Benzoic Acid Enhances Embryo Implantation through LIF-Dependent Expression of Integrin $\alpha V\beta 3$ and $\alpha V\beta 5$

Hee-Jung Choi$^1\dag$, Tae-Wook Chung$^1\dag$, Mi-Ju Park$^1$, Hyung Sik Kim$^2$, Sooseong You$^1$, Myeong Soo Lee$^3$, Bo Sun Joo$^1$, Kyu Sup Lee$^3$, Keuk-Jun Kim$^5$, Gabbine Wee$^6$, Choong-Yong Kim$^6$, Cheorl-Ho Kim$^7$, and Ki-Tae Ha$^1\ast$

$^1$School of Korean Medicine and Healthy Aging, Korean Medicine Research Center, Pusan National University, Yangsan 50612, Republic of Korea
$^2$Laboratory of Molecular Toxicology, School of Pharmacy, SungKyunKwan University, Suwon 16419, Republic of Korea
$^3$Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea
$^4$Department of Obstetrics and Gynecology, Pusan National University Hospital, Busan 49241, Republic of Korea
$^5$Department of Clinical Pathology, DaeguGyeongbuk University, Gyeongsan 38547, Republic of Korea
$^6$Laboratory Animal Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 41061, Republic of Korea
$^7$Department of Biological Science, Sungkyunkwan University, Suwon 16419, Republic of Korea

Embryo implantation is the crucial step for a successful pregnancy. Diverse factors, including adhesion molecules, growth factors, and cytokines are important for embryo implantation through improving endometrial receptivity. Benzoic acid (BA), a component of various plants, has been shown to have antifungal and antioxidant effects. However, the effect of BA on embryo implantation remains unknown. Here, we showed the contribution of BA for the enhancement of endometrial receptivity through the leukemia inhibitory factor (LIF)-dependent increase of integrin $\alpha V$, $\beta 3$, and $\beta 5$ expression. Furthermore, in vivo study using a mifepristone-induced implantation failure model showed that BA definitely improves the numbers of implantation embryos. Taken together, we suggest that BA has a novel function for embryo implantation through the up-regulation of LIF-mediated integrins, and may be a candidate for therapeutic medicine to increase the pregnancy rate.

Keywords: Benzoic acid, leukemia inhibitory factor, integrin, adhesion, endometrial receptivity, implantation
Benzoic acid (BA) is a simple aromatic carboxylic acid that is available synthetically as well as naturally by isolating from the wide varieties of plants as a source of secondary metabolites [16, 17]. Previous studies demonstrated that BA has antifungal and antioxidant effects [16]. In addition, its sodium salt derivative, sodium benzoate, was reported to have an inhibitory effect on D-amino acid oxidase, DNA synthesis, drug-induced cytotoxicity, and ROS-dependent immune responses (such as responses involving natural killer cells and neutrophils) [17-22]. In vivo studies showed that sodium benzoate has a protective effect on gentamicin-induced renal failure and ethanol-induced gastric lesions [23, 24]. Although there are general concerns regarding BA toxicity, it has recently been reported that BA has no chronic, genetic, reproductive, or developmental toxicity when it administered orally [16]. However, there are currently no reports investigating the effect of BA on improving endometrial receptivity.

In this study, we examined the effect of BA on the expression of LIF and adhesion molecules in endometrial Ishikawa cells, and the adhesion of trophoblastic JAr cells to Ishikawa cells. In addition, the in vivo effect of BA on embryo implantation was also evaluated using a mifepristone-induced implantation failure model.

Fig. 1. Effect of benzonic acid (BA) on enhancement of cell adhesion and expression of adhesion molecules.
(A) Structural representation of BA. (B) Ishikawa cells were cultured at indicated concentrations of BA for 24 h. The viability of the cells was measured using MTT assay. (C) Ishikawa cells were treated with 50 μM of BA for 48 h. Fluorescence-labeled JAr cells were added to the Ishikawa cells. After incubation and washing, the number of adhered JAr cells was calculated and expressed as a fold of control (Mean ± SD). **p < 0.01 compared between two groups. (D) Ishikawa cells were treated with 50 μM of BA for 24 h. The expression levels of ITGAV, ITGB1, ITGB3, ITGB4, ITGB5, CD44s, E-cadherin, ICAM-1, and L-selectin, were examined by RT-PCR. (E) Ishikawa cells were treated with 50 μM of BA for 48 h. The expression levels of surface sLeA and sLeX were measured by FACS analysis with specific antibodies for sLeA and sLeX structures, respectively.
Materials and Methods

Materials

BA was obtained from Sigma-Aldrich (USA; purity >99.5%); its structure is shown in Fig. 1A. Mifepristone (RU; progesterone receptor antagonist) was purchased from Sigma-Aldrich.

