Effects of bisphenol A and 4-tert-octylphenol on embryo implantation failure in mouse

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Abbreviations

Bisphenol A, (BPA; 4,4'-(propane-2,2-diyl)diphenol); OP, 4-tert-octylphenol; E2, 17β-Estradiol, P4, progesterone; Endocrine disrupting chemicals, (EDs); TRPV5 and TRPV6, Transient receptor potential cation channel subfamily V (TRPV) member 5 and 6; PMCA1, Plasma membrane Ca^{2+}-ATPase 1; NCX1, Na^{+}/Ca^{2+} exchanger 1; LIF, Leukemia inhibitory factor; HOXA-10, homebox gene member 10; MUC1, mucin 1; ERα and ERβ, estrogen receptor alpha and beta, PR- and PR-B; progesterone receptor and progesterone receptor B.

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Abstract: Miscarriage due to blastocyst implantation failure occurs in up to two-thirds of all miscarriage cases in human. The calcium ion has been shown to be involved in many cellular signal transduction pathways as well as in the regulation of cell adhesion, which is necessary for the embryo implantation process. Exposure to endocrine-disrupting chemicals (EDs) during early gestation results in disruption of intrauterine implantation and uterine reception, leading to implantation failure. In this study, ovarian estrogen (E2), bisphenol A (BPA), or 4-tert-octylphenol (OP), with or without ICI 182,780 (ICI) were injected subcutaneously from gestation day 1 to gestation day 3 post-coitus. The expression levels of the calcium transport genes were assessed in maternal uteri and implantation sites. The number of implantation sites was significantly low in the OP group, and implantation sites were absent in the E2 and EDs+ICI groups. There were different calcium transient transport channel expression levels in uterus and implantation site samples. The levels of *TRPV5* and *TRPV6* gene expression were significantly increased by EDs with/without ICI treatment in uterus. Whereas, TRPV5 and TRPV6 gene expression were significantly lower in implantation sites samples. *NCX1* and *PMCA1* mRNA levels were significantly decreased by OP and BPA in the implantation site samples. Compared to vehicle treatment in uterus, both the *MUC1* mRNA and protein levels were markedly high in all but the BPA group. Taken together, these results suggest that both BPA and OP can impair embryo implantation through alteration of calcium transport gene expressions and by affecting uterine receptivity.

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1. Introduction

The implantation of a blastocyst into a receptive uterus is part of a multifaceted process that includes embryo implantation, decidualization, and vascular modification [1, 2], which are key events in the establishment of a successful pregnancy, and the success of each event is a prerequisite for advancement to the next stage. Blastocyst implantation occurs within a limited period when blastocyst competency is coordinated with the receptivity of the uterus. Any disturbance of this coordination can induce unsuccessful or flawed implantation. In human, around 75% of pregnancy losses are due to implantation failure [3].

Progesterone and estrogen are the principal hormones that directly affect uterine receptivity, acting through nuclear estrogen (ERα) and progesterone receptor (PR) [1]. Both E2 and PR can regulate the production of cytokines, growth factors, homeobox transcription factors, and cyclooxygenase-derived prostaglandins. These factors are crucial during uterine preparation for implantation and post-implantation decidualization and include leukemia inhibitory factor (LIF) [4], HOXA10 (a member of developmentally regulated AbdB subclass of the homeobox gene), and the adhesion molecule mucin 1 (MUC1) [5-9]. Such factors are molecules that have important roles in implantation and during pregnancy [2]. Thus, the disruption of the genes associated with these factors may lead to infertility and implantation failure in various species including human. Both HOXA10 and LIF are essential for the endometrium development during implantation [10, 11]. LIF and its receptor have been shown to be reduced in the endometrium of infertile women [11-13]. Moreover, LIF deficiency results in the loss of embryos during early pregnancy in murine [12-14]. A low expression of *HOXA10* mRNA results in a decreased implantation rate [15]; whereas, downregulation of *MUC1* mRNA and its protein prior to implantation allows the embryo attach to the maternal uterus [5, 16, 17]. Furthermore, LIF and MUC1 were shown to enhance the calcium ion level in an *in vitro* experiment [18, 19].

The calcium ion has important roles in many aspects of a living organism. However, the role of the calcium ion in female reproductive organs is not fully described. During pregnancy, the calcium ion is involved in a variety of crucial process including fertilization, decidualization, and implantation [20-22]. The transient receptor potential cation channel, subfamily V (TRPV) member 5, as well as TRPV6, the Na^{+}/Ca^{2+} exchanger 1 (NCX1), and the plasma membrane Ca^{2+}-ATPase (PMCA1) appear to have critical roles in calcium ion absorption [23]. These proteins are

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found in the apical membranes of intestinal and renal epithelial cells and have been proposed as mediators of calcium ion uptake during transcellular transport [24]. Recently, we reported that the calcium ion influx genes *TRPV5* and *TRPV6* are expressed in the uterine endometrial region, while the calcium ion efflux genes *NCX1* and *PMCA1* were detected in the basolateral membranes of the uterus [25]. These observations suggest that these calcium ion transport genes not only have a critical role in calcium ion transport in the duodenum and kidney but also in the uterus. In addition, TRPV6 has been reported to be a key element in controlling calcium transport between the embryo and placenta in uterus during pregnancy [26, 27]. Furthermore, TRPV6 and PMCA1 are involved in specific uterine functions, including fetal implantation in human [28]. However, the roles of the calcium channel during the implantation stage have not been elucidated.

During the last few decades, there has been a growing concern about the effect of exposure to environmental endocrine-disrupting chemicals (EDs) on the reproductive system. Recent studies have shown that EDs are associated with adverse reproductive health outcomes in human, including infertility, implantation failure, and pregnancy loss [29, 30]. Bisphenol A (BPA; 4,4'-(propane-2,2-diyl)diphenol) and 4-tert-octylphenol (OP; 4-(1,1,3,3-tetramethylbutyl)phenol) are common EDs and are reported to have weak estrogenic activities. Exposure to BPA and OP during early pregnancy has resulted in implantation failure [31-33]. Moreover, our recent study showed that both BPA and OP could modulate the calcium channel during pregnancy [34, 35]. In addition, BPA exposure had increased the expression of MUC1 [36] and decreased the expression of HOXA10, both at the mRNA and protein levels [37], during early pregnancy. Thus, we hypothesize that BPA and OP, through their estrogenic activity, can affect the calcium channel and disrupt the expression of pregnancy-related genes such as *HOXA10*, *LIF*, and *MUC1* during the implantation stage. In addition, previous studies have indicated that these genes are expressed in endometrium or are regulated for endometrial receptivity in the uterus. Regardless, successful implantation requires the synchronized development of both the embryo and the endometrium. In the present study, we investigate the effects of EDs on both the uterus and the blastocyst implantation site.

