Administration of Herb Formulation Enhanced Blastocyst Implantation via IkB Activation in Mouse Endometrium

Songhee Jeon  
Chonnam National University

Quan Feng Liu  
Dongguk University

Hua Cai  
Chonnam National University

Ha Jin Jeong  
Chonnam National University

Su-Hyun Kim  
Sangji University

Dong-Il Kim  
Dongguk University

Ju-Hee Lee (✉️ jh1548@dongguk.ac.kr)  
Dongguk University  https://orcid.org/0000-0003-4147-9794

Research

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Abstract

Background

Baelan Chagsang Bang (BCB), consisting of a mixture of 11 herbs, may be prescribed as a reproductive function supplement to improve ovulation and implantation during the treatment of infertility and recurrent abortion in Oriental Medicine. This study aimed to investigate the effects and mechanisms of action of water-extracted BCB on endometrial receptivity and blastocyst implantation in a mifepristone (RU486)-induced implantation failure model.

Methods

In *in vitro*, antioxidant potentials of BCB were evaluated using DPPH and superoxide anion radical scavenging assays and DCFH-DA 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. BCB was administrated from 7 days before pregnancy until the end of the experiment. They were housed with male mice until pregnancy was confirmed. On day 4 of pregnancy, RU486 (4 mg/kg) was injected subcutaneously to induce implantation failure.

Results

Administration of the BCB water extract increased the number of newborn pups in BCB-treated mice compared to that of sham-treated mice and improved the number of implantation sites in pregnant mice despite RU486 injection. BCB increased the protein expression level of cyclooxygenase-2 and inducible nitric oxide synthase through IκB activation. Moreover, the expression level of matrix metalloproteinases (MMPs) at the uterus implantation site was up-regulated in the BCB-treated group compared to that of the RU486-treated group. To determine the mechanisms of action of BCB, the anti-oxidative effect of BCB was examined via *in vitro* assay. The BCB water extract showed strong anti-oxidative effects in the DPPH assay and the superoxide anion free-radical scavenging activity assay.

Conclusion

Our findings provide evidence that BCB has therapeutic potential against poor endometrial receptivity.

Background

Implantation is the process by which the embryo (blastocyst stage) attaches to the endometrium in early pregnancy and is only allowed in a very short period called the implantation window. This process is regulated by a complex and precise interaction between the embryo and the endometrium [1]. This interaction involves differentiation to form a receptive endometrium by various biological factors of the embryonic and maternal origin, including cytokines, growth factors, and adhesion molecules [2]. Implantation failure is considered as a result of the embryo defects itself, 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]. Embryo implantation failure is one of the leading causes of unsatisfactory outcomes. Traditional Oriental medicine has been using herbal medicine, acupuncture, and moxibustion to treat female infertility [8–10]. Recently, herbal medicine has emerged as complementary and alternative medicine as obtained scientific evidence for its efficacy in improving endometrial receptivity [11, 12].

In Oriental medicine, kidney deficiency, Qi and blood deficiencies, and liver congestion are considered to be the causes of infertility [13]. Thus, the most important treatments for infertility involve tonifying the kidney, reinforcing Qi, and nourishing the blood to induce ovulation, improve oocyte quality, and generate a suitable endometrial environment [14]. BaelanChagsangBang (BCB) has been prescribed based on the information in the book Dongeuibogam [8], as a supplement for reproductive system functions that can be used to improve ovulation and implantation during treatment for infertility and recurrent abortion. BCB consists of a mixture of 11 herbs; 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.

Mifepristone (also known as RU486) is a potent antiprogesterone and antiglucocorticosteroid agent in humans and can be used orally as an abortion pill [15]. It also has been known to be effective as an emergency contraceptive [16]. Its action mechanism involves antagonism of the intracellular receptors of progesterone and glucocorticosteroids. During medical abortion procedures, the blockage of progesterone receptors by RU486 induces endometrial decidual degeneration, cervical softening and dilatation, endogenous prostaglandin release, and increase myometrial sensitivity to the prostaglandins stimulating contraction. Furthermore, it decreased production of human chorionic gonadotropin (hCG), which in turn results in decreased production of progesterone by the corpus luteum. RU486 has been often used to induce embryo implantation failure or polycystic ovary syndrome in experimental animal models [11, 17–19].

