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ARTICLE *in* PLANTA MEDICA · MARCH 2009

Impact Factor: 2.15 · DOI: 10.1055/s-2009-1216525

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Teratogenic Effects of Blue Cohosh (*Caulophyllum thalictroides*) in Japanese Medaka (*Oryzias latipes*) Are Probably Mediated through GATA2/EDN1 Signaling Pathway

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Received June 17, 2010

Blue cohosh (*Caulophyllum thalictroides*) (BC) has been used widely to induce labor and to treat other uterine conditions. However, the safety and effectiveness of this herbal product has not yet been evaluated by the US Food and Drug Administration (FDA). Several conflicting reports indicated that the root extract of BC is a teratogen and, by some unknown mechanisms, is able to induce cardiovascular malfunctions in new-born babies. To understand the mechanism, we have used Japanese medaka (*Oryzias latipes*) embryo-larval development as the experimental model and the methanolic extract of BC root as the teratogen. The embryo mortality, hatching efficiency, and morphological abnormalities in craniofacial and cardiovascular systems are considered for the evaluation of BC toxicity. Our results indicate that BC is able to disrupt cardiovascular and craniofacial cartilage development in medaka embryo in a dose and developmental stage-specific manner. Moreover, embryos in precirculation are to some extent more resistant to BC than ones with circulation. By using subtractive hybridization, we have observed that *gata2* mRNA was differentially expressed in the circulating embryos after BC treatment. As GATA-binding sequences are required for the expression of the *endothelin1* (*edn1*) gene and *edn1* expressed in blood vessels and craniofacial cartilages, we have extended our investigations to *edn1* gene expression regulation by BC. We found that *edn1*, *furin1*, and endothelin receptor A (*ednrA*) genes are developmentally regulated; endothelin converting enzyme mRNA (*ece1*) maintained a steady-state level throughout development. Circulating medaka embryos (3 days post fertilization, dpf) exposed to BC (10 μg/mL) for 48 h have increased levels of *gata2*, *ece1*, and preproenodthelin (*preproedn1*) mRNA contents; however, other mRNAs (*furin* and *ednrA*) remained unaltered. Therefore, the enhanced expression of *gata2* mRNA followed by *ece1* and *preproedn1* mRNA by BC might be able to induce vasoconstriction and cardiovascular defects and disrupt craniofacial cartilages in medaka embryos. We conclude that cardiovascular and craniofacial defects in medaka embryogenesis by BC are probably mediated through a GATA2-EDN1 signaling pathway.

Introduction

Blue cohosh (*Caulophyllum thalictroides*) is an indigenous perennial herb found in the northeastern part of the United States. It is one of the important perennial medicinal herbs used by Native Americans for the treatment of rheumatism, dropsy, colic, sore throat, cramp, epilepsy, hysterics, and inflammation of the uterus (*1, 2*). It was also used as an emmenagogue and parturifacient producing diuretic, diaphoretic, and expectorant effects (*3, 4*). Moreover, it was also recommended for use by women with amenorrhea and dysmenorrhea (*5*). It was listed in the *United States Pharmacopoeia* as a labor inducer (*6, 7*). In recent years, a survey on midwives in the United States showed that approximately 64% of them were reported to use Blue Cohosh (BC) as a labor-inducing aid (*6, 8*). Although there is no recommended dose of BC in the scientific literature, BC was typically administered as a tincture during labor at 5 drops every 4 h or 10 drops in hot water every 2 h (*8*). A few reports have shown that the maternal drinking of BC tea may cause cardiovascular dysfunction, such as myocardial infarction, heart failure, and perinatal stroke, in the newborn babies (*9*−*13*). The exact causes of these cardiovascular defects are yet to be known; however, the safety and efficacy of BC during pregnancy and lactation are now in question. BC is also used in certain dietary supplement products available commercially (*14*). The doses recommended for these products (dried roots or extracts of rhizomes of BC) are contained in the product labels. Chemical analysis of the root extract of BC identified many compounds belonging to the alkaloids and triterpene saponin groups (*15*−*18*). The calculated amounts of saponins and alkaloids present in the dietary supplement preparations of BC are varied from 1−75 mg/day for alkaloids and 9−420 mg/day for saponins (*14*). Pharmacological studies identified that the glycosides of BC are able to stimulate smooth muscle cells and constrict coronary blood vessels (*19*). In an in vitro study, Madgula et al. (*20*) have also observed that the alkaloid fraction of BC is able to inhibit cytochrome P450 enzymes including

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10.1021/tx100205a © 2010 American Chemical Society
Published on Web 07/23/2010

Table 1. List of Primers Used in qPCR

gene	sense (5′-3′)	antisense (5′-3′)	product (bp)	GenBank accession/ensembl number
edn1	GATCCCAAACGAGCGTGAGC	CGCAATGATGAACCTATTC	646	ENSORLT 00000011634
gata2	CACCACCCTATCCCCACCTAC	GTTGGACATCTTCCTGTTGCG	484	NM_001104886
ece1	GAACTCGTCTGTGGAGGCCTTC	GAGTTGGAGATGGTGCCGATG	344	ENSORLT00000021333
ednrA	CAGCACAAGTGCATGAGGAATG	GATGGCTTCAGGAACGGCCAGG	348	ENSORLT00000009302
furin1	CCATTCTGGATGATGGCATTG	CAGCTGGCACTGTAAATGTGG	310	ENSORLT00000014949

CYP2C19, 3A4, 2D6, and 1A2. Earlier studies indicate that *N*-methylcytisine extracted from BC paralyzes the ganglion cells of the cardiac vagus in frogs and dogs (*21*). Taspine, an alkaloid purified from BC roots, decreased the expression of VEGF, bFGF, Bcl22, and Bax in mouse S180 sarcoma cells and induced apoptosis in A549 cell lines (*22*−*24*). Other reports indicate that triterpene monodesmosides isolated from BC roots are cytotoxic to HL-60 cells (*18*); moreover, caulophine possesses antimyocardial ischemia activity (*25*).

Although most of the biological studies of BC were conducted either in mammalian models (*26*) or in specific cell lines (*23*−*25*) and all these studies indicate that BC or its components are toxic in nature, no conclusive results have been drawn from these studies. Therefore, more studies in various different models are necessary. The use of different models with emphasis on mechanisms will strengthen or weaken the confidence in the use of BC as a dietary supplement or as a labor inducer. To our knowledge, only one study was done in a nonmammalian model frog (*21*). Studies focusing on the mechanism of action of these plant products at the cellular level are few. As BC is able to induce teratogenic effects in rat embryos in vitro (*26*), we have evaluated the teratogenic potency of BC in a fish model, medaka (*Oryzias latipes*). The embryo-larval development of this fish (medaka) has been used as a model organism to evaluate teratogenic potency of many organic and inorganic compounds (*27*−*33*). The transparency of the chorion, the short generation time and ease of maintaining the fish in the laboratory are some of the advantages of using medaka embryogenesis as a model to evaluate the teratogenic effects of a compound. Our results indicate that BC is a potent teratogen, able to induce cardiovascular and craniofacial defects during development, and that the teratogenic effects are probably mediated through the GATA2-EDN1 signaling pathway.

Experimental Procedures

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Mississippi.

Plant Material. Roots of BC (*Caulophyllum thalictroides*) were purchased from Mountain Rose Herbs (www.mountainroseherbs.com) on June 26, 2006, authenticated by Dr. V. Joshi at the National Center for Natural Products Research, University of Mississippi, where a voucher specimen (No. 2973) has been deposited. Root powder (4.0 kg) was extracted with MeOH (4.0 L × 24 h × 4) at room temperature (*17*)• The combined extracts were evaporated under reduced pressure to a brown powder (592 g). A stock solution (100 mg/mL) was prepared in DMSO (Sigma-Aldrich, St. Louis, MO) and diluted to the desired concentration with medaka embryo hatching solution (17 mM NaCl, 0.4 mM KCl, 0.36 mM $CaCl_2$, and 0.6 mM $MgSO_4$) before use.