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2. Materials and methods

2.1. Chemical

The BPA and 17β-Estradiol were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while the ICI 182,780 (ICI) was obtained from Tocris Bioscience (Bristol, UK). The OP was obtained from Fluka Chemie (Seoul, Republic of Korea). Stock solutions were diluted with corn oil (Sigma-Aldrich, St. Louis, MO, USA). Chicago Skye Blue 6B were bought from Sigma Chemical (St. Louis, MO, USA).

2.2. Animals and treatments

Female ICR mice (9-weeks-old, 25–30g) were purchased from Samtaco (Osan, Gyeonggi, Republic of Korea). Mice were housed in polycarbonate cages under controlled environment conditions with a 12 h light/dark cycle, a constant temperature of 23°C ± 1°C, and a relative humidity of 50% ± 10%. The mice were fed a diet of AIN-76A and tap water. After a 1-week adaption period, female mice were mated with adult ICR male mice overnight, and the presence of a vaginal plug was checked the following morning. The day a vaginal plug was observed was set as gestation day (GD) 0.5.

From GD 0.5 to GD 3.5, the pregnant mice were randomly divided into seven groups (n = 8 mouse/group). Mice were given subcutaneous (s.c.) injection of corn oil (vehicle [VE] group), or ovarian estrogen (E2; 40 μg/kg/day; positive control group), or BPA (100 mg/kg/day), or OP (100 mg/kg/day) dissolved in corn oil (Sigma-Aldrich). Mice in three additional groups (E2+ICI, BPA+ICI, and OP+ICI) received s.c injection of ICI. (4 mg/kg) 30 mins before treatment with E2, BPA, or OP. The BPA dose was selected based on a study of pregnant s.c. BPA-exposed mice in which 100 mg/kg BPA did not produce an effect on the reproductive organs [38]. A similar OP dose was selected to allow comparison with the BPA results. Five mice of each group were sacrificed 24 h after final treatment (GD 4.5) and uterus tissue was collected. The remaining three mice were sacrificed 3–5 min after Chicago Blue dye injection on GD 5.5. In all groups, the implantations were counted, and implantation tissue samples were collected. All animal experimental procedures were approved by Chungbuk National University Institutional Animal Care and Use Committee (IACUC) (project identification code: CBNUA-1108-17-01, approval date 1st July 2017)

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2.3. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from uterus and implantation site samples by using Trizol reagent (Ambion, Austin, TX, USA) and the concentration of total mRNA was determined by measuring the absorbance at 260 nm. First-strand complementary DNA (cDNA) was produced from 1 μg of total mRNA by reverse transcription (RT) using Moloney murine leukemia virus reverse transcriptase (iNtRON Bio, Gyeonggi-do, Korea) and random primers (9-mers; TaKaRa Bio., Shiga, Japan). The cDNA template (1 μL) was assayed by applying SYBR-ROX (TaKaRa Bio) real-time PCR according to the manufacturer's protocols. Primer sequences were presented in Table 1. The qRT-PCR was carried out for 40 cycles using the following schedule: denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. During the exponential phase of PCR amplification, the reaction cycle at which PCR products exceeded the fluorescence intensity was the threshold cycle (CT). The CT value was determined automatically from the exponential phase of the delta (Δ) CT fluorescence detection graph. The expression of a target gene was quantified relative to that of the internal vehicle gene (18S ribosomal RNA) based on a comparison of CTs at a constant fluorescence intensity. Expression of 18s was not significantly altered under all experimental conditions. The amount of transcript present was inversely related to the observed CT, and the CT was expected to increase by one for every 2-fold dilution in the amount of transcript. Relative expression (R) was calculated using the equation $R = 2^{-(\Delta CTsample - \Delta CTcontrol)}$. To determine a normalized arbitrary value for each gene, every data point was normalized to the control gene, as well as to the respective controls.

2.4. Western-blot analysis

Proteins were extracted from mouse uterus and implantation site samples by using Pro-prep solution (InTron, Seoul, Korea) according to the manufacturer's protocol. A 20 μg cytosolic protein sample was separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the product transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Taunton, MA, USA). The membrane was then blocked in TBS-T containing 5% skim milk for 60 min, then incubated overnight in primary antibodies for: TRPV5 (Santacruz Biotechnology, dilute 1:3000), TRPV6 (Alomone labs, catalog: ACC-036, diluted 1:3000), PMCA1 (Swant, dilute 1:3000), NCX1 (Abcam, dilute 1:3000), MUC1 (Abcam, dilute

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1:3000), or β-actin (Cell Signaling Technology, diluted 1:3000). After washing with TBS-T buffer, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, Cell Signaling Technology 1:3000; anti-mouse, Cell Signaling Technology 1:3000; anti-hamster, Jackson Human Research, 1:300) for 1 h at room temperature. The membrane was then washed four times for 10 min each with TBS-T. Enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology) with a charge-coupled device was used to detect antibody binding. Using the Chemi Doc equipment, GenGnome 5 (Syngene, Cambridge, UK. The optical density of the target band was analyzed by using Image J software (NIH, Bethesda, MD, USA).

2.5. Statistical analysis

All statistical analyses were performed by applying one-way ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons. Data were analyzed using GraphPad Prism software. The results are presented as means ± SEM. A $p < 0.05$ was considered statistically significant. All combination treatment group results were compared to the VE group and the individual treatment group results.

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3. Results

3.1. Effect of EDs on blastocyst implantation

Initially, we examined the effect of BPA and OP on embryo implantation. Statistical analysis performed on the combined data from GD 5.5 showed that the number of implantation sites in pregnant mice treated with OP (100 mg/kg) from GD 0.5 to GD 3.5 was significantly lower than that in VE-treated mice. There was no significant difference between the numbers of implantation sites in the BPA (100mg/kg) and VE groups (Figure 1), but the implantation sites had different appearances in the two groups. Both BPA and OP substantially reduced implantation site growth and size (Supplementary Figure 1). There were no implantation sites observed when mice were treated with E2, E2+ICI, BPA+ICI, or OP+ICI. These results suggest that both BPA and OP affected blastocyst implantation.

