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Black Cohosh Extracts and Powders Induce Micronuclei, a Biomarker of Genetic Damage, in Human Cells

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Abstract

Black cohosh extract (BCE) is a widely used dietary supplement marketed to women to alleviate symptoms of gynecological ailments, yet its toxicity has not been well characterized. The National Toxicology Program (NTP) previously reported significant increases in micronucleated erythrocytes in peripheral blood of female Wistar Han rats and B6C3F1/N mice administered 15–1000 mg BCE/kg/day by gavage for 90 days. These animals also developed a dose-dependent non-regenerative macrocytic anemia characterized by clinical changes consistent with megaloblastic anemia. Both micronuclei (MN) and megaloblastic anemia can arise from disruption of the folate metabolism pathway. The NTP used in vitro approaches to investigate whether the NTP's test lot of BCE, BCEs from various suppliers, and root powders from BC and other cohosh species, were genotoxic in general, and to gain insight into the mechanism of action of BCE genotoxicity. Samples were tested in human TK6 lymphoblastoid cells using the In Vitro MicroFlow[®] MN assay. The NTP BCE and a BC extract reference material (XRM) were tested in the MultiFlow[®] DNA Damage assay, which assesses biomarkers of DNA damage, cell division, and cytotoxicity. The NTP BCE and several additional BCEs were tested in bacterial mutagenicity assays. All samples induced MN when cells were grown in physiological levels of folic acid. The NTP BCE and BC XRM produced activity patterns consistent with an aneugenic mode of action. The NTP

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STATEMENT OF AUTHOR CONTRIBUTIONS

SLS and KLW designed the study and analyzed the data. CDS contributed to experimental design and conducted the Ames and in vitro micronucleus tests. KGS collected the in vitro micronucleus test data. SDD and SMB contributed to experimental design, conducted the MultiFlow DNA Damage assays, and analyzed data SSA and GEK analyzed the in vitro micronucleus data. SW acquired test articles and directed chemical analyses of cohosh sample composition. SLS wrote the manuscript with contributions from SSA, SDD, GEK, CDS, SW, and KLW. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST DECLARATION

SMB and SDD are employed by Litron Laboratories. Litron has patents covering the flow cytometry-based assays described in this manuscript and sells commercial kits based on these procedures: *In Vitro* MicroFlow; and MultiFlow DNA Damage Kit—p53, γH2AX, Phospho-Histone H3.

BCE and 5 additional BCEs were negative in bacterial mutagenicity tests. These findings show that black cohosh preparations induce chromosomal damage and may pose a safety concern.

Keywords

Micronucleus assay; folic acid; *Actaea racemosa*; dietary supplement; aneugen

INTRODUCTION

The manufacture and sale of botanical products, some of which are considered by the Food and Drug Administration (FDA) to be dietary supplements, is a multibillion dollar industry in the United States. In accordance with the 1994 Dietary Supplement Health Education Act, although the FDA regulates dietary supplements based on Good Manufacturing Practices, pre-market safety assessments of dietary supplements are not required by the FDA unless they contain a "new dietary ingredient" defined as a dietary ingredient not sold in the United States in a dietary supplement before October 15, 1994" [Dietary Supplement Health and Education Act of 1994]. Since the late 1990s, the National Toxicology Program (NTP) has included botanicals in its toxicity testing program due to public concern about their safety [Abdel-Rahman et al., 2011; NTP 2017].

The roots of black cohosh (*Actaea racemosa*, previously known as *Cimicifuga racemosa*) are used as a dietary supplement. Black cohosh can be purchased in a variety of forms, but is most typically available as a black cohosh extract (BCE) that is sold as pills, tinctures, teas, and concentrates. These supplements may contain the BCE alone or BCE blended with other botanicals. BCE is among the top-ten selling botanicals in the United States, with annual sales of just under 50 million dollars each year between 2012 and 2014 [Gafner, 2016]. Black cohosh extract is marketed to older women as an alternative to hormone replacement therapy for amelioration of menopausal symptoms. It is also marketed to younger women for relief from a variety of gynecological ailments including, for example, premenstrual syndrome (PMS) and menstrual cycle irregularity. This usage pattern suggests that BCE should have estrogenic effects; however, in vivo and in vitro studies indicate that BCE neither stimulates nor inhibits estrogenic signaling [Einer-Jensen et al., 1996; Lupu et al., 2003; Mercado-Feliciano et al., 2012; Rachon et al., 2008; Seidlova-Wuttke et al., 2003]. With regard to the efficacy of BCE in reducing menopausal symptoms, a systematic review of published results from randomized, controlled trials concluded that BCE was no more effective than placebo [Laakmann et al., 2012]. It has been proposed that BCE may provide relief from menopausal symptoms to some consumers through analgesic effects or effects on mood attributable to one or more constituents of the extract [Reame et al., 2008; Rhyu et al., 2006; Zhang et al., 2017].