Cell Culture

The established human endometrial Ishikawa cell line was provided by Dr. Jacques Simard (CHUL Research Center, Canada). The cells were maintained using Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO₂. The human trophoblastic JAr cells were obtained from the Korean Cell Line Bank (Korea) and cultured in RPMI-1640 (Welgene) containing 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA).

Cell Viability Assay

The cytotoxicity caused by BA was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Ishikawa cells were cultured in 24-well plates with BA (indicated concentrations) for 24 h. The cells were then washed with PBS, and MTT (0.5 mg/ml) was added to each well. After incubation for 4 h, the supernatants were removed. DMSO/EtOH (1:1) solution was added to dissolve the purple formazan in living cells, and measurements were obtained at 540 nm with a microplate reader (SpectraMax M2; Molecular Devices, USA). The percentage of living cells was calculated against untreated cells.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from Ishikawa cells using RiboEx (GeneAll, Korea). An equal amount of total RNA (1 μg) from each sample was then subjected to reverse transcription with oligo-dT primers using M-MLV reverse transcriptase (Enzynomics, Korea). The cDNA was amplified by PCR using AccuPower PCR PreMix (Bioneer Co., Korea). The primers used in this study are shown in Table 1.

Flow Cytometric Analysis

The cell surface sialyl Lewis A (sLeA) and sialyl Lewis X (sLeX) were determined by flow cytometric analysis as described previously [25]. Detached cells were incubated with their respective antibodies on ice for 2 h, and then incubated with FITC-conjugated goat anti-mouse IgM. Washed cells were analyzed by flow cytometry (FACS Canto II; BD Biosciences, USA). The level of antigen synthesized on the cell surface was expressed as the mean fluorescence intensity. The antibodies for sLe A (121SLE) and sLe X (CSLEX1) were purchased from EXBIO Praha (Czech Republic) and BD Pharmingen, respectively.

Western Blot Analysis

Equal amounts of total proteins (20 μg) from Ishikawa cells exposed to BA (indicated concentrations) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes blocked by 5% skim-milk/TBS-T solution were incubated with anti-LIF (Santa Cruz Biotechnology Inc., USA) and anti-β-actin (Sigma-Aldrich) antibodies. After the reaction with appropriate secondary antibodies linked with horseradish peroxide, the signals were visualized using the ECL chemiluminescence system (GE Healthcare, UK).

Cell Adhesion Assay

Ishikawa cells (1.5 × 10⁶ cells) were seeded into 6 well plates and cultured for 24 h. The medium was replaced and the cells were incubated in serum-free DMEM containing BA and/or purified recombinant human LIF receptor antagonist (hLA) [26] for 48 h. The JAr cells were labeled with 5-chloromethylfluoresceindiacetate (CMFDA) fluorescence dye (CellTracker Green; Life Technologies, USA) for 15 min at 37°C. The labeled JAr cells were then washed in 1× PBS and gently added onto Ishikawa cell monolayers in JAr growth medium. After gently shaking at 40 rpm for 30 min at 37°C, the cells were washed to exclude non-binding JAr cells. The attached JAr cells, visualized by using a fluorescent microscope

Table 1. The primers used in this study.

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>LIF</td>
<td>Forward: 5'-GGCCCGGACACCCAT AGACG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCACGGCGCATCAGGTGA AA-3'</td>
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<td>ITGAV</td>
<td>Forward: 5'-ATGCTTCCATGTAATCCACAAAGAT-3'</td>
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<td></td>
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<td>ITGB1</td>
<td>Forward: 5'-GTCGTGTTGAGTGGACAC-3'</td>
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<td></td>
<td>Reverse: 5'-GCGGGGTTAATTGTTGCCA-3'</td>
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<td>Reverse: 5'-AAAGGGATTTGCGACGTGTACT-3'</td>
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<tr>
<td>ICAM-1</td>
<td>Forward: 5'-CAGTGCAATCATCAGCTTTGCCG-3</td>
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<td>Reverse: 5'-GCTGCCCATACACAGTGAGCAC-3'</td>
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<td>L-selectin</td>
<td>Forward: 5'-AACCCATGAACTGGCAAAG-3</td>
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<td>β-Actin</td>
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<tr>
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<td>Reverse: 5'-TCCTTCTGCAATCCAGTGCGC-3'</td>
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Knockdown of LIF by shRNA

To knock down endogenous hLIF, lentiviral shRNA constructs were obtained from Open Biosystems (Thermo Fisher Scientific). The lentiviral vector encoding shRNA (3 μg) was transfected into 293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). After 48 h, supernatant harboring lentiviruses was collected and then infected into Ishikawa cells. These cells were selected using 3 μg/ml puromycin over 1 week. The knockdown efficiency of the shRNAs against LIF expression was verified by western blot analysis.

