 114-116] A significant increase in DNA damage as measured by the comet assay was observed by Seo et al.[116]; however, the DNA damage occurred at a high BCE concentration, was transient, and returned to near-baseline levels after 2 hours of exposure. Seo et al.[116] concluded the results did not contradict the negative conclusions for clastogenic activity drawn from other tests.[114; 115] Further, Seo et al.[116] concluded that black cohosh induces chromosomal alterations, manifested as micronuclei, via an aneugenic mechanism that involves microtubule destabilization during mitosis, as observed by Bernacki et al.[115] Rodent carcinogens constitute a high proportion of the compounds that conclusively induce micronuclei in rodents.[163] However, aneugens are not DNA-reactive and typically are assumed to have a threshold effect for carcinogenicity, such that below a certain level of exposure there is a low risk of tumor induction via a genotoxic mechanism of action.[176] Since a positive carcinogenic effect was not observed at the doses used in the present NTP studies, this threshold might not have been reached. The destabilization of tubulin induced by BCE may play a role in the induction of megaloblastic anemia observed in this and other NTP studies[2; 55] with female rats and mice exposed to BCE. Another compound, colchicine, is also associated with development of megaloblastic anemia resulting from a functional cobalamin deficiency,[115; 177] and colchicine is a well-characterized aneugen. The role of tubulin in uptake and intracellular distribution of cobalamin has not yet been characterized.[115; 178; 179] Taken together, the accumulated evidence to date points to an aneugenic, threshold mechanism of action for induction of micronuclei by BCE. Because the tubulin-destabilizing, genotoxic component of BCE has not yet been identified, it is difficult to evaluate the relevance of these genotoxic findings to humans as the internal concentrations of the active component cannot be compared.

No neoplasms were considered related to BCE administration in female mice. Nonneoplastic lesions related to BCE administration over 2 years were identified in the liver (necrosis) and thyroid gland (follicle dilation). These lesions were minimal to moderate in severity, and incidences were increased primarily in the highest dosed group (1,000 mg/kg/day). The incidence of liver necrosis (of minimal severity) was significantly increased in female mice in the highest dosed group. In previous 3-month gavage studies, mild liver necrosis was noted in female rats but not in female mice.[55] The lack of changes in biomarkers or any microscopic changes related to the liver in previous studies in B6C3F1/N mice administered the same BCE doses for 3 months,[55] combined with the minimal necrosis observed in these 2-year studies,

suggest limited hepatotoxicity of BCE in rodents. Follicular dilation in the thyroid gland is a common age-related lesion observed in rodents[180]; however, given the connection of estrogen to thyroid gland function,[181] the significant increase could be related to disrupted estrogen hormonal balance or signaling from BCE.

Conclusions

Under the conditions of these 2-year gavage studies, there was *equivocal evidence of carcinogenic activity* of black cohosh root extract (BCE) in female Hsd:Sprague Dawley® SD® rats based on marginal increases in the incidence of uterine squamous cell papillomas. There was *no evidence of carcinogenic activity* of perinatal BCE exposure in male Hsd:Sprague Dawley® SD® rats at maternal doses of 75, 250, or 750 mg/kg/day.

There was *no evidence of carcinogenic activity* of BCE in female B6C3F1/N mice at doses of 30, 100, 300, or 1,000 mg/kg/day.

Dose-related nonneoplastic lesions were observed in the uterus and ovary in rats and in the liver and thyroid gland in female mice. A significant decrease in litter size was observed in rats.

Interim hematological evaluations and micronucleus assays in female mice showed disruption of normal erythropoiesis and increased frequency of micronucleated erythrocytes at 3 months; at 12 months, the same effects were observed with similar severity and frequency.

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Appendix A. Chemical Characterization and Dose Formulation Studies

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A.1. Procurement and Characterization

A.1.1. Black Cohosh Root Extract

Black cohosh root extract (BCE) was obtained from PlusPharma, Inc. (Vista, CA) in a single lot (3012782). The lot was manufactured by Frutarom Switzerland Ltd. (Wädenswil, Switzerland). According to the manufacturer's certificate of analysis, lot 3012782 was a 50% aqueous ethanolic extract standardized to 7.8% (w/w) total triterpene glycosides. Identity, purity, and stability analyses were conducted by the study laboratory Battelle (Columbus, OH). Reports on analyses performed in support of the BCE studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

The BCE test lot was a light-brown powder. It was confirmed to be authentic black cohosh by multiple laboratories using high-performance thin-layer chromatography and DNA barcoding. Fourier transform infrared (IR) spectroscopy detected the presence of phenolic compounds, as well as plant components such as sugars, terpenes, and glycosides. Proton-induced X-ray emission spectroscopy analysis conducted by Element Analysis Corporation (Lexington, KY) indicated that the BCE was composed almost entirely of carbon (69.5%), hydrogen (11.7%), and oxygen (15.4%). Potassium (2.5%), magnesium (0.3%), and calcium (0.3%) were also detected. Water content in lot 3012782 was determined to be <7.9% via Karl Fisher analysis (Prevalere Life Sciences, Inc., Whitesboro, NY) and weight loss on drying.

Analysis of the extract by liquid chromatography (LC) coupled with quadrupole time-of-flight mass spectrometry (MS) or quadrupole ion trap MS identified up to 39 constituents belonging to triterpene glycoside, phenolic acids, and alkaloid classes. Twenty-four constituents were identified with high confidence, whereas the remaining 15 constituents were classified with tentative identification due to lower confidence stemming from lack of authentic standards, low levels present to obtain a representative spectrum, presence of multiple isomers, or less than ideal library matches. The constituents definitively identified and for which authentic standards are available (caffeic acid, ferulic acid, isoferulic acid, actein, 23-epi-26-deoxyactein [27-deoxyactein], cimifugin, allocryptopine, cimicifugoside H-1, cimiracemoside C, 26-deoxycimicifugoside, magnoflorine, and prim-o-glucosylcimifugin) were quantified by standard addition[182] followed by LC coupled with tandem mass spectrometry. Quantitation of analytes by LC-MS/MS showed that the extract contained (w/w) 0.28% caffeic acid, 0.04% ferulic acid, 0.70% isoferulic acid, 0.47% actein, 2.09% 23-epi-26-deoxyactein (27-deoxyactein), 2.13% cimiracemoside C, 0.08% 26-deoxycimicifugoside, and 0.01% magnoflorine. Cimifugin, prim-o-glucosylcimifugin, allocryptopine, and cimifugoside H-1 were not quantifiable and were estimated to be <0.01% in the extract. The measured constituents accounted for approximately 5.8% of the total extract weight.

An aliquot of the test lot was evaluated for environmental contaminants by Covance Laboratories, Inc. (Madison, WI). The heavy metals arsenic, cadmium, and lead were present at low levels, below the threshold limits for botanical dietary supplements (5, 0.3, and 10 ppm, respectively).[119; 120] Antimony and mercury were not present above the limits of quantitation. Ochratoxin and zearalenone were not present in concentrations at or above the limits of quantitation. Of >300 pesticides screened, only chlorpropham (0.012 ppm) and 2-phenylphenol (0.016 ppm) were detected, and these were present at concentrations below the lowest respective U.S. Environmental Protection Agency residue tolerance levels (0.06 and 5 ppm,

respectively).[121; 122] Aflatoxins B_1, B_2, G_1, and G_2 and the pesticide imazilil could not be accurately detected because of matrix interferences. All other pesticides were determined to be below the limits of quantitation.

A comparison of high-performance liquid chromatography (HPLC)-charged aerosol detection chromatographic profiles of BCE lot 3012782 with root extract reference material and a black cohosh product available to the public in the United States showed that the chemical profiles were similar.

Accelerated stability studies were conducted using HPLC with ultraviolet (UV) detection (Table A-1) with isoferulic acid as a marker. Samples of lot 3012782 were stored sealed in amber glass bottles for 14 days at frozen (−20°C), refrigerated (5°C), room (25°C), and elevated (60°C) temperatures. Stability was confirmed for at least 14 days at ≤25°C. Further details identifying the test lot as BCE, as well as a thorough description of its composition, are available in a separate publication by Waidyanatha et al.[123]

Six plastic bags of the BCE test lot were originally received. Each was double bagged and rolled for 5 minutes to homogenize. All bags were then transferred to 4 L plastic bottles. Samples were removed and stored to serve as analytical standards (room temperature), reference standards (−20°C), and an archive sample (−20°C). The remaining bulk test article was sealed and stored at room temperature. Reanalysis of the bulk chemical, using isoferulic acid as the marker, was performed once (mice) or three times (rats) before the start of the study, four times during the study, and one time after study termination using HPLC/UV (Table A-1). No degradation of the bulk test article was detected by analysis of the marker constituent, comparison of the constituent profile with frozen reference material, or the appearance of the bulk material.

A.1.2. Methylcellulose

Methylcellulose used to make the 0.5% aqueous vehicle for gavage formulations was obtained from Spectrum (Gardena, CA) in two lots (2CB0045 and 2AJ0439). The identity of methylcellulose was confirmed by the study laboratory using IR spectroscopy. The IR spectra were consistent with a reference spectrum from the Wiley Hummel Surfactants Library (No. 1003) (Figure A-2). Methoxy content was measured by Galbraith Laboratories, Inc. (Knoxville, TN). The average methoxy contents of lots 2CB0045 and 2AJ0439 were 31.0% and 30.8%, respectively. Methoxy content was reanalyzed at 6-month intervals during the study to determine the methoxy group content. Lot 2AJ0439 was acceptable for use. Lot 2CB0045 was approved for use by the National Toxicology Program (NTP) despite having a methoxy group value approximately 0.3% above the NTP acceptance criterion of 31.5% at multiple measurement intervals.

Water from the City of Columbus municipal supply was deionized at Battelle and used to prepare the 0.5% (w/v) aqueous methylcellulose vehicle. Other than analyses conducted by the City of Columbus to assure the acceptability of the water as a potable supply, no further analyses of the municipal water supply or the deionized water supply were performed.

A.2. Preparation and Analysis of Dose Formulations

Dose formulations of BCE (lot 3012782) in 0.5% aqueous methylcellulose were prepared at the study laboratory following the protocols outlined in Table A-2. For the rat study, formulations were prepared at 0, 15, 50, and 150 mg/mL (38 preparations; May 2012–June 2014). For the mouse study, formulations were prepared at 0, 3, 10, 30, and 100 mg/mL (36 preparations; March 2012–April 2014).