Exposure and Experimental Setup. Methods of animal maintenance, egg collection, RNA preparation and purification, quantitative real-time RT-PCR (qRT-PCR), cloning, and sequencing of the PCR products were previously described (*30*−*33*). Briefly, adult male and female medaka orange-red varieties (3−4 months old, breeding) were maintained at 25 °C in a balanced salt solution (BSS, 17 mM NaCl, 0.4 mM KCl, 0.3 mM $MgSO_4$, and 0.3 mM $CaCl_2$) with a standard diet and a 16 L/8D photoperiod. Fertilized embryos were collected in the morning (9 a.m.) of the experimental day, separated mechanically from the clutch, and examined under a binocular microscope for the removal of unfertilized, older, and damaged embryos. Viable embryos were maintained 1 egg/mL hatching solution in a 48-well culture plate at 25 ± 1 °C with 16 L/8D photoperiod. BC (0−20 μg/mL) was added to the culture medium at different stages of development starting from ∼2 h postfertilization (hpf) (Iwamatsu stage 3−4) (*34*) to 3 days postfertilization (dpf), and discontinued after 48 h of addition or 6 dpf depending upon the nature of the experiment. The embryos were examined daily for developmental changes (cardiovasculature, thrombi, heart rate, and active circulation) under a phase-contrast microscope (AO Scientific Instruments) with a 50% static renewal of the medium (when BC was not required in the medium). The heart rate of the embryos (2 dpf to 6 dpf) was counted under the microscope for 3 min, and the data (heart rate) were expressed as heart rate per min. The embryonic development was classified after Iwamatsu (*34*).

Quantitative Real-Time PCR (qPCR). Total RNA was extracted from whole embryos by using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and the prepared RNA freed from genomic DNA by treating with DNase I (*31*). Quantification of the target gene mRNAs was done by qPCR. We performed qPCR in two steps. First, purified genomic DNA-free RNA (2.5 μg) was reverse transcribed to cDNA by superscript III (Invitrogen, Carlsbad, CA) following the protocol provided in the kit. The cDNA was left at −80 °C until analysis. Quantitative mRNA analyses of *preproedn1*, *ednrA*, *ece1*, *furin1*, and *gata2* mRNA were done from the prepared cDNA by qPCR based on a previously published method (*29, 31*). See the Supporting Information (the MIQE Table) included in the manuscript for details of the qPCR procedure. Briefly, 1 μL of cDNA was added to a reaction mixture (2× buffer from Qiagen, 10 μL; gene specific forward and reverse primers 50 pM each; 400× diluted SYBR Green I from Sigma-Aldrich, 0.2 μL; the final volume was adjusted to 20 μL by nuclease-free water). The primers used for the amplification of target genes are listed in Table 1. The standards for each of the target genes were amplified by PCR, using the same sense and antisense primers (Table 1) used in qPCR analysis. The cDNA prepared from normal hatchings (10 dpf) was used as the template. The amplified PCR product was purified elecrophoretically in 1% agarose gel containing ethidium bromide (0.01%), eluted, and quantified spectrophometrically (Nanodrop 2000c, Thermo Fisher Scientific, Waltham, MA). The standards were aliquoted into separate tubes and stored at −80 °C until use. During qPCR analysis (DNA Engine Opticon 2), the standards were diluted to the requisite concentration (copy numbers) with nuclease free water and used for the preparation of a standard curve. The reaction conditions were initial denaturation at 95 °C for 3 min, one cycle, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 1 min (the annealing temperature for *gata2*, *preproedn1*, *ece1*, and *ednrA* is 60 °C and for *furin1*, 52 °C), extension at 72 °C for 2 min, and fluorescence data collection for 1 s. After the end of each cycle, the samples were incubated again for 1 s at 78 °C, and a second set of fluorescence data collection was made to prevent error due to the formation of primer−dimers, if any. A final extension of one cycle at 72 °C for 10 min was made. The melting curve was constructed by plotting fluorescence data (1 s) against temperature (65 to 95 °C with an interval of 0.2 °C and a holding time is 0.5 s). Each reaction was performed in duplicate. The quantification cycle or Cq was set at the point manually with the best R^2 value which ranged from 0.80−0.99 for standard curves. This Cq was applied to all wells for consistent analysis of standards and individual samples. Standards were run each time in every set of qPCR analysis. The copy numbers of mRNA in each tube were determined

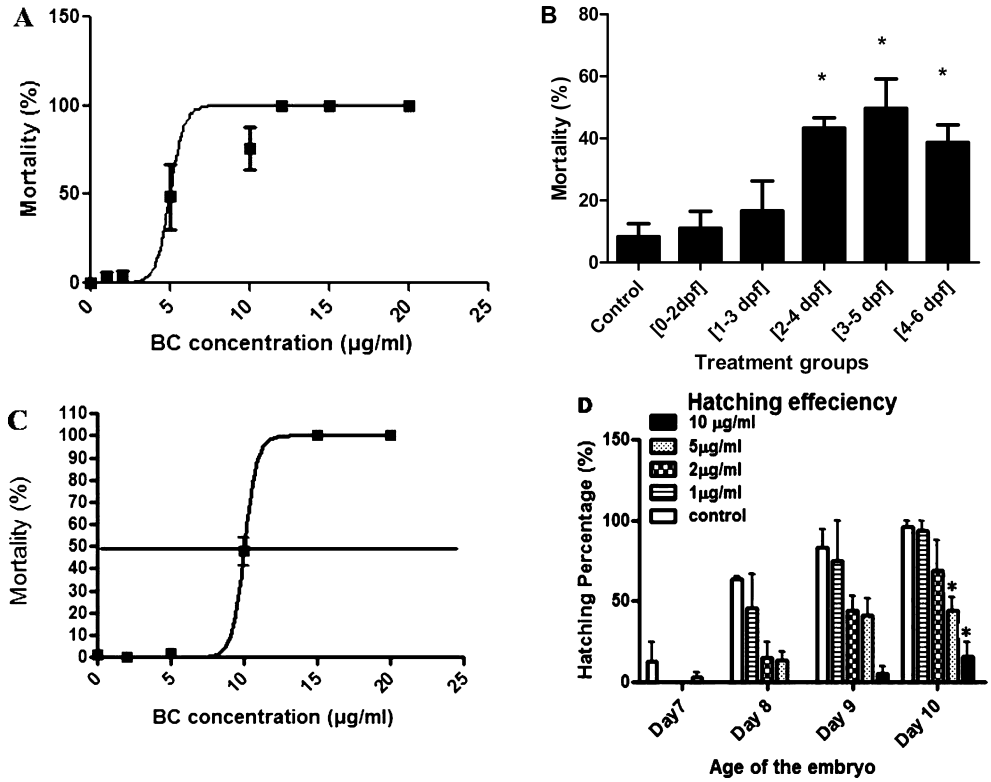

Figure 1. BC-mediated mortality in medaka embryos. Fertilized eggs of medaka within 2 hpf (A), 0−4 dpf (B), or 3 dpf (C) were exposed to BC (0−20 μg/mL) either for 6 dpf (A) or 0−20 μg/mL BC for 48 h (B and C), and the effect on mortality was assessed at 10 dpf. Each group consists of 8 embryos. The experiment was repeated 5 times. The LC_{50} was calculated to be 5.40 ± 1.33 μg/mL with an r^2 of 0.8681 for A and for 10.04 ± 1.06 μg/mL with an r^2 of 0.9836 for C by log transformed data using nonlinear regression (curve-fit) (GraphPad Prism). Each point represents the mean mortality percentage ± SEM ($n = 5$). (B) Embryos of early developmental stages (0−1 dpf) are more resistance to BC than the embryos of late stages (2−4 dpf). Each bar is the mean ± SEM of 5−8 observations. (D) Hatching efficiency of medaka embryos developmentally exposed to BC is presented in a histogram. Medaka embryos 3 dpf were exposed to BC (0−10 μg/mL) for 48 h. The hatching was recorded from 7 dpf. Each bar is the mean ± SEM of 8 observations. Statistical analyses of day 10 data indicate that BC at 5 and 10 μg/mL concentration is able to significantly reduce ($p < 0.05$) the hatching of medaka embryos. Asterisks (*) indicate that the data were significantly different ($p < 0.05$) from the controls.

by using a software program, MJ Opticon Monitor Analysis software, version 3.1, Bio-Rad laboratories, Hercules, CA. The data were expressed as the copy number of target gene mRNA/ng of total RNA.