Black cohosh extract was nominated to the NTP by the National Cancer Institute and the National Institute for Environmental Health Sciences due to widespread exposure, a few case reports of liver toxicity, and a lack of human or animal studies demonstrating its safety [Mercado-Feliciano et al., 2012]. Short-term toxicity tests conducted by the NTP showed that BCE had no effect in the uterotrophic assay in mice nor did it affect estrous cycling in

mice or rats, although the first estrous cycle was slightly delayed in rats at the highest dose (1000 mg BCE/kg/day). However, in genotoxicity tests integrated into sub-chronic (90-day) toxicity studies, BCE significantly increased the frequency of micronucleated reticulocytes (MN-RET) in female Wistar-Han rats and MN-RET and micronucleated erythrocytes (MN-E) in female B6C3F1/N mice in a dose-dependent manner [Mercado-Feliciano et al., 2012]. Micronuclei (MN) are biomarkers of structural and/or numerical chromosomal alterations, and as such, may indicate potential for a compound to cause cancer, birth defects, or infertility. Due to these findings in both mice and rats, the NTP initiated additional studies of BCE, including a 2-year rodent cancer bioassay (presently on test). As part of the cancer bioassay, subsets of female B6C3F1/N mice were evaluated at 90 days and 1 year for induction of MN. The finding that BCE induces MN-RET and MN-E was replicated at both time points [NTP and CEBS 2017]. Lastly, a follow-up study conducted by the NTP using the same lot of BCE replicated the finding of significant induction of MN in female B6C3F1/N mice exposed to 1000 mg/kg BCE for 90 days [Cora et al., 2017].

In addition to induction of MN, rats and mice in the initial 90-day study and subsequent studies developed a non-regenerative macrocytic anemia that showed clinical characteristics of megaloblastic anemia, which arises from impairment of the folate 1-carbon metabolism pathway [Wickramasinghe, 2006]. MN can also be induced by folate or cobalamin deficiency [Everson et al., 1988; Fenech, 1999; MacGregor et al., 1997], suggesting that the adverse apical outcomes observed in rodents could be due to a shared etiology. Low folic acid (FA) is associated with increased risk for various types of cancers [Duthie, 1999] and increased risk for neural tube defects during fetal development [MRC Vitamin Study Research Group, 1991]. Thus, use of BCE products may pose a risk for genotoxic damage, as well as adverse pregnancy outcomes.

A complicating factor in the interpretation of the literature on BCE, and botanicals in general, is that the chemical composition of the tested material can vary for many reasons, including plant growth conditions, manufacturing processes, and whether the material was adulterated during the manufacturing process. Furthermore, well over 100 chemical constituents have been detected in various BCE preparations [Nikolic et al., 2015; Wang et al., 2011] and attribution of biological effects to specific constituents is an area of ongoing investigation.

In this work, we tested a variety of cohosh extracts and powders from different suppliers, including the lot procured for previous NTP studies (referred to as NTP BCE), and powders from other cohosh species that were available at the NTP in the in vitro MN assay using p53-proficient, human TK6 lymphoblastoid cells. These MN studies were conducted using standard cell culture medium containing either a supraphysiological (3000 nM) concentration of folic acid (FA), or a cell culture medium with a more physiologically relevant concentration of FA (120 nM) to model normal human folic acid levels. In addition, we investigated whether BCE might act through a clastogenic or an aneugenic mode-of-action (MoA) using the MultiFlow DNA Damage assay [Bryce et al., 2016, 2017]. Along with investigating the potential for chromosomal alterations, we evaluated the mutagenic potential of various cohosh extracts in bacterial assays (Ames test).

MATERIALS AND METHODS

Procurement and Analysis of Cohosh Samples

Cohosh samples were obtained from various suppliers (Table I). A black cohosh extract reference material (BC XRM) and vouchered botanical reference materials (VBRMs) for Chinese (*Actaea dahurica*), red (*Actaea rubra*), and yellow (*Actaea podocarpa*) cohosh root powders were obtained from ChromaDex (Irvine, CA, USA). Because the NTP BCE was selected in part due to the similarity of its chromatographic profile to that of a popular commercial black cohosh extract, Remifemin® [Mercado-Feliciano et al., 2012], Remifemin® tablets (Enzymatic Therapy Inc., Green Bay, WI) were analyzed for comparison. Standards of known cohosh constituents, including triterpene glycosides (cimifugin, prim-o-glucosylcimifugin (a.k.a. cimifugin glycoside), cimicifugoside H1, actein, 27-deoxy actein (also known as 23-epi-26-deoxyactein), cimicracemoside C, and 25-O-acetylcimigenol-3-O--D-xylopyranoside (abbreviated as acetylcimigenol xyloside) and polyphenols (isoferulic acid, ferulic acid, and caffeic acid) were obtained from either ChromaDex (Irvine, CA, USA) or Sigma-Aldrich (St Louis, MO). All samples and standards were stored at -20 °C.

All samples and Remifemin® tablets, except VBRMs of other cohosh species, were extracted using either 70:30 (for polyphenol analysis) or 80:20 (for triterpene glycoside analysis) methanol:water. Extracts were analyzed by high performance liquid chromatography (HPLC) with either charged aerosol detection (for triterpene glycosides) or with ultraviolet detection (for polyphenols) along with standards for aligning retention times. The amount of sample used for extraction was the same across all samples. The number of Remifemin® tablets used for the extraction was based on the amount of BCE on the label; tablets were ground prior to extraction. The peaks for fukinolic acid, cimicifugic acid A, and cimicifugic acid B (also polyphenols) in cohosh samples were identified by making comparisons to peaks in the profile for the black cohosh reference material in Jiang et al. [2011] that were nearly identical to our BC XRM profile. The percent (%) area under the peak (relative to the total chromatographic peak area) for the 13 constituents mentioned above was used to organize and compare cohosh samples and Remifemin® tablets using the Partek Genomics Suite 6.6 hierarchical clustering discovery tool. The analysis functions chosen for the discovery tool were Euclidean dissimilarity, the average linkage method was used for calculating the distance between two clusters, and hierarchical agglomerative clustering was used for the clustering method.