In the present study, we investigated the effects of a BCB water extract on endometrial receptivity and embryo implantation 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. Their voucher specimens are stored at the College of Korean Medicine, Dongguk University. To prepare the BCB extract, a mixture of the 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* in the ratio 8:16:10:4:4:2:4:4:3:2, respectively, was pulverized and extracted twice with 10 volumes of water at 85–90 °C for 3 h. The extract was then passed through using a 50 µm filter paper and the filtrate was concentrated by vacuum evaporation at 60 °C. Finally, the concentrate was lyophilized; the yield was 6.5%.

Table 1
Composition of BaelanChagsangBang

| Latin name               | Scientific name                        | Used part | Voucher specimen   | Ratio |
|--------------------------|----------------------------------------|-----------|--------------------|-------|
| Cuscutae Semen           | *Cuscuta chinensis* Lam. (Convolvulaceae) | Seed      | DUMCKM2015-100     | 8     |
| Dioscoreae Rhizoma       | *Dioscorea batatas* Decne.             | Rhizome   | DUMCKM2015-036     | 16    |
| Rubi Fructus             | *Rubus coreanus* Miq. (Rosaceae)       | Fruit     | DUMCKM2015-110     | 10    |
| Ginseng Radix            | *Panax ginseng* C.A.Mey. (Araliaceae)  | Root      | DUMCKM2015-054     | 4     |
| Lycii Fructus            | *Lycium chinense* Mill. / *Lycium barbarum* L. (Solanaceae) | Fruit     | DUMCKM2015-008     | 4     |
| Angelicae Gigantis Radix | *Angelica gigas* Nakai (Umbelliferae)  | Root      | DUMCKM2015-015     | 2     |
| Perillae Folium          | *Perilla frutescens* L. Britton (Labiatae) | Leaf     | DUMCKM2015-055     | 4     |
| Amomi Fructus            | *Amomum villosum* Lou. (Zingiberaceae) | Fruit     | DUMCKM2015-034     | 4     |
| Artemisiae Argyi Folium  | *Artemisia argyi* Lev. et Vant.        | Leaf      | DUMCKM2015-101     | 4     |
|                          | *Artemisia princeps* Pamp.             |           |                    |       |
|                          | *Artemisia montana* (Nakai) Pamp. (Compositae) | | | |
| Zingiberis Rhizoma Crudus| *Zingiber officinale* Roscoe (Zingiberaceae) | Rhizome   | DUMCKM2015-004     | 3     |
| Zizyphi Fructus          | *Zizyphus jujuba* Mill. var. inermis Rehder (Rhamnaceae) | Fruit     | DUMCKM2015-016     | 2     |

BCB analysis by HPLC
The BCB extract was analyzed its marker compounds using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a dual pump, an autosampler, a thermostatted column oven, and a diode-array spectrophotometric detector and controlled by the 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 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. The 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 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), and incubated at 37 °C in a humidified incubator (5% CO₂/95% air; Thermo Fisher Scientific, Langenselbold, Germany). Cell viabilities were evaluated using a thiazolyl blue tetrazolium bromide (MTT) assay. CHO-K1 cells were plated at a density of 5–7 × 10^3 cells per well in 96-well plates and after incubating for 12 h, 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 before adding 1.5 mM of 4-vinylcyclohexene diepoxide (VCD; Sigma-Aldrich) and then incubated for a further 24 h. Viable cells were stained with MTT solution (0.2 mg/mL, Sigma-Aldrich) for 3 h, and then the formazan crystals were completely dissolved by adding 100 µL dimethyl sulforxide. 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 the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radicals was evaluated as previously described [20], and reductions in their 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:

\[
\text{Scavenging effect (\%)} = \frac{[(\text{control abs.} - \text{sample abs.})/ \text{control abs.}]}{\times 100.}
\]

**Superoxide anion free-radical scavenging activity assay**

The ability of BCB to scavenge the superoxide anion free-radical was determined by applying the method described previously [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 was pre-incubated at room temperature for 3 min. Subsequently, 0.5 U/mL xanthine oxidase was added to the mixture. The solution was reacted at room temperature for 20 min, and absorbance measured at 560 nm.