Cartilage Staining. Hatchlings at 10 dpf were used for cartilage staining by 0.1% Alcian Blue (Sigma-Aldrich, St. Louis, MO) following previously published methods (*29, 33*) with minor modifications. Hatchlings (8−10 per group) were anesthesized with 0.1% MS 222 (Sigma-Aldrich, St.Louis, MO) in hatching solution. They were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBT) at 4 °C. Fixed samples were washed twice in water and then treated for 10 min in 5% TCA. After a brief washing in acid−ethanol (0.37% HCl, 70% ethanol), embryos were stained with 0.1% Alcian Blue for 1 h. Excess stain was removed by washing in acid−ethanol, then the samples were transferred to hydrogen peroxide (1%) for 12−24 h for clearing. Another wash in Glycerol−KOH (50% glycerol and 0.25% KOH) was performed prior to storing the stained samples at 4 °C in 70% glycerol. Digital images of the entire skeleton, head skeleton (neurocranium and splanchnocranium), lower jaw including Meckel's cartilage (MC), and tail cartilages of 8−10 hatchlings in different orientations (dorsal, ventral, and lateral) were captured on an Olympus B-Max 40 microscope at constant magnification. The linear length of these cartilages and bones were measured from the captured images with Optimas 6 image analysis software (Media Cybernetics, Sliver Springs, MD). After staining with Alcian blue, the ethmoid plate (EP) with lamina orbitonasalis, paired trabeculae (TB), anterior orbital cartilage (AOC), posterior orbital cartilage (POC), epiphyseal bar (EB), basilar plate (BP) with anterior (ABC) and posterior (PBC) basicranial commissures, hypophyseal plate (HP) with paired polar cartilages (PC), and auditory capsules in the neurocranium are clearly visible. In the splanchnocranium, Meckel's cartilage (MC), pterygoid processes (PT), quadrate (QU), hyosymplectic (HYS), hyoid (HYO), basihyal (BH), ceratohyal (CH), 4 basibranchials (BB), 3 pairs of hypobranchials (HBR), 5 pairs of ceratobranchials (CBR), the fourth epibranchial (EBR), and the fourth hypobranchial (HBR) are also identifiable. The nomenclature of the cartilages was published previously (*33*) and obtained from Langille and Hall (*35*). During morphometrical analysis, the length of the entire skeleton, neurocranium, EP, AOC, and EB are found to be linear. In the splanchnocranium, the linear lengths of QU, BH, CH, BB (1−4), and CBR (1−4) were measured directly (linear). In the case of BB, the first three cartilages are not visualized separately; therefore, the lengths of the first three BB are the cumulative; the fourth one was visible separately and therefore measured individually. In the fifth CBR, an imaginary line was drawn joining the pharyngeal teeth of the left and right CBR (5th), and the distance was measured from the center. The bones of the lower jaw (MC) are also curved. Therefore, the length of the lower jaw was determined as the distance between the meeting points of two MC and the midpoint of an imaginary line drawn between the two ends of the MC. The POC, auditory capsule, PBC in the neurocranium, and PT, HYS, and HYO in the splanchnocranium were not considered for morphometric analysis because of their irregular shape. Therefore, the total cartilages considered for morphometric analysis in the head skeleton are 18 (considering BB1−3 as a single cartilage).

Subtractive Hybridization. Subtractive hybridization (SH) was performed using 1.5 μg of RNA extracted from the control and BC treated (10 μg/mL, embryos were exposed 3−5 dpf) embryos on 6 dpf with a BD Bio Science PCR subtraction kit (BD Bioscience, San Jose, California), following the manufacturer's protocol. This PCR based technique allows differentially expressed

genes to be isolated by comparing the expression between control and treatment groups at a specific stage of development. It involves a subtractive hybridization step, in which one population is supplied in excess (driver), to saturate (and subtract) all similar sequences found in the second (tester) cDNA population. After hybridization, only genes unique to the tester cDNA population (differentially expressed genes) are retrieved. In our study, two rounds of subtractive hybridization were undertaken; in the first round, control RNA served as a driver, and in the second round, BC (10 μg/mL; 3−5 dpf)-treated RNA served as the driver. Differentially expressed cDNAs from both rounds of SH were viewed in 1% agarose gel electrophoresis containing 0.01% ethidium bromide. Differentially expressed bands (3 visible bands identified) were cut manually and the amplified cDNA was extracted by using a gel purification kit (Qiagen, Valencia, CA). The purified cDNA was cloned into pGEM-T Easy vector system (Promega, Madison, WI) and transformed into JM 109 strains of *Escherichia coli*. The cells were plated on LB medium containing ampicillin and incubated at 37 °C overnight. The white positive colonies (4 colonies for each cDNA) were grown at 37 °C in 3 mL of LB media with ampicillin. The plasmid DNA was prepared by using a Miniprep DNA purification system (Promega, Madison, WI). The nucleotide sequences of the plasmids (4 plasmids for each cDNA, both directions) were analyzed by using a CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter, Fullerton, CA, USA) in a Beckman-Coulter CEQ 8000 Genetic Analysis System. For identifying the gene, the obtained nucleotide sequences were matched with the sequences reported in GenBank or with other databases. Among the analyzed cDNA, only *gata2* sequences were matched with the sequences reported in GenBank (GenBank Accession AB183298). The other two cDNAs were matched with vector DNA.

***In Situ* Hybridization.** An *in situ* hybridization technique was used to identify the expression of *edn1*, *gata2*, *furin1*, and *ece1* genes at specific sites following the methods described by Sinomiya et al. (*36*) and Conlon (*37*) with some modifications. Both control and treated embryos were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 1× PBS (8.0 mM sodium phosphate, 1.9 mM potassium phosphate, 2.7 mM KCl, and 0.14 M NaCl in 2× H_2O, pH 7.4) with 0.1% Tween 20 (Sigma-Aldrich, St.Louis, MO) (PBST) at 4 °C overnight. The chorion and yolk were removed from the fixed embryos with the help of watchmaker's forceps under a binocular microscope. The fixed embryos were washed in PBS and distilled water, then transferred to a graded series of methanol, finally stored in 100% methanol, and left at −20 °C until analysis. On the assay day, the stored embryos were transferred to PBS after washing in graded methanol (100%−30%). The embryos were incubated at room temperature with Proteinase K (5 μg/mL) for 30 min and then refixed in 4% PBST for an additional 20 min. The embryos were transferred to the prehybridization buffer (50% formamide, 0.1% Tween-20, 5 mg/mL Torula Yeast RNA type VI, and 50 μg/mL heparin in 20× SSC) for 1 h at 64 °C, then transferred to the hybridization buffer (prehybridization buffer with 1 μg/mL antisense probe labeled with digoxigenin) overnight. The embryos were washed at 64 °C in 2× SSC and 0.2× SSC, 30 min each, followed by washing three times in PBST, and transferred to the blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN). The embryos were incubated overnight at 4 °C with 1:3000 diluted antidigoxigenin antibody coupled with alkaline phosphatase (Roche Molecular Biochemicals) in 10% blocking buffer. After the desired period, the embryos were washed four times in PBST and treated for 20 min with coloration buffer (0.1 M NaCl, 50 mM $MgCl_2$, and 0.1 M Tris-HCl, pH 9.5) and finally with nitro blue tetrazolium (NBT). After the development of the color, the embryos were washed in PBST, fixed in 4% paraformaldehyde, and stored in 70% glycerol for microscopic observation. The photographs were taken with an Olympus B-max 40 microscope at fixed magnification.

Statistical Analysis. The data were analyzed using one way ANOVA with posthoc Tukey's multiple comparison test. Two way ANOVA with Bonferroni post-test was used for hatching efficiency, heart rate, and active circulation analysis considering the concentration of BC and time required for response as the two factors. The results were expressed as the mean ± SEM with $p < 0.05$ considered as significant.

Results

Effects of BC on Morphological Features of Japanese Medaka.

Sensitivity Test. Fertilized medaka eggs within 2 h of collection (Iwamatsu stage 4−10), were exposed to 1, 2, 5, 10, and 20 μg/mL of BC for 6 days. The calculated LC_{50} value for BC in embryos at 10 dpf as determined from five separate experiments was 5.719 ± 1.33 μg/mL with 0−6 dpf constant exposure (media changed once everyday), followed by clean hatching (6−10 dpf) solution (Figure 1A). Embryo survivability and hatching were severely affected at BC concentrations ≥15 μg/mL with 100% mortality occurring at a concentration >20 μg/mL (Figure 1A).

To determine the critical period of BC toxicity during embryogenesis, embryos at different developmental stages (Iwamatsu stages 4−32) were exposed to only one concentration of BC (10 μg/mL) for a short duration (48 h with a one-time change of BC at 24 h), and the mortality was determined 10 dpf. It was observed that the embryos of late stages of development (Iwamatsu stages 27−32) were more sensitive to BC than the embryos in early stages (Iwamatsu stages 4−24) (Figure 1B).