Animals

Male and female C57BL/6 mice, inbred in a specific pathogen-free facility (7–8 weeks old, weight 20–22 g), were purchased from Orient Bio, Co. (Korea). They were bred separately and had free access to water and a standard diet with a 12 h light:12 h dark cycle. All experimental procedures were examined and approved by the Animal Research Ethics Committee at the Pusan University of Korea (No. PNU-2015-0921).

Animal Models and Treatment

The embryo implantation failure model was performed as described previously [27]. Briefly, all 24 female mice were randomly divided into three groups: a control group, an RU group, and an RU with BA group. The female mice belonging to the RU with BA group were administered BA (2.5 mg/kg/day) using an oral Zonde needle, whereas the mice belonging to the control and RU groups received the same amount of PBS. Seven days after BA treatment, all females were caged with males (ratio 2:1) at 6pm, and day 1 of pregnancy was defined by the presence of vaginal plugs the following morning. The mice belonging to the RU group and the RU with BA group were injected subcutaneously with 0.08 mg/0.1 ml RU solution, whereas the mice belonging to the control group were injected with the same volume of corn oil on day 4 of pregnancy at 9 am. Seven days after RU treatment, all mice were sacrificed and both uterine horns were excised to determine the number of implantation sites. The implanted embryos on each uterine were recorded.

Statistical Analysis

The difference between the mean values of two groups was determined by a Student t-test using GraphPad Prism (GraphPad Software, USA). The minimum significance level was set at a p value of 0.05. All experiments, except for the animal studies, were independently performed at least three times.

Results

BA Increases the Adhesion of JAr Cells onto Ishikawa Cells and the Expression of Integrin αV, β3, and β5 in Ishikawa Cells

BA did not show significant levels of cytotoxicity up to 500 μM, as measured by MTT assay (Fig. 1B). As shown in Fig. 1C, BA treatment (50 μM) clearly enhanced the adhesion of human trophoblastic JAr cells to endometrial Ishikawa cells. Next, we investigated the effect of BA on the expression of the adhesion molecules, including integrins, CD44, E-cadherin, ICAM-1, sLeA, and sLeX, which are reported as regulators of adhesion between the endometrium and trophoblast [13, 14, 28–30]. The mRNA levels of integrin αV (ITGAV), β3 (ITGB3), and β5 (ITGB5) showed a considerable increase in Ishikawa cells after BA treatment, whereas the expression levels of integrin β1 (ITGB1), β4 (ITGB4), CD44, E-cadherin, ICAM-1, L-selectin, sLeA, and sLeX were not significantly induced by BA (Figs. 1D and 1E).

BA-Increased LIF Expression Is Mediated by ERα Pathway

We examined the expression of cytokines, including IL-11 and LIF, well-known as regulators of the adhesion molecules involved in embryo implantation [4, 7]. The result showed that BA increases the expression of LIF in endometrial Ishikawa cells. However, the expression of IL-11 was not increased by BA treatment (Fig. 2A). Next, we examined the effect of BA on the expression of LIF in human endometrial Ishikawa cells. When Ishikawa cells were stimulated with BA, both the mRNA and protein levels of LIF were dramatically increased in a dose-dependent manner (Fig. 2B). To examine the contributions of MAPK, NF-κB, and PI3K to BA-induced LIF expression, we pre-incubated Ishikawa cells with SP600125 (JNK inhibitor), LY294002 (PI3K inhibitor), SB203580 (p38MAPK inhibitor), U0126 (MEK/ERK inhibitor), or Bay11-7082 (NF-κB inhibitor), before BA treatment. BA-induced LIF expression was not reduced by any of these inhibitors (Fig. S1). Previous studies have shown that p53 and estrogen up-regulate the expression of LIF [31-33], and ERα plays an essential role during embryo implantation [34]. Our data showed that the expression of p53 was not changed by BA treatment in Ishikawa cells (Fig. S2). The results shown in Fig. 2C demonstrated that pre-treatment with ICI 182,780, an antagonist for ERα, clearly blocked BA-stimulated LIF expression at both the mRNA and protein levels. In addition, BA-stimulated nuclear translocation of ERα was reduced by pre-incubation of ICI 182,780 in Ishikawa cells (Fig. 2D).