The homogeneity and stability of the dose formulations were determined by the study laboratory using HPLC/UV (Table A-1). Isoferulic acid was used as the marker for BCE formulation analysis. Homogeneity was evaluated using 3, 20, 100, and 200 mg/mL formulations and was confirmed at these doses. Stability of the 3, 30, and 100 mg/mL formulations was investigated using marker constituents actein, 27-deoxyactein, isoferulic acid, or cimiracemoside C prior to and following study termination. Stability studies were conducted at room temperature (approximately 25°C) and refrigerated temperature (approximately 5°C). Stability of the 3, 30, and 100 mg/mL dose formulations was confirmed for up to 42 days in sealed, refrigerated, amber glass bottles. In addition, a simulated animal room stability study was conducted with the 3, 30, and 100 mg/mL dose formulations stored at room temperature for 7 days and exposed to simulated usage conditions. These formulations were stable under simulated animal room conditions for at least 3 hours. The formulations for the 2-year rat and mouse studies were stored in sterile, clear glass, straight-sided bottles under inert headspace at 2°C–8°C (refrigerated).

Analyses of preadministration and postadministration dose formulations for the 2-year studies of BCE were conducted by the study laboratory using HPLC/UV (Table A-1), with isoferulic acid as the marker. All preadministration dose formulations for BCE were within 10% of the target concentrations (Table A-3, Table A-4). All postadministration samples in the rat and mouse studies were within 10% of the target concentrations, with one exception from the mouse study: the 3 mg/mL formulation concentration prepared on November 7, 2013, was below the limit of quantitation (BLOQ) when analyzed on December 12, 2013 (Table A-3, Table A-4). To investigate this result, the following samples from the same formulation batch were analyzed: an archive sample collected at the time of formulation preparation (stored frozen), a retention sample collected at the time of formulation receipt (stored frozen), and two additional bottles that had been used to dose animals (stored refrigerated). All samples were within 5% of the preadministration value.

To determine whether degradation was an issue with other formulation batches, the 3 mg/mL formulation in the next set of formulations (prepared December 19, 2013) was measured both immediately before and after administration to mice. All resulting concentrations were within 10% of the target values (Table A-4). Furthermore, all other 3 mg/mL postadministration values throughout the entire 2-year study were within 10% of the target values. Therefore, the BLOQ findings from the original animal room analysis of this batch were considered anomalous and unlikely to affect the study results.

The dose formulations were used within 42 days during the first 11 months of the studies. Before dose administration on April 13, 2013, unidentified particles resembling mold were observed in the 15 mg/mL rat dose formulation and the 3 and 10 mg/mL mouse dose formulation. To determine whether storing the formulation frozen would prevent mold growth, an experiment was conducted by storing formulations prepared May 2, 2013, at approximately −20°C in

polycarbonate jars for 26 days. The frozen formulations were then thawed and analyzed using HPLC/UV (Table A-1). All dose formulations were within 10% of the target concentrations, and the results indicated no differences between refrigerated and frozen samples (Table A-3, Table A-4). Following this finding, subsequent dose formulations were used within 21 days and included backup formulations that were stored frozen. There were no further reports of mold growth in the dose formulations during the 2-year studies.

Table A-1. Liquid Chromatography Systems Used in the Two-year Gavage Studies of Black Cohosh Root Extract

Chromatography	Detection System	Column	Mobile Phase
System A			
High-performance liquid chromatography	Ultraviolet (317 nm)	Phenomenex Hypersil Phenyl (250 mm × 4.6 mm ID, 5 µm particle size)	A: 1% Formic Acid:ACN (90:10) B: 1% Formic Acid:ACN (40:60) Gradient program: A:B 100:0 to 40:60 in 30 min; 40:60 to 0:100 in 30 min; 0:100 to 100:0 in 0.1 min, hold at 100:0 for 9.9 min 1.0 mL/min flow rate

ID = internal diameter; ACN = acetonitrile.

Table A-2. Preparation and Storage of Dose Formulations Administered to Rats and Mice in the Two-year Gavage Studies of Black Cohosh Root Extract

Preparation
For the 0 mg/mL formulation, blank 0.5% (w/v) aqueous methylcellulose vehicle was used. For remaining formulations, the appropriate amount of black cohosh root extract was weighed into a tared mixing container that had been previously calibrated to the appropriate volume necessary for the formulation concentration. With the mixing container still on the balance, enough vehicle was added to wet the test article and create a slurry without excess foaming. Vehicle was then slowly added to the mixing container to the final target weight depending on the density of each formulation. The resulting mixture was checked to confirm the final weight agreed with the final volume calibration mark on the mixing container. A stir bar was added to each mixing container, and the formulations were stirred on a stir plate for approximately 10 minutes at a speed that produced a vortex without creating excess foam. To ensure homogeneity, the formulations were capped and stirred on a stir plate for approximately 2 hours or until the batch was visibly homogenous. The formulations were dispensed into the appropriate number of labeled 250 mL (rat) or 120 mL (mouse) sterile, clear glass, straight-sided bottles. The bottles were filled with an inert headspace and sealed with Teflon®-lined lids.
Chemical Lot Number
3012782 (PlusPharma, Inc., Vista, CA)
Maximum Storage Time
42 days
Storage Conditions
Stored in sterile, clear glass, straight-sided bottles under inert headspace at 2°C to 8°C (refrigerated).
Study Laboratory
Battelle (Columbus, OH)

Table A-3. Results of Analyses of Dose Formulations Administered to Rats in the Perinatal and Two-year Gavage Study of Black Cohosh Root Extract

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
May 3, 2012	May 3, 2012	0	BLOQ	NA
		50	49.1 ± 0.8	−1.9
		100	153 ± 1	1.8
July 26, 2012	July 27, 2012	0	BLOQ	NA
		15	15.2 ± 0.1	1.3
		50	50.2 ± 0.2	0.4
		150	153 ± 1	1.8
September 20, 2012	September 20, 2012	0	BLOQ	NA
		15	15.1 ± 0.1	0.9
		50	49.8 ± 0.2	−0.4
		150	145 ± 1	−3.6
December 13, 2012	December 11, 2012	0	BLOQ	NA
		15	14.7 ± 0.1	−1.8
		50	49.5 ± 0.6	−1.0
		150	150 ± 1	0.0
February 7, 2013	February 7, 2013	0	BLOQ	NA
		15	15.2 ± 0.1	1.1
		50	50.2 ± 0.1	0.4
		150	149 ± 2	−0.4
May 2, 2013	May 2, 2013	0	BLOQ	NA
		15	15.4 ± 0.1	2.7
		50	51.3 ± 0.1	2.6
		150	152 ± 1	1.1
July 11, 2013	July 11, 2013	0	BLOQ	NA
		15	15.0 ± 0.1	−0.2
		50	49.7 ± 0.1	−0.5
		150	150 ± 1	−0.2
September 12, 2013	September 12, 2013	0	BLOQ	NA
		15	15.1 ± 0.1	0.4
		50	49.0 ± 1.5	−2.1
		150	148 ± 1	−1.6
November 7, 2013	November 6, 2013	0	BLOQ	NA
		15	15.2 ± 0.0	1.3

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
		50	50.2 ± 0.3	0.4
		150	153 ± 1	2.0
December 19, 2013	December 19, 2013	0	BLOQ	NA
		15	14.3 ± 0.3	−4.9
		50	50.5 ± 0.4	1.1
		150	146 ± 1	−2.4
January 30, 2014	January 31, 2014	0	BLOQ	NA
		15	14.9 ± 0.2	−0.9
		50	45.7 ± 0.4	−8.7
		150	164 ± 2	9.1
April 16, 2014	April 17, 2014	0	BLOQ	NA
		15	14.3 ± 0.1	−4.9
		50	49.3 ± 0.3	−1.3
		150	143 ± 1	−4.5
June 16, 2014	June 16, 2014	0	BLOQ	NA
		15	14.8 ± 0.0	−1.3
		50	50.4 ± 0.4	0.8
		150	145 ± 0	−3.3
Animal Room Samples				
May 3, 2012	June 6, 2012	0	BLOQ	NA
		15	15.0 ± 0.1	0.0
		50	49.8 ± 0.2	−0.3
		150	153 ± 0	2.0
September 20, 2012	October 25, 2012	0	BLOQ	NA
		15	15.7 ± 0.1	4.5
		50	53.0 ± 0.2	5.9
		150	155 ± 3	3.1
May 2, 2013	May 28, 2013	0	BLOQ	NA
		15	15.2 ± 0.0	1.3
		50	52.3 ± 0.3	4.7
		150	158 ± 1	5.3
		0 Freezer[c]	BLOQ	NA
		15 Freezer[c]	15.4 ± 0.1	2.4
		50 Freezer[c]	51.7 ± 1.3	3.4
		150 Freezer[c]	155 ± 2	3.1

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
November 7, 2013	December 12, 2013	0	BLOQ	NA
		15	14.9 ± 0.1	−0.9
		50	49.0 ± 0.1	−2.1
		150	155 ± 1	3.1
December 19, 2013	January 13, 2014	0	BLOQ	NA
		15	14.1 ± 0.1	−6.2
		50	51.6 ± 0.3	3.1
		150	152 ± 1	1.1
June 16, 2014	June 30, 2014	0	BLOQ	NA
		15	14.9 ± 0.0	−0.7
		50	50.0 ± 0.1	−0.1
		150	148 ± 2	−1.6

BLOQ = below the limit of quantification; NA = not applicable.
[a]Date first chromatograms were acquired.
[b]Data are presented as mean ± standard deviation.
[c]These samples were analyzed to determine whether storing the formulation frozen would prevent mold growth. This experiment was conducted after unidentified particles resembling mold were observed in formulations on April 13, 2013.