To determine the LC_{50} value in late stages of development, 3 dpf embryos (Iwamatsu stages 27−28) were exposed to 2, 5, 10, 15, and 20 μg/mL BC for 48 h, and the mortality was determined 10 dpf. The calculated LC_{50} value for BC from 5 independent experiments was 10.05 ± 1.06 μg/mL (Figure 1C), which is approximately 2-fold higher than that in the previous experiments (0−6 dpf constant exposure).

Furthermore, we have also determined the effect of BC on the hatching efficiency of medaka embryos (Iwamatsu stages 27−28) during development. In our culture conditions (28 ± 1 °C, 16 L/8D), medaka embryos started to hatch 7+ dpf (∼175 hpf), and the peak hatching was observed on 10 dpf. BC inhibited hatching efficiency of medaka embryos (Iwamatsu stages 27−28) in a dose-dependent manner (Figure 1D). More than 90% of the control and the embryos exposed to 1 μg/mL BC were hatched on 10 dpf; however, a dose-specific reduction in hatching was observed (2−10 μg/mL). The embryos exposed to 10 μg/mL BC had an ∼20% successful hatch.

Effect on Cardiovascular System. The teratogenic effects of BC on the cardiovasculature of medaka embryos were observed microscopically and assessed on the basis of thrombi formation, reduction in heart rates, and delay in the onset of circulation (Figure 2). During medaka embryogenesis, the cardiovasculature became functional at the 16 somite stage ∼44 hpf (heart beat starts ∼44 hpf, Iwamatsu stage 24), and the onset of circulation begins at ∼50 hpf (Iwamatsu stage 25, 18−19 somites). It was observed that embryos exposed to BC showed thrombi mostly in the heart and blood vessels. However, thrombi were also seen in other regions of the embryonic body, such as in the brain and eyes (Figure 2A−C). Control embryos were devoid of thrombi. Furthermore, embryos were treated with 10 μg/mL BC for different periods, and thrombus percentage was calculated on 6 dpf; It was found that thrombus percentage for late-stage treatments (3−5 dpf and 4−6 dpf) was significantly different (increased) from that for early stage (0−2, 1−3, and 2−4 dpf) groups (Figure 2D).

Heart rate was examined in embryos once daily from 2 dpf (24 h before the treatment of BC) to 6 dpf after exposing the

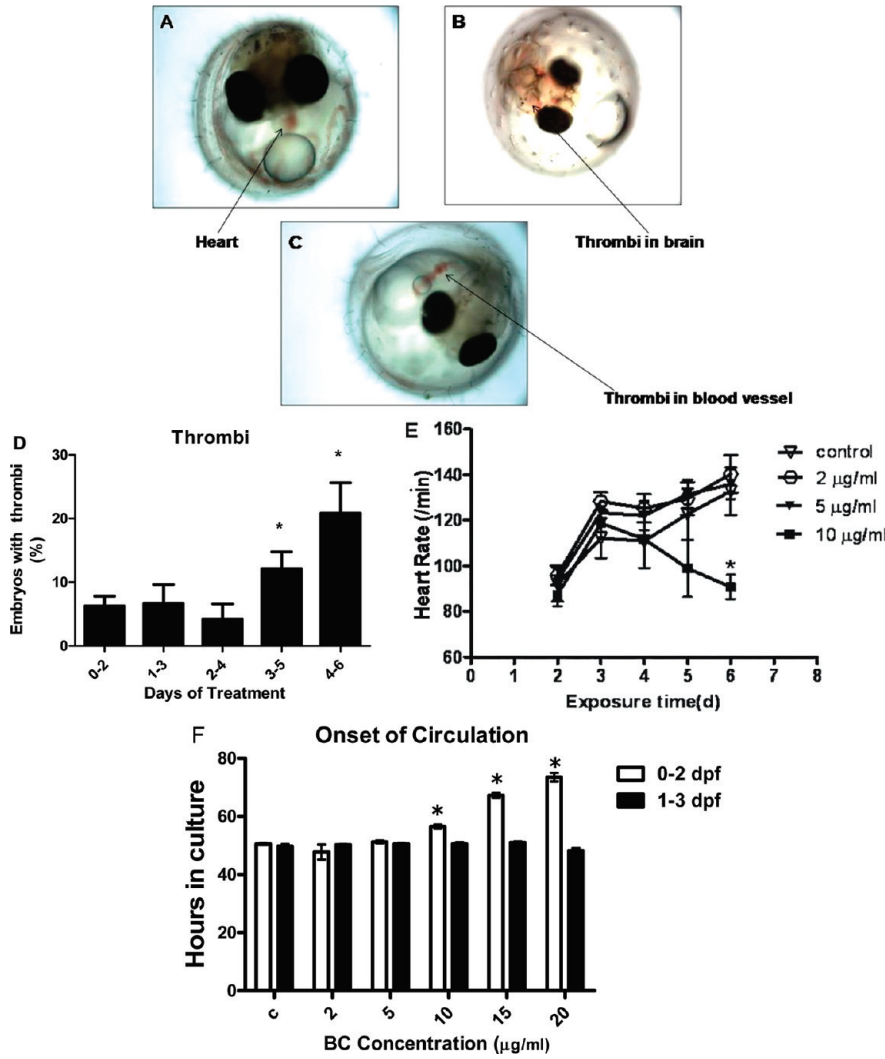

Figure 2. Effect of BC on cardiovascular development in Japanese medaka. (A−C) Representative figures showing the formation of thrombi in different body parts. Fertilized eggs at 3 dpf were exposed to BC (10 μg/mL) for 48 h, and the photographs at fixed magnification were taken on 6 dpf. (A) Control (no thrombi); (B) thrombi in the head; (C) thrombi in the blood vessels. (D) The occurrence of thrombi observed in embryos exposed to BC for 5 different days of development are presented in histograms. Each bar is the mean ± SEM of 3 independent observations. Bar heads with asterisks (*) indicate that the data were significantly different ($p < 0.05$) from those of 0−2 dpf groups. (E) The heart rate in medaka embryos after BC treatment. Embryos (3 dpf) were exposed to BC (0−10 μg/mL), and the heart rates were recorded once everyday until 6 dpf. The values are the mean ± SEM of 4 observations. Asterisks (*) indicate that the values are significantly different from those of the controls ($p < 0.05$). (F) The time required for the onset of active blood circulation in the embryos after BC treatment is presented in histograms. The embryos were exposed to BC (0−20 μg/mL) for 48 h. Each group contains 8−16 embryos. Each bar is the mean ± SEM, and the bar heads with asterisks (*) indicate that the values were significantly different from those of the the corresponding controls.

embryos with 2, 5, and 10 μg/mL BC (BC treatment started at 3 dpf and continued to 6 dpf; the media were changed once daily, and the heart rate was counted 1 h post-BC treatment). It was observed that the heart rate was not significantly different between the control and BC-treated (2, 5, and 10 μg/mL) groups until 4 dpf. However, from 5 dpf onward the embryos exposed to10 μg/mL BC had significantly lower heart rates than the control, 2, and 5 μg/mL BC-treated groups (Figure 2E).

The onset of circulation in medaka embryos exposed to 2, 5, and 10 μg/mL BC in early stages (Iwamatsu stages 4−20) were also examined (Figure 2F). Generally, in a medaka embryo, the circulation of blood through blood vessels occurs at ∼50 hpf (*34, 38*). We have observed that in our culture conditions, medaka embryos started circulation at ∼50 hpf. However, once the circulation started (embryos of 50 hpf or older), treatment with BC (10 μg/mL) did not change the circulation status of the embryo. Therefore, BC was given to the embryos before the onset of circulation (treatment started on 0, 1, and 2 dpf and continued for 48 h with one time change of the medium after 24 h). It was observed that BC (10 μg/mL) was able to delay the onset of circulation in embryos exposed to BC for 0−2 dpf. However, it had no effect if BC was added to the embryos after 24 hpf (1−3 dpf) (Figure 2F).