3.2. Effects of BPA and OP on TRPV5, TRPV6, PMCA1, *and* NCX1 *expressions in maternal uterus and implantation sites*

To determine the effects of BPA and OP on the regulation of calcium ion transport during implantation in the uterus and at implantation sites, TRPV5, TRPV6, PMCA1 and NCX1 expression levels were quantified by both real-time-PCR and western-blot assays. In uterus samples, there were marked increases in mRNA levels of *TRPV5* in the E2, E2+ICI, BPA+ICI, OP, and OP+ICI groups (around 2279%, 210%, 165%, 203%, and 187%, respectively) over that in the VE group (Figure 2a). The *TRPV6* mRNA levels were also higher in the E2, E2+ICI, BPA+ICI, and OP+ICI groups (by 2800%, 2414%, 160%, and 274%, respectively) than in the VE group (Figure 2b). Furthermore, TRPV5 and TRPV6 protein levels were markedly higher in all groups than that in the VE group (Supplementary Figure 2a,b). The expressions of *TRPV5* and *TRPV6* mRNA in uteri were higher in BPA+ICI group than in the BPA group. The expressions of *PMCA1* mRNA were significantly lower in the E2, E2+ICI, and OP+ICI groups (around 30% lower) than in the VE group (Figure 2c). The expression of *PMCA1* mRNA in uteri were lower in the OP+ICI group than in the OP group. mRNA levels of *NCX1* were significantly lower in the E2 and E2+ICI groups than in the VE group, but it was higher in the OP group than in the VE group (Figure 2d). In contrast, NCX1 protein levels were significantly high in the E2 and OP+ICI group (Supplementary Figure 2d).

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The mRNA expression levels of *TRPV5*, *TRPV6*, *PMCA1*, and *NCX1* were markedly low in the BPA- and OP-treated implantation sites (Figure 2e-h). Moreover, the protein levels of TRPV5 and TRPV6 were significantly low in the OP group (Supplementary Figure 2e,f). Treatment with BPA also decreased the TRPV5 and TRPV6 protein levels but not significantly (Supplementary Figure 2e,f). Protein levels of PMCA1 and NCX1 were also not significantly decreased by BPA or OP treatment (Supplementary Figure 2g,h). These results suggest that BPA and OP treatments lead to abnormal expressions of calcium channel genes in both uterus and implantation sites.

3.3. Effect of BPA and OP on MUC1 *expression in maternal uterus and implantation sites*

To determine the effects of BPA and OP on embryo attachment, *MUC1* expression was measured by using both real-time PCR and western-blot assays. As a barrier to implantation, MUC1 expression has an important role in embryo attachment with downregulation of MUC1 expression being necessary for successful implantation [17]. Both *MUC1* mRNA and MUC1 protein levels were markedly higher in the E2, E2+ICI, BPA+ICI, OP, and OP+ICI groups (approximate 3230%, 1314%, 283%, 175%, and 270%, respectively) than in the VE group (Figure 3a,b). The BPA+ICI and OP+ICI combination treatments produced higher both MUC1 mRNA and protein expression in uteri than that in the BPA- and OP-alone group. These results suggest that OP could impair implantation through the abnormal expression of *MUC1* in uterus. Following implantation success, MUC1 expression was either downregulated or was completely absent (Figure 3c,d). These results suggest that BPA or OP exposure can result in disruption of uterine receptivity. Combined exposure to ICI and BPA or OP can lead to a substantial reduction in embryo attachment.

3.4. Effects of BPA and OP on HOXA-10 *and* LIF *expression in maternal uterus and implantation sites*

To confirm the effects of BPA and OP on both the maternal uterus and the implantation site, the expressions of the *HOXA10* development factor gene and the *LIF* growth factor gene were measured. The expression of *HOXA10* mRNA was significantly decreased in uteri of the E2, E2+ICI, BPA+ICI, and OP+ICI groups than in the uteri of the VE group (Figure 4a). The BPA+ICI and OP+ICI combination treatments reduced lower *HOXA10* mRNA expression in uteri than that in the BPA- and OP-alone group. Moreover, it was markedly lower in the implantation sites of the

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OP group than in those of the VE group (Figure 4c). There was no significant difference in *HOXA10* expression levels in the implantation sites of the BPA and VE groups.

The expressions of *LIF* mRNA in uterus were markedly lower in the E2+ICI group (93% lower), BPA+ICI and OP+ICI groups (both 97% lower), E2 group (84% lower), and OP group (39% lower) than in the VE group. There was no significant difference in *LIF* mRNA levels between the BPA and VE groups (Figure 4b). The expression of *LIF* mRNA in uteri was significantly lower in the BPA+ICI and OP+ICI combination treatments than that in the BPA- and OP-alone group. Moreover, *LIF* mRNA levels at implantation sites were significantly lower in the BPA (70% lower) and OP (85% lower) groups than in the VE group (Figure 4d). These results suggest that both BPA and OP affect development and growth factor expression in both the maternal uterus and implantation sites.

3.5. Effects of BPA and OP on estrogen and progesterone receptor in maternal uterus and implantation sites

Real-time PCR results showed that the expressions of *ERα* mRNA in uterus samples were significantly lower in the E2 and E2+ICI groups than in the VE group (Figure 5a). Treatment of BPA and OP with/without ICI did not change the expression of *ERα* mRNA. The expression of *ERα* mRNA were significantly lower in the E2+ICI combination treatments than in the E2-alone, but it was higher in the OP+ICI combination treatments than in the OP group. Moreover, mRNA level of *PR* in uteri of the E2, E2+ICI, BPA+ICI, and OP+ICI groups were markedly lower than that in uteri of the VE group (lower by 45%, 65%, 35%, and 50%, respectively) (Figure 5b). The expression of *PR-B* mRNA was significantly lower in uteri of the E2, E2+ICI, BPA+ICI, OP, and OP+ICI groups (lower by 35%, 64%, 35%, 45%, and 47%, respectively) than in the VE group. However, there was no significant difference between the BPA and VE groups (Figure 5c). The E2+ICI, BPA+ICI and OP+ICI combination treatments markedly reduced *PR* and *PR-B* mRNA expression in uteri than that in the E2-, BPA- and OP-alone group.

At the implantation sites, there were no significant differences in the mRNA levels of *PR* or *PR-B* between the BPA- or OP-treated groups and the VE group (Figure 5d-f). mRNA level of *ERα* of the OP group was significantly lower than that in VE group. These results suggest that BPA and OP can affect blastocyst implantation through modulation of PR in the maternal uterus.

