Research Square

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Administration of a herbal formulation enhanced blastocyst implantation via IκB activation in mouse endometrium

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Abstract

Background: BaelanChagsangBang (BCB), a herbal formulation consisting of eleven herbs, may be prescribed as a reproductive functional supplement to improve ovulation and implantation during the treatment of infertility and recurrent abortion in Korean Medicine. This study aimed to investigate the effects and action mechanisms of water-extracted BCB on endometrial receptivity and blastocyst implantation under normal conditions and in a mifepristone (RU486)-induced implantation failure murine model.

Methods: *In vitro*, the antioxidant potentials of BCB were evaluated using DPPH and superoxide anion radical scavenging assays and a DCFH-DA assay, and the cytotoxic and cytoprotective effects of BCB were confirmed using an MTT assay. *In vivo*, C57BL/6 female mice (n = 6 per group) orally received BCB (300 mg/kg/day), a dose similar to that used clinically, from 7 days before pregnancy until the end of the experiment. On day 4 of pregnancy, RU486 (4 mg/kg) was injected subcutaneously to induce implantation failure. The effect of BCB on embryo implantation was evaluated by implantation rate analysis, histological examination, and western blotting of uterus tissues.

Results: BCB water extract showed strong anti-oxidative and cytoprotective effects *in vitro*. *In vivo* administration of BCB water extract increased the number of newborn pups in BCB-treated mice versus sham-treated mice under normal conditions and improved the number of implantation sites in pregnant mice despite RU486 injection. BCB increased the protein levels of cyclooxygenase-2 and inducible nitric oxide synthase through IκB activation. Moreover, the expression levels of matrix metalloproteinases (MMPs) at uterus implantation sites were up-regulated in the BCB-treated group as compared with those in the RU486-treated group.

Conclusion: These results show BCB improved embryo implantation through IκB activation in our mouse model and suggest that BCB has therapeutic potential in the context of poor endometrial receptivity.

Background

Implantation is a process by which the embryo (blastocyst stage) attaches to endometrium in early pregnancy and is only allowed during a short period called the ‘implantation window’. This process is regulated by complex and precise interactions between the embryo and endometrium [1], which involve differentiation to form a receptive endometrium under the influences of various biological factors of embryonic and maternal origin that include cytokines, growth factors, and adhesion molecules [2]. Implantation failure is considered to be the result of embryo defects, decreased endometrial receptivity, and embryo-uterine dialogue failure [3]. Infertility is an increasing problem among couples of reproductive age, and over the past three decades, an increasing number of married women have sought pregnancy assistance due to infertility [4-6]. Although assisted reproduction techniques (ART) have been widely used for the clinical treatment of infertility resulting from benign gynecological disorders or primary unexplained infertility, subsequent pregnancy rates remain unsatisfactory [4, 7]. In traditional Eastern

medicine, herbal medicines, acupuncture, and moxibustion are used to treat female infertility [8, 9], and recently, due to accumulated scientific evidence, herbal medicine has emerged as an effective, complementary, alternative medicine for improving endometrial receptivity [10, 11].

In Eastern medicine, kidney deficiency, Qi and blood deficiencies, and liver congestion are considered to be the causes of infertility [12]. Thus, the most important treatments for infertility involve tonifying kidneys, reinforcing Qi, and nourishing blood to induce ovulation, improve oocyte quality, and generate a suitable endometrial environment [13]. BaelanChagsangBang (BCB) has been prescribed based on Wontogotae-tang in Bangyakhappyeon (a classical textbook of traditional Korean medicine) and Sutaehwan (Shou Tai Wan or Shoutai Pill) in *Yixue Zhongzhong Canxilu* (Records of Tradition Chinese and Western Medicine in Combination), as a supplement for reproductive system functions that can be used to improve ovulation and implantation during treatment for infertility and recurrent abortion [6, 14, 15]. BCB consists of a mixture of eleven herbs, that is, the seeds of *Cuscuta chinensis* Lam. (Dodder seed), the rhizomes of *Dioscorea japonica* Thunb., the fruits of *Rubus coreanus* Miq., the roots of *Panax ginseng* C. A. Mey., the fruits of *Lycium chinense* Mill., the roots of *Angelica gigas* Nakai, the leaves of *Perilla frutescens* L. Britton, the fruits of *Amomum villosum* Lour., the leaves of *Artemisia princeps* Pamp., the rhizomes of *Zingiber officinale* Roscoe, and the fruits of *Zizyphus jujuba* Mill. var. *inermis* Rehder. These BCB constituents are the main herbs used to tonify kidneys, reinforce Qi, and nourish blood. Although this prescription is used clinically to treat female infertility, experimental evidence obtained from *in vitro* and *in vivo* laboratory studies is lacking [6]. Mifepristone (also known as RU486) is a potent antiprogesterone agent, which can be used orally to induce abortion [16] or as an effective emergency contraceptive [17], and has often been used to induce embryo implantation failure or polycystic ovary syndrome in experimental animal models [10, 18, 19]. In the present study, we investigated the effects of a BCB water extract on endometrial receptivity and embryo implantation under normal conditions and in an RU486-induced mouse model of implantation failure.

Methods

Preparation of BCB

The constituents of BCB are shown in Table 1. All required herbs or herbal parts were purchased from Humanherb (Daegu, Korea), a good manufacturing practice (GMP)-certified Korean herbal medicine supplier. Voucher specimens are stored at the College of Korean Medicine, Dongguk University. To prepare the BCB extract, a mixture of the dried requisite parts of *Cuscutae Semen*, *Dioscoreae Rhizoma*, *Rubi Fructus*, *Ginseng Radix*, *Lycii Fructus*, *Angelicae Gigantis Radix*, *Perillae Folium*, *Amomi Fructus*, *Artemisiae Argyi Folium*, *Zingiberis Rhizoma Crudus*, and *Zizyphi Fructus* (weight ratio 8:16:10:4:4:2:4:4:4:3:2, respectively) was pulverized and extracted twice with 10 volumes of water at 85-90°C for 3 h. The extract was then passed through a 50 μm filter paper, and the filtrate was concentrated

by vacuum evaporation at 60°C, lyophilized to yield BCB extract (yield 6.5% based on herbal mixture weight).