Effects on Skeletal System. Teratogenic effects of BC were further examined in the craniofacial and tail cartilages (Figure 3A−H) of medaka hatchlings (10 dpf). Medaka embryos (Iwamatsu stages 27−28) were exposed to 2, 5, and 10 μg/mL BC for 48 h (3−5 dpf), and the hatchlings on 10 dpf were stained in Alcian blue and used for cartilage analysis. In medaka hatchlings, the Alcian blue positive regions (cartilages) on 10 dpf were observed only in the skull and tail (*29, 33*). Analysis of the data indicate that the total length of the skeleton of the BC-treated hatchlings (10 μg/mL) was smaller compared to that of the controls (Figure 3A−E); however, the values were significant only in embryos treated with 10 μg/mL BC (Figure 3H). No morphological difference in the cartilages of the tail region (hypural, parhypural, and epural cartilages) was observed (Figure 3F,G). Furthermore, the linear length of the head

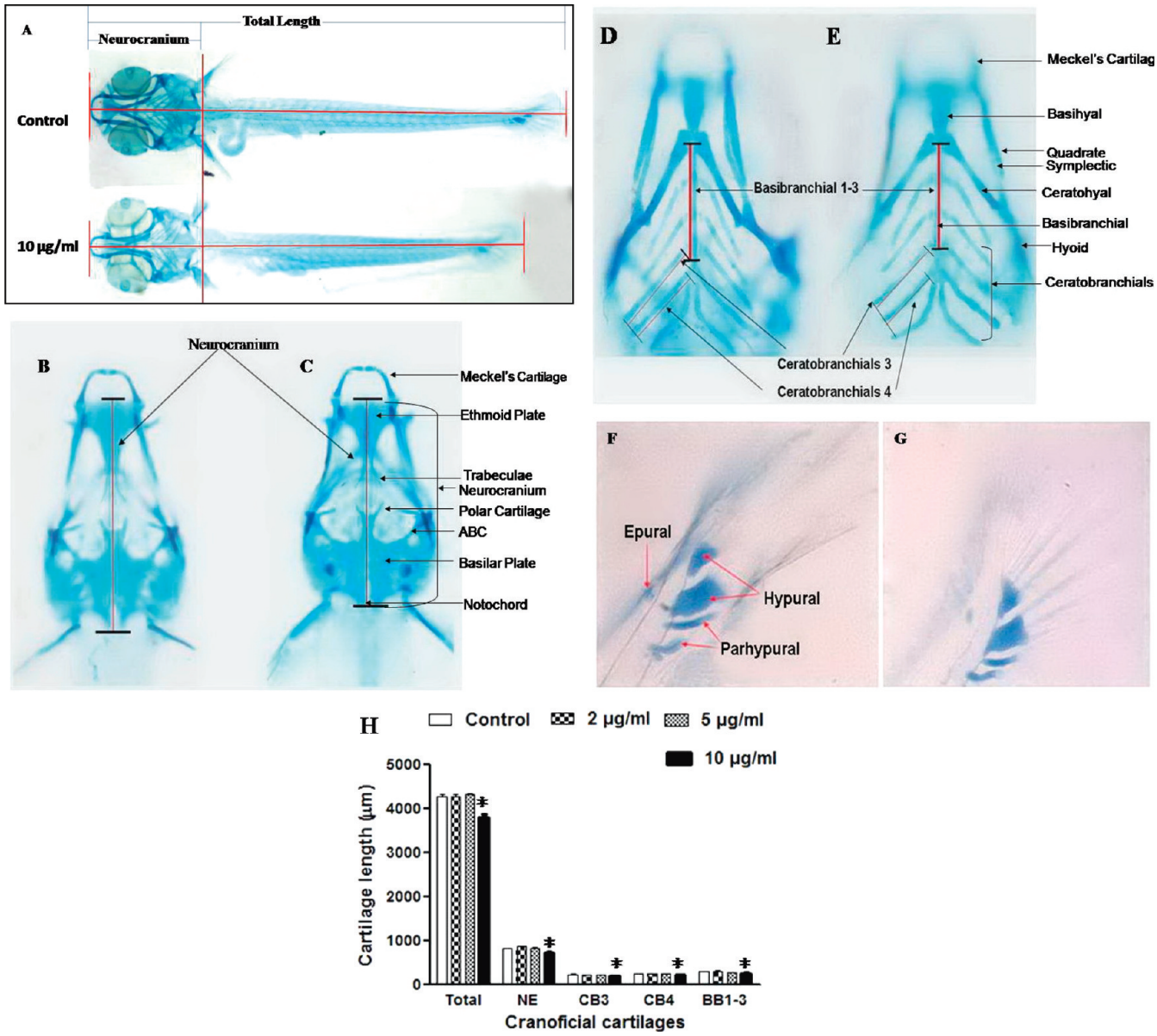

Figure 3. Effect of BC on cartilage development of medaka. (A−E) Representative figures showing the effects of BC on total skeletal length and neurocranial cartilages of medaka hatchlings developmentally exposed to BC. Hatchlings (10 dpf) were stained with Alcian Blue. (A) Total skeleton of control and embryos exposed to 10 μg/mL BC; (B) neurocranium (control); (C) embryos treated with 10 μg/mL BC for 3−5 dpf. (D) Bronchial cartilages of splanchnocranium (control); (E) bronchial cartilages of the splanchnocranium (BC 10 μg/mL). (F,G) Representative figures of tailfin cartilages of medaka hatchlings with Alcian Blue positive cartilages. (F) Control; (G) BC treatment (10 μg/mL). No significant differences in tail cartilages are seen. (H) Effect of BC (0−10 μg/mL) on the total length, linear length of the neurocranium, basibranchials (1−3), and ceratobranchials (CB) 3 and 4 (CB3, CB4) of medaka hatchlings 10 dpf are presented in the histogram. Alcian Blue stained hatchlings were measured from photomicrographs using Optimus image analysis software. The results are the mean ± SEM of 10−15 observations. Bars with asterisks (*) indicate that the data are significantly different from those of the corresponding controls. Other skeletal parameters are reduced in length but not significantly different from those of the corresponding controls.

skeleton (neurocranium) was significantly reduced in the BC-treated group (10 μg/mL) compared to that in the controls (Figure 3H). To find a BC sensitive cartilage in the skull (neurocranium and splanchnocranium), we have further measured the linear length of several cartilages (total 18) located either in the neurocranium or in the splanchnocranium. Our results indicate that the majority of the cartilages are reduced in length in the hatchlings exposed to BC (10 μg/mL); however, the data are significantly different ($p < 0.05$) from those of the controls only in basibranchials 1−3 (BB1−3) and in ceratobranchials 3 and 4 (CB3 and CB4) (Figure 3B−E and H).

Biochemical Analysis. To find a BC-sensitive gene, we used subtractive hybridization analysis in the RNA extracted from medaka embryos (Iwamatsu stages 27−28) exposed to BC (10 μg/mL) for 48 h and the corresponding controls (vehicle-treated). It was observed that BC (10 μg/mL) was able to differentially express *gata2* mRNA in these embryos. GATA2 is a transcription factor required for hematopoietic stem cell survival and neuronal development in vertebrates (*39*). Moreover, GATA-binding sequences in the promoter region are required for the expression of endothelin (EDN-1) (*40*). Therefore, we have analyzed the expression of *edn-1* mRNA in medaka embryos under identical conditions.

The qPCR data indicate that *gata2*, *preproedn1*, *furin*, *ece1*, and *ednrA* mRNA expressions were developmentally regulated in medaka (Figure 4); however, the expression patterns of these mRNAs were found to be different (Figure 4). *Gata2*, *preproedn-1*, *furin*, and *ece-1* mRNAs but not *ednrA* were detectable in fertilized eggs (0 dpf). Maximum amount of *gata2* mRNA was detected in 0 dpf embryos, and then a sharp decrease