Preparation of Cohosh Samples for Genetic Toxicity Tests

Samples were stored at -20 °C prior to use. Cohosh extracts and root powders were weighed into amber vials using aseptic methods. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) was added to the vials to prepare stock concentrations of 25 mg/ml for in vitro MN assays and 60 mg/ml for bacterial mutagenicity assays. Samples were mixed in DMSO overnight at room temperature on a rocker. Serial dilutions using fresh DMSO were made on the day of use. Cohosh root powders (Table I) did not completely dissolve and were tested as suspensions. The final concentration of DMSO in cell culture did not exceed 1%.

In Vitro Micronucleus Assay

Human TK6 lymphoblastoid cells were obtained from the American Type Culture Collection® (ATCC®) (catalog # CRL-8015) and were determined to be free of mycoplasma contamination using the LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO). Cells were grown in a humidified chamber at 37 °C in the presence of $6 \pm 1\%$ CO₂.

Folic acid was dissolved in 1 M sodium bicarbonate and was added to FA-free RPMI 1640 medium (catalog # R1145, Sigma-Aldrich, St. Louis, MO) to a final concentration of either 120 or 3000 nM FA. Medium was supplemented with 0.3 g/L L-glutamine, 1% Pluronic F-68™, 0.5% sodium pyruvate, and antibiotics (penicillin at 20 Units/mL and streptomycin at 20 µg/mL). Medium was also supplemented with 10% fetal bovine serum (FBS), dialyzed to remove FA (catalog # 12105C, Sigma-Aldrich). The reduction of FA in dialyzed FBS was confirmed via testing at Quest Diagnostics™. The contribution of FA from FBS in the complete medium was < 1 nM.

TK6 cells were grown in T75 flasks containing medium with either 120 or 3000 nM FA for 72 h. Cells were transferred to 12-well plates at 2×10^5 cells/well in 3 ml medium and incubated for another 24 h (in either 120 or 3000 nM FA) before exposure to cohosh samples. Cells were initially exposed to the NTP BCE continuously for 24 h before harvest. Following this initial test, the cells were exposed to the NTP BCE with or without 10% phenobarbital/benzoflavone-induced male Sprague Dawley rat liver S9 and co-factors (S9 mix) (Moltox, Boone, NC, USA) for 4 h, then washed with PBS warmed to 37 °C, and allowed to grow in fresh media for an additional 20 h before harvest. Cells were exposed to other cohosh samples for 24 h in the absence of S9 mix. Each cohosh sample was tested in TK6 cells at 7 – 8 concentrations, selected based on results of a range finding study conducted with the NTP BCE sample. If the recommended limit of cytotoxicity ($55\% \pm 5\%$) was not reached in cells cultured in medium containing 120 nM FA, the samples were retested using higher concentrations, with 2 concentrations overlapping the initial concentration range. DMSO was the vehicle control, mitomycin C (100 ng/mL) (Sigma-Aldrich, St. Louis, MO) dissolved in water was the positive control in test conducted without S9 mix. Cyclophosphamide (3 µg/mL) (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO was used as the positive control in tests conducted in the presence of S9 mix. The osmolality and pH of the media (120 or 3000 nM FA) were not altered by the cohosh samples compared to the DMSO vehicle control (data not shown).

A flow cytometry-based high content MN and cytotoxicity assay was performed using the *In Vitro* MicroFlow kit (Litron Laboratories, Rochester, NY). Sample preparation, staining, and other methods were performed according to the manufacturer's instructions with minor modifications. Data were collected using a Becton-Dickinson FACSCalibur 2 laser, 4-color instrument. Unless limited by cytotoxicity, 20,000 ($\pm 2,000$) cells from each sample were analyzed for frequency of MN (%MN). Cytotoxicity was measured as the relative survival of cells from treated cultures compared to cells from solvent control cultures using ratios of counted nuclei to inert latex microspheres (counting beads) added to each sample.

Data points for which cell survival was less than 45% of the control were excluded from analysis. To maintain the overall significance level at 0.05, tests for trend and pairwise differences were considered statistically significant if one-sided $P < 0.025$ ($= 0.05/2$).

For %MN, Levene's test was used to determine if variances were equal across dose groups. If variances were equal, linear regression was used to test for trend and Williams' test was used to test for pairwise differences from the control group. In the presence of unequal variances, Jonckheere's test was used to test for trend and Dunn's test was used to test for pairwise differences from the control group.

A result was considered positive if the trend test was significant and at least one dose group was significantly increased compared to the control, or if 2 or more dose groups were significantly increased compared to the control. A response was considered equivocal if only the trend test was significant or a single dose group was significantly increased over the control. In the absence of either a significant trend or a significantly increased dose group, the result was considered negative.