BCB analysis by HPLC

Marker compounds in BCB extract were analyzed using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a dual pump, an autosampler, a temperature regulated column oven, a diode-array spectrophotometric detector, and Chromeleon 6.8 chromatography management system software [20]. Ellagic acid (Sigma-Aldrich, St. Louis, Mo, USA) and chlorogenic acid (Sigma-Aldrich) were used as standards. Component separations were achieved on a VDSpher C-18 column (VDSoptilab, Germany) maintained at 30°C. The column was step-gradient eluted using 0.3% trifluoroacetic acid (A) and acetonitrile (B) at a flow rate of 0.8 mL/min as follows: 10% B for 0-25 min, 60% B for 25-30 min, 100% B for 30-36 min, and 10% B for 36-40 min. Ellagic acid and chlorogenic acid were detected at 254 nm and 340 nm, respectively.

Cell culture and cell viability

Chinese hamster ovary (CHO)-K1 cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in DMEM (Dulbecco's modified Eagle's medium; WELGENE, Gyeongsan, Republic of Korea), supplemented with 10% fetal bovine serum (WELGENE) and 100 U/mL penicillin/100 μg/mL streptomycin (Thermo Fisher Scientific, Grand Island, NY, USA) at 37°C in a humidified incubator (5% CO_2/95% air; Thermo Fisher Scientific, Langenselbold, Germany). Cell viabilities were evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide (MTT) assay. Briefly, CHO-K1 cells were plated at a density of $5–7\times10^3$ cells per well in 96-well plates, incubated for 12 h, and treated with various concentrations of BCB (10–500 μg/mL) for 24 h. In other experiments, cells were pretreated with 50–500 μg/mL of BCB for 4 h, 1.5 mM of 4-vinylcyclohexene diepoxide (VCD; Sigma-Aldrich) was added, and cells were incubated for a further 24 h. Viable cells were stained with MTT solution (0.2 mg/mL, Sigma-Aldrich) for 3 h. Formazan crystals were completely dissolved by adding 100 μL dimethyl sulfoxide, and absorbances were measured at 540 nm using a microplate reader (Tecan, Research Triangle Park, NC, USA).

DPPH radical scavenging activity assay

The ability of BCB to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals was evaluated as previously described [20], and reductions in free radical levels were quantified by measuring absorbance (abs.) at 540 nm. Briefly, various concentrations of BCB (5–500 μg/mL) were mixed with 0.3 mM DPPH

ethanol solution and reacted for 30 min in the dark. Absorbances were measured at 515 nm and DPPH free-radical scavenging activities were calculated as follows:

Scavenging effect (%) = [(control abs. – sample abs.)/ control abs.] × 100.

Superoxide anion free-radical scavenging activity assay

The ability of BCB to scavenge superoxide anion free radicals was determined as previously described [20]. Briefly, BCB (5–500 μg/mL) was added to a solution containing 30 mM EDTA (pH 7.4), 30 mM hypoxanthine, and 1.42 mM nitro blue tetrazolium and pre-incubated at room temperature for 3 min. Xanthine oxidase (0.5 U/mL) was then added and allowed to react at room temperature for 20 min. Absorbances were measured at 560 nm.

Measurement of reactive oxygen species (ROS)

A DCFH-DA (2',7'-dichlorofluorescein diacetate) assay was used to assess intracellular ROS levels. CHO-K1 cells were plated on a black 96-well plate at 1×10^4 cells/well and incubated with 100 μM hydrogen peroxide (H_2O_2) in the presence or absence of BCB (100 μg/mL). After removing medium, 10 μM DCFH-DA was added to each well, and mixtures were incubated at 37°C for 30 min. Fluorescence intensities were measured at excitation and emission wavelengths of 480 and 530 nm, respectively, using a fluorescence microplate reader (SpectraMAX Gemini, Molecular Devices, Sunnyvale, CA, USA).

Animal experimental design and treatment

Male and female C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). Animals were bred separately by gender and given free access to drinking water and a standard diet and were maintained in a controlled environment under a 12 h light/dark cycle. All experimental procedures were performed according to the guidelines issued by the Animal Research Ethics Committee at Dongguk University Animal Center (IACUC-2016-001).

Experiment 1: Eighteen 4-week-old female mice were randomly divided into three groups: Control group, BCB 100 group, and BCB 300 group. The BCB 100 and BCB 300 groups were orally administered BCB at 100 or 300 mg/kg daily, respectively, for 30 days. At 6 pm local time on day 8 of the 30-day treatment period, all females were exposed to mating with 6-week-old males (ratio 2:1). Days on which vaginal plugs were first discovered, were designated as day 1 of pregnancy. The number of pups born to each mouse was recorded.

Experiment 2: Eighteen 4-week-old female mice were randomly divided into three groups: Control group, RU486 group, and RU486 plus BCB 300 group. Mice in the RU486 plus BCB 300 group were treated with BCB 300 mg/kg for 18-21 days. At 6 pm on day 8 during the 30-day treatment period, all females were exposed to mating with 6-week-old males (ratio 2:1). Days on which vaginal plugs were first discovered, were designated as day 1 of pregnancy. On day 4 of pregnancy, mice in the RU486 and RU486 plus BCB 300 groups were injected subcutaneously with RU486 solution (4 mg/kg, 0.08 mg/100 μL), whereas control group mice were injected with corn oil as vehicle. Seven days after RU486 injection, mice were sacrificed, uterine horns were excised, and numbers of implanted embryos in each uterine horn were counted.