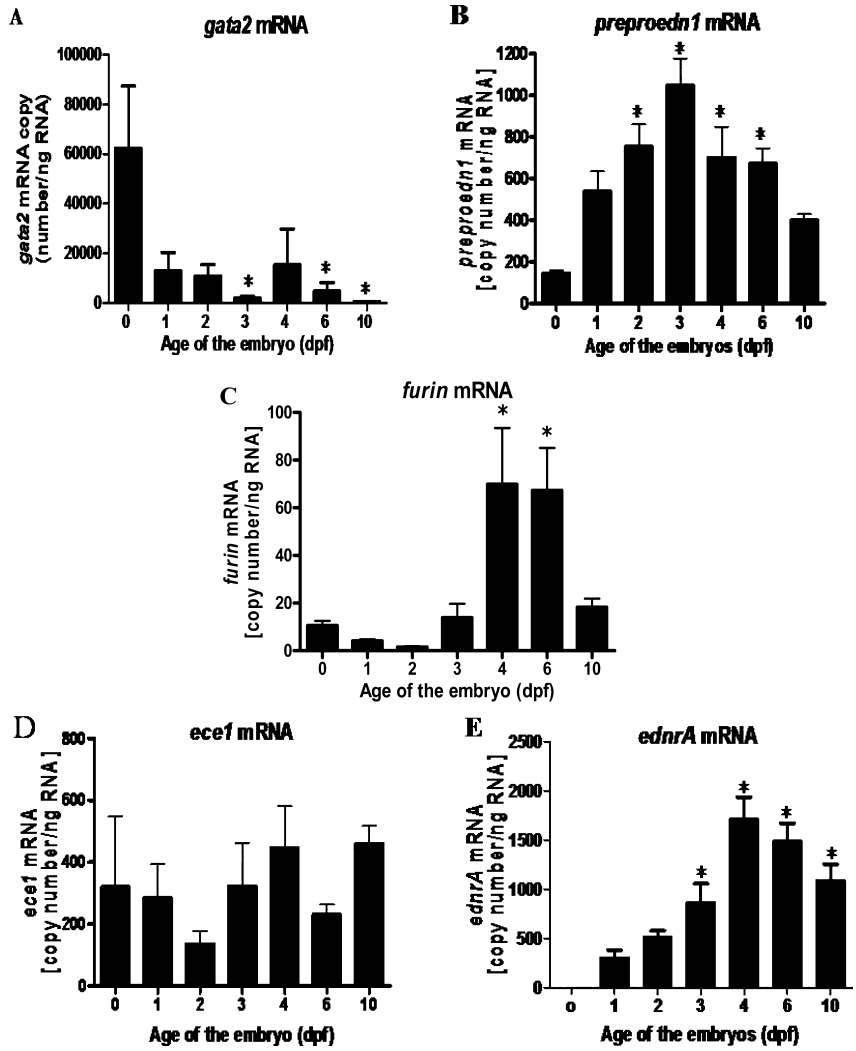

Figure 4. Developmental expression of *gata2*, *preproedn1*, *ednrA*, *ece1*, and *furin1* mRNA in medaka embryos. (A) *gata2*, (B) *preproedn1*, (C) *furin*, (D) *ece1*, and (E) *ednrA*. Total RNA was prepared from 6−8 pooled medaka embryos or hatchlings (10 dpf), reverse transcribed, and analyzed by qPCR. The data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each bar is the mean ± SEM of 4 observations. Bar heads with asterisks (*) indicate that the data are significantly different ($p < 0.05$) from the zero dpf data. All of these mRNA maintained a distinct pattern during medaka embryogenesis.

in 1 dpf and there after until hatching (Figure 4A) was observed. In contrast to *gata2*, *preproedn-1* mRNA was increased gradually and reached a peak on 3dpf (Figure 4B). *Furin* mRNA was very low until 3 dpf and then increased 4 and 6 dpf (Figure 4C). *Ece1* mRNA maintained a steady-state level throughout embryogenesis (Figure 4D). Although e*dnrA* mRNA was undetectable in 0 dpf embryos, like *preproedn1* mRNA, it increased during development from 3 dpf and then maintained a steady-state until hatching (Figure 4E).

The 3 dpf embryos were exposed to BC (2, 5, and 10 μg/mL) for 48 h and used for *gata2*, *preproedn1*, *furin1*, *ece1*, and *ednrA* mRNA analysis by qPCR techniques (Figures 5−9). It was observed that compared to controls *gata2*, *preproedn1*, and *ece1* mRNA levels were changed (significantly increased) in embryos treated with 10 μg/mL BC (Figures 5A, 6A, and 8A); however, the other two doses (2 and 5 μg/mL) were unable to induce any alteration in these mRNA levels. Moreover, *furin* and *ednrA* mRNA contents remained unaltered after BC (2, 5, and 10 μg/mL) treatment (Figures 7 and 9).

We used ISH techniques to detect the mRNA expression pattern of *gata2*, *preproedn1*, *furin*, and *ece1* mRNAs in medaka embryos after BC exposure. Although ISH is more qualitative than qPCR, it was observed that *gata2* mRNA was expressed in the hind brain and ear regions of the 5 dpf embryos (Figure 5B,C, Iwamatsu stages 30−32); however, *preproedn1* mRNA expression was detected only in the gill regions (Figure 6B), *furin1* mRNA in the whole brain (Figure 7B), and *ece1* in the somites (Figure 8B). Treatment of BC (10 μg/mL) appears to enhance the expression of *gata2* (Figure 5C), *preproedn1* (Figure 6C), and *ece1* (Figure 8C) mRNAs but not the *furin1* mRNA (Figure 7C).

Discussion

It is evident from the present experimental results that the methanolic extracts of the rhizomes of BC are toxic and able to induce teratogenic effects in medaka embryos in a dose and developmental stage-specific manner (Figures 1−3). This is the first report of a fish developmental model being used to evaluate the teratogenic potency of BC *in vitro*. Previously, a rat embryo culture model was tested (*26*) and concluded that a methanolic extract of BC (500 μg/mL) was nontoxic and unable to produce significant teratogenic effects; however, both ethyl acetate- and *n*-butanol fractions were lethal at 250 and 500 μg/mL concentrations, respectively. Among pure compounds isolated from BC extracts, only *N*-methylcytisine was able to show teratogenic effects; taspine was found to be most toxic but nonteratogenic.

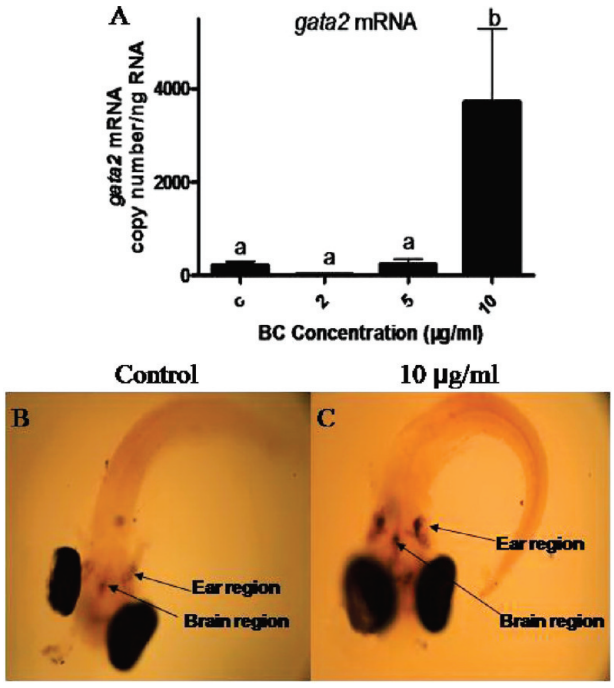

Figure 5. Effect of BC on gata2 mRNA expression in medaka embryos. (A) Medaka embryos were exposed to BC 3−5 dpf. Total RNA was extracted, reverse transcribed, and analyzed by qPCR. Data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each bar is the mean ± SEM of 4 observations. Different superscript letters indicate that the data are significantly different from each other. (B and C) gata2 mRNA analysis by in situ hybridization. (B) control; (C) BC (10 μg/mL). BC (10 μg/mL) was able to induce gata2 mRNA expression in medaka.

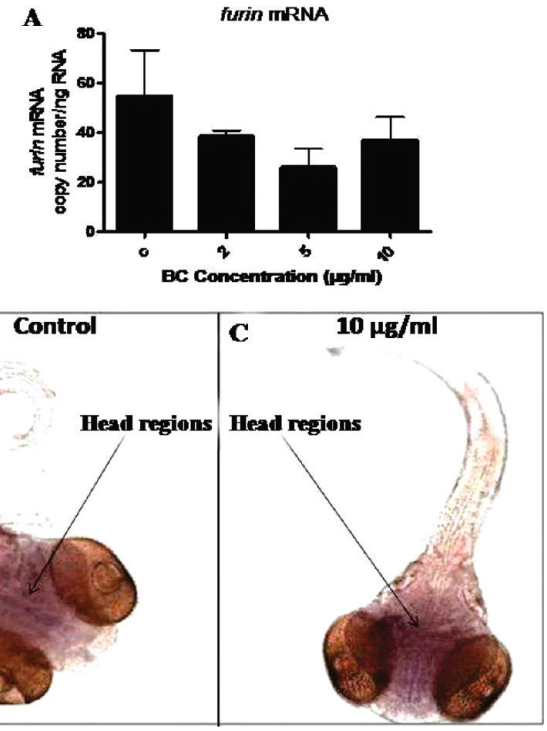

Figure 7. Effect of BC on *furin* mRNA expression in medaka embryos. (A) Medaka embryos were exposed to BC (2−10 μg/mL) 3−5 dpf. Total RNA was extracted, reverse transcribed, and analyzed by qPCR. Data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test. Each bar is the mean ± SEM of 4 observations. Values are not significantly different from those of the controls. (B and C) *furin* mRNA analysis by in situ hybridization. (B) control; (C) BC (10 μg/mL). Expression of *furin* mRNA was observed in the head region of both control and BC-treated (10 μg/mL) embryos.