Table A-4. Results of Analyses of Dose Formulations Administered to Mice in the Two-year Gavage Study of Black Cohosh Root Extract

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
March 8, 2012	March 9, 2012	0	BLOQ	NA
		3	3.10 ± 0.01	3.3
		10	10.2 ± 0.1	2.3
		30	30.4 ± 0.1	1.3
		100	101 ± 1	1.3
May 3, 2012	May 3, 2012	0	BLOQ	NA
		3	2.88 ± 0.04	−4.0
		10	10.2 ± 0.0	2.0
		30	29.9 ± 0.0	−0.3
		100	92.2 ± 0.3	−7.8
July 26, 2012	July 27, 2012	0	BLOQ	NA
		3	2.96 ± 0.01	−1.5
		10	10.1 ± 0.0	1.0
		30	30.5 ± 0.0	1.7
		100	101 ± 0	1.0

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
September 20, 2012	September 20, 2012	0	BLOQ	NA
		3	3.00 ± 0.03	−0.1
		10	10.1 ± 0.1	1.0
		30	30.3 ± 0.2	0.9
		100	98.8 ± 2.0	−1.2
December 13, 2012	December 11, 2012	0	BLOQ	NA
		3	3.03 ± 0.03	1.0
		10	10.0 ± 0.01	0.0
		30	29.4 ± 0.3	−2.0
		100	99.8 ± 0.2	−0.2
February 7, 2013	February 7, 2013	0	BLOQ	NA
		3	3.02 ± 0.02	0.8
		10	10.1 ± 0.0	1.0
		30	30.6 ± 0.3	2.1
		100	100 ± 1	0.3
May 2, 2013	May 2, 2013	0	BLOQ	NA
		3	3.07 ± 0.02	2.5
		10	10.2 ± 0.1	2.3
		30	30.4 ± 0.1	1.3
		100	102 ± 1	2.0
July 11, 2013	July 11, 2013	0	BLOQ	NA
		3	2.92 ± 0.03	−2.7
		10	10.0 ± 0.1	0.2
		30	30.1 ± 0.1	0.2
		100	99.7 ± 0.2	−0.3
September 12, 2013	September 12, 2013	0	BLOQ	NA
		3	3.02 ± 0.02	0.8
		10	9.96 ± 0.04	−0.4
		30	30.2 ± 0.3	0.6
		100	99.6 ± 0.4	−0.4
November 7, 2013	November 6, 2013	0	BLOQ	NA
		3	2.96 ± 0.03	−1.4
		10	10.2 ± 0.1	2.3
		30	30.6 ± 0.1	2.1
		100	97.7 ± 0.3	−2.3

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
December 19, 2013	December 19, 2013	0	BLOQ	NA
		3	3.20 ± 0.02	6.7
		10	9.84 ± 0.02	−1.6
		30	28.9 ± 0.2	−3.8
		100	97.8 ± 0.2	−2.2
January 30, 2014	January 31, 2014	0	BLOQ	NA
		3	2.74 ± 0.02	−8.8
		10	10.1 ± 0.0	1.0
		30	29.0 ± 0.3	−3.3
		100	94.1 ± 2.3	−5.9
Animal Room Samples				
March 8, 2012	April 17, 2012	0	BLOQ	NA
		3	3.09 ± 0.01	3.0
		10	10.2 ± 0.01	2.3
		30	29.8 ± 0.1	−0.7
		100	102 ± 1	2.3
September 20, 2012	October 25, 2012	0	BLOQ	NA
		3	2.77 ± 0.02	−7.6
		10	10.3 ± 0.1	3.3
		30	30.8 ± 0.2	2.6
		100	104 ± 1	4.0
May 2, 2013	May 28, 2013	0	BLOQ	NA
		3	3.16 ± 0.04	5.3
		10	10.1 ± 0.1	1.3
		30	30.3 ± 0.2	0.9
		100	102 ± 1	1.7
		0 Freezer[c]	BLOQ	NA
		3 Freezer[c]	3.13 ± 0.02	4.2
		10 Freezer[c]	10.2 ± 0.0	2.0
		30 Freezer[c]	30.1 ± 0.2	0.2
		100 Freezer[c]	102 ± 1	2.3
November 7, 2013	December 12, 2013	0	BLOQ	NA
		3	BLOQ	NA
		10	9.92 ± 0.31	−0.8
		30	30.9 ± 0.1	2.9

Date Prepared	Date Analyzed[a]	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[b]	Difference from Target (%)
		100	98.3 ± 1	−1.7
		3 Archive[d]	2.91 ± 0.01	−2.9
		3 Frozen[d]	2.85 ± 0.01	−5.0
		3 Batch 37[d]	2.98 ± 0.03	−0.5
December 19, 2013	January 13, 2014	0	BLOQ	NA
		3 Pre[e]	2.94 ± 0.02	−2.0
		3 Post[f]	2.97 ± 0.00	−1.0
		10	10.3 ± 0.1	2.7
		30	30.3 ± 0.0	1.0
		100	103 ± 1	2.7

BLOQ = below the limit of quantification; NA = not applicable.
[a]Date first chromatograms were acquired.
[b]Data are presented as mean ± standard deviation.
[c]These samples were analyzed to determine whether storing the formulation frozen would prevent mold growth. This experiment was conducted after unidentified particles resembling mold were observed in formulations on April 13, 2013.
[d]These samples were analyzed following receipt of the results for the November 7, 2013, batch showing BLOQ for the 3 mg/mL formulation. The "3 Archive" sample was originally collected by the chemistry group at the time of formulation preparation and stored frozen, the "3 Frozen" sample was collected by the toxicology group at the time of formulation receipt and stored frozen, and the "3 Batch 37" sample was from additional bottles of the 3 mg/mL formulation from batch 37 that were stored refrigerated.
[e]Sample taken immediately prior to administration to animals.
[f]Sample taken after administration to animals.