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4. Discussion

The timing window for blastocyst implantation is a crucial period in the establishment of a successful pregnancy. Abnormal events before, during, or immediately after implantation can result in poor pregnancy rates in many species, including human. In the present study, we investigated the effect of EDs during the initial pregnancy process. We also investigated the expressions of calcium-related genes and the relationships of their expression to the development of a successful implantation. Indeed, the calcium ion is a crucial element in living organisms from fertilization onward. Additionally, the regulation of calcium ion homeostasis clearly has important roles in the process of implantation. In recent decades, many researchers have reported on the role of the calcium ion during life, particularly during pregnancy. However, the roles of the calcium ion in early pregnancy have not been fully described.

By exerting estrogenic activity, EDs can interfere with the normal endocrine process, causing adverse pregnancy outcomes [39]. BPA and OP are among the most disruptive EDs and are widely used as plasticizers in consumer products. Several studies showed that high-dose BPA or OP treatment can result in a lowering of implantation rates in murine [33, 40, 41]. Moreover, high exposure to BPA has been associated with miscarriage in human [42]. In this study, exposure to EDs resulted in a reduction or loss of implantation success and an abnormal appearance of the implantation site. Notably, direct exposure to ICI has been shown to lead to a complete loss of implantation [43]. Thus, the complete absence of implantation sites in all groups treated with ICI.

The calcium ion is a crucial element in the development and physiology of living organisms and is involved in a variety of important pregnancy-related events, including fertilization, decidualization, and implantation [20]. The calcium ion acts as an intracellular messenger to regulate a diverse range of biological processes related to cellular function, such as gene transcription, proliferation, differentiation, necrosis, and apoptosis [44-46]. Thus, maintenance of calcium ion activity in the uterus and at the implantation site is necessary for successful implantation. Calcium ion entry via the calcium channels contributes to calcium ion mobilization, and four cell membrane calcium channel factors, TRPV5, TRPV6, PMCA1, and NCX1 are reported to have critical roles in the major steps of calcium ion transport. TRPV5 and TRPV6 are members of the transient receptor potential cation channel subfamily V gene family and are responsible for the influx of calcium ion into cells during transcellular absorption [47, 48]. Plasma membrane Ca^{2+}-ATPase 1 (PMCA1) and Na^{+}/Ca^{2+} exchanger 1 (NCX1) have important roles in the outflow of cytosolic calcium ions from cells [49, 50] and the maintenance of calcium ion level

in the cytoplasm [51, 52]. Additionally, TRPV5, TRPV6, PMCA1, and NCX1 are not only observed in duodenum and kidney but also in uterus [25, 28, 53]. Moreover, they have been reported to have important roles in placental calcium ion transport and regulation [54]. These observations suggest that an abnormal expression of a calcium transport gene may induce abnormal maternal–fetal calcium ion transport. For example, exposure to BPA or OP from GD 11.5 to GD 16.5 has induced a decrease in TRPV6 and PMCA1 expression in mouse placenta [35]. Also, BPA and OP can disrupt the expression of calcium transport genes in kidney and duodenum of pregnant mice [34]. In this study, BPA, OP, and E2 exposure at the initial stage of pregnancy resulted in upregulation of TRPV5 and TRPV6 expressions in uterus. Cotreatment with ICI strictly increased the mRNA expressions of *TRPV5* and *TRPV6*. Interestingly, the expression of *TRPV5*, *TRPV6*, *PMCA1*, and *NCX1* were significantly decreased in the implantation sites of the BPA- and OP-treated groups. These results suggest that exposure to EDs-alone or -combine to ICI during early pregnancy can result in an increase in the intracellular calcium ion level and affect the blastocyst implantation site. Moreover, intracellular calcium ion overload can result in necrotic or apoptotic cell death [55].

The implantation stage is initiated after embryo attachment to the maternal uterus. During attachment, adhesion is the initial step and involves apposition and adhesion of the hatched blastocyst to the uterine luminal epithelium. MUC1 is a transmembrane glycoprotein belonging the mucin family and is expressed at the apical surface of uterine epithelia and various organs. As MUC1 is a barrier to embryo implantation, MUC1 downregulation or absence prior to implantation is a prerequisite for uterine receptivity in many species [5, 16, 17, 56] with downregulation allowing embryo attachment and leading to successful implantation. EDs have been reported to induce the expression of MUC1 and chronic exposure to BPA has resulted in the upregulation of MUC1 during early pregnancy [36]. In the present study, the expression of *MUC1* in uterus was markedly increased by the EDs tested. These results indicate that EDs can affect uterine receptivity and result in failure of blastocyst attachment and implantation. Recently, MUC1 has been shown to increase the expression of TRPV5 in kidney [57]. These results suggest that the expression of TRPV5 and TRPV6 may involve the expression of MUC1 in uterus during pregnancy.

Implantation success requires the synchronized development of both the embryo and the endometrium. Homeobox gene transcription factors are reported to be crucial for endometrial development and embryo implantation in both mouse and human [10]. *HOXA10*, a member of developmentally regulated AbdB subclass of the homeobox gene, is essential for embryo survival

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and implantation [58]. Peak *HOXA10* and *HOXA11* expressions are first observed during the window of endometrial receptivity. Decreased *HOXA10* expression leads to a decreased implantation rate; furthermore, *HOXA10* expression is significantly low in cases of recurrent miscarriage and recurrent implantation failure [15]. Recently, it was reported that BPA exposure results in downregulation of *HOXA10* [37]. In this study, the expression of HOXA10 mRNA in uterus was markedly low in E2 and EDs+ICI groups. *HOXA10* mRNA expression in the implantation site was significantly lower in the OP groups.

Among the cytokines, *LIF* (an interleukin-6 (IL-6) cytokine) is reported to be important for implantation in mouse [4, 59]. *LIF* is expressed in both the embryo and the endometrium and is involved in various processes during the implantation period such as blastocyst development, endometrial differentiation, blastocyst attachment, and invasion of the endometrium [4, 11]. *LIF* deficiency can significantly decrease the survivability of embryos during the cell and morula stages and can affect blastocyst development [14]. Furthermore, several studies have suggested that *LIF* and *LIF* receptor expressions are significantly low in the endometrium of infertile women [12, 13, 60]. In this study, *LIF* mRNA levels were significantly low in the E2, E2+ICI, BPA+ICI, OP, and OP+ICI groups. Those results support the observed lack of implantation in those groups. Moreover, downregulation of HOXA10 and LIF may be associated with the different appearances of the implantation sites visualized in this study. These results suggest that treatment with EDs can induce the loss of embryo survival via inhibition of the development and growth of the implantation site and through inhibition of uterine preparation.