Western blot analysis

For western blotting, uterus tissue was homogenized in protein lysis buffer consisting of 50 mM Tris-base (pH 7.5), 2 mM EDTA, 1% glycerol, 150 mM NaCl, 10 mM NaF, 10 mM Na-pyrophosphate, 1% NP-40 and protease inhibitors. Cell lysates (30 μg) were loaded and electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% skim milk in 0.1% TBS-T for at least 1 h, membranes were incubated with anti-phospho-IκB-α, anti-IκB-α, anti-β-actin (Cell Signaling Technology, Beverly, MA, USA), anti-MT1-MMP, anti-MMP2, anti-COX-2, or anti-iNOS (Abcam, Cambridge, MA, USA) for 16 h at 4°C. After washing with 0.05% TBS-T, membranes were treated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h, and after washing with 0.05% TBS-T, bands were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific). Band images were obtained using the ChemiDoc^{TM} XRS+ system (Bio-Rad, Hercules, CA, USA) and band intensities were analyzed by using Image Lab^{TM} software version 2.0.1 (Bio-Rad).

Hematoxylin and eosin (H&E) staining

For histological examination, uteri were removed, fixed in 10% formalin overnight, and dehydrated in 70% ethanol. Tissues were then paraffin-embedded, sectioned, and stained with H&E and smooth muscle actin. Three randomly selected sections were chosen for histopathological analysis.

Statistical analysis

Data were analyzed using the Student's *t*-test or by one-way ANOVA, and the significances of differences between means were determined using Dunnett's test or Tukey–Kramer's multiple comparison test. Null hypotheses of no intergroup differences were rejected for p -values of < 0.05. Results are presented as means ± SDs (*in vitro*) or means ± standard errors (*in vivo*), and the analysis was performed using SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Results

Antioxidant and cytoprotective effects of BCB extract

To assess the antioxidant effects of BCB extract, we first evaluated its free-radical scavenging activities. As shown in Fig. 1, BCB extract exhibited strong, dose-dependent, scavenging activities against DPPH and the superoxide anion radical. Next, the cytotoxic effect of BCB on CHO-K1 cells was evaluated using the MTT assay. Results showed that BCB at concentrations up to 500 μg/mL had no toxic effect on CHO-K1 cells (Fig. 2a). To examine the cellular antioxidant capacity of BCB, intracellular ROS levels were measured in H_2O_2-treated CHO-K1 cells. H_2O_2 treatment significantly increased intracellular ROS levels in CHO-K1 cells as compared with non-treated controls and this increase was suppressed by pretreating cells with 100 μg/mL BCB (Fig. 2b).

In addition, we evaluated whether BCB extract could exert cytoprotective effects using a cell-based screening system that we previously established [21]. VCD (4-vinylcyclohexene diepoxide), an occupational and environmental chemical of interest, can act as an ovotoxicant due to its ability to destroy ovarian follicles selectively [22]. To investigate the protection afforded by BCB extract against VCD, CHO-K1 cells were pretreated with BCB extract at various concentrations (50–500 μg/mL). As shown in Fig. 2c, BCB pretreatment significantly protected CHO-K1 cells against VCD-induced ovotoxicity at all concentrations but with maximal effect at 100 μg/mL.

BCB administration increased the *in vivo* implantation rate in mice

In order to determine the efficacy of BCB treatment on pregnancy rate under normal conditions, female mice were administrated 100 or 300 mg/kg of BCB daily for 30 days, after which, they were mated with normal male mice. Examination showed no significant difference between these two groups in terms of numbers of vaginal plugs (Fig. 3a). However, numbers of pups were significantly higher for 300 mg/kg BCB-treated mice than for 100 mg/kg BCB-treated mice or control mice (Fig. 3b). The food efficiency ratios of mice fed normal or experimental diets were not significantly different (Fig. 3c).

Next, we investigated whether the administration of 300 mg/kg BCB daily might promote blastocyst implantation *in vivo* in our RU486 (4 mg/kg) induced mouse implantation failure model. Numbers of live embryos were recorded on day 8 of pregnancy. The mean number of implanted embryos was markedly lower in the RU486-treated group (0.97 ± 0.98) than in the control group (6.66 ± 0.33) but significantly higher in the RU486 plus BCB group (3.16 ± 1.51) than in the RU486 group (Fig. 4a, b). These results show that BCB improved blastocyst implantation in mice under normal conditions and in our RU486-induced implantation failure model.

BCB inhibited matrix metalloproteinase (MMP) down-regulation in RU486-treated uteri

To determine whether BCB affects trophoblast invasion into endometrium, we examined the expression levels of MMP2 and membrane-type 1 MMP (MT1-MMP, also as known MMP14) in mouse uterus tissues. RU486 reduced the expression levels MMP2 and MT1-MMP, and BCB inhibited these down-regulations (Fig. 5b, c). To confirm the effect of BCB treatment on endometrium development, H&E staining of uterus tissue was carried out. Mice in the vehicle-treated control group showed intact endometria, but mice treated with RU486 exhibited damaged/destroyed endometria. However, BCB treatment protected against this RU486-induced destruction of endometrium (Fig. 5a).

BCB induced COX-2, iNOS, and IκBα expressions in RU486-treated uteri

In order to investigate the mechanisms responsible for the protective effect of BCB against RU486, we examined the inductions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthetase (iNOS) in uterus tissues. COX-2 is important for initiating decidualization [23], whereas iNOS is associated with the differentiation process during decidualization [24]. RU486 significantly reduced both COX-2 and iNOS expressions in uterus tissues, but BCB treatment inhibited these reductions (Fig. 6a). Since NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation is associated with implantation, we examined the expression levels of IκBα in uterus tissues. As shown in Fig. 6b, neither RU486 nor BCB changed the expression of IκBα, but BCB treatment significantly increased IκBα phosphorylation.