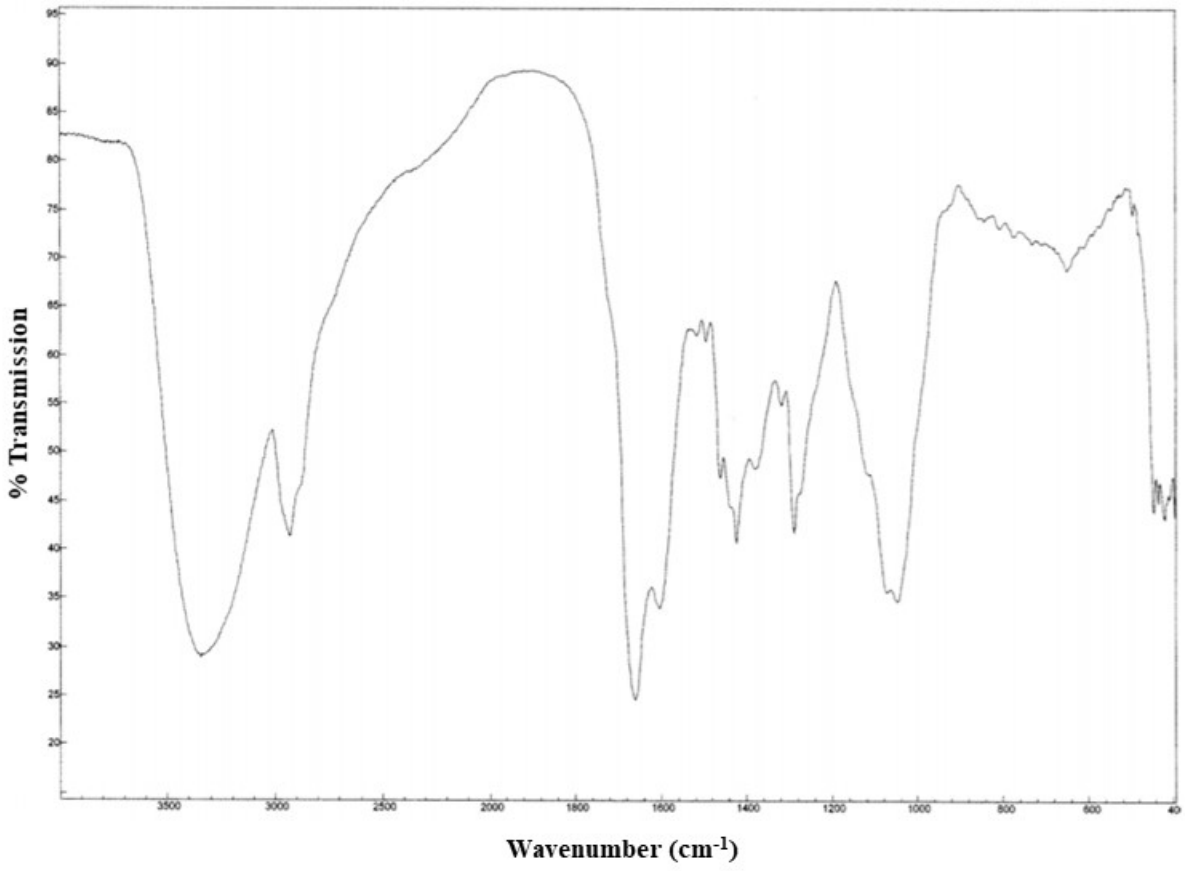

Figure A-1. Infrared Absorption Spectrum of Black Cohosh Root Extract

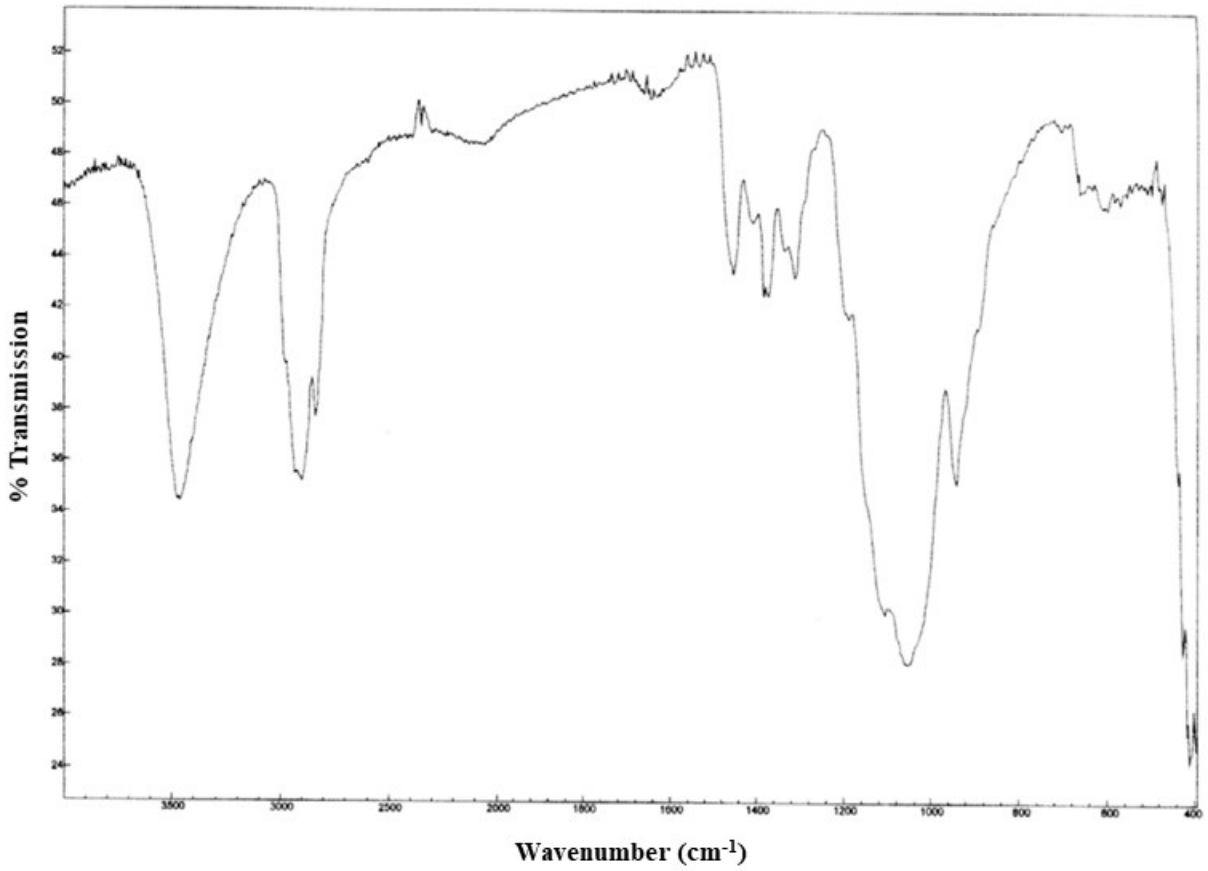

Figure A-2. Infrared Absorption Spectrum of Methylcellulose (Lot ZW0053)

Appendix B. Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and NTP-2000 Rat and Mouse Ration

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Tables

B.1. NIH-07 Feed

Table B-1. Ingredients of NIH-07 Rat Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	23.00
Ground #2 Yellow Shelled Corn	24.25
Wheat Middlings	10.0
Oat Hulls	0.0
Alfalfa Meal (Dehydrated, 17% Protein)	4.0
Purified Cellulose	0.0
Soybean Meal (47% Protein)	12.0
Fish Meal (62% Protein)	10.0
Corn Oil (without Preservatives)	0.0
Soy Oil (without Preservatives)	2.5
Dried Brewer's Yeast	2.0
Calcium Carbonate (USP)	0.5
Vitamin Premix[a]	0.25
Mineral Premix[b]	0.15
Calcium Phosphate, Dibasic (USP)	1.25
Sodium Chloride	0.5
Choline Chloride (70% Choline)	0.10
Dried Skim Milk	5.0
Dried Molasses	1.50
Corn Gluten Meal (60% Protein)	3.00
Methionine	0.0

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table B-2. Vitamins and Minerals in NIH-07 Rat Ration

	Amount[a]	Source
Vitamins		
Vitamin A	6,062 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	5,070 IU	D-activated animal sterol
Vitamin K	3.1 mg	Menadione sodium bisulfite complex
Vitamin E	22 IU	α-Tocopheryl Acetate
Niacin	33 mg	–
Folic Acid	2.4 mg	–

	Amount[a]	Source
d-Pantothenic Acid	19.8 mg	d-Calcium pantothenate
Riboflavin	3.8 mg	–
Thiamine	11 mg	Thiamine mononitrate
B_{12}	50 µg	–
Pyridoxine	6.5 mg	Pyridoxine hydrochloride
Biotin	0.15 mg	d-Biotin
Minerals		
Iron	132 mg	Iron sulfate
Zinc	18 mg	Zinc oxide
Manganese	66 mg	Manganese oxide
Copper	4.4 mg	Copper sulfate
Iodine	2.0 mg	Calcium iodate
Cobalt	0.44 mg	Cobalt carbonate

[a]Per kg of finished diet.

Table B-3. Nutrient Composition of NIH-07 Rat Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	23.3 ± 0.698	22.4–23.9	4
Crude Fat (% by Weight)	5.53 ± 0.096	5.4–5.6	4
Crude Fiber (% by Weight)	3.248 ± 0.205	3.1–3.55	4
Ash (% by Weight)	6.09 ± 0.285	5.83–6.44	4
Amino Acids (% of Total Diet)			
Arginine	1.278 ± 0.343	0.258–1.49	11
Cystine	0.307 ± 0.059	0.153–0.372	11
Glycine	1.065 ± 0.289	0.217–1.31	11
Histidine	0.482 ± 0.121	0.125–0.553	11
Isoleucine	0.914 ± 0.233	0.214–1.03	11
Leucine	1.873 ± 0.485	0.423–2.13	11
Lysine	1.140 ± 0.345	0.111–1.32	11
Methionine	0.453 ± 0.117	0.102–0.515	11
Phenylalanine	1.023 ± 0.245	0.286–1.12	11
Threonine	0.850 ± 0.228	0.168–0.961	11
Tryptophan	0.259 ± 0.064	0.076–0.326	11
Tyrosine	0.801 ± 0.200	0.209–0.894	11
Valine	1.055 ± 0.264	0.262–1.17	11

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Essential Fatty Acids (% of Total Diet)			
Linoleic	2.436 ± 0.489	0.199–3.77	11
Linolenic	0.367 ± 0.397	0.214–1.56	11
Vitamins			
Vitamin A (IU/kg)	5,438 ± 369	5,140–5,900	4
α-Tocopherol (ppm)	6,097 ± 20,067	31.36–66,600	11
Thiamine (ppm)[a]	14.65 ± 2.588	12.1–18.1	4
Riboflavin (ppm)	13.54 ± 4.438	4.2–19.8	11
Niacin (ppm)	95.02 ± 16.30	51.9–112.0	11
Pantothenic Acid (ppm)	40.69 ± 12.76	3.8–51.1	11
Pyridoxine (ppm)[a]	11.74 ± 4.81	0.42–19.7	11
Folic Acid (ppm)	2.38 ± 0.571	1.37–3.09	11
Biotin (ppm)	0.300 ± 0.187	0.0–0.638	11
B_{12} (ppb)	45.27 ± 15.14	4.0–61.6	11
Choline (as Chloride) (ppm)	1,719.0 ± 386.0	700.0–2,200.0	11
Minerals			
Calcium (%)	1.034 ± 0.048	0.966–1.08	4
Phosphorus (%)	0.871 ± 0.047	0.816–0.929	4
Potassium (%)	0.762 ± 0.226	0.088–0.88	11
Chloride (%)	0.656 ± 0.102	0.411–0.8	11
Sodium (%)	0.409 ± 0.112	0.318–0.721	11
Magnesium (%)	0.171 ± 0.053	0.0162–0.218	11
Iron (ppm)	353.3 ± 117.5	35.7–469.0	11
Manganese (ppm)	82.88 ± 27.28	3.53–104.0	11
Zinc (ppm)	58.75 ± 20.3	4.74–89.2	11
Copper (ppm)	12.91 ± 4.73	0.683–21.1	11
Iodine (ppm)	1.647 ± 1.088	0.0–3.45	11
Chromium (ppm)	3.95 ± 0.035	3.89–4.0	9
Cobalt (ppm)	0.470 ± 0.296	0.01–0.963	11

[a]As hydrochloride.

Table B-4. Contaminant Levels in NIH-07 Rat Ration

	Mean ± Standard Deviation	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.407 ± 0.093	0.315–0.535	4
Cadmium (ppm)	0.067 ± 0.011	0.057–0.082	4
Lead (ppm)	0.138 ± 0.070	0.10–0.243	4
Mercury (ppm)	0.016 ± 0.006	0.012–0.024	4
Selenium (ppm)	0.427 ± 0.068	0.337–0.493	4
Aflatoxins (ppb)[a]	<5.0	–	4
Nitrate Nitrogen (ppm)[b]	17.63 ± 7.40	10.4–24.4	4
Nitrite Nitrogen (ppm)[a,b]	<0.61	–	4
BHA (ppm)[a,c]	<1.0	–	4
BHT (ppm)[a,c]	<1.0	–	4
Aerobic Plate Count (CFU/g)	<10.0	–	4
Coliform (MPN/g)	<3.0	–	4
Escherichia coli (MPN/g)[a]	<10.0	–	4
Salmonella sp. (MPN/g)	Negative	–	4
Total Nitrosamines (ppb)[d]	6.15 ± 4.28	0.0–9.8	4
N,Nitrosodimethylamine (ppb)[d]	1.65 ± 1.97	0.0–3.9	4
N,Nitrosopyrrolidine (ppb)[d]	4.5 ± 4.14	0.0–9.8	4
Pesticides (ppm)			
α-BHC[a]	<0.01	–	4
β-BHC[a]	<0.02	–	4
γ-BHC[a]	<0.01	–	4
δ-BHC[a]	<0.01	–	4
Heptachlor[a]	<0.01	–	4
Aldrin[a]	<0.01	–	4
Heptachlor Epoxide[a]	<0.01	–	4
DDE[a]	<0.01	–	4
DDD[a]	<0.01	–	4
DDT[a]	<0.01	–	4
HCB[a]	<0.01	–	4
Mirex[a]	<0.01	–	4
Methoxychlor[a]	<0.05	–	4
Dieldrin[a]	<0.01	–	4
Endrin[a]	<0.01	–	4

	Mean ± Standard Deviation	Range	Number of Samples
Telodrin[a]	<0.01	–	4
Chlordane[a]	<0.05	–	4
Toxaphene[a]	<0.10	–	4
Estimated PCBs[a]	<0.20	–	4
Ronnel[a]	<0.01	–	4
Ethion[a]	<0.02	–	4
Trithion[a]	<0.05	–	4
Diazinon[a]	<0.10	–	4
Methyl Chlorpyrifos	0.054 ± 0.032	0.02–0.096	4
Methyl Parathion[a]	<0.02	–	4
Ethyl Parathion[a]	<0.02	–	4
Malathion	0.040 ± 0.039	0.02–0.10	4
Endosulfan I[a]	<0.01	–	4
Endosulfan II[a]	<0.01	–	4
Endosulfane Sulfate[a]	<0.03	–	4

All samples were irradiated.
BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.
[a]All values were below the detection limit. The detection limit is given as the mean.
[b]Sources of contamination include alfalfa, grains, and fish meal.
[c]Sources of contamination include soy oil and fish meal.
[d]All values were corrected for percent recovery.