BA Increases the Expression of Adhesion Molecules and Endometrial Receptivity through LIF–Dependent Pathway

As shown in Fig. 3A, we knocked down LIF gene expression in Ishikawa cells by infection with lentiviral vector harboring shRNA for LIF (shLIF). BA increased the...
expression levels of LIF protein in pLKO.1-transfected Ishikawa cells, but not in shLIF. To further examine whether the expression of BA-induced integrins was increased by an LIF-dependent pathway or not, we confirmed the mRNA levels of ITGAV, ITGB3, and ITGB5 in pLKO.1- or shLIF-transfected cells after BA treatment. The expression levels of ITGAV, ITGB3, and ITGB5 mRNAs were markedly decreased in BA-induced shLIF-transfected cells compared with that in BA-induced pLKO.1 cells (Figs. 3A and 3B). Despite of BA stimulation, the numbers of attached JAr cells were consistently reduced in shLIF-transfected cells, compared with BA-treated pLKO.1 cells (Fig. 3C). LIF binds to its receptor, LIFR, expressed on the endometrial epithelium, which consequently facilitates blastocyst attachment and implantation [5]. Thus, to investigate whether the enhanced attachment of trophoblast to BA-induced Ishikawa cells is a result of ligation of LIF to LIFR, the activation of LIFR was blocked by its antagonist, hLA. With pre-treatment of hLA prior to BA stimulation, the receptivity of Ishikawa cells toward JAr cells was significantly reduced (Fig. 3D). These results clearly demonstrate that the LIF/LIFR pathway is crucial for adhesion between Ishikawa cells and JAr cells.

**Effect of BA on the Implantation Sites of Pregnant Mice**

To improve endometrial receptivity, female mice were treated with BA (2.5 mg/kg/day) for part of the experiment (17 days). To interrupt the pregnancy, an antagonist of progesterone receptor, RU, was injected after mouse mating on day 3. At 7 days after subcutaneous injection of RU, the number of live embryos from each group was recorded. Compared with the control group (8.50 ± 0.19), the implantation sites were clearly depleted in female mice of the RU group (Figs. 4A and 4B). The number of implantation sites in the RU plus BA group (5.75 ± 1.42) was significantly increased compared with that of the RU group (1.86 ± 1.13). The results clearly show that BA improves the implantation of the blastocyst in the RU-induced
implantation failure model. In addition, the results from biochemical analysis, including serum concentrations of creatinine, BUN, AST, and ALT, clearly indicated that the oral dose was not toxic to female mice (Fig. S3).

**Discussion**

Recently, the focus for infertility treatment has been shifting from the possibility of achieving good quality
embryos to improving the receptivity of the endometrium [35, 36]. Excitingly, more efficient technologies are being developed to assess endometrial receptivity. The endometrial receptivity array, a diagnostic tool for infertility treatment, has recently been developed based on the knowledge of endometrial receptivity markers, including LIF, prostaglandins, and adhesion molecules [35]. However, to date, infertility treatments based on the improvement of endometrial receptivity to increase implantation efficiency have still not been developed. The improvement of implantation efficiency is an issue that has not yet been resolved by assisted reproductive technology [37]. Owing to this point, we are struggling to find out novel and effective agents that increase endometrial receptivity, to provide new alternatives for infertility treatments. Zhang et al. [38] reported that Scutellaria baicalensis and its ingredient baicalin increased the adhesion of trophoblast cells to endometrial cells through enhancing endometrial receptivity via the elevation of Wnt/β-catenin signaling-mediated FUT4 expression in endometrial cells, and facilitated embryo implantation in vivo [38]. In our previous report, Paeonia lactiflora increased the expression of adhesion molecules,
including integrin β3 and β5, by inducing p38 and ERK signaling-mediated LIF expression, leading to an increase in trophoblast adhesion to the endometrium, and improved embryo implantation in an implantation failure mouse model [27]. Furthermore, we confirmed that *P. lactiflora* contained gallic acid, catechin, methyl gallate, paeoniflorin, and BA. Even though BA is not a major indication component of *P. lactiflora*, it showed the highest induction of LIF expression among the components of *P. lactiflora* [27]. Thus, we investigated whether BA improves the embryo implantation by inducing endometrial receptivity. Here, we are the first to report that BA increases the endometrial receptivity through LIF-dependent regulation of integrin αV, β3, and β5 expression (Fig. 4C).