Both ovarian estrogen (E2) and progesterone (P4) are principal hormones involved in the preparation of the uterus for embryo implantation and in the maintenance of pregnancy [1, 61]. Their synchronized production enables the blastocyst to attach and initiate the implantation process through their effects on uterine structure and function. Embryonic estrogen is considered important for embryo implantation in pigs, guinea pig, rabbits and hamster [1]. E2 and P4 affect the uterus primarily through their nuclear receptors, E2 receptor alpha (*ERα*) and beta (*ERβ*), and via *P4* and *P4* receptor B (*PR-B*), respectively. In mouse, *ERα* is important for uterine receptivity and embryo implantation [62]. In addition, the lack of both *PR* and *PR-B* results in infertility by affecting the function of ovary and uterus. EDs are described as synthetic compounds that mimic natural estrogens, and they can bind to nuclear *ERα*. In addition, *MUC1* and *LIF* expression are regulated by P4 [6], while *HOXA10* and *HOXA11* are upregulated by E2 and P4 [7]. These results suggest

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that EDs primarily affect embryo implantation, attachment, and survival through the modulation of *PR*, and *PR-B*.

Throughout experiment, ICI treatments showed the combined effect to BPA and OP. The BPA+ICI and OP+ICI combination strictly increase the expression of TRPV5, TRPV6 and MUC1 genes. In addition, they also markedly decrease the mRNA expression of HOXA10, LIF, PR and PR-B than that in the BPA- and OP-alone. However, the effects of ICI-exposure during implantation period is not fully understood; therefore, we will focus on assessing ICI-exposure alone in future studies.

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5. Conclusions

These results show that BPA and OP regulate the expression of TRPV5, TRPV6, PMCA1, and NCX1 in both maternal uterus and embryo implantation sites during the implantation stage in mouse. Additionally, they disrupt orchestration of embryo–uterine cross-talk by modulating the expression of *MUC1*, *LIF*, and *HOXA10* genes. Moreover, there are several reports showing that *LIF* and *MUC1* can regulate the calcium transient transport channel [18, 19]. Thus, overexpressions of TRPV5 and TRPV6 appear to be involved in the downregulation of *LIF* and upregulation of *MUC1* in uterus. As a consequence, BPA and OP can reduce the implantation rate. In conclusion, BPA, OP, and E2 can have negative effects on the embryo implantation and survival by disrupting the calcium transient transport channel and by affecting growth and development factors through the progesterone receptor.

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Author contributions

Dinh Nam Tran, Changhwan Ahn, and Eui-Bae Jeung designed the experiments. Dinh Nam Tran performed the experiments. Dinh Nam Tran analyzed the data. Dinh Nam Tran wrote the paper. Eui-Man Jeung and Yeong-Min Yoo revised the manuscript. Eui-Bae Jeung conceived and supervised the study.

Conflicts of Interest

The author declared no conflict of interest.

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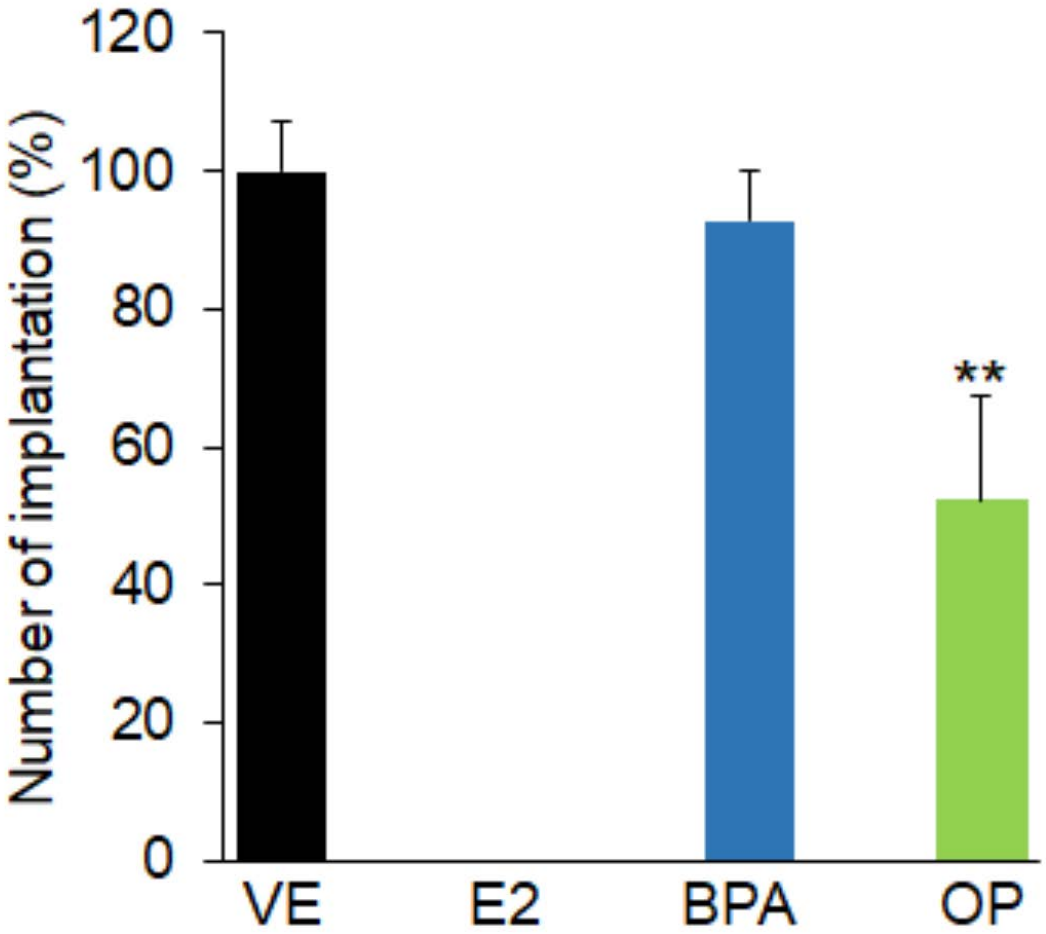

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593 **Figure 1. E2, BPA, and OP result in loss of implantation sites**

594 Pregnant mice at gestation day 5.5 (GD 5.5) were sacrificed 48 h after final injection. Implantation
595 sites in uteri were detected by application of Chicago Sky Blue 5 min before sacrifice. All
596 implantation sites in the control group were detected as distinct blue bands. The number of
597 implantation sites was significantly low in the OP group and there were no sites in the E2 group.
598 N = 3 mice per group. Statistical significance was determined by one-way ANOVA with the
599 Bonferroni correction test. *$p < 0.05$ and **$p < 0.01$ *vs*. control group.