MultiFlow DNA Damage Assay

As for the MN assay, human TK6 lymphoblastoid cells were obtained from the ATCC® (catalog # CRL-8015). Cells were grown in a humidified atmosphere at 37 °C with 5% CO₂. The culture medium consisted of RPMI 1640 with 200 µg/mL sodium pyruvate, 200 µM L-glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, and 10% heat-inactivated horse serum. On the day of treatment, cells were adjusted to 2×10^5 /mL and 198 µL were aliquoted into wells of a round-bottom 96-well plate. Solvent (DMSO) and ten concentrations of cohosh test articles were added from 100× stock solutions for a final DMSO concentration of 1% v/v. A concurrent positive control, methotrexate, was studied at ten concentrations. Top concentrations were chosen based on preliminary dose-range finding experiments (data not shown). Whereas four solvent control wells were prepared, each cohosh and methotrexate concentration was studied in duplicate.

Treated cells were incubated at 37 °C for 24 h. At 4 and 24 h time points, cells were resuspended with pipetting and 25 µL from each well were combined in a new 96-well plate containing 50 µL/well of pre-aliquoted working MultiFlow Kit reagents. As described by Bryce et al. [2016, 2017], the MultiFlow reagents produce detergent-liberated nuclei that are brought into contact with anti-gamma-H2AX-Alexa Fluor® 647 to detect DNA double strand breaks, anti-phospho-histone H3-PE as a mitotic cell marker, and anti-p53-FITC as a DNA damage response biomarker. A fluorescent nucleic acid dye and RNase, in combination with a known number of fluorescent microspheres (counting beads), provide the means to enumerate nuclei as well as cell cycle effects that include polyploidization. After at least 30 min of incubation at room temperature, flow cytometric analysis was performed using a Miltenyi Biotec MACSQuant® Analyzer 10 flow cytometer with integrated 96-well MiniSampler device. Flow cytometric parameters associated with gating logic, placement of scoring regions, and other specifics have been described in detail previously [Bryce et al., 2016, 2017].

The biomarker response data generated by the MultiFlow DNA Damage assay were analyzed using two 4-factor multinomial logistic regression models that have been described previously [Bryce et al., 2016, 2017]. Briefly, clastogen and aneugen detection algorithms have been developed from MultiFlow data generated with a diverse set of reference chemicals. The algorithms provide clastogen and aneugen probability scores for each concentration tested, and as shown previously, these models can be successfully applied to new MultiFlow data that were not part of the algorithm-building process. In this manner, MultiFlow data and associated analytics supply insights into test chemicals' likely genotoxic MoA.

Benchmark Dose (BMD) Values

The BMD and lower and upper limits (BMDL and BMDU, respectively) were calculated in BMDEExpress2 software (version 2.00765, available at <https://github.com/auerbachs/BMDEExpress-2.0>). Data were pre-filtered for response prior to being loaded into BMDEExpress2. To compare between estimated BMD values most effectively and avoid model-based biasing of BMD estimates, the data were only fit to the Hill model (US EPA Version 2.18 model executable). Settings for the BMD modeling were as follows: Maximum interactions: 250, Confidence level: 95, Constant Variance was assumed. BMD, BMDL and BMDU estimates were reported for all cohosh samples along with associated model parameters and fit statistics (fit Log Likelihood and Global Goodness of fit P-value). If models demonstrated convergent values they were considered adequate for use for the purposes of our study; however, the dose-response data for BCE sample 1B did not meet the criteria for the global goodness of fit parameter for modeling in BMDEExpress2 and the BMD values should be interpreted with caution for this sample.

Bacterial Mutagenicity Assays

Six cohosh extracts, NTP BCE (1A), 2A, BC XRM, 3B, 1C, and 1F (listed in Table I), were tested in bacterial mutagenicity assays using *Salmonella typhimurium* strains TA100 and TA98, and *Escherichia coli* strain WP2 *uvrA* pKM101. Samples were tested in triplicate using a pre-incubation protocol at 0, 187.5, 375, 750, 1500, 3000, and 6000 µg/plate, with and without induced rat liver S9 mix. Colonies were counted using an automated colony counter (Sorcerer/Ames Study Manager system, Perceptive Instruments, Surrey, UK). A complete description of the NTP testing protocol for bacterial mutagenicity assays can be accessed at <https://ntp.niehs.nih.gov/testing/types/genetic/invitro/sa/index.html>

The bacterial mutagenicity assay results were considered to be positive if a sample induced a reproducible, dose-related increase in histidine- or tryptophan-independent (revertant) colonies. Results were considered to be negative if no increase in revertant colonies was observed. Results that were not dose-related, reproducible, or lacked sufficient magnitude to support a determination of mutagenicity were considered to be equivocal.