Zhao et al. [39] reported that the alteration of LIF and integrin β3 plays an essential role in increasing endometrial receptivity during embryo implantation. In addition to this, according to our recent study, the expression of integrin αV, β3, and β5 increased in an LIF-dependent manner. Furthermore, blocking the action of integrin αV/β3 and αV/β5 by treatment of neutralizing antibodies disrupted the adhesion between Ishikawa cells and JAr cells [40]. The results in this study showed good agreement to these previous reports.

In maternal reproduction, it has been reported that the function of p53 is crucial to the regulation of LIF expression [33]. In addition, LIF expression is regulated through the induction of mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), and phosphoinositide-3-kinase (PI3K) pathways [41-43]. The interaction between p53 and MAPKs are bidirectional (i.e., MAPKs phosphorylate and activate p53). p53 is also an upstream activator for transcriptional activation of MAPKs [44]. The NF-κB and PI3K signaling pathways also crosstalk with p53 [45, 46]. Thus, to determine which signaling pathways is actually responsible for BA-induced LIF expression, several inhibitors were tested. Contrary to our expectations, the BA-induced LIF expression was not decreased by these inhibitors against known signaling pathways (Fig. S1). In addition, the expression of p53 was not significantly affected by BA treatment (Fig. S2). To explore another pathway accounting for BA stimulation of LIF expression, we focused on the function of steroid hormones. According to several reports, estrogen is involved in the regulation of uterine LIF production during embryo implantation through ERα [31, 47]. When Ishikawa cells were pretreated with ICI 182,780, a high affinity antagonist for ER, the expression of LIF and nuclear translocation of ERα were significantly reduced in BA-treated cells (Figs. 2C and 2D). Therefore, our data clearly showed that ERα is required for BA-stimulated LIF expression.

Several previous reports showed that LIF is up-regulated at the period of implantation windows in uterine cells, including the uterine epithelium, glands, and endometrial stromal cells of several species, such as mouse, rabbit, and human [48, 49]. In addition, LIF has been known to be a crucial factor for successful implantation in mammals, including humans [4, 50]. RU is a steroidal anti-progestogen that was used as an abortifacient drug in the early stages of pregnancy. Furthermore, RU reduces endometrial glandular LIF expression at the expected time of implantation, and inhibits human blastocyst attachment to an in vitro endometrial three-dimensional cell [51, 52]. Thus, to evaluate the effect of BA on implantation efficiency in in vivo experiments, we used the RU-induced implantation depletion model that was adopted in our previous study [27]. The results from in vivo study suggest that BA enhanced LIF expression through the steroid hormone-independent pathway, which ultimately leads to improved embryo implantation. Further extensive experiments to elucidate the exact mechanism underlying BA-induced LIF expression are still needed.

BA, a well-known natural compound harboring an aromatic acid structure, has been used as a preservative in the cosmetics and foods industries [53]. According to a report on safety assessment by the US Food and Drug Administration, BA is generally recognized as safe in foods. The World Health Organization has also recommended 5 mg/kg as an acceptable daily intake of BA [16]. Our data also showed that BA is not severely toxic to endometrial Ishikawa cells. In the in vivo study, female mice were orally administered BA (2.5 mg/kg/day) for 17 days using a Zonde needle. The dose is one half of the daily intake of BA as a food additive. Our results of biochemical indices, such as serum concentrations of creatinine, BUN, AST, and ALT, also clearly indicated that the oral dose was not toxic to female mice. The teratogenic effect of BA is controversial. Previous studies showed that 600 mg/kg of BA produced significant results in hamsters, but was negative in two rat studies at up to 500 mg/kg/day [16]. These dose are much higher than that of this study. Therefore, we suggest that BA has relative low toxicity in both in vitro and in vivo experiments from the dose used for this study. However, more extensive toxicological studies are needed to evaluate the toxicity of BA on reproduction and development.

In conclusion, BA effectively increased the expression of ITGAV, ITGB3, and ITGB5 cell adhesion molecules in an LIF-dependent manner. In addition, BA enhanced adhesion...
of trophoblastic JAr cells to endometrial Ishikawa cells. Furthermore, BA improved the implantation of blastocysts in the RU-induced implantation failure mouse model. These findings suggest that BA could provide a novel and alternative therapeutic option by increasing the pregnancy rate.

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References