Identification of compounds in BCB extract

To enable control of BCB extract quality, HPLC analysis was performed using ellagic acid from *Rubi Fructus* and chlorogenic acid from *Lycii Fructus* as controls. The peaks of these two standards appeared at 15.53 min and 11.66 min, respectively (Fig. 7). Concentrations of both acids in BCB extract were determined using standard calibration curves. The amounts of ellagic and chlorogenic acids present in the BCB extract were 3.78 and 0.79 mg/g, respectively.

Discussion

The present study demonstrates that the administration of a hot water extract of BCB can increase numbers of newborn pups as compared with sham-treated mice under normal conditions and increase the number of implantation sites in pregnant mice treated with RU486. Of the 11 ingredients of BCB, *Cuscutae Semen* and *Rubi Fructus* are often used to treat female infertility as the main herbs used to tonify kidneys in traditional medicine. *Cuscutae Semen* has been shown to increase the number of ovulating ovaries significantly in mice, and *Rubi Fructus* has been reported to improve female gonadal function imbalance and follicle numbers and sizes [25-28]. *Artemisiae Argyi Folium* is also used to treat female infertility and gonadal function imbalance [29]. Moreover, *Ginseng Radix* and *Lycii Fructus* have effects that include the reinforcement of Qi and Yin and the enhancement of embryo implantation and

endometrial environment [30, 31]. *Angelicae Gigantis Radix* is an effective treatment for infertility due to its ability to nourish blood [32, 33]. *Dioscoreae Rhizoma* has been shown to prevent abortion, reinforce essences, and tonify kidneys [34], and *Perillae Folium* and *Amomi Fuctus* are representative herbal medicines that can be used to reduce the risk of abortion under threatening conditions [35]. Therefore, the improvement in blastocyst implantation rate observed after the administration of BCB extract may be due to tonifying kidneys, reinforcing Qi, and nourishing the blood. Furthermore, these complementary properties of BCB constituents would appear to be beneficial for embryo implantation. Although we did not elucidate the mechanism responsible for the beneficial effects of BCB on infertility, these findings strongly suggest that BCB has therapeutic potential in the context of poor endometrial receptivity.

NF-κB exists in cytoplasm in the form of inactive NF-κB/IκB complexes formed binding to IκBα [36], and NF-κB activation results from IκBα degradation induced by IκBα phosphorylation and is known to be crucial for mouse embryos development beyond the 2-cell stage [37]. Levels of NF-κB generally increase in the human endometrium premenstrually and during early pregnancy, and these increases may regulate molecules essential for implantation [38, 39]. In the present study, RU486 reduced mean numbers of implanted embryos and IκBα activation, but BCB administration induced the phosphorylation of IκBα in RU486-treated uterus tissues, suggesting that BCB might improve implantation rates by activating the NF-κB/IκBα pathway. However, how BCB regulates IκBα activation in endometrium remains unknown.

NF-κB is critical for the induction of both iNOS and COX-2 in various cells including gingival fibroblasts and endometrial stromal cells [40, 41], and the expression of pro-inflammatory cytokines in uterus throughout the estrous cycle is necessary for embryo receptivity and successful blastocyst implantation [42]. Decidualization describes a series of changes in uterus during the early stages of pregnancy that are necessary for placenta formation and fetal development [43, 44]. In particular, COX-2 is important for the initiation of decidualization and its deletion suppressed fertilization, implantation, and decidualization in a COX-2 deficient mouse model [23]. Nitric oxide (NO) is a key mediator of various physiological functions including vascular functions and inflammatory responses [45], and has been reported to play a crucial role during implantation and pregnancy establishment [46, 47]. In addition, NO may regulate the growth and development of preimplantation embryos [48, 49]. NO is generated by three isoforms of nitric oxide synthase (NOS), and up-regulations of cytokine-inducible NOS (iNOS) and endothelial NOS (eNOS) have been reported in pregnant rodent uteri [50]. In the present study, we also observed that BCB prevented the RU486-induced down-regulations of COX-2 and iNOS in RU486-treated mouse uteri, which suggests BCB-induced IκBα activation might induce COX-2 and iNOS to support implantation and decidualization.

iNOS has also been reported to play an important role in promoting trophoblast invasion and to be particularly abundant at the leading edge of migrating trophoblasts [51], and NO production by iNOS has been shown to regulate trophoblast invasion [51]. Matrix metalloproteinases (MMPs) are indicators of the blastocyst trophoblast invasion of endometrial cells, and MMP expression levels are correlated with trophoblast invasiveness [52]. MMP2 (also as known gelatinase A) is known to degrade ECM and is expressed in endometrium within three days of the onset of blastocyst implantation, which suggests

MMP2 might be the primary mediator of endometrium invasion by blastocysts [51]. MT1-MMP, another key MMP, has different functions, which include the degradation of various ECM components and the activation of proMMP-2. Based on this knowledge, we investigated whether BCB affects trophoblast invasion into endometrium. We found that BCB inhibited the down-regulations of MMP2 and MT1-MMP protein levels in RU486-treated uteri and protected against RU486-induced destruction of the endometrium.

ROS, oxidative stress, and antioxidants have been implicated in the establishment and progression of pregnancy including embryo implantation, placental differentiation, and embryo growth [53, 54]. However, ROS can also cause pathological conditions within the female reproductive system [55]. Excess ROS in follicles may overwhelm follicular fluid antioxidant defense, directly damage oocytes, cause defective fertilization, and even when fertilization has been achieved, oxidative stress-induced apoptosis may result in embryo fragmentation, implantation failure, abortion, impaired placentation, or congenital abnormalities [56]. Nicol *et al*. (2000) reported glucose 6-phosphate dehydrogenase prevented oxidative stress-induced embryopathies [57], and more recently, Qin *et al*. (2016) reported that dehydroepiandrosterone improves endometrium receptivity and enhances embryo implantation by inhibiting the generation of intracellular ROS in endometrial stromal cells [58]. In concert with these findings, in the present study, BCB showed strong scavenging activities against DPPH and superoxide anion radicals and inhibited ROS production in CHO-K1 cells treated with H_2O_2. Notably, ellagic acid and chlorogenic acid in BCB have strong antioxidant effects [59, 60]. Although the present study did not demonstrate that the mechanism of action of BCB is directly related to antioxidant activity, evidence suggests that antioxidant activity is a contributory factor.