RESULTS

Chemical Screening of Black Cohosh Samples

All samples were screened using chromatographic methods and the % area under the peak (relative to the total chromatographic peak area) for known triterpene glycosides and several polyphenols were estimated. The chemical signatures of the cohosh samples were organized in the form of a heatmap (Fig. 1). Each cell is the % area under the peak for each constituent. The Y-axis dendrogram shows 2 major clades. The top clade is populated with cohosh samples that have a black cohosh chemical signature. This clade includes the BC XRM, the BCE that was genotoxic in NTP short-term toxicity studies (NTP BCE, 1A), a second lot from the same supplier (2A), 3 lots of Remifemin® tablets (125742, 430291, and 066211), a BCE from another supplier (1D), and the black cohosh root powder sample (1G). Notably, the root powder (1G) in the top clade is clustered separately from the extracts. Although Remifemin® tablets were not tested in the MN assay or in bacterial mutagenicity tests due to solubility issues and difficulty in selecting an appropriate negative control, they are included in Figure 1 to demonstrate the similarity of a standardized manufactured product with samples available from suppliers. The bottom clade is distinct from the top clade due in part to the presence of cimifugin and cimicifugoside H1, which are constituents associated with Chinese cohosh [Jiang et al., 2011]. Therefore, extracts in the bottom clade that had been obtained by the NTP as BCEs appear to be adulterated with Chinese cohosh. Consistent with this observation, 1F was obtained as a Chinese cohosh extract and is clustered separately in the bottom clade. Taken together, Figure 1 illustrates the variability of BCEs procured at the level of the supplier, which may be due in part to adulteration with Chinese cohosh. The limited analysis of cohosh constituents conducted at the NTP does not exclude the possibility that the cohosh samples may be adulterated with other plant materials.

Micronucleus Assay: Results with NTP BCE Sample

TK6 cells were analyzed for induction of MN after 24 h of exposure to the NTP BCE, the same material that induced MN in mice and rats [Mercado-Feliciano et al., 2012]. BCE induced significant, dose-dependent increases in %MN when cells were grown in medium containing 120 nM FA (Fig. 2A). A similar response was observed when cells were grown in 3000 nM FA; however, the absolute increase in %MN was reduced compared to the magnitude of the response in 120 nM FA (Fig. 2A). In the presence of S9 mix, induction of MN was attenuated in cells grown in either FA concentration (Fig. 2B).

To help clarify whether the lower %MN observed in the presence of S9 was due to detoxification of BCE or to a shorter exposure time, cells were exposed to BCE for 4 h in the absence of S9, followed by 20 h of growth before harvest (Fig. 3). A significant, dose-dependent increase in %MN was observed in cells cultured in 120 nM FA for 4 h in the absence of S9. In contrast, only the highest dose of BCE (500 µg/ml) showed a significant, but very small induction of MN when cells were cultured in 3000 nM FA.

Also, the NTP BCE had a greater effect on induction of MN when cells were exposed for 24 h versus 4 h. For example, exposure to BCE for 24 h (120 nM FA without S9) produced a

4.8-fold increase in MN at a concentration of 125 $\mu\text{g/ml}$ (5.43 ± 0.7 %MN with BCE versus 1.12 ± 0.3 %MN for the vehicle control, Fig. 2A), while exposure to BCE for 4 h followed by 20 h in fresh medium produced a 2-fold increase in MN at the same concentration (2.28 ± 0.02 %MN with BCE versus 1.12 ± 0.3 %MN for the vehicle control, Fig. 3).

Micronucleus Assay: Results with Other Cohosh Samples

TK6 cells were exposed to various cohosh samples for 24 h (without S9) at several concentrations, with 250 $\mu\text{g/ml}$ as the top concentration. Some cohosh samples were tested at higher concentrations in 120 nM FA in order to reach the limit of cytotoxicity for the assay (55 ± 5 % reduction in cell viability). All cohosh samples induced significant increases in MN when grown in 120 nM FA (Table II). The NTP BCE and lot 2C significantly also significantly induced MN when grown in 3000 nM FA (Table II). MN data that support the positive calls for each of the cohosh samples that were tested are available in the Supporting Information, Tables 1 – 16.

Benchmark Dose Analysis: Ranking Cohosh Samples by Potency

To examine whether cohosh samples showed differences in potency, the Hill curve model in BMDEExpress2 software was used to calculate the BMD with lower (BMDL) and upper (BMDU) limits for each sample. The samples were ranked from the lowest to the highest BMDL (Fig. 4). Based on this ranking, although the samples varied in potency, the variation was less than an order of magnitude. The NTP BCE was the most potent sample. In general, root powder suspensions tended to be less potent inducers of MN than the extracts, although extract 1E was the least potent sample overall. There did not appear to be a relationship between potency and adulteration of black cohosh with Chinese cohosh in our set of samples.

MultiFlow DNA Damage Assay

To gain further insight into MoA for MN induction by these cohoshes, we tested the NTP BCE that was genotoxic in mice and rats along with the BC XRM in the MultiFlow assay. The graphs in Figure 5 show the probability at each concentration as to whether the data for γH2AX , p53 translocation, phospho-histone H3, and polyploidy are predictive of clastogenic or aneugenic MoA in the MultiFlow assay, overlaid with relative nuclei count, a measure of cytotoxicity. The dose-response data for the biomarkers that underwent computational modeling to assign MoA in Figure 5 are available in Supporting Information Figure S2. Methotrexate, an inhibitor of dihydrofolate reductase (DHFR) that was used as a positive control, showed the expected pattern of response predictive of a clastogenic MoA (Fig. 5A), with induction of γH2AX and translocation of p53 to the nucleus at 4 and 24 h accompanied by a reduction in phospho-histone H3-positive events (Supporting Information Fig. S2). In contrast, both BCEs showed a pattern of response consistent with an aneugenic MoA (Fig. 5B, C), i.e., an increase in phospho-histone H3-positive events, translocation of p53 to the nucleus at 24 h (but not 4 h), and no induction of γH2AX (Supporting Information Fig. S2). A comparison of the responses for the two BCEs and methotrexate to well-characterized aneugens, clastogens, and non-genotoxicants used to develop the 4-factor multinomial logistic regression models for aneugenicity and clastogenicity is presented in Supporting Information Figure S3.