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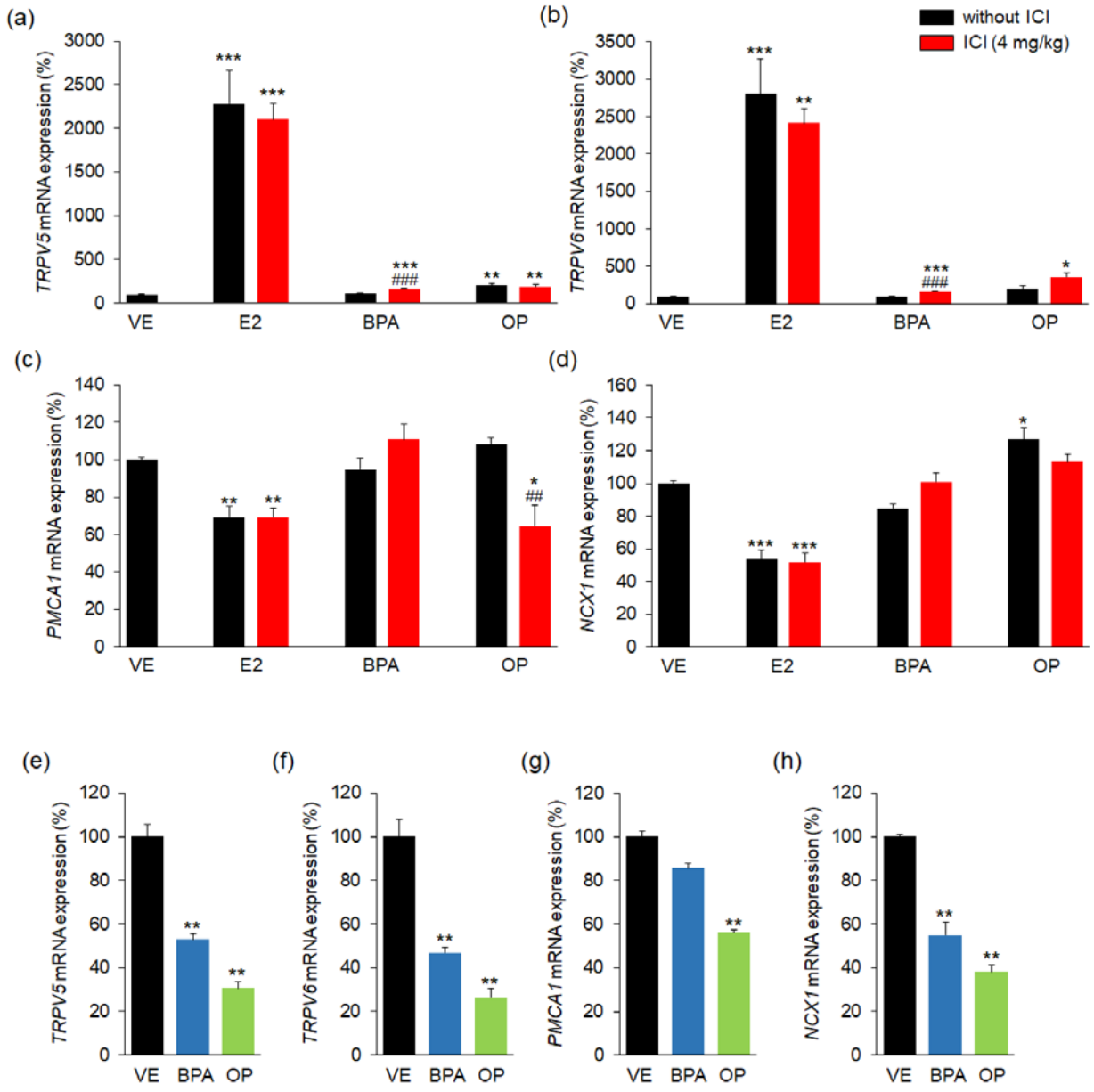

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Figure 2. E2, BPA, and OP change the expressions of *TRPV6*, *TRPV5*, *PMCA1*, and *NCX1* in maternal uterus and implantation sites

Mice were sacrificed at GD 4.5 (24 h after final injection) to collect uterus tissues and at GD 5.5 (after 48 h after final injection) to collect implantation sites. The mRNA expressions of calcium transporter channel genes in uterus and implantation sites were assessed. The mRNA levels of *TRPV6*, *TRPV5*, *PMCA1*, and *NCX1* genes were measured by using real-time PCR and were normalized to that of 18S ribosomal RNA (RN18S). In uterus, (a) the expressions of *TRPV5* mRNA were significantly high in the E2, E2+ICI, BPA+ICI, OP, and OP+ICI groups. (b) *TRPV6* mRNA level changes were similar to those for *TRPV5* expression but there was no significant change in the OP group. mRNA expression of *TRPV5* and *TRPV6* in uteri were higher in BPA+ICI group than in the BPA group. (c and d) mRNA level of *PMCA1* and *NCX1* were significantly decreased by E2 and E2+ICI. In implantation sites, (e, f, g, and h) the mRNA levels of *TRPV6*, *TRPV5*, *PMCA1*, and *NCX1*, respectively, were markedly low in all groups. N = 5 mice per group for uterus, N = 3 mice per group for implantation sites. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. $^{*}p < 0.05$ *vs*. VE, $^{\#}p < 0.05$ *vs*. EDs. Treatments: E2; 40 µg/kg/day, BPA; 100 mg/kg, OP; 100 mg/kg, ICI; 4 mg/kg.