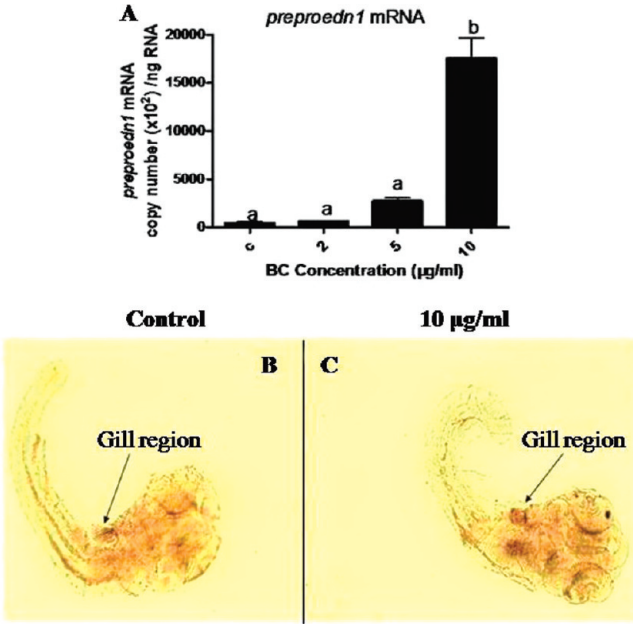

Figure 6. Effect of BC on preproendothelin1 mRNA expression in medaka embryos. (A) Medaka embryos were exposed to BC (2, 5, and 10 μg/mL) 3−5 dpf. Total RNA was extracted, reverse transcribed, and analyzed by qPCR. Data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each bar is the mean ± SEM of 4 observations. Different superscript letters indicate that the data are significantly different from each other. (B and C) *preproendothelin1* mRNA analysis by in situ hybridization. (B) control; (C) BC (10 μg/mL). BC (10 μg/mL) was able to induce *preproendothelin1* mRNA expression in medaka.

Other compounds, such as thalictroidine, anagyrine, and α-isolupanine, were nonteratogenic (0−80 μg/mL). Recently, Madgula et al. (*20*) have observed that a methanolic extract of BC was unable to induce alteration in several cytochrome P450 enzyme activities *in vitro*; however, alkaloid fractions including *N*-methylcytisine and anagyrine were able to inhibit these enzyme activities.

There are three case reports in the scientific literature where human maternal ingestion of BC might have cause neonatal congestive heart failure (*9*−*13*). Therefore, we are interested in examining the effects of BC on the cardiovasculature of medaka embryos during development. Because of the transparency of the chorion, the development of the heart and blood vessels can easily be studied in medaka embryogenesis. Moreover, the cardiovascular development and function is well characterized in medaka embryos (*38*). The teratogenic effects of BC on the cardiovasculature of medaka embryos were evaluated both in precirculating and circulating stages after exposing the embryos with BC for 48 h. In the 0 (∼ 2hpf) and 1 dpf groups, the formation of heart and blood vessels was not visible, but in the 3 and 4 dpf groups, both heart and blood vessels were functioning. Therefore, the embryos of the 0−2 dpf (0−48 hpf) period were considered as precirculating, and those from 2−4 dpf (50−96 hpf) were considered as circulating embryos.

Our data indicate that BC (10 μg/mL, 48 h) was able to induce thrombi (Figure 2A−C) more in the circulating embryos than in the precirculating ones (Figure 2D). The heart-rate was evaluated in embryos treated with 2, 5, and 10 μg/mL BC from 2−6 dpf, and the heart rate was recorded once every day, 1 h

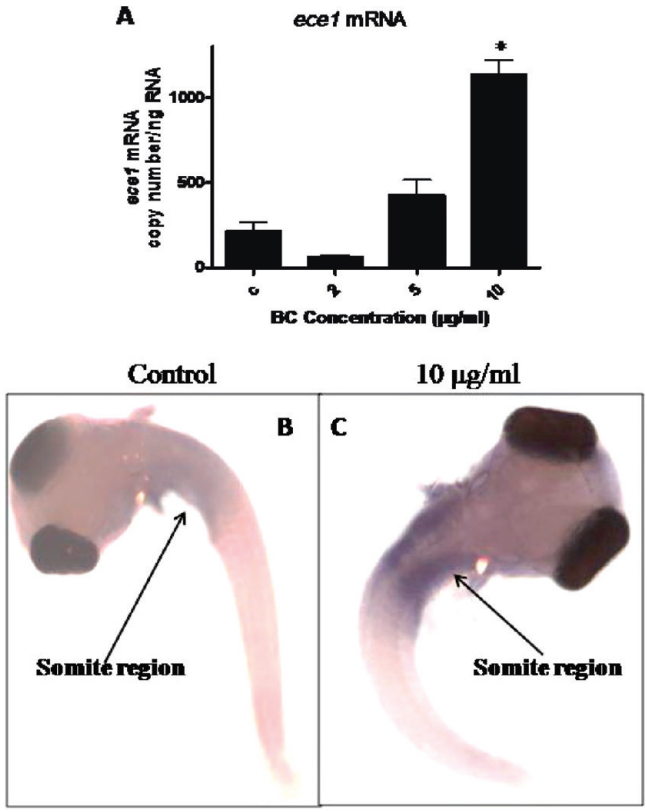

Figure 8. Effect of BC on *ece1* mRNA expression in medaka embryos. (A) Medaka embryos were exposed to BC 3−5 dpf. Total RNA was extracted, reverse transcribed, and analyzed by qPCR. Data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each bar is the mean ± SEM of 4 observations. Asterisks (*) indicate that the value is significantly different from those of the controls. (B and C) *ece1* mRNA analysis by in situ hybridization. (B) control; (C) BC (10 µg/mL). BC was able to induce *ece1* mRNA expression in BC-treated (10 µg/mL) medaka.

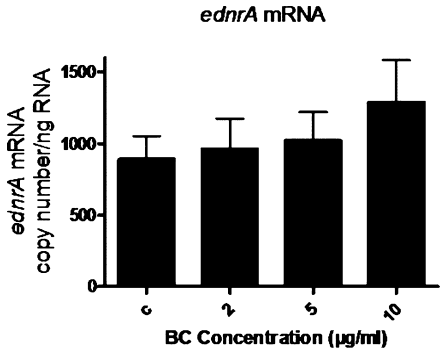

Figure 9. Effect of BC on *ednrA* mRNA expression in medaka embryos. (A) Medaka embryos were exposed to BC 3−5 dpf. Total RNA was extracted, reverse transcribed, and analyzed by qPCR. Data were analyzed by one-way ANOVA followed by posthoc Tukey's multiple comparison test. Each bar is the mean ± SEM of 4 observations. There was no significant difference between the control and BC-treated embryos.

after BC treatment (Figure 2E). The data indicate that in medaka embryos the heart rate increased with the advancement of age (99 ± 4 beats/min in 2 dpf and 133 ± 18 beats/min in 6dpf, $n = 4$), and BC at higher doses (10 µg/mL) was able to reduce the heart rate. The inhibition was not significantly different from that of the control and BC-treated embryos until 4 dpf. Furthermore, the circulation status of the precirculating medaka embryos (0 and 1 dpf) was examined from the 2−4 dpf period (Figure 2F). It was observed that the onset of circulation was delayed in the 0 dpf group by BC in a dose-dependent manner (10−20 µg/mL); however, in the 1 dpf group, the onset of circulation was normal, and no significant effect of BC was observed (Figure 2F). From these three parameters (thrombi, heart rate, and the time of the onset of circulation), it was observed that the effects of BC are specific to the developmental status of the embryos. In early stages when the embryos were in precirculating stages, BC was not very effective in initiating blood cell aggregation and delayed the onset of the circulation process; however, the circulation status (whether precirculating or not) has no effect on the heart rate (reduced in 10 µg/mL BC groups in 6 dpf irrespective of the circulation status). In the first two parameters (thrombi and onset of circulation), the treatment with BC was continued only for 48 h; however, in heart rate studies the treatment continued throughout the experiments (2−6 dpf). Moreover, from the present data we are unable to analyze the mechanism of thrombi formation in medaka, but we expect that BC probably activates the clotting factors and causes the platelets to aggregate in medaka embryos, which might be the cause of thrombus formation. Reports indicate that BC contains vasoactive glycosides and alkaloids which are able to produce toxic effects on the myocardium of laboratory animals (*21*). Moreover, BC may constrict coronary arteries and decrease the flow of oxygen to the heart (*9*−*13*). Taken together, it may be concluded that BC is able to induce adverse effects in cardiovasculature and that these effects are probably mediated by its alkaloid/saponin components.