Bacterial Mutagenicity Testing

Of the six BCEs that were tested in bacterial mutagenicity assays using *S. typhimurium* strains TA100 and TA98, and *E. coli* strain WP2 *uvrA* pKM101, with and without S9, all were negative except for the NTP BCE, which was equivocal in strain TA98 with 10% rat liver S9. In addition, no cytotoxicity was observed for the 6 samples in any strain. The results of these assays can be accessed at the NTP CEBS database [NTP CEBS, 2017].

DISCUSSION

BCE is a widely used botanical product marketed to women to alleviate symptoms of menopause and PMS. The NTP tested BCE due to widespread exposure and a lack of safety testing. Studies conducted by the NTP showed that BCE induced MN and a non-regenerative, megaloblastic anemia in mice and rats [Mercado-Feliciano et al., 2012]. Induction of MN and the type of anemia that was observed may have a shared etiology, which is impairment of the folate metabolism pathway. In this work, we showed that the sample of BCE tested by the NTP in vivo also induced MN in TK6 cells in vitro (Fig. 2, Fig. 3, and Table II). We extended this finding by testing a number of different BCEs and powders obtained from various suppliers and from different species of cohoshes in TK6 cells and found that all of these samples induced MN (Table II), and their BMDLs were not very different from the BMDL for the NTP BCE, the most potent sample (Fig. 4). Notably, all results were positive when TK6 cells were first conditioned to a concentration of folic acid (120 nM) that is more physiologically relevant to human blood levels (Table II). These results suggest that the powdered roots of cohosh plants may contain a genotoxic component that is active under a variety of preparation methods. MN are biomarkers of chromosomal damage (clastogenicity) or changes in chromosome number (aneugenicity). Using the MultiFlow assay, we found that the NTP BCE and an extract reference material (Table I) produced biomarker activation signatures indicative of aneugenic activity in TK6 cells (Fig. 5 and Supporting Information Figs. S2 and S3).

It has long been known that disruption of the folate metabolism pathway results in both induction of MN and development of non-regenerative megaloblastic anemia [reviewed in Wickramasinghe, 1995]. In this pathway, folate (vitamin B9) supplies methyl groups that are needed for DNA synthesis and DNA methylation, and cobalamin (vitamin B12) is a co-factor for an enzymatic reaction that generates intermediates that are essential for both processes. When cells are deficient for these vitamins, there is a reduction in the availability of folate-derived methyl groups for conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, which leads to incorporation of uracil into DNA in place of thymine. It is thought that this disruption of thymidylate synthesis leads to a dysregulation of nuclear and cytoplasmic maturation that underlies the megaloblastic phenotype of affected red blood cells. Excess incorporation of uracil also leads to DNA damage in the form of double strand DNA breaks that, if not repaired, can be detected as MN or as DNA migration the comet assay [Blount and Ames, 1995; Blount et al., 1997; Duthie and Hawdon, 1998; Duthie and McMillan, 1997]. Furthermore, broken chromosomes are commonly observed in metaphase spreads taken from the bone marrow of patients deficient for folate and/or cobalamin [Das et al., 1986; Heath, 1966]. Taken together, we expected

BCEs to show a clastogenic signature in the MultiFlow assay. However, both the NTP BCE and the BC XRM did not activate H2AX, and both samples produced patterns of biomarker activation that are associated with aneugenic chemicals. In contrast, methotrexate, a positive control compound that affects folate metabolism by inhibiting DHFR, produced a distinct clastogenic signature in the MultiFlow assay. That BCE might be acting through an aneugenic MoA was surprising and requires further investigation. However, this finding does not exclude the possibility that BCE affects the folate metabolism pathway, as excess FA in cell culture medium appeared to attenuate the BCE-induced MN response (Table II). Furthermore, culturing human cells in low levels of folic acid can lead to chromosome loss [Beetstra et al., 2005; Ni et al., 2010; Wang et al., 2004], and, as recently reported by the NTP, BCE may target the folate metabolism pathway by causing dysregulation of cobalamin in mice [Cora et al., 2017].

Interestingly, colchicine, an alkaloid that induces aneugenic effects by inhibiting the polymerization of microtubules, has also been associated with induction of megaloblastic anemia when used as a chemotherapeutic [Leite and Hoogstraten, 1977]. The mechanism by which colchicine induces megaloblastic anemia has not been well characterized, but may occur by disrupting uptake of cobalamin. Expression of the cubilin receptor, which takes up intrinsic factor-cobalamin complexes from the small intestine, was reduced in guinea pigs exposed to colchicine [Stopa et al., 1979] and colchicine also reduced expression of the transcobalamin receptor, which takes up transcobalamin II-cobalamin complexes from the circulation, in Caco-2 cells [Bose et al., 2007]. The cubilin and transcobalamin receptors are both endocytic receptors and may rely on microtubules for intracellular localization. Colchicine was originally extracted from the corms of *Colchicum autumnale* (autumn crocus), and has also been extracted from the tuber portion of *Gloriosa superba* L. (climbing lily), another member of the *Colchicaceae* family. To date, the *Ranunculaceae* family of plants, to which *Actaea* plants belong, has been identified as one of the top three producers of alkaloid compounds [Cordell et al., 2001], and a variety of alkaloids have been identified in black cohosh extracts [Nikolic et al., 2015]. It may be possible that roots from cohosh plants contain colchicine or a similar compound that could both disrupt uptake of cobalamin and induce micronuclei. Notably, a microarray study of liver tissue from rats treated with black cohosh extract produced a pattern of gene expression with some similarity to those produced by tubulin-binding vinca alkaloids [Einbond et al., 2012].