Conclusions

We investigated the effects of a hot water extract of BCB on endometrial receptivity in a mouse model of RU486-induced implantation failure. Administration of BCB extract increased numbers of newborn pups as compared with sham-treated mice under normal conditions and increased the number of implantation sites in pregnant mice treated with RU486. In addition, BCB increased the expressions of COX-2 and iNOS via IκBα phosphorylation and up-regulated MMP2 and MT1-MMP levels in uterus implantation sites in RU486-treated mice. Furthermore, BCB exhibited strong anti-oxidative effects in DPPH and superoxide anion free-radicals scavenging assays. These findings show that BCB, a herbal medicine, improved embryo implantation via IκB activation in our murine model and suggest BCB has potential use as a treatment for female infertility.

Abbreviations

BCB: BaelanChagsangBang; CHO: Chinese hamster ovary; COX-2: cyclooxygenase-2; DCFH-DA: 2',7'-dichlorofluorescein diacetate; DMEM: Dulbecco's modified Eagle's medium; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ECM: extracellular matrix; EGF: epidermal growth factor; eNOS: endothelial nitric oxide synthase; H&E: hematoxylin and eosin; IκB: inhibitor of NF-κB; iNOS: inducible nitric oxide synthetase; MMP: matrix metalloproteinase; MT1-MMP: membrane-type 1 MMP; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NO: nitric oxide; NOS: nitric oxide synthase; ROS: reactive oxygen species; VCD: vinylcyclohexene diepoxide

Declarations

Acknowledgments

Not applicable.

Authors' contributions

DIK as the principal director and study supervision were responsible for the design of the study and obtained funding. JHL, SJ, and QFL participated in the study design and experiments and wrote the manuscript. HC, HJJ, and SHK carried out the experiments and the statistical analysis. All authors participating in the preparation of the manuscript approved the final version.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal experiments were approved beforehand by the Institutional Animal Care and Use Committee of Dongguk University (Approval No. IACUC-2016-001).

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Figures

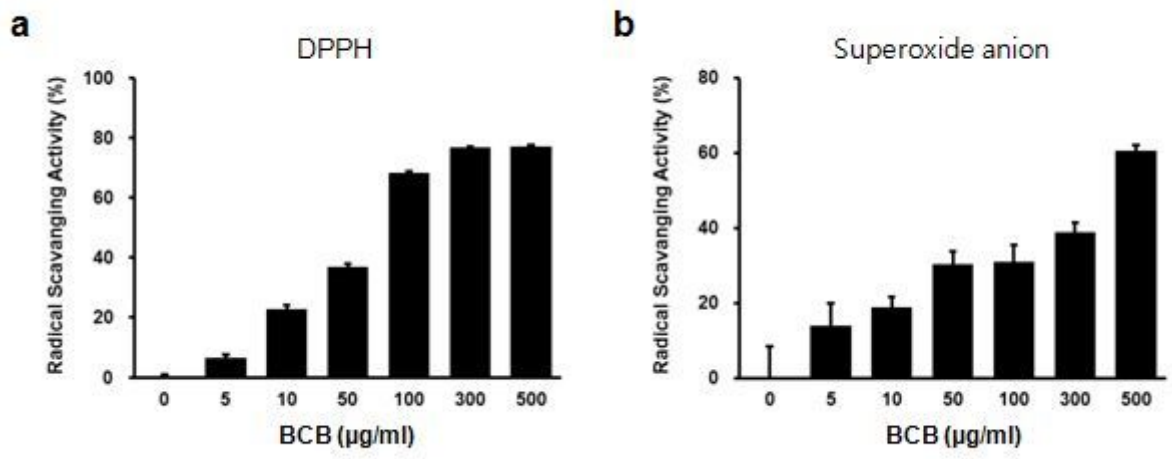

Figure 1

Free radical scavenging activities of BCB extract. a DPPH radical scavenging activity of BCB extract. b Superoxide anion free-radical scavenging activity of BCB extract.