B.2. NTP-2000 Feed

Table B-5. Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	23.00
Ground #2 Yellow Shelled Corn	22.44
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	4.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0

Ingredients	Percent by Weight
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table B-6. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration

	Amount[a]	Source
Vitamins		
Vitamin A	4,000 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	1,000 IU	D-activated animal sterol
Vitamin K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	–
d-Pantothenic Acid	10 mg	d-Calcium pantothenate
Riboflavin	3.3 mg	–
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	d-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished diet.

Table B-7. Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	14.6 ± 0.434	13.9–15.5	32
Crude Fat (% by Weight)	8.42 ± 0.328	7.7–9.2	32
Crude Fiber (% by Weight)	9.35 ± 0.671	8.09–11.8	32
Ash (% by Weight)	4.89 ± 0.133	4.66–5.14	32
Amino Acids (% of Total Diet)			
Arginine	0.806 ± 0.074	0.67–0.97	30
Cystine	0.220 ± 0.021	0.15–0.25	30
Glycine	0.702 ± 0.037	0.62–0.8	30
Histidine	0.341 ± 0.069	0.27–0.68	30
Isoleucine	0.548 ± 0.039	0.43–0.66	30
Leucine	1.096 ± 0.062	0.96–1.24	30
Lysine	0.070 ± 0.103	0.31–0.86	30
Methionine	0.409 ± 0.041	0.26–0.49	30
Phenylalanine	0.623 ± 0.046	0.471–0.72	30
Threonine	0.513 ± 0.041	0.43–0.61	30
Tryptophan	0.156 ± 0.026	0.1–0.2	30
Tyrosine	0.423 ± 0.065	0.28–0.54	30
Valine	0.666 ± 0.039	0.55–0.73	30
Essential Fatty Acids (% of Total Diet)			
Linoleic	3.939 ± 0.233	3.49–4.55	30
Linolenic	0.306 ± 0.030	0.21–0.368	30
Vitamins			
Vitamin A (IU/kg)	3,941 ± 664	2,820–5,450	32
Vitamin D (IU/kg)	1,000[a]	–	–
α-Tocopherol (ppm)	2,376 ± 12,602	13.6–69,100	30
Thiamine (ppm)[b]	9.86 ± 13.0	5.8–81.0	32
Riboflavin (ppm)	8.17 ± 2.792	4.2–17.5	30
Niacin (ppm)	79.19 ± 8.497	66.4–98.2	30
Pantothenic Acid (ppm)	26.33 ± 10.87	17.4–81.0	30
Pyridoxine (ppm)[b]	9.719 ± 2.018	6.44–14.3	30
Folic Acid (ppm)	1.6 ± 0.440	1.15–3.27	30
Biotin (ppm)	0.330 ± 0.097	0.2–0.704	30
B_{12} (ppb)	50.06 ± 34.24	18.3–174.0	30
Choline (as Chloride) (ppm)	2,572 ± 634	1,160–3,790	30

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Minerals			
Calcium (%)	0.906 ± 0.061	0.697–1.02	32
Phosphorus (%)	0.556 ± 0.0233	0.51–0.615	32
Potassium (%)	0.668 ± 0.287	0.626–0.733	30
Chloride (%)	0.391 ± 0.44	0.3–0.517	30
Sodium (%)	0.194 ± 0.027	0.153–0.283	30
Magnesium (%)	0.217 ± 0.053	0.185–0.49	30
Iron (ppm)	190.43 ± 36.11	135–311	30
Manganese (ppm)	50.02 ± 9.27	21.0–73.1	30
Zinc (ppm)	56.81 ± 25.25	42.5–184	30
Copper (ppm)	7.61 ± 2.46	3.21–16.3	30
Iodine (ppm)	0.514 ± 0.217	0.0–0.972	30
Chromium (ppm)	1.119 ± 1.157	0.33–3.97	30
Cobalt (ppm)	0.219 ± 0.150	0.086–0.864	30

[a]From formulation.
[b]As hydrochloride.

Table B-8. Contaminant Levels in NTP-2000 Rat and Mouse Ration

	Mean ± Standard Deviation	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.206 ± 0.045	0.143–0.312	32
Cadmium (ppm)	0.064 ± 0.080	0.0150–0.54	32
Lead (ppm)	0.161 ± 0.202	0.066–1.19	32
Mercury (ppm)	0.011 ± 0.003	0.01–0.026	32
Selenium (ppm)	0.172 ± 0.024	0.097–0.23	32
Aflatoxins (ppb)[a]	<5.0	–	32
Nitrate Nitrogen (ppm)[b]	17.47 ± 8.02	10.0–45.9	32
Nitrite Nitrogen (ppm)[a,b]	<0.61	–	32
BHA (ppm)[c]	1.12 ± 0.665	1.0–4.76	32
BHT (ppm)[c]	1.03 ± 0.156	1.0–1.88	32
Aerobic Plate Count (CFU/g)	14.844 ± 18.556	10–110	32
Coliform (MPN/g)	<3	–	32
Escherichia coli (MPN/g)[a]	<10	–	32
Salmonella sp. (MPN/g)	Negative	–	32
Total Nitrosamines (ppb)[d]	8.66 ± 4.70	0.0–19.9	32
N-Nitrosodimethylamine (ppb)[d]	1.46 ± 1.21	0.0–4.8	32
N-Nitrosopyrrolidine (ppb)[d]	7.28 ± 4.10	0.0–18.6	32

	Mean ± Standard Deviation	Range	Number of Samples
Pesticides (ppm)			
α-BHC[a]	<0.01	–	32
β-BHC[a]	<0.02	–	32
γ-BHC[a]	<0.01	–	32
δ-BHC[a]	<0.01	–	32
Heptachlor[a]	<0.01	–	32
Aldrin[a]	<0.01	–	32
Heptachlor Epoxide[a]	<0.01	–	32
DDE[a]	<0.01	–	32
DDD[a]	<0.01	–	32
DDT[a]	<0.01	–	32
HCB[a]	<0.01	–	32
Mirex[a]	<0.01	–	32
Methoxychlor[a]	<0.05	–	32
Dieldrin[a]	<0.01	–	32
Endrin[a]	<0.01	–	32
Telodrin[a]	<0.01	–	32
Chlordane[a]	<0.05	–	32
Toxaphene[a]	<0.01	–	32
Estimated PCBs[a]	<0.20	–	32
Ronnel[a]	<0.01	–	32
Ethion[a]	<0.02	–	32
Trithion[a]	<0.05	–	32
Diazinon[a]	<0.10	–	32
Methyl Chlorpyrifos	0.113 ± 0.141	0.02–0.686	32
Methyl Parathion[a]	<0.02	–	32
Ethyl Parathion[a]	<0.02	–	32
Malathion	0.099 ± 0.091	0.02–0.344	32
Endosulfan I[a]	<0.01	–	32
Endosulfan II[a]	<0.01	–	32
Endosulfan Sulfate[a]	<0.03	–	32

All samples were irradiated.
BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.
[a]All values were below the detection limit. The detection limit is given as the mean.
[b]Sources of contamination include alfalfa, grains, and fish meal.
[c]Sources of contamination include soy oil and fish meal.
[d]All values were corrected for percent recovery.

Appendix C. Sentinel Animal Program

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Tables

C.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that might affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or exposed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For these toxicology and carcinogenesis studies, blood samples were collected from each sentinel animal and allowed to clot, and the serum was separated. Additionally, fecal samples were collected and tested for endoparasites and *Helicobacter* species. All samples were processed appropriately with serology and *Helicobacter* testing performed by IDEXX BioResearch (formerly Rodent Animal Diagnostic Laboratory [RADIL], University of Missouri), Columbia, MO, for determination of the presence of pathogens. Evaluation for endo- and ectoparasites was performed in-house by the testing laboratory.

The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed (Table C-1, Table C-2).

C.2. Results

Rats: Positive for endoparasites, pinworms (*Syphacia* spp.). All other test results were negative.

Mice: All test results were negative.

Table C-1. Methods and Results for Sentinel Animal Testing in Male and Female Rats

Collection Time Points	Two-year Study						
	Quarantine[a]	Perinatal[b]	1 Month[c]	6 Months	12 Months	18 Months	Study Termination
Number Examined (Males/Females)	0/10	0/10	5/5	5/5	5/5	5/5	5/5
Method/Test							
Multiplex Fluorescent Immunoassay (MFI)							
Kilham rat virus (KRV)	−	−	−	−	−	−	−
Mycoplasma pulmonis	−	−	−	−	−	−	−
Pneumonia virus of mice (PVM)	−	−	−	−	−	−	−
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	−	−	−	−	−	−	−
Rat minute virus (RMV)	−	−	−	−	−	−	−
Rat parvo virus (RPV)	−	−	−	−	−	−	−
Rat theilovirus (RTV)	−	−	−	−	−	−	−
Sendai	−	−	−	−	−	−	−
Theiler's murine encephalomyelitis virus (TMEV)	−	−	−	−	−	−	−
Toolan's H-1	−	−	−	−	−	−	−
Immunofluorescence Assay (IFA)							
Pneumocystis carinii	NT	NT	NT	−	NT	NT	NT
In-house Evaluation							
Endoparasite evaluation (evaluation of cecal content)	−	−	−	NT	+	+	NT
Ectoparasite evaluation (evaluation of perianal surface)	−	−	−	NT	+	+	NT

− = negative; NT = not tested; + = positive.
[a]Age-matched nonpregnant females.
[b]Time-mated females that did not have a litter; 3.5 weeks after arrival.
[c]F_1 animals tested 4 weeks after start of chronic phase.