Although the NTP BCE was equivocal in strain TA98 (only with S9), it was negative in strain TA100 and *E. coli* WP2, and an additional 5 cohosh samples that were selected based on chemical screening, supplier, possible adulteration with Chinese cohosh, and potency in the in vitro MN assays were negative in bacterial mutagenicity tests. These results suggest that it is unlikely that BCEs contain direct acting mutagens, and are consistent with the finding that BCEs appear to induce MN through an aneugenic mode of action.

Chromatographic analyses of the various cohosh samples illustrated chemical differences among the samples and showed that some BCEs appear to be adulterated with Chinese cohosh (Fig. 1). A limitation of the current study is the inability to directly relate the amounts of the chemical markers shown in Figure 1 to the potencies of the cohosh samples shown in Figure 4, as it is not known whether dissolving the extracts and powders in DMSO

may have differentially affected the solubility of these various chemical markers in culture medium. Furthermore, a correlative analysis of each of the 13 chemical markers identified in our samples with potencies in the MN assay may be uninformative, as more than 100 constituents have been reported to be present in BCEs [Nikolic et al., 2015; Wang et al., 2011], and the possibility remains that an unknown but active constituent could co-vary with a known, but inactive, constituent.

Despite the difficulty in relating what is known about the chemical composition of the cohosh samples to results in the MN assay, it should be noted that caffeic acid and ferulic acid, 2 of the 13 chemical markers used to characterize the cohosh samples (Fig. 1), have been tested for genotoxicity. Caffeic acid, classified by the International Agency for Research on Cancer as “possibly carcinogenic to humans” (Group 2B) [IARC Working Group, 1993], was positive for induction of micronucleated polychromatic erythrocytes in male B6C3F1/N mice exposed by intraperitoneal injection in NTP studies [NTP CEBS, 2017]. Results for the genotoxic effects of caffeic acid in vitro have been mixed. Caffeic acid induced MN in rat hepatoma cells, but was negative in the comet assay [Maistro et al., 2011], and showed little to no induction of MN in human HepG2 or Hep3B cells [Majer et al., 2004]. Ferulic acid induced MN in rat hepatoma cells [Maistro et al., 2011]. It may be possible that these constituents were responsible for the observed induction of MN by BCE in TK6 cells; however, both caffeic and ferulic acid also induced chromosomal aberrations in Chinese hamster ovary cells (cytotoxicity levels not reported) [Stich et al., 1981], suggesting a clastogenic mode of action for these chemicals, in contrast to the biomarker signatures consistent with aneugenicity that were produced by the BCEs in the MultiFlow assay. Given the considerable chemical heterogeneity of cohosh preparations, it is unlikely that caffeic or ferulic acid were present in quantities similar to the concentrations of these chemicals that were required to induce genotoxic effects in vivo or in vitro.

While cohosh materials obtained at the level of the supplier may reasonably be suspected to have genotoxic activity, it remains to be investigated whether this observation can be extended to commercial cohosh products, such as pills, tinctures, powders, or teas. Our results also suggest that there may be species-specific detoxification of the genotoxic component, as the induction of MN by the NTP BCE was attenuated in the presence of rat S9 (Fig. 2B) and in the in vivo studies conducted by the NTP, rats were less affected than mice with regard to induction of MN and anemia [Mercado-Feliciano et al., 2012]. The recommended daily dose for BCE is typically 40 mg/day, or 0.67 mg/day for a 60 kg woman. The lowest dose at which MN were significantly induced in mice in the NTP studies was 250 mg/kg/day [Mercado-Feliciano et al., 2012]. Using allometric scaling [Reagan-Shaw et al., 2008], the human equivalent dose is 20 mg/kg, which is 30-fold greater than the recommended human dose. Presently, the NTP is collaborating with the NIEHS Clinical Research Unit to investigate whether the effects observed in animal studies might also be observed in women who routinely use BCE products. This cross-sectional study is evaluating the effects of BCE exposure using several endpoints, including frequencies of MN-RETs, levels of folate and cobalamin, and a number of hematological measures. BCE products used by study participants will be analyzed by the NTP, and future analyses may potentially include a more comprehensive panel of constituents in cohosh preparations.

More detailed data on the chemical composition of cohosh extracts and powders may be helpful in identifying the genotoxic constituent(s) in these complex mixtures.

CONCLUSIONS

Little is known about the potential toxicity of botanical dietary supplements such as black cohosh preparations, and differences in the chemical composition of similar botanical products complicates the interpretation and extension of experimental findings. Taken together, the results of our in vitro and previous in vivo tests indicate that root preparations, both powders and extracts, from different species of cohosh plants contain a genotoxicant that can alter chromosomal number, and may pose a safety concern for users of black cohosh-containing botanical products, particularly for women of child-bearing age.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Heatmap analysis of cohosh samples using hierarchical clustering. Each cell depicts the % area under the peak for the 13 constituents used to characterize cohosh samples.