**Measurement of reactive oxygen species (ROS)**
A DCFH-DA (2',7'-dichlorofluorescein diacetate) assay was used to assess intracellular ROS. CHO-K1 cells were plated on a black 96-well plate at $1 \times 10^4$ 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 the mixture incubated at 37 °C for 30 min. Fluorescence intensities were measured at 480 nm excitation and 530 nm emission wavelengths, by 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 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 treated with BCB at 100 mg/kg or 300 mg/kg daily, respectively, for 30 days. At 6 pm local time on day 8 during the 30-day treatment period, all females were exposed to mating with 6-week-old males (ratio 2:1). Day on which vaginal plugs were first discovered, was 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. The mice in the RU486 plus BCB 300 group were treated with BCB 300 mg/kg for 18–21 days. At 6 pm local time on day 8 during the 30-day treatment period, all females were exposed to mating with 6-week-old males (ratio 2:1). Day on which vaginal plugs were first discovered, was designated as day 1 of pregnancy. On day 4 of pregnancy, the 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 a vehicle. Seven days after RU486 injection, the 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 onto nitrocellulose membranes. After blocking with 5% skim milk in 0.1% TBS-T for over 1 h, the 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, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h. After washing with 0.05%
TBS-T, the bands were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific). Band images were obtained by using the ChemiDoc™ XRS + system (Bio-Rad, Hercules, CA, USA) and band intensities were analyzed by using Image Lab™ software version 2.0.1 (Bio-Rad).

**Hematoxylin and eosin (H&E) staining**

For histological examination, uteri were removed and fixed in 10% formalin overnight, followed by dehydration in 70% ethanol. The tissues were paraffin embedded, then 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 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 difference were rejected when $p$-values were $< 0.05$. Results are presented as means ± SD (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**

ROS, oxidative stress, and antioxidants have been implicated in the establishment and progression of pregnancy including embryo implantation, placental differentiation, and embryo growth [21, 22]. Recently, a study reported that dehydroepiandrosterone improves endometrium receptivity and enhances embryo implantation by inhibiting the generation of intracellular ROS in endometrial stromal cells [23]. On that basis, we hypothesized that the beneficial effects of BCB are probably due to its antioxidant effect. To assess the antioxidant effects of the BCB extract, we first evaluated its free-radical scavenging activities. As shown in Fig. 1, the BCB extract exhibited strong scavenging activities against DPPH and superoxide anion radical in a dose-dependent manner. Next, the cytotoxicity of BCB on CHO-K1 cells was evaluated by using the MTT assay. The results showed that BCB at concentrations up to 500 µg/mL had no toxicity on CHO-K1 cells (Fig. 2a). To examine the cellular antioxidant capacity of BCB, intracellular ROS levels were measured in H$_2$O$_2$-treated CHO-K1 cells. Compared to the non-treated controls, the H$_2$O$_2$ treatment significantly increased intracellular ROS levels in CHO-K1 cells, and this increase was suppressed by pretreating cells with 100 µg/mL BCB (Fig. 2b).

In addition, we evaluated whether the BCB extract could exert a cytoprotective effects using a cell-based powerful screening system that we previously established [24]. VCD, an occupational and environmental chemical of interest, can act as an ovotoxicant due to its ability to destroy ovarian follicles selectively [25]. 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 indicated concentrations but with maximal effect at 100 µg/mL.

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

In order to elucidate the efficacy of BCB treatment on pregnancy rate under normal conditions, female mice were administrated 100 mg/kg or 300 mg/kg of BCB daily for 30 days, after which, the female mice were mated with normal male mice. Upon examination, there was no significant difference between these two groups in terms of numbers of vaginal plugs (Fig. 3a). However, the number of pups was 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 the normal or experimental diets were not significantly different (Fig. 3c).

Next, we investigated whether the administration of 300 mg/kg BCB might promote blastocyst implantation in vivo in our mouse implantation failure model that had been induced by an RU486 (4 mg/kg) injection. The numbers of live embryos were recorded on day 8 of pregnancy. 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 was 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 our RU486-induced implantation failure model.