Table C-2. Methods and Results for Sentinel Animal Testing in Female Mice

Collection Time Points	Two-year Study					
	Quarantine	1 Month[a]	6 Months	12 Months	18 Months	Study Termination
Number Examined (Males/Females)	5/5	5/5	5/5	5/5	5/5	5/5
Method/Test						
Multiplex Fluorescent Immunoassay (MFI)	–	–	–	–	–	–
Ectromelia virus	–	–	–	–	–	–
Epizootic diarrhea of infant mice (EDIM)	–	–	–	–	–	–
Lymphocytic choriomeningitis virus (LCMV)	–	–	–	–	–	–
Mycoplasma pulmonis	–	–	–	–	–	–
Mouse hepatitis virus (MHV)	–	–	–	–	–	–
Mouse norovirus (MNV)	–	–	–	–	–	–
Mouse parvovirus (MPV)	–	–	–	–	–	–
Minute virus of mice (MVM)	–	–	–	–	–	–
Pneumonia virus of mice (PVM)	–	–	–	–	–	–
Reovirus (REO3)	–	–	–	–	–	–
Sendai	–	–	–	–	–	–
Theiler's murine encephalomyelitis virus (TMEV) GDVII	–	–	–	–	–	–
Immunofluorescence Assay (IFA)						
Mouse norovirus (MNV)	NT	NT	NT	NT	NT	–
Polymerase Chain Reaction (PCR)						
Helicobacter species	NT	NT	NT	NT	–	NT

– = negative; NT = not tested.
[a]Animals tested 4 weeks after start of study.

Appendix D. Genetic Toxicology

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Tables

D.1. Evaluation Protocol

National Toxicology Program (NTP) reports consider biological as well as statistical factors to determine an overall assay result. For an individual assay, the statistical procedures for data analysis are described in the following protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these samples and/or among laboratories. In such cases, all the data are critically evaluated with attention given to possible protocol variations in determining the weight of evidence for an overall conclusion of chemical activity in an assay. For in vitro assays conducted with and without exogenous metabolic activation, results obtained in the absence of activation are analyzed separately from results obtained in the presence of activation. The summary table in the abstract of this Technical Report presents the Division of Translational Toxicology's (DTT's) scientific judgment regarding the overall evidence for activity of the chemical in an assay.

D.2. Bacterial Mutagenicity

D.2.1. Bacterial Mutagenicity Test Protocol

Testing procedures were modified from those originally reported by Zeiger et al.[183] Coded samples of lot 0501-11854 of black cohosh root extract (BCE) were incubated with the *Salmonella typhimurium* (TA97, TA98, TA100, TA102, TA104, TA1535, TA1537) tester strains. Coded samples of lot 3012782, the same chemical lot used in the 3- and 12-month bioassays, were incubated with the *S. typhimurium* (TA98, TA100) and *Escherichia coli* WP2 *uvrA* (pKM101) tester strains. Incubations took place either in buffer or S9 mix (metabolic activation enzymes and cofactors from phenobarbital/benzoflavone-induced male Sprague Dawley rat liver) for 20 minutes at 37°C. Top agar supplemented with *L*-histidine (or tryptophan for the *E. coli* strain) and *d*-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- or tryptophan-independent mutant colonies arising on these plates were counted after incubation for 2 days at 37°C.

Each trial consisted of up to three plates of concurrent positive and negative controls and at least five doses of BCE. All strains were tested up to the previous assay limit dose of 10,000 µg/plate in the earlier study (Table D-1) and the current assay limit dose of 6,000 ug/plate in the recent study (Table D-2). Almost all trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine- or tryptophan-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose-related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed after chemical treatment. No minimum percentage or fold increase is required for a chemical to be judged positive or weakly positive, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

D.2.2. Results

Results were negative in the initial assay that used both hamster and rat liver S9 over a dose range of 100–10,000 µg/plate in six strains of *S. typhimurium* or a dose range of 3.3–

10,000 µg/plate in *S. typhimurium* TA97 (Table D-1). The BCE lot used in the present studies was not mutagenic in two strains of bacteria, *S. typhimurium* TA100 and *E. coli* WP2 *uvrA* (pKM101), but it was judged to be equivocal in *S. typhimurium* TA98 in the presence of induced rat liver S9 (Table D-2).

Table D-1. Mutagenicity of Black Cohosh Root Extract in Bacterial Tester Strains (Lot 0501-11854)[a]

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 30% Rat S9	With 10% Hamster S9	With 10% Hamster S9	With 30% Hamster S9
TA97										
	0	125 ± 11.0	179 ± 7.3	124 ± 5.9	157 ± 17.0	153 ± 2.1	152 ± 7.3	176 ± 20.1	154 ± 13.8	155 ± 12.8
	3.3	–	–	–	–	–	–	–	178 ± 10.0	–
	10	–	–	–	–	–	–	–	145 ± 5.8	–
	33	–	–	–	–	–	–	–	151 ± 4.0	–
	100	138 ± 7.1	165 ± 6.7	125 ± 5.2	154 ± 4.5	175 ± 9.0	152 ± 6.5	221 ± 8.2	141 ± 7.9	157 ± 8.1
	333	138 ± 6.4	190 ± 13.9	124 ± 5.7	146 ± 9.9	153 ± 13.4	158 ± 6.9	202 ± 9.4	154 ± 6.9	150 ± 12.9
	1,000	127 ± 2.3	184 ± 8.1	121 ± 6.2	161 ± 6.0	179 ± 1.0	168 ± 6.1	218 ± 5.9	–	169 ± 10.4
	3,333	142 ± 2.1	227 ± 5.6	129 ± 7.2	160 ± 4.1	171 ± 25.4	157 ± 11.9	147 ± 7.6	–	145 ± 9.1
	10,000	132 ± 6.8	191 ± 2.7	125 ± 5.8	201 ± 16.6	196 ± 6.7	160 ± 3.8	187 ± 12.9	–	133 ± 6.0
Trial Summary		Negative	Equivocal	Negative	Negative	Negative	Negative	Equivocal	Negative	Negative
Positive Control[b]		865 ± 101.2	669 ± 28.6	778 ± 74.4	1,358 ± 30.3	1,100 ± 78.8	609 ± 4.4	1,439 ± 127.9	1,384 ± 89.3	1,331 ± 37.8
TA98										
	0	14 ± 1.5	17 ± 1.7	–	22 ± 4.1	–	16 ± 1.8	28 ± 3.8	–	24 ± 4.5
	100	12 ± 2.9	23 ± 0.9	–	19 ± 3.2	–	16 ± 3.8	24 ± 4.1	–	22 ± 2.6
	333	11 ± 1.2	17 ± 2.4	–	23 ± 1.8	–	18 ± 3.3	28 ± 4.6	–	18 ± 0.9
	1,000	17 ± 3.5	20 ± 1.2	–	20 ± 3.0	–	18 ± 0.3	14 ± 1.9	–	17 ± 2.6
	3,333	15 ± 0.9	16 ± 1.2	–	24 ± 1.9	–	20 ± 3.3	25 ± 1.0	–	21 ± 0.6
	10,000	12 ± 0.9	22 ± 3.2	–	20 ± 1.9	–	15 ± 3.8	25 ± 1.5	–	24 ± 1.2
Trial Summary		Negative	Negative	–	Negative	–	Negative	Negative	–	Negative
Positive Control		101 ± 6.4	92 ± 4.0	–	329 ± 42.4	–	367 ± 19.7	1,240 ± 58.5	–	1,360 ± 27.0

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 30% Rat S9	With 10% Hamster S9	With 10% Hamster S9	With 30% Hamster S9
TA100										
	0	185 ± 5.2	199 ± 11.9	–	185 ± 7.5	–	194 ± 2.6	190 ± 16.2	–	174 ± 3.8
	100	158 ± 17.0	221 ± 8.2	–	195 ± 9.2	–	201 ± 12.9	210 ± 7.7	–	213 ± 6.4
	333	199 ± 12.3	221 ± 13.7	–	187 ± 4.5	–	217 ± 21.7	211 ± 16.3	–	199 ± 4.6
	1,000	164 ± 2.6	238 ± 5.9	–	218 ± 8.0	–	233 ± 15.5	225 ± 14.0	–	227 ± 11.8
	3,333	169 ± 1.2	192 ± 5.9	–	214 ± 27.9	–	189 ± 14.4	212 ± 5.7	–	209 ± 9.2
	10,000	173 ± 7.5	207 ± 20.5	–	219 ± 10.4	–	206 ± 10.7	243 ± 4.8	–	233 ± 4.9
Trial Summary		Negative	Negative	–	Negative	–	Negative	Negative	–	Negative
Positive Control		642 ± 25.5	668 ± 15.3	–	843 ± 58.4	–	1,450 ± 184.5	647 ± 24.4	–	1,641 ± 68.0
TA102										
	0	314 ± 7.8	209 ± 3.2	–	274 ± 3.5	–	350 ± 3.6	297 ± 11.6	–	367 ± 4.2
	100	304 ± 14.9	263 ± 17.7	–	252 ± 15.9	–	334 ± 16.2	235 ± 22.9	–	350 ± 55.2
	333	302 ± 9.8	216 ± 6.1	–	265 ± 26.4	–	374 ± 16.6	265 ± 16.7	–	376 ± 8.7
	1,000	316 ± 19.5	236 ± 13.2	–	289 ± 7.1	–	343 ± 19.9	266 ± 26.5	–	333 ± 20.8
	3,333	309 ± 4.4	178 ± 17.2	–	319 ± 20.0	–	368 ± 13.9	304 ± 19.7	–	371 ± 6.0
	10,000	269 ± 17.2	210 ± 6.9	–	317 ± 12.3	–	399 ± 14.2	266 ± 11.3	–	313 ± 11.0
Trial Summary		Negative	Negative	–	Negative	–	Negative	Negative	–	Negative
Positive Control		1,022 ± 4.5	847 ± 55.3	–	1,189 ± 98.2	–	1,034 ± 27.1	992 ± 78.4	–	1,162 ± 21.9