Fig. 2.

TK6 cells were incubated with the NTP BCE for 24 h in cell culture medium containing either 120 or 3000 nM FA in the absence (**A**) or presence (**B**) of S9. Each concentration was evaluated using triplicate wells. Error bars represent one standard error above the mean.

Significant trend tests: 120 nM FA, $P = 0.004$; 3000 nM FA, $P < 0.001$; 120 nM FA with S9, $P < 0.001$. For pairwise comparisons, * $P < 0.025$, ** $P < 0.01$

Fig. 3.

TK6 cells were incubated with the NTP BCE for 4 h in cell culture medium containing either 120 or 3000 nM FA. Each concentration was evaluated using duplicate wells. Error bars represent one standard error above the mean. Significant trend test: 120 nM FA, $P < 0.001$. For pairwise comparisons, * $P < 0.025$, ** $P < 0.01$

Fig. 4. BMD analysis of cohosh sample dose-response curves ranked in order of lowest to highest BMDL. *The dose-response data for BCE sample 1B did not meet the criteria for the goodness of fit parameter when analyzed using BMDExpress2 software.

Fig. 5.

TK6 cell MultiFlow DNA Damage assay predictive algorithm results at each concentration for γ H2AX, phospho-histone H3, p53 translocation, and polyploidy after 4 or 24 h of exposure, and cytotoxicity after 24 h of exposure. Methotrexate is shown as a positive control for clastogenicity (A), NTP BCE (B) and BC XRM (C). Assays conducted with TK6 cells.

Table I

Test article, supplier, lot number, and identifier

Powdered Extracts	Supplier	Lot Number	Identifier
<i>Actaearacemosa</i> (black cohosh), XRM ^a	ChromaDex	ASB-00030148-005	BC XRM
Black cohosh ^b	PlusPharma, Inc.	3012782	1A (NTP BCE)
Black cohosh	Extracts Plus, Inc.	3012781	2A
Black cohosh, 2.5% triterpene glycosides	Gojira Fine Chemicals, LLC	331501	1B
Black cohosh, 2.5% triterpene glycosides	Gojira Fine Chemicals, LLC	331502	2B
Black cohosh, 2.5% triterpene glycosides	Gojira Fine Chemicals, LLC	331503	3B
Black cohosh, 2.5% triterpene glycosides	Maypro Industries, LLC	BC15240103	1C
Black cohosh, 2.5% triterpene glycosides	Maypro Industries, LLC	TP20141210-R	2C
Black cohosh, 2.5% triterpene glycosides	Spectrum Chemicals	RP0712	1D
Black cohosh	BOC Sciences	B14J0808	1E
Black cohosh, 2.5% triterpene glycosides	Amax NutraSource, Inc.	BCO2.5-A2401090310	1F
Root Powders	Supplier	Lot Number	
Black cohosh	Stryka Botanicals Co., Inc.	01541C	1G
<i>Acteadahurica</i> (Chinese cohosh), VBRM ^c	ChromaDex	ASB-00030669-134	CC VBRM
<i>Actaearubra</i> (red cohosh), VBRM	ChromaDex	ASB-00030669-134	RC VBRM
<i>Actaeapodocarpa</i> (yellow cohosh), VBRM	ChromaDex	ASB-00030837-051	YC VBRM

^aExtract reference material^bSample that produced a dose-dependent, significant induction of MN in mice and rats in NTP studies [Mercado-Feliciano et al., 2012]^cVouchered botanical reference material

Table II

Micronucleus assay results for black cohosh samples (3000 versus 120 nM folic acid in cell culture medium)

Identifier – Powdered Extracts	Cohosh Chemical Signature ^a	3000 nM FA Medium ^b	120 nM FA Medium ^b	120 FA Medium ^c	Conclusion
BC XRM	Black	Negative	Negative ^d	Positive	Positive
1A (NTP BCE ⁶)	Black	Positive	Positive	Positive	Positive
2A	Black	Equivocal	Positive	Positive	Positive
1B	Black & Chinese	Negative	Positive	Positive	Positive
2B	Black & Chinese	Equivocal	Positive	Positive	Positive
3B	Black & Chinese	Equivocal	Positive	Positive	Positive
1C	Black & Chinese	Equivocal	Positive	Positive	Positive
2C	Black & Chinese	Positive	Positive	Positive	Positive
1D	Black & Chinese	Negative	Equivocal ^d	Positive	Positive
1E	Black & Chinese	Negative	Negative ^d	Positive	Positive
1F	Chinese	Negative	Negative ^d	Positive	Positive
Identifier – Root Powders					
1G	Black	Negative	Negative ^d	Positive	Positive
CC VBRM	Chinese	Equivocal	Positive	Positive	Positive
RC VBRM	Red	Negative	Positive	Positive	Positive
YC VBRM	Yellow	Negative	Negative ^d	Positive	Positive

^aTentative classification based on a limited analysis

^b24 h exposure; 10, 25, 50, 75, 100, 125, 175, and 250 µg/ml

^c4 h exposure; 125, 250, 300, 400, 500, 750, and 1,000 µg/ml

^dDid not reach limit of cytotoxicity; retested at higher concentrations

^eSample that produced a dose-dependent, significant induction of MN in mice and rats in NTP studies [Mercado-Feliciano et al., 2012]