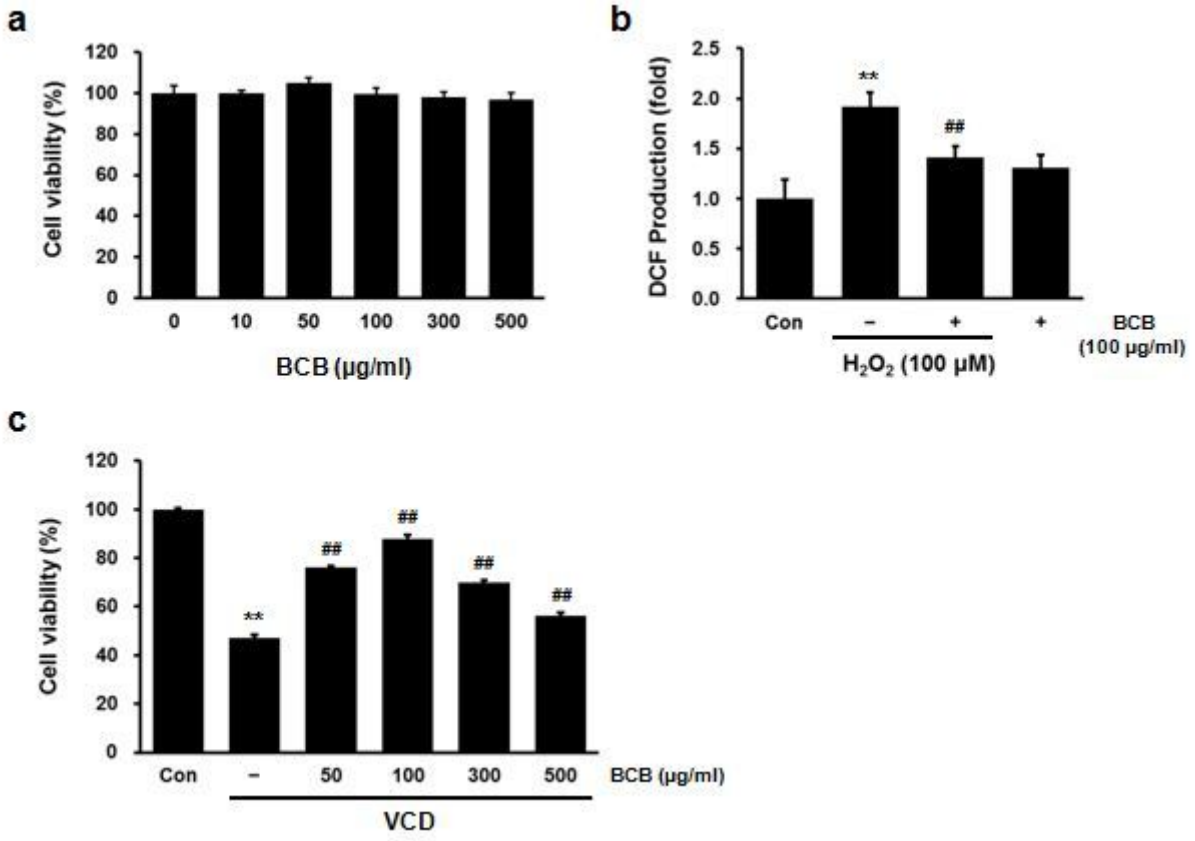

Figure 2

Effects of BCB on ROS generation and its ovotoxicity in CHO-K1 cells. a Cytotoxicity of BCB in CHO-K1 cells. Cells were treated with various concentrations of BCB extract (10, 50, 100, 300, or 500 μg/mL) for 24 h. Cell viabilities were measured using an MTT assay. Results are presented as percentages of vehicle-treated controls. b Effect of BCB extract on ROS generation. CHO-K1 cells were treated with 100 μM H2O2 with or without 100 μg/mL BCB. Fold increases in ROS versus vehicle-treated controls, were determined by measuring DCF fluorescence intensities. c Effect of BCB extract on ovotoxicity. CHO-K1 cells were pretreated with different concentrations (50–500 μg/mL) of BCB for 2 h and then treated with 1.5 mM VCD for 24 h. (significant vs. vehicle-treated controls, ** $p < 0.01$; significant vs. VCD or H2O2 treated cells, ## $p < 0.01$)

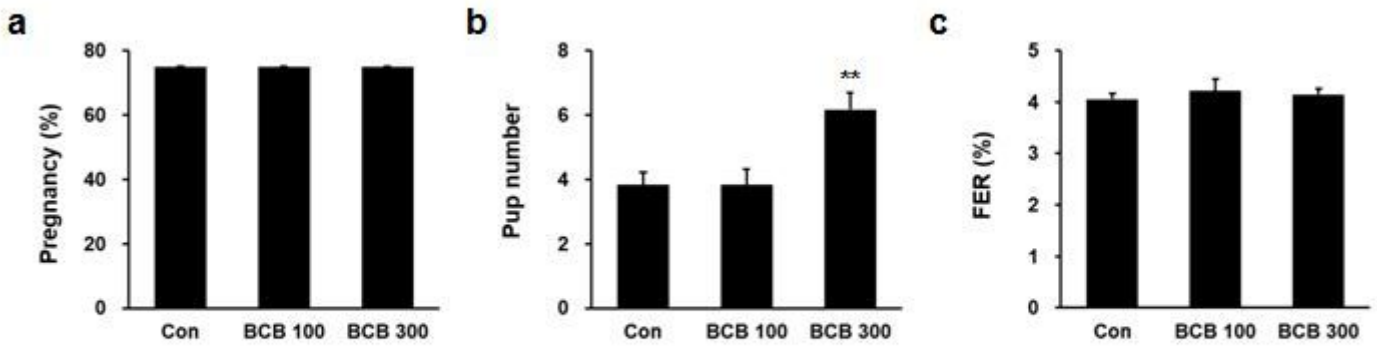

Figure 3

Effects of BCB extract on pregnancy rate and number of newborn pups in normal mice. Female mice were administrated 100 mg/kg or 300 mg/kg of BCB daily for 30 days. After BCB treatment, female mice were mated with normal male mice. Pregnancy rates were measured by counting vaginal plugs (a) and numbers of pups (b). c Food efficiency ratios (FER), expressed as percentages of body weight gains on food intake. Data are presented as means ± SEMs. Groups are: Control (n = 6), BCB 100 mg/kg (BCB 100, n = 6), and BCB 300 mg/kg (BCB 300, n = 6). ** $p < 0.001$ vs. controls.