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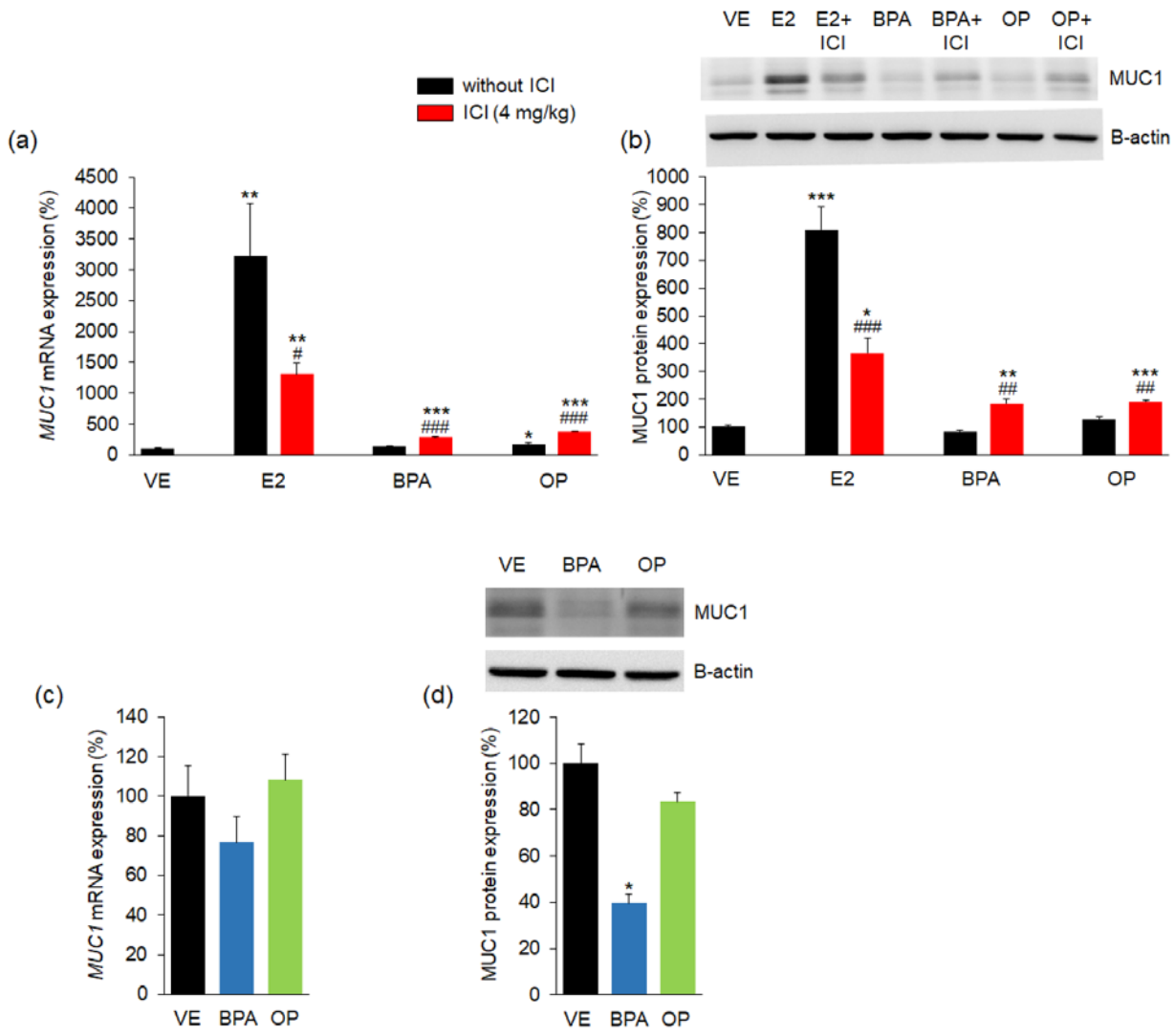

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Figure 3. BPA, OP, and E2 increased the expression of *MUC1* in maternal uterus and implantation sites

The expression of *MUC1* was measured by real-time PCR and was normalized to that of 18S ribosomal RNA (RN18S). Expression of MUC1 protein was investigated by western blotting and was normalized by β-actin. Histograms show quantification of western blots. In uterus, (a) the expressions of *MUC1* mRNA in maternal uterus were markedly high in the E2, OP, and EDs+ICI groups. (b) Protein levels of MUC1 were significantly high after E2, and EDs+ICI administration. Both mRNA and protein levels of MUC1 were significantly higher in the combination BPA+ICI and OP+ICI than that in BPA- and OP-alone. In implantation sites, (c) there were no differences in the expression of *MUC1* mRNA. (d) Protein level of MUC1 was significantly decreased by BPA treatment. N = 5 mice per group for uterus, N = 3 mice per group for implantation sites. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. $^{*}p < 0.05$ *vs*. VE, $^{\#}p < 0.05$ *vs*. EDs.

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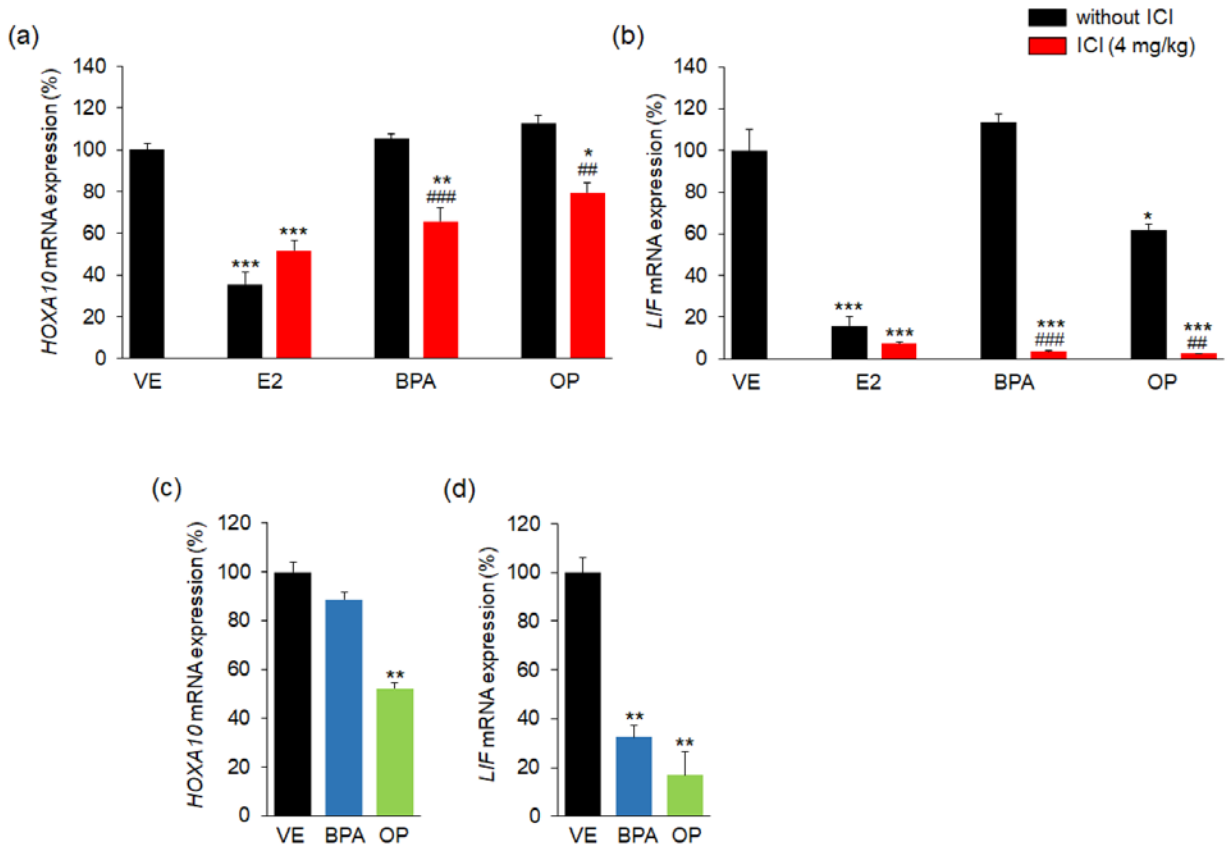