The teratogenic effect of BC was further evaluated by examining the craniofacial cartilage development in medaka (Figure 3A−G). These parameters are considered as important features for the evaluation of teratogenesis in both mammalian and nonmammalian models (*41*−*43*). The cartilaginous and bony elements in the head skeleton of medaka embryos and hatchlings were described previously (*33, 35*). The development of the cartilaginous head skeleton starts at 5 dpf (Iwamatsu stage 33) and is complete at hatching (*35*). Therefore, we have examined the hatchlings (10 dpf) after staining with Alcian blue (*29*), and the embryos were pretreated 3 dpf with 2, 5, and 10 µg/mL BC for 48 h. Previously, we have observed that medaka hatchlings (10 dpf) showed Alcian blue positive regions (cartilages) only in the head-skeleton and tail (*29*). Staining of these hatchlings (10 dpf) with alizarin red did not show any positive regions (Bone). We, therefore, restricted our studies to cartilages and considered only the head and tail cartilages for the evaluation of teratogenesis. As observed previously, both the head-skeleton and tail were positive to Alcian blue, and there was no loss or absence of cartilages either in the head-skeleton or in the tail of the hatchlings treated with BC during embryogenesis (Figure 3A−G). In general, the total length of the skeleton was reduced compared to that in the controls, and the mean data were significantly different from those of the vehicle-treated controls only in the embryos exposed to 10 µg/mL BC (Figure 3H). A similar result was observed in the linear length of neurocranium (significantly reduced only in embryos exposed to 10 µg/mL BC compared to that in the controls). Further analysis of individual cartilages in the head-skeleton showed that the linear lengths of BB1−3, CB3, and CB4 were significantly reduced at 10 µg/mL BC concentrations (Figure 3H). Other cartilages appeared to be reduced in length; however, the data were not significantly different from those of the controls. Therefore, we conclude that the reduction of the total length of the embryos as well as the neurocranium by BC is a cumulative effect of reduction of all the craniofacial cartilages rather than the shortening of one particular cartilage.

After observing the significant effects of BC on cardiovascular and craniofacial features of medaka embryos, we have extended our investigation to the cellular level. We have used a subtractive hybridization technique to find a suitable BC-sensitive gene in medaka. This technique has been used for determining the expression of functional genes in many model organisms (*44*). We have identified *gata2*, a zinc finger transcription factor, as a possible BC-sensitive gene in medaka. Gata2 is an essential hematopoietic factor that is also expressed prominently in the nervous system (*45*). Moreover, both gata1 and gata2 were upregulated by a natural product *Panax notoginosides* in human bone marrow cells *in vitro* (*46*). Expression studies with *Xenopus* and gene targeting experiments with mice have established that *gata2* has a very early role in hematopoietic cell development (*47, 48*). Studies in zebrafish showed that gata2 expressed very early during embryogenesis (5-somite stage) and even before the expression of gata1 (*49*). Other studies indicate that GATA-binding sequences are required for the optimal expression of the endothelial cell-specific gene, including EDN1, a peptide hormone with potent vasoconstrictor properties (*50, 51*). This vasoactive peptide (EDN1) is also synthesized in nonvascular tissues (*52*). Production of EDN1 is regulated by numerous stimuli and the enhanced level of EDN1 may contribute to the progression of heart failure (*53*) via myocardial ischemia. Moreover, genetic and pharmacological studies demonstrate that EDN1 signaling patterns the ventral facial skeleton in mice, chicken, and fish (*54*). In zebrafish (*Danio rerio*), the ventral jaw structures are severely reduced and fused to their dorsal counterparts in *edn1* mutants (*54, 55*). We therefore studied the effect of BC on *gata2*, and several other genes participated in *edn1* mRNA expression and function in the medaka embryo (Figures 5−9).

Our studies with the expression of *gata2*, *preproedn1*, *ednrA*, *ece1*, and *furin1* genes during medaka development indicate that these genes are developmentally regulated and that all are expressed in 3 dpf embryos when they were initially exposed to BC. Our analyses by qPCR and/or ISH techniques showed that BC is able to induce *gata2* and *edn1* mRNA, and suggest a possibility that the cardiovascular and craniofacial defects caused by BC in medaka are probably mediated by gata2−edn1 signaling systems (Figures 5 and 6). EDN1 is a 21-residue peptide, a potent vasoconstrictor, secreted and synthesized by vascular endothelial cells (*56*−*59*). In vertebrates, EDNs are expressed as preproendothelin (preproEDN), which initially consists of ∼200 amino acids (aa), and are cleaved by furin-like enzymes (*60*) into an inactive 38 aa proendothelin (proEDN or big-EDN) (*61*). Further cleavage of proEDN by the enzyme endothelin-converting enzyme (ECE-1 and/or ECE-2) produces 21 aa endothelin (*62, 63*). In medaka, preproEDN consists of 195 aa (Ensemble number ENSORLT00000011634), and endothelin-like immunoreactivity was found in the hypothalamo-neurohypophysal system, caudal neurosecretory system, and the gill and kidney tissues (*64*).

The *edn1* mRNA we have measured is preproendothelin1 mRNA. The increase of *ece1* mRNA by BC suggests that preproEDN 1 should be converted to proEDN or big EDN in medaka embryos after BC treatment. Our data also showed that *furin* and *ednrA* mRNAs remained unaltered after BC treatment. However, developmental studies indicate that *furin* mRNAs were the highest in embryos during 4−6 dpf of development and that *ednrA* mRNA reached a peak level during this period. Therefore, it is expected that these two mRNAs are already in the elevated state and that further enhancement is not necessary for these biological effects. Expression of *furin* mRNA in medaka (adult tissues and embryos during development) has been studied previously (*65*). It was observed that in adult fish *furin* mRNA was expressed in a wide variety of tissues including the brain, heart, intestine, kidney, liver, spleen, testis, and small growing follicles in the ovary. However, in large ovarian follicles, the ovulated unfertilized oocytes, and the embryos up to 72 hpf, *furin* mRNA expression remained very low (*65*), which is in agreement with our data on *furin* mRNA expression during development (Figure 7). Furthermore, the *preproedn1* gene played a critical role in the development of jaw and opercular bones in fish (*66, 67*). Our studies with the skull cartilages of medaka indicate that both neurocranial and splanchnocranial cartilages are shorter in length in BC-treated hatchlings compared to those in the controls. Furthermore, *in situ* hybridization analysis indicates significant expression of *preproedn1* mRNA in the gill region (Figure 5B). In zebrafish, *edn1* expression was identified in the pharyngeal arches, and the expression of this gene is necessary for the correct patterning of pharyngeal cartilage development (*55*). Therefore, the enhancement of *edn1* mRNA by BC may cause craniofacial and cardiovascular abnormalities in the embryo, the teratogeneic features that are observed in several varieties of birth defects.

Conclusions

In this study, we have observed that BC interrupted medaka embryogenesis and produced an abnormal phenotype, which identifies BC as a potent teratogen. Moreover, the induction of *gata2* mRNA followed by *edn1* mRNA by BC indicates that the teratogenic response of BC is probably mediated by the Gata2-End1 signaling pathway.

Acknowledgment. This work was supported by the United States Food and Drug Administration (FDA) (FD-U-002071-01), National Center of Natural Product Research, Environmental Toxicology Research Program, and the Department of Pharmacology of the University of Mississippi.

Supporting Information Available: qPCR analysis as per Minimum Information for Publication of Quantitative real-Time PCR Experiments (MIQE) guide lines. This material is available free of charge via the Internet at http://pubs.acs.org.

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TX100205A