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An ethanolic extract of black cohosh causes hematological changes but not estrogenic effects in female rodents

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ABSTRACT

Black cohosh rhizome (*Actaea racemosa*) is used as a remedy for pain and gynecological ailments; modern preparations are commonly sold as ethanolic extracts available as dietary supplements. Black cohosh was nominated to the National Toxicology Program (NTP) for toxicity testing due to its widespread use and lack of safety data. Several commercially available black cohosh extracts (BCE) were characterized by the NTP, and one with chemical composition closest to formulations available to consumers was used for all studies. Female B6C3F1/N mice and Wistar Han rats were given 0, 15 (rats only), 62.5 (mice only), 125, 250, 500, or 1000 mg/kg/day BCE by gavage for 90 days starting at weaning. BCE induced dose-dependent hematological changes consistent with a non-regenerative macrocytic anemia and increased frequencies of peripheral micronucleated red blood cells (RBC) in both species. Effects were more severe in mice, which had decreased RBC counts in all treatment groups and increased micronucleated RBC at doses above 125 mg/kg. Dose-dependent thymus and liver toxicity was observed in rats but not mice. No biologically significant effects were observed in other organs. Puberty was delayed 2.9 days at the highest treatment dose in rats; a similar magnitude delay in mice occurred in the 125 and 250 mg/kg groups but not at the higher doses. An additional uterotrophic assay conducted in mice exposed for 3 days to 0.001, 0.01, 0.1, 1, 10, 100 and 500 mg/kg found no estrogenic or anti-estrogenic activity. These are the first studies to observe adverse effects of BCE in rodents.

Introduction

Black cohosh (*Actaea racemosa*, previously *Cimicifuga racemosa*) is a perennial woodland plant native to North America. The rhizome has traditionally been used as a remedy for inflammatory pain, neuralgia, and many gynecological ailments, including pre-menstrual discomfort, dysmenorrhea, premature labor, "difficult" labor, pain after childbirth, and perimenopausal symptoms (American Herbal Pharmacopoeia, 2002; Dugoua et al., 2006). Because of this usage pattern, there is potential for long-term exposure of women during childbearing years and perimenopause. Modern preparations are most commonly sold as dried ethanolic extract tablets or capsules, sometimes formulated in combination with other herbs and used as dietary supplements. The recommended intake of black cohosh extract (BCE) is 40 mg/day (~0.5 mg/kg/day) (American Herbal Pharmacopoeia, 2002). Black cohosh was nominated to the National Toxicology Program (NTP) for general toxicity testing by both the National Cancer Institute and National Institute of Environmental Health Sciences due to its widespread use and lack of human or animal studies in the published literature demonstrating its safety. Both Institutes recommended that safety studies also focus on possible reproductive and developmental toxicities.

The current scientific literature suggests that black cohosh may be a liver toxicant; evaluation of its historic uses and chemical constituents suggests that it also has the potential for cardiac and reproductive toxicity. Several cases of human liver injury have been associated with black cohosh use, with pathologies ranging from transient autoimmune hepatitis (Pierard et al., 2009; Zimmermann et al., 2010) to centrilobular necrosis consistent with severe drug-induced liver injury (Guzman et al., 2009; Pierard et al., 2009). However at least two meta-analyses of black cohosh clinical data found no evidence of liver toxicity (Naser et al., 2011; Teschke and Schwarzenboeck, 2009). Only one clinical report has associated heart disease (bradycardia) with black cohosh use (McKenzie and Rahman, 2010). The use of black cohosh during labor and lactation

Abbreviations: BCE, black cohosh extract; PND, postnatal day; MN, micronucleus; RET, reticulocytes; MN-RET, micronucleated reticulocytes; MNE, micronucleated erythrocytes; SEM, standard error of the mean.

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is of concern because experimental data on reproductive and developmental effects is lacking (Dugoua et al., 2006).

While currently there is no adequate toxicity data for black cohosh, several studies have tried to elucidate its therapeutic mode of action. BCE appears to reduce luteinizing hormone secretion in ovariectomized rats but not in perimenopausal women (Chung et al., 2007; Jacobson et al., 2001; Jarry and Harnischfeger, 1985; Nappi et al., 2005; Rachon et al., 2008; Reame et al., 2008; Seidlova-Wuttke et al., 2003); demonstrations of estrogenic/anti-estrogenic activity of black cohosh are inconclusive (Jarry and Harnischfeger, 1985; Jarry et al., 2003). However, neurotransmitter activities that can modify activity of the hypothalamic-pituitary-gonadal (HPG) axis at the CNS level (reviewed by Rivier and Rivest, 1991), including dopaminergic, serotonergic, and opioid activity, have been observed in vitro (Jarry et al., 2003; Powell et al., 2008; Rhyu et al., 2006).

Given the scientific literature to date, NTP designed studies focused on subchronic toxicity of BCE with special emphasis on possible toxic effects in the liver and reproductive systems. Here we report the results of subchronic toxicity studies in female weanling rats and mice, peripheral blood micronucleus tests for evaluation of chromosomal damage in rats and mice, and uterotrophic assay in juvenile CD-1 mice, a sensitive and well characterize model of estrogenic activity (Newbold et al., 2001). The studies were conducted to characterize the general toxicity of BCE and address suspected estrogenic/anti-estrogenic activity. While typical NTP subchronic studies use adult animals, in these subchronic studies exposures were started at weaning in order to assess the effects of BCE on pubertal endpoints as well as general toxicology endpoints usually evaluated in NTP subchronic studies.