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

Matrix metalloproteinases (MMPs) are indicators of blastocyst trophoblast invasion of endometrial cells, and MMP expression levels are correlated with the invasiveness of trophoblasts. Generally, MMPs are secreted into extracellular matrix (ECM) and hydrolyze ECM [26, 27]. In this study, we examined the expression levels of MMP2 and membrane-type 1 MMP (MT1-MMP, MMP14) in mouse uterus tissues. RU486 reduced the expression levels MMP2 and MMP14, 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. The 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 induction of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthetase (iNOS) in the uterus tissues. COX-2 is important for the initiation of decidualization [28], whereas iNOS is associated
with the differentiation process during decidualization [29]. RU486 significantly reduced both COX-2 and iNOS expressions in uterus tissues, but BCB treatment inhibited these reductions (Fig. 6a). In human endometrium, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) levels are typically elevated during the premenstrual phase and during early pregnancy and these increases may regulate molecules vital for implantation [30, 31]. The NF-κB is sequestered in the cytoplasm as an inactive complex bound with an inhibitor of NF-κB (IκB) and is activated by separation and degradation of IκB [32–34]. Thus, we examined the expression levels of IκBα in uterus tissues. As shown in Fig. 6b, neither RU486 nor BCB changed the expression of IκBα; however, BCB treatment significantly increased IκBα phosphorylation.

**Identification of compounds in BCB extract**

To control the quality of the BCB extract, 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 11.66 min for chlorogenic acid and 15.53 min for ellagic acid (Fig. 7). Concentrations of both acids in the BCB extract were determined by applying the standard calibration curves. The amounts of ellagic and chlorogenic acids present in the BCB extract used in this study were 3.78 mg/g and 0.79 mg/g, respectively.

**Discussion**

The present study demonstrates that the administration of a hot water extract of BCB can increase the number of newborn pups as compared with sham-treated mice and can improve the number of implantation sites in pregnant mice treated with RU486.

BCB contains the 11 herbs; *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.* Among those, *Cuscutae Semen* is often used to treat female infertility in traditional medicine and has been shown to significantly increase the number of ovulating ovaries in mice [35]. *Cuscutae Semen, Dioscoreae Rhizoma,* and *Rubi Fructus* are the main herbs used to tonify kidneys, and *Rubi Fructus* is also used to treat female infertility [35, 36]. Furthermore, *Rubi Fructus* has been reported to improve female gonadal function imbalance and follicle numbers and sizes [37, 38]. In addition, *Dioscoreae Rhizoma* has been shown to prevent abortion, reinforce essences, and tonify kidneys [39]. Moreover, *Ginseng Radix* and *Lycii Fructus* have effects that include the reinforcing of Qi and Yin and improve embryo implantation and the endometrial environment [40, 41]. *Angelicae Gigantis Radix* is also an effective treatment for infertility due to its ability to nourish blood [42, 43]. *Perillae Folium* and *Amomi Fuctus* are representative herbal medicine that may be used to reduce the risk of abortion under threatening conditions [44]. *Artemisiae Argyi Folium* is also a representative herbal medicine and used to treat female infertility and gonadal function imbalance [45]. These previous findings strongly suggest BCB has therapeutic potential in the context of poor endometrial receptivity.
Decidualization is a series of changes in uterus during the early stages of pregnancy that are necessary for placenta formation and fetal development [46–48]. Decidualization occurs only near the embryos in mice, whereas this process occurs periodically, regardless of the presence or absence of an embryo in humans [49]. During decidualization, uterine epithelium falls apart and is followed by extensive proliferation and angiogenesis in the subepithelial stroma [46]. The signal transduction required for successful decidualization is generated by two types of cell–cell interactions: embryo–epithelial and epithelial–stromal [46]. COX-2 and epidermal growth factor (EGF) family members including heparin binding-EGF (HB-EGF) are activated in luminal epithelium surrounding blastocyst and induce activation of Bmp2 (bone morphogenic protein 2) and Wnt4 (wingless-related mouse mammary tumor virus integration site 4) resulting in stromal cell growth [46, 50]. COX-2 deletion suppresses implantation and decidualization as well as fertilization in the COX-2 deficiency mouse model [28]. In the present study, we also demonstrated that BCB can prevent the RU486-induced down-regulation of COX-2 in the RU486-treated mouse uterus, which suggests BCB-induced COX-2 may be involved in implantation and decidualization.

Nitric oxide (NO) is a key mediator of various physiological functions including vascular functions and inflammatory responses [51, 52], and has been reported to play a crucial role during implantation and pregnancy establishment [53–55]. In addition, it has been reported that NO may regulate the growth and development of preimplantation embryos [56–58]. NO is generated by three isoforms of nitric oxide synthase (NOS). Up-regulation of cytokine-inducible NOS (iNOS) and endothelial NOS (eNOS) have been found in pregnant rodent uteri [59, 60]. In this study, we demonstrated that BCB administration increased the expression of iNOS in RU486-treated mouse uteri, suggesting that BCB-induced iNOS may enhance implantation and decidualization.