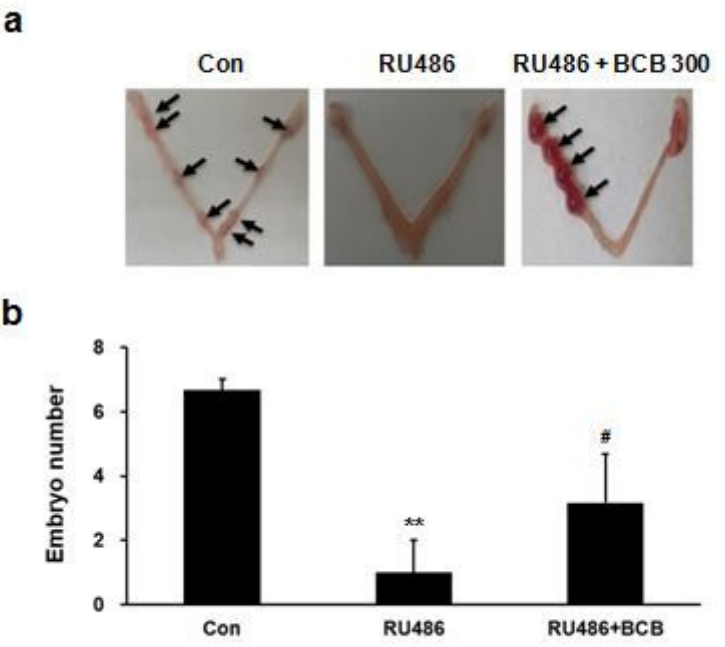

Figure 4

Effects of BCB on in vivo implantation rate in the RU486-induced implantation failure model. Female mice were administrated 300 mg/kg BCB for 18-21 days. On day 8, all females were mated with males, and days when vaginal plugs were first detected were designated as day 1 of pregnancy. The RU486 and RU486 plus BCB 300 groups were injected subcutaneously with RU486 solution (4 mg/kg, 0.08 mg/100 μL), while controls were injected with corn oil as vehicle on day 4 of pregnancy. Seven days after RU486 injection, mice were sacrificed, and uterine horns were excised. a Representative photographs of uterine horns showing embryo implantation sites. b Quantification of implanted embryos in each uterine horn. Data are presented as means ± SEMs. Groups are: the control group (n = 6), the RU486 group (RU486, n = 6) and the RU486 plus BCB 300 group (RU486+BCB 300, n = 6). ** p < 0.001 vs. controls.

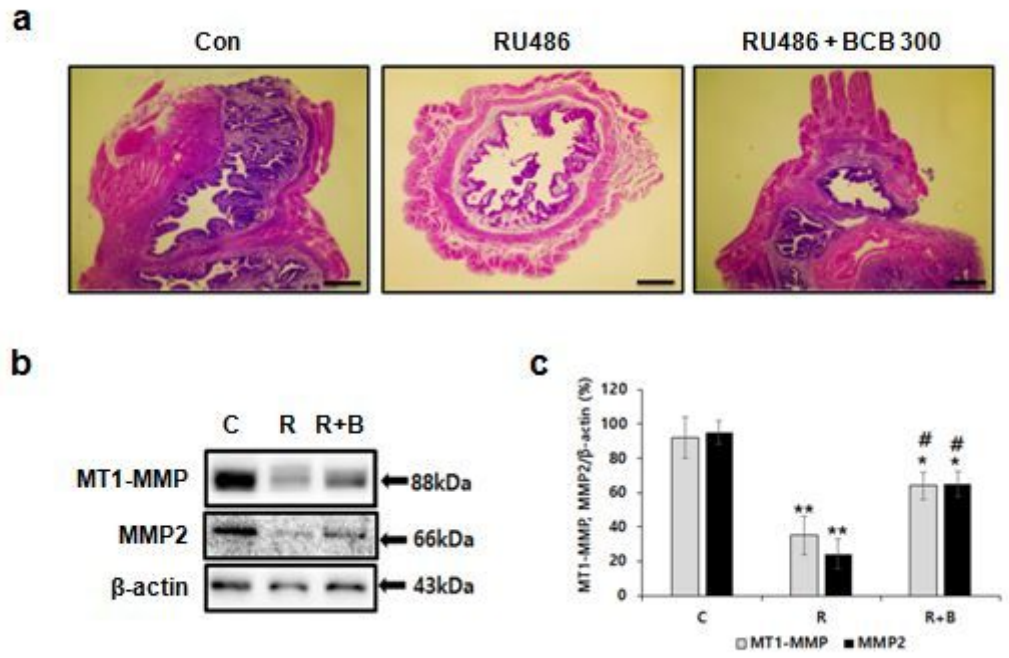

Figure 5

BCB protected against RU486-induced matrix metalloproteinase (MMP) down-regulation in uteri. Female mice were administrated 300 mg/kg BCB for 18-21 days. On day 8, all females were mated with males, and days when vaginal plugs were first detected were designated as day 1 of pregnancy. On day 4 of pregnancy, animals in the RU486 and RU486 plus BCB 300 groups were injected subcutaneously with RU486 solution (4 mg/kg, 0.08 mg/100 μL), while controls were injected with corn oil as vehicle. Seven days after RU486 injection, mice were sacrificed, and uterus tissue was dissected. Endometria were stained with H&E (a). Uterus tissues were homogenized and immunoblotted with MT1-MMP and MMP2 antibodies (b). Protein levels were normalized versus β-actin (c). Data are presented as means ± SEMs. Groups are: the control group (C, n = 3), the RU486 group (R, n = 3), and the RU486 plus BCB 300 group (R+B, n = 3). ** p < 0.01, * p < 0.05 vs. controls, ## p < 0.01, # p < 0.05 vs. the RU486 group.