Materials and methods

Chemical characterization. Chemical procurement and characterization was conducted at Battelle Memorial Institute (Columbus, OH) under NTP contract #N01-ES-55551. Single component standards used in the chromatographic characterization were purchased from Chromadex (Irvine CA) and Sigma-Aldrich (St. Louis, Mo). The test article, black cohosh (CAS 84776-26-1) dried extract (BCE) lot number 3012782 (extracted using 1:1 ethanol/water) was obtained from PlusPharma, Inc. (Vista, CA) in six plastic bags. Three bags were randomly selected, combined in a larger high density polyethylene (HDPE) bag, and homogenized by rolling for 5 min. Each of the 3 bags was sampled and subjected to Karl Fischer, proton-induced X-ray emission (PIXE) spectroscopy, and infrared analysis. The remaining 3 bags were similarly homogenized and combined, and each of the larger bags was divided into multiple 4-L opaque HDPE bottles, which were sealed with lids and stored at approximately 25 °C. All homogeneity samples gave consistent analytical results, indicating that potassium (2.5%), magnesium (0.3%), and calcium (0.3%) were the significant inorganic components. Infrared reference spectra for black cohosh were not available, but sample spectra showed that absorption bands were consistent with the presence of phenolic compounds, as well as plant components such as sugars, terpenes, and glycosides. Karl Fischer analysis determined water content between 3.50 and 4.11%. Weight loss on drying indicated a loss of 7.9%. High performance liquid chromatographic analysis with ultraviolet detection (HPLC/UV), HPLC with evaporative light scattering detection (HPLC/ELSD), and HPLC with mass spectrometry (HPLC/MS) were used to generate chromatographic profiles and identify components using retention time matching and spectral information. Seven compounds typical of BCE, caffeic acid, ferulic acid, isoferulic acid, cimiracemoside A, cimicifugin, actein, and 27-deoxyactein were identified. Standard addition experiments (HPLC/UV detection) also confirmed the presence of caffeic acid, ferulic acid and isoferulic acid, but did not confirm the presence of formononetin in the BCE samples. For both the rat and mouse 90-day studies, the stability of the BCE test article relative to a frozen reference sample, using isoferulic acid as a marker, was determined before study start, once during the study, and following euthanization. No degradation was observed.

Dose formulations. Dose formulations for the 90-day studies were prepared by mixing the dry BCE powder in 0.5% methylcellulose (CAS 9004-67-5) purchased from Spectrum Chemical Mfg. Corp (Gardena, CA) for approximately 2 h on a stir plate at concentrations of 0, 3 (rats only), 6.25 (B6C3F1/N mice only), 12.5, 25, 50, 100 and 200 mg/mL. Formulations were stored refrigerated (approximately 5 °C), stirred at room temperature for a minimum of 2 h before use, and used within 43 days (chemical stability was established for 43 days using isoferulic acid as a marker). Pre- and post-administration analyses by liquid chromatography with UV detection (using benzophenone as an internal standard and isoferulic acid as a marker for BCE concentration) confirmed that all dose formulations varied by <4.0% from target concentrations. Dose formulations for the uterotrophic assay were prepared by mixing the dry BCE powder in corn oil.

Immature CD-1 mouse uterotrophic assay. Uterotrophic studies were conducted at the National Institute of Environmental Health Sciences (NIEHS, Research Triangle Park, NC). Animal procedures complied with NIEHS/NIH animal care guidelines. Female CD-1 mice from the NIEHS breeding colony were weaned on postnatal day 17 (PND 17) as described previously (Newbold et al., 2001). Mice consumed ad libitum fresh reverse osmosis/deionized water and NIH-31 feed (Thigpen et al., 1999). Estradiol (50 μg/kg/day, a concentration expected to produce 50% of maximum response) and/or BCE (0.001, 0.01, 0.1, 1, 10, 100 and 500 mg/kg) were administered by subcutaneous injection once a day on PND 17, 18 and 19 using a 25 gauge needle (except for the 100 and 500 mg/kg doses a 23 gauge needle was required). Mice were killed by cervical dislocation on PND 20; body and uteri weight (wet weight) were recorded.

Animals and animal husbandry for 90-day study. Since BCE dietary supplements are used almost exclusively by women, subchronic (90-day) studies were conducted in female rats and mice only. The studies were conducted in compliance with the Food and Drug Administration's Good Laboratory Practice Regulations (21 CFR 58) at Battelle Memorial Institute (Columbus, OH) under NTP contract #N01-ES-55536. Time-mated Wistar Han rats were received from Charles River Laboratories (Raleigh, NC) on gestation day (GD) 14. B6C3F1/N mouse dams with litters were received on postnatal day (PND) 8 from Taconic Farms (Germantown, NY). On arrival, rats and mice were quarantined for 13 days. The date of birth was designated PND 0. Litters were randomly standardized to a maximum of eight pups (three males and five females when possible) on PND 4, and litters containing fewer than 3 female pups were removed from the study. On PND 21, pups were weaned and dams were removed from the study. Male weanlings were also removed from the study except twenty male rats (10 per rack using 2 racks) and 3 male mice (3 per rack using 1 rack) were housed (up to three per cage for rats, one per cage for mice) in the same study room as the corresponding females to ensure the regular cyclicity of study animals. After weaning, females were randomly assigned to six treatment groups and housed five per cage. Irradiated NIH-07 (gestation and lactation phases) or NTP-2000 (13-week phase) wafer feed (Zeigler Brothers, Inc., Gardners, PA) and tap water were provided ad libitum. Animals were euthanized by carbon dioxide asphyxiation.

Animal care for all studies. Animal use was in accordance with the U.S. Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Animals were treated humanely and with regard for alleviation of pain and distress. All animals were housed in facilities accredited by the Association for Assessment