NF-κB induces COX-2 expression in various cells including gingival fibroblasts and endometrial stromal cells [61–63], and the expression of pro-inflammatory cytokines in uterus throughout the estrous cycle is necessary for embryo receptivity and successful blastocyst implantation [64]. NF-κB dimers are sequestered in the cytoplasm by binding with IκBα and hence inactivated [65]. Activation of NF-κB occurs via IκBα degradation, which is initiated by IκB kinase complex-induced phosphorylation of IκBα. NF-κB activation is known to be crucial for development beyond the 2-cell stage of mouse embryos [66]. Furthermore, the deregulation of IκBα has been associated with the aging of mouse and bovine oocytes [67–70]. In the present study, we observed BCB administration induced the phosphorylation of IκBα in uterus tissues, suggesting that BCB might improve implantation rates via the NF-κB/IκBα activation pathway.

ROS have beneficial effects on biological functions but can also produce pathological conditions within the female reproductive system [71]. ROS concentration may also have major effects on the implantation and fertilization of eggs [72]. Recently, Qin et al. (2016) reported that dehydroepiandrosterone improves endometrium receptivity and enhance embryo implantation by inhibiting the generation of intracellular ROS in endometrial stromal cells [23], and Nicol et al. (2000) reported glucose 6-phosphate dehydrogenase prevented oxidative stress-induced embryopathies [73]. In concert with those findings, in
this study, BCB showed strong scavenging activities against DPPH and the superoxide anion radicals, and inhibited ROS production in CHO-K1 cells treated with H$_2$O$_2$. These results suggest that the therapeutic effect of BCB against infertility may be due to antioxidant activity.

**Conclusions**

In the present study, 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 the BCB extract increased the numbers of newborn pups as compared with sham-treated mice and improved the number of implantation sites in pregnant mice treated with RU486. In addition, BCB increased the expressions of COX-2 and iNOS via IkBα phosphorylation, and up-regulated MMP levels in uterus implantation sites in RU486 + BCB group as compared with RU486-treated group. Furthermore, BCB exhibited strong antioxidative effects in DPPH and superoxide anion free-radicals scavenging assays. These findings show that BCB, a herbal medicine, has potential use as a treatment for 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; IkB:inhibitor of NF-κB; iNOS:inducible nitric oxide synthetase; MMP:matrix metalloproteinase; MTT:thiazolyl blue tetrazolium 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](image)

**Figure 1**

The free radical scavenging activities of the BCB extract. a DPPH radical scavenging activity of the BCB extract. b Superoxide anion free-radical scavenging activity of the BCB extract.
Effects of BCB on ROS generation and 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 viability was measured using an MTT assay. Results are presented as percentages of vehicle-treated control. 

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 compared to the vehicle-treated control, 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, followed by treatment with 1.5 mM VCD for 24 h. (significant vs. vehicle-treated control, **p<0.01; significant vs. VCD or H2O2 treatment, ##p<0.01)
Figure 3

Effects of BCB extract on pregnancy rate and the number of newborn pups in normal mice. Female mice were administrated 100mg/kg or 300 mg/kg of BCB in daily for 30 days. After BCB treatment, female mice were mated with normal male mice. Pregnancy rate was measured by counting vaginal plugs (a) and the number of pups (b). c Food efficiency ratio (FER) expressed as the percentage of the body weight gain per food intake. Data are mean ± SEM values. Groups are: Control (n=6), BCB 100mg/kg (BCB 100, n=6), and BCB 300mg/kg (BCB 300, n=6). **p < 0.001 vs. control.
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 the first detection of vaginal plug was 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/100µL), while the control group was injected with corn oil as a 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 with embryo implantation sites. b Quantification of implanted embryos on each uterine horn. Data are mean ± SEM values. 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. control.
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 the first detection of vaginal plug was designated as day 1 of pregnancy. On day 4 of pregnancy, the RU486 and RU486 plus BCB 300 groups were injected subcutaneously with RU486 solution (4 mg/kg, 0.08 mg/100µL), while the control group was injected with corn oil as a vehicle. Seven days after RU486 injection, mice were sacrificed, and uterus tissue 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 that of β-actin (c). Data are mean ± SEM values. 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. control, ##p<0.01, # p<0.05 vs. the RU486 group.
Figure 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 mean ± SEM values. 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. control, # 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.