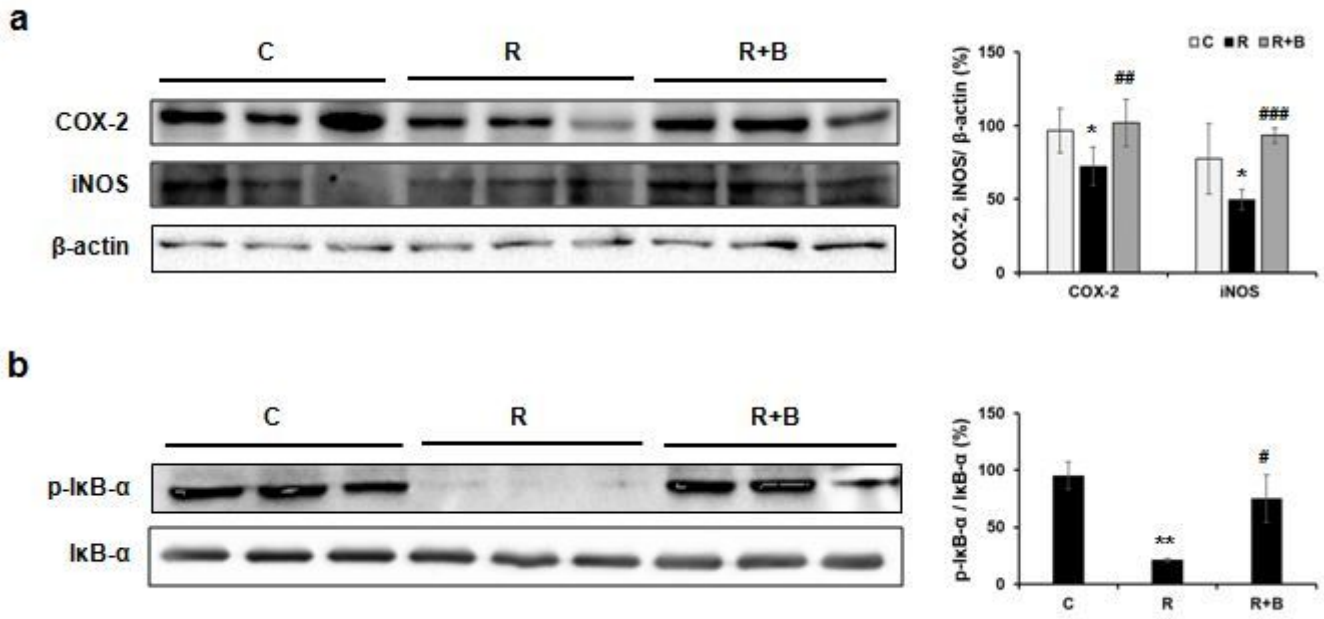

Figure 6

6 BCB induced the expressions of COX-2, iNOS, and IκBα in RU486-treated mouse uteri. Seven days after RU486 injection, uterus tissues were dissected and immunoblotted with COX-2 and iNOS. Protein levels were normalized versus β-actin (a). Uterus tissue lysates were immunoblotted for phosphorylated IκBα and total IκBα and normalized versus total IκBα (b). Data are presented as means ± SEMs. Groups are: the control group (c, n = 3), the RU486 group (R, n = 3), and the RU486 plus BCB 300 group (R+B, n = 3). ** $p < 0.01$ vs. controls, # $p < 0.05$ vs. the RU486 group.

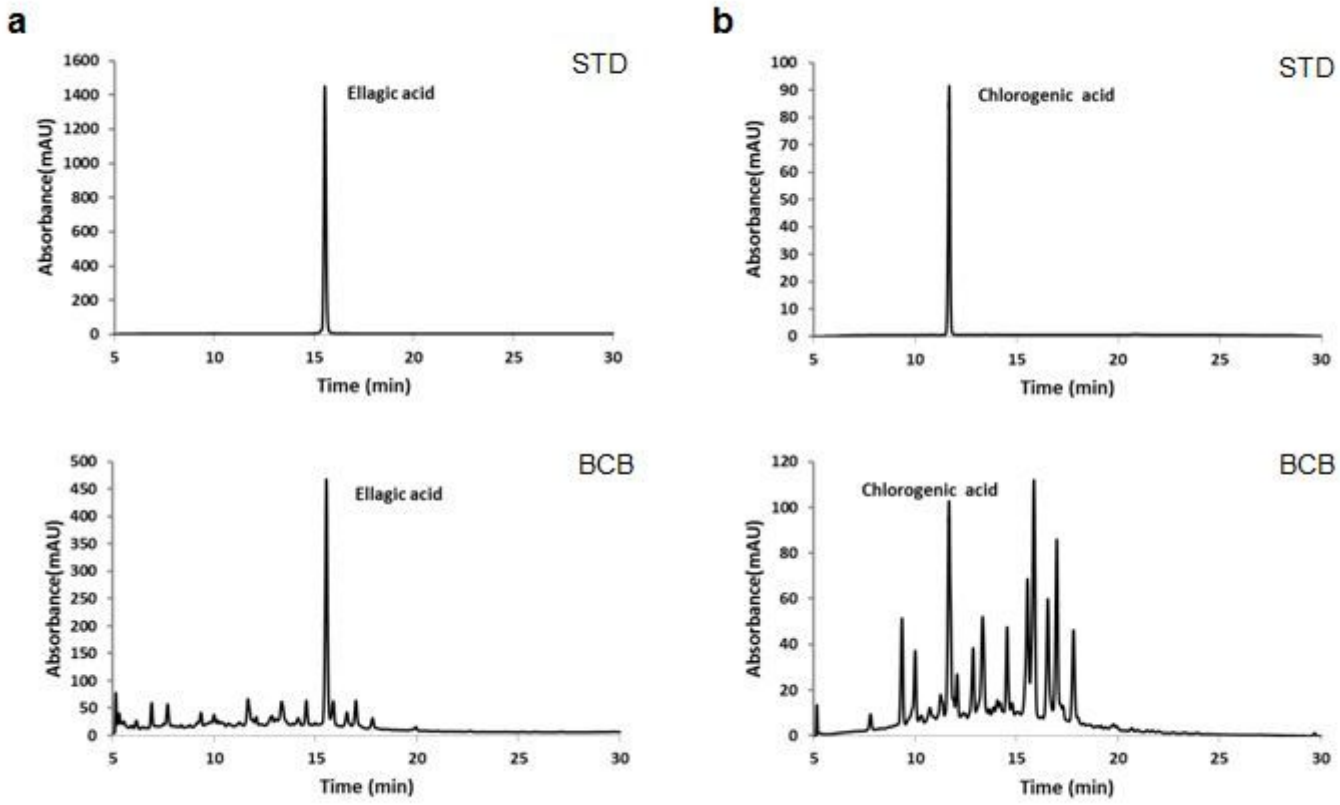

Figure 7

Representative HPLC chromatogram of the BCB water extract. Two compounds, ellagic acid (a) and chlorogenic acid (b) were chosen as marker compounds for quality control purposes. The retention times of ellagic and chlorogenic acids were 15.53 and 11.66 min, respectively.