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 30% Rat S9	With 10% Hamster S9	With 10% Hamster S9	With 30% Hamster S9
TA104										
	0	270 ± 9.0	225 ± 4.4	–	279 ± 7.0	–	271 ± 21.9	245 ± 10.6	–	256 ± 8.5
	100	312 ± 4.5	247 ± 3.9	–	232 ± 32.4	–	255 ± 12.5	248 ± 10.1	–	271 ± 5.9
	333	311 ± 16.7	212 ± 10.7	–	221 ± 13.8	–	271 ± 4.9	211 ± 11.7	–	267 ± 24.0
	1,000	336 ± 8.5	249 ± 12.5	–	260 ± 9.7	–	284 ± 4.7	253 ± 13.9	–	250 ± 9.6
	3,333	291 ± 8.5	233 ± 4.1	–	250 ± 11.6	–	291 ± 16.8	249 ± 5.5	–	252 ± 13.9
	10,000	302 ± 15.9	223 ± 7.9	–	302 ± 13.7	–	295 ± 5.6	265 ± 6.4	–	234 ± 13.7
Trial Summary		Negative	Negative	–	Negative	–	Negative	Negative	–	Negative
Positive Control		976 ± 14.0	834 ± 20.1	–	1,470 ± 21.6	–	1,567 ± 7.2	943 ± 15.0	–	849 ± 56.7
TA1535										
	0	20 ± 4.9	14 ± 1.8	24 ± 5.1	21 ± 2.4	–	25 ± 2.9	19 ± 1.9	–	21 ± 1.9
	100	26 ± 2.1	14 ± 3.2	19 ± 1.0	25 ± 1.9	–	20 ± 1.2	21 ± 1.2	–	23 ± 3.2
	333	17 ± 2.0	14 ± 2.6	22 ± 3.8	23 ± 0.9	–	21 ± 2.7	24 ± 2.9	–	23 ± 0.7
	1,000	26 ± 1.5	14 ± 2.7	24 ± 3.5	17 ± 3.2	–	24 ± 1.8	20 ± 0.7	–	24 ± 2.6
	3,333	28 ± 2.1	15 ± 1.8	20 ± 3.5	24 ± 2.1	–	34 ± 3.5	17 ± 0.7	–	16 ± 1.7
	10,000	37 ± 3.3	11 ± 1.0	22 ± 2.7	21 ± 1.7	–	26 ± 2.4	21 ± 0.7	–	22 ± 1.0
Trial Summary		Equivocal	Negative	Negative	Negative	–	Negative	Negative	–	Negative
Positive Control		316 ± 28.7	183 ± 10.2	379 ± 27.2	221 ± 18.2	–	177 ± 6.5	99 ± 8.2	–	317 ± 35.9

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 30% Rat S9	With 10% Hamster S9	With 10% Hamster S9	With 30% Hamster S9
TA1537										
	0	–	–	–	7 ± 0.9	–	–	–	–	–
	100	–	–	–	5 ± 1.5	–	–	–	–	–
	333	–	–	–	5 ± 1.5	–	–	–	–	–
	1,000	–	–	–	4 ± 0.9	–	–	–	–	–
	3,333	–	–	–	4 ± 0.7	–	–	–	–	–
	10,000	–	–	–	7 ± 0.9	–	–	–	–	–
Trial Summary		–	–	–	Negative	–	–	–	–	–
Positive Control		–	–	–	365 ± 33.5	–	–	–	–	–

[a]Studies performed at BioReliance Corporation. Data are presented as revertants/plate (mean ± standard error) from up to three plates; 0 µg/plate served as the solvent control (dimethyl sulfoxide).
[b]The positive controls in the absence of metabolic activation were 9-aminoacridine (TA97), 4-nitro-o-phenylenediamine (TA98), sodium azide (TA100 and TA1535), mitomycin C (TA102), and methyl methane sulfonate (TA104). The positive controls for metabolic activation were 2-aminoanthracene (TA97, TA98, TA100, TA104, TA1535, and TA1537) and sterigmatocystin (TA102).

Table D-2. Mutagenicity of Black Cohosh Root Extract in Bacterial Tester Strains (Lot 3012782)[a]

Strain	Concentration (µg/plate)	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA98					
	0	14 ± 2.1	17 ± 3.0	19 ± 0.6	26 ± 0.9
	100	15 ± 4.5	16 ± 5.0	22 ± 3.4	27 ± 2.0
	250	18 ± 2.7	18 ± 1.7	30 ± 3.2	27 ± 3.1
	500	13 ± 2.2	19 ± 0.9	30 ± 1.5	28 ± 1.5
	1,500	25 ± 0.6	16 ± 3.2	33 ± 4.7	29 ± 4.7
	3,000	23 ± 5.0	18 ± 1.2	36 ± 5.4	43 ± 4.3
	6,000	26 ± 4.0	22 ± 5.5	31 ± 4.8	45 ± 3.5
Trial Summary		Negative	Negative	Equivocal	Equivocal
Positive Control[b]		621 ± 38.9	607 ± 9.0	1,471 ± 56.0	1,509 ± 27.9
TA100					
	0	83 ± 4.7	88 ± 9.0	83 ± 3.5	86 ± 8.7
	100	69 ± 6.4	76 ± 8.5	89 ± 5.0	87 ± 3.2
	250	85 ± 3.0	79 ± 3.5	87 ± 3.5	87 ± 1.2
	500	71 ± 11.4	79 ± 2.3	86 ± 3.3	91 ± 3.9
	1,500	78 ± 5.9	87 ± 5.9	85 ± 5.5	95 ± 3.9
	3,000	86 ± 3.7	83 ± 4.6	87 ± 8.8	91 ± 9.3
	6,000	74 ± 8.0	95 ± 7.9	98 ± 5.0	97 ± 2.2
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		556 ± 11.1	595 ± 10.5	444 ± 25.0	487 ± 5.0
***Escherichia coli* WP2 *uvrA* (pKM101)**					
	0	137 ± 4.6	137 ± 4.3	183 ± 6.2	205 ± 26.9
	100	140 ± 6.7	138 ± 9.3	176 ± 12.1	198 ± 7.7
	250	128 ± 6.4	138 ± 0.3	162 ± 7.6	185 ± 12.9
	500	139 ± 1.0	144 ± 5.7	158 ± 24.2	184 ± 3.3
	1,500	144 ± 2.6	129 ± 8.6	174 ± 6.7	186 ± 1.8
	3,000	130 ± 3.5	146 ± 3.3	180 ± 3.3	190 ± 5.9
	6,000	155 ± 6.5	148 ± 7.5	172 ± 1.2	201 ± 10.2
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		1,328 ± 73.7	1,662 ± 102.6	1,183 ± 79.1	1,680 ± 35.1

[a]Studies performed at Integrated Laboratory Systems, LLC. Data are presented as revertants/plate (mean ± standard error) from two plates; 0 µg/plate served as the solvent control (dimethyl sulfoxide).
[b]The positive controls in the absence of metabolic activation were 2-nitrofluorene (TA98), sodium azide (TA100), and 4-nitroquinolone-N-oxide (*E. coli*). The positive controls for metabolic activation were 2-aminoanthracene (TA98, *E. coli*) and benzo[a]pyrene (TA100).

D.3. Micronucleus Assay

D.3.1. Peripheral Blood Micronucleus Test Protocol

At the 3-month and 12-month interim evaluations of BCE, blood samples (approximately 200 µL) were collected from female mice, placed in ethylenediaminetetraacetic acid (EDTA)-coated tubes, and shipped overnight to the testing laboratory. Upon arrival, blood samples were fixed in ultracold methanol using a MicroFlowPLUS Kit (Litron Laboratories, Rochester, NY) according to the manufacturer's instructions. Fixed samples were stored in a −80°C freezer until analysis. Thawed blood samples were analyzed for frequency of micronucleated immature erythrocytes (i.e., reticulocytes or polychromatic erythrocytes [PCEs]) and mature erythrocytes (i.e., normochromatic erythrocytes [NCEs]) using a flow cytometer[184]; both the mature and the immature erythrocyte populations can be analyzed separately by employing special cell surface markers to differentiate the two cell types. In mice, both the mature and immature erythrocyte populations can be evaluated for micronucleus frequency because the mouse spleen does not sequester and eliminate damaged erythrocytes. Damaged erythrocytes achieve steady state in the peripheral blood of mice after 4 weeks of continuous exposure. Approximately 20,000 PCEs and 1×10^6 NCEs were analyzed per animal for frequency of micronucleated cells, and the percentage of immature erythrocytes (% PCE) was calculated as a measure of bone marrow toxicity resulting from BCE exposure.

Prior experience with the large number of cells scored using flow-cytometric scoring techniques[185] suggests it is reasonable to assume that the proportion of micronucleated reticulocytes is approximately normally distributed. The statistical tests selected for trend and for pairwise comparisons with the vehicle control group depend on whether the variances among the groups are equal. The Levene test at $\alpha = 0.05$ is used to test for equal variances. In the case of equal variances, linear regression is used to test for a linear trend with dose, and the Williams test[142; 143] is used to test for pairwise differences between each dosed group and the vehicle control group. In the case of unequal variances, the Jonckheere test[147] is used to test for linear trend, and the Dunn test[146] is used for pairwise comparisons of each dosed group with the vehicle control group. To correct for multiple pairwise comparisons, the p value for each comparison with the vehicle control group is multiplied by the number of comparisons made. In the event that this product is >1.00, it is replaced with 1.00. Trend tests and pairwise comparisons with the vehicle control group are considered statistically significant at $p \leq 0.025$.

In the micronucleus test, it is preferable to base a positive result on the presence of both a positive trend as well as at least one significantly elevated dosed group compared with the corresponding vehicle control group. In addition, historical control data are used to evaluate the biological significance of any observed response. Both statistical significance and biological significance are considered when arriving at a call. The presence of either a positive trend or a single significant dosed group generally results in an equivocal call. The absence of both a trend and any significant differences between dosed groups and the vehicle control group results in a negative call. Ultimately, the scientific staff determines the final call after considering the results of statistical analyses, reproducibility of any effects observed (in acute studies), and the magnitudes of those effects.

D.3.2. Results

At the 3-month interim evaluation, a significant, dose-related increase in micronucleated erythrocytes was observed (Table 12). At the 12-month evaluation, a significant, dose-related increase in the frequencies of micronucleated erythrocytes was also observed, and the magnitude of response was similar to that observed at the 3-month evaluation (Table 12). No significant changes were observed in the percentage of immature erythrocytes (% PCE) in the BCE-dosed mice at either evaluation.