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632 **Figure 4. E2, BPA, and OP decreased the expression of *HOXA10* and *LIF* in maternal uterus**

633 **and implantation sites**

634 The expressions of *HOXA10* and *LIF* genes were measured by real-time PCR and were normalized

635 to that of 18S ribosomal RNA (RN18S). In uterus, (a) mRNA levels of *HOXA10* were significantly

636 different after E2 and EDs+ICI treatment. (b) mRNA levels of *LIF* were significantly low in the

637 E2, OP, and EDs+ICI groups. Both mRNA levels of *HOXA10* and *LIF* were significantly low in

638 the combination BPA+ICI and OP+ICI than that in BPA- and OP-alone. In implantation sites, (c)

639 the mRNA level of *HOXA10* was significantly decreased by OP treatment. (d) mRNA levels of

640 *LIF* were markedly lowered by BPA or OP treatment. N = 5 mice per group for uterus collection,

641 N = 3 mice per group for implantation sites. Statistical significance was determined by one-way

642 ANOVA with the Bonferroni correction test. $^{*}p < 0.05$ *vs*. VE, $^{\#}p < 0.05$ *vs*. EDs.

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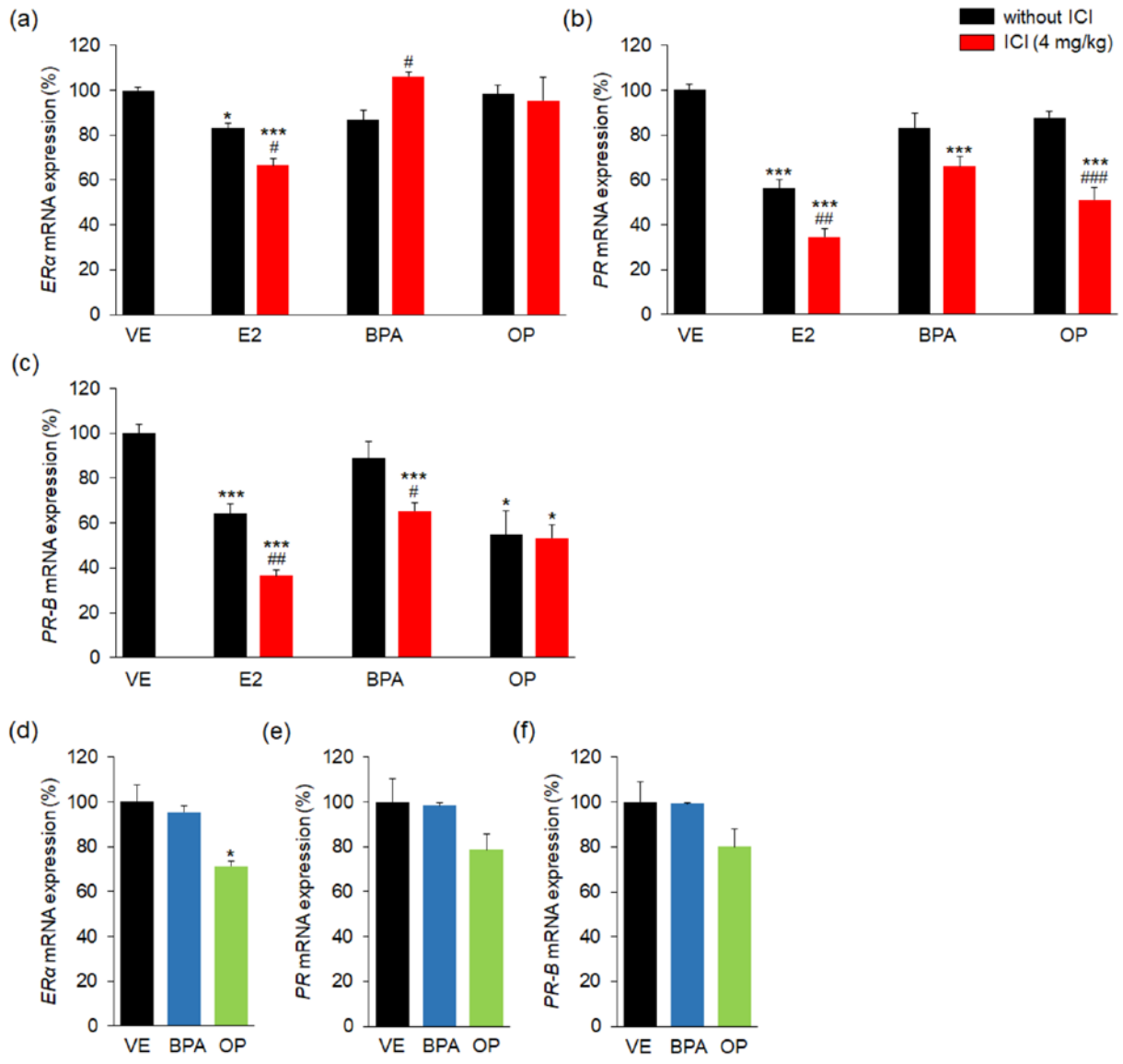

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Figure 5. E2, BPA, and OP effects and estrogen and progesterone receptor expressions in maternal uterus and implantation sites

The expression levels of *ERα, PRα*, and *PRβ* genes were measured by real-time PCR and normalized to that of 18S ribosomal RNA (RN18S). In uterus, (a) mRNA levels of *ER* were not changed by BPA or OP with/without ICI. (b) mRNA levels of *PR* were significantly low in the E2 and EDs+ICI groups. (c) mRNA levels of *PR-B* were markedly low in all groups, except the BPA group. In implantation sites, (d, e, and f) the expressions of *ERα* mRNA were significantly decreased by OP. N = 5 mice per group for uterus, N = 3 mice per group for implantation sites. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. $^{*}p < 0.05$ *vs*. VE, $^{\#}p < 0.05$ *vs*. EDs.

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