Appendix E. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-TR-603.[164]

E.1. Perinatal and Two-year Study – Rats

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Individual Pup Census and Litter Weight by Sex Data
0005803_Individual_Pup_Census_and_Litter_Weight_by_Sex_Data.xlsx

E.2. Two-year Study (Three-month Interim Evaluation) – Mice

E01 – Animal Removal Summary by Treatment Group
0005804_E01_Animal_Removal_Summary_by_Treatment_Group_3_month_sac.pdf

E02 – Animals Removed from Experiment
0005804_E02_Animals_Removed_from_Experiment_3_month_sac.pdf

E03 – Growth Curves
0005804_E03_Growth_Curves_3_month_sac.pdf

E04 – Mean Body Weights and Survival Table
0005804_E04_Mean_Body_Weights_and_Survival_Table_3_month_sac.pdf

E05 – Clinical Observations Summary
0005804_E05_Clinical_Observations_Summary_3_month_sac.pdf

P02 – Incidence Rates of Neoplasms by Anatomic Site
0005804_P02_Incidence_Rates_of_Neoplasms_by_Anatomic_Site_3_month_sac.pdf

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site
0005804_P03_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_3_month_sac.pdf

P04 – Neoplasms by Individual Animal
0005804_P04_Neoplasms_by_Individual_Animal_3_month_sac.pdf

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)
0005804_P05_Incidence_Rates_of_Neoplasms_by_Anatomic_Site_Systemic_Lesions_Abridged_3_month_sac.pdf

P06 – Organ Weights Summary
0005804_P06_Organ_Weight_Summary_3_month_sac.pdf

P08 – Statistical Analysis of Primary Tumors
0005804_P08_Statistical_Analysis_of_Primary_Tumors_3_month_sac.pdf

P09 – Non-Neoplastic Lesions by Individual Animal
0005804_P09_Non-Neoplastic_Lesions_by_Individual_Animal_3_month_sac.pdf

P10 – Statistical Analysis of Non-Neoplastic Lesions
0005804_P10_Statistical_Analysis_of_Nonneoplastic_Lesions_3_month_sac.pdf

P11 – Statistical Analysis of Survival Data
0005804_P11_Statistical_Analysis_of_Survival_Data_3_month_sac.pdf

P12 – Organ Weights by Individual Animal
0005804_P12_Organ_Weights_by_Individual_Animal_3_month_sac.pdf

P13 – Organ Weights by Treatment Group
0005804_P13_Organ_Weights_by_Treatment_Group_3_month_sac.pdf

P14 – Individual Animal Pathology Data
0005804_P14_Individual_Animal_Pathology_Data_3_month_sac.pdf

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)
0005804_P17_Neoplasms_By_Individual_Animal_Systemic_Lesions_Abridged_3_month_sac.pdf

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades
0005804_P18_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_with_Average_Severity_Grades_3_month_sac.pdf

P40 – Survival Curves
0005804_P40_Survival_Curves_3_month_sac.pdf

E.3. Two-year Study (12-month Interim Evaluation) – Mice

E01 – Animal Removal Summary by Treatment Group
0005804_E01_Animal_Removal_Summary_by_Treatment_Group_12_month_sac.pdf

E02 – Animals Removed from Experiment
0005804_E02_Animals_Removed_from_Experiment_12_month_sac.pdf

E03 – Growth Curves
0005804_E03_Growth_Curves_12_month_sac.pdf

E04 – Mean Body Weights and Survival Table
0005804_E04_Mean_Body_Weights_and_Survival_Table_12_month_sac.pdf

E05 – Clinical Observations Summary
0005804_E05_Clinical_Observations_Summary_12_month_sac.pdf

P02 – Incidence Rates of Neoplasms by Anatomic Site
0005804_P02_Incidence_Rates_of_Neoplasms_by_Anatomic_Site_12_month_sac.pdf

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site
0005804_P03_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_12_month_sac.pdf

P04 – Neoplasms by Individual Animal
0005804_P04_Neoplasms_by_Individual_Animal_12_month_sac.pdf

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)
0005804_P05_Incidence_Rates_of_Neoplasms_by_Anatomic_Site_Systemic_Lesions_Abridged_12_month_sac.pdf

P06 – Organ Weights Summary
0005804_P06_Organ_Weight_Summary_12_month_sac.pdf

P08 – Statistical Analysis of Primary Tumors
0005804_P08_Statistical_Analysis_of_Primary_Tumors_12_month_sac.pdf

P09 – Non-Neoplastic Lesions by Individual Animal
0005804_P09_Non-Neoplastic_Lesions_by_Individual_Animal_12_month_sac.pdf

P10 – Statistical Analysis of Non-Neoplastic Lesions
0005804_P10_Statistical_Analysis_of_Nonneoplastic_Lesions_12_month_sac.pdf

P11 – Statistical Analysis of Survival Data
0005804_P11_Statistical_Analysis_of_Survival_Data_12_month_sac.pdf

P12 – Organ Weights by Individual Animal
0005804_P12_Organ_Weights_by_Individual_Animal_12_month_sac.pdf

P13 – Organ Weights by Treatment Group
0005804_P13_Organ_Weights_by_Treatment_Group_12_month_sac.pdf

P14 – Individual Animal Pathology Data
0005804_P14_Individual_Animal_Pathology_Data_12_month_sac.pdf

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)
0005804_P17_Neoplasms_By_Individual_Animal_Systemic_Lesions_Abridged_12_month_sac.pdf

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades
0005804_P18_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_with_Average_Severity_Grades_12_month_sac.pdf

P40 – Survival Curves
0005804_P40_Survival_Curves_12_month_sac.pdf

E.4. Hematology, Bone Marrow, and Spleen Weights for Two-year Study (3- and 12-month Interim Evaluations) – Mice

Abnormal Metarubicytes in Bone Marrow
G00058B04_Abnormal_Metarubicytes_in_Bone_Marrow.pdf

PA06 – Organ Weights Summary
0005804_PA06_Organ_Weights_Summary.pdf

PA43 – Hematology Summary
0005804_PA43_Hematology_Summary.pdf

PA50 – Bone Marrow Smear Summary
0005804_PA50_Bone_Marrow_Smear_Summary.pdf

E.5. Two-year Study – Mice

E.5.1. Data Tables

E01 – Animal Removal Summary by Treatment Group
0005804_E01_Animal_Removal_Summary_by_Treatment_Group_Core.pdf

E02 – Animals Removed from Experiment
0005804_E02_Animals_Removed_from_Experiment_Core.pdf

E03 – Growth Curves
0005804_E03_Growth_Curves_Core.pdf

E04 – Mean Body Weights and Survival Table
0005804_E04_Mean_Body_Weights_and_Survival_Table_Core.pdf

E05 – Clinical Observations Summary
0005804_E05_Clinical_Observations_Summary_Core.pdf

P02 – Incidence Rates of Neoplasms by Anatomic Site
0005804_P02_Incidence_Rates_of_Neoplasms_by_Anatomic_Site.pdf

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site
0005804_P03_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_Core.pdf

P04 – Neoplasms by Individual Animal
0005804_P04_Neoplasms_by_Individual_Animal_Core.pdf

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)
0005804_P05_Incidence_Rates_of_Neoplasms_by_Anatomic_Site_Systemic_Lesions_Abridged_Core.pdf

P08 – Statistical Analysis of Primary Tumors
0005804_P08_Statistical_Analysis_of_Primary_Tumors_Core.pdf

P09 – Non-Neoplastic Lesions by Individual Animal
0005804_P09_Non-Neoplastic_Lesions_by_Individual_Animal_Core.pdf

P10 – Statistical Analysis of Non-Neoplastic Lesions
0005804_P10_Statistical_Analysis_of_Nonneoplastic_Lesions_Core.pdf

P11 – Statistical Analysis of Survival Data
0005804_P11_Statistical_Analysis_of_Survival_Data.pdf

P14 – Individual Animal Pathology Data
0005804_P14_Individual_Animal_Pathology_Data_Core.pdf

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)
0005804_P17_Neoplasms_By_Individual_Animal_Systemic_Lesions_Abridged_Core.pdf

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades
0005804_P18_Incidence_Rates_of_Nonneoplastic_Lesions_by_Anatomic_Site_with_Average_Severity_Grades_Core.pdf

P40 – Survival Curves
0005804_P40_Survival_Curves_Core.pdf

E.5.2. Individual Animal Data

Female Individual Animal Body Weight Data All Animals
0005804_Female_Individual_Animal_Body_Weight_Data_All_Animals.xls

Female Individual Animal Clinical Observations
0005804_Female_Individual_Animal_Clinical_Observations.xls

Female Individual Animal Neoplastic Pathology Data
0005804_Female_Individual_Animal_Neoplastic_Pathology_Data.xls

Female Individual Animal Non-Neoplastic Pathology Data
0005804_Female_Individual_Animal_Nonneoplastic_Pathology_Data.xls

Female Individual Animal Survival Data
0005804_Female_Individual_Animal_Survival_Data.xls

Female Individual Animal Terminal Body Weight Data
0005804_Female_Individual_Animal_Terminal_Body_Weight_Data.xls

Individual Animal Bone Marrow Smear Data
G00058B04_Individual_Animal_Bone_Marrow_Smear_Data.xlsx

Individual Animal Hematology Data
G00058B04_Individual_Animal_Hematology_Data.xlsx

Metarubicytes Data
0005804_Metarubicytes_Data.xlsx

E.6. Genetic Toxicology

E.6.1. Two-year Study (Three-month Interim Evaluation) – Mice

G04 – In Vivo Micronucleus Summary Data
G00058C_G04_In_Vivo_Micronucleus_Summary_Data_3_Month_Interim.pdf

Individual Animal In Vivo Micronucleus Data
G00058C_Individual_Animal_In_Vivo_Micronucleus_Data_3_Month_Interim.xlsx

E.6.2. Genetic Toxicity Study G00058D

G06 Ames Summary Data
G00058D_G06_Ames_Summary_Data.pdf

E.6.3. Genetic Toxicity Study A13815

G06 Ames Summary Data
A13815_G06_Ames_Summary_Data.pdf

E.6.4. Two-year Study (12-month Interim Evaluation) – Mice

G04 – In Vivo Micronucleus Summary Data
G00058E_G04_In_Vivo_Micronucleus_Summary_Data_12_Month_Interim.pdf

Individual Animal In Vivo Micronucleus Data
G00058E_Individual_Animal_In_Vivo_Micronucleus_Data_12_Month_Interim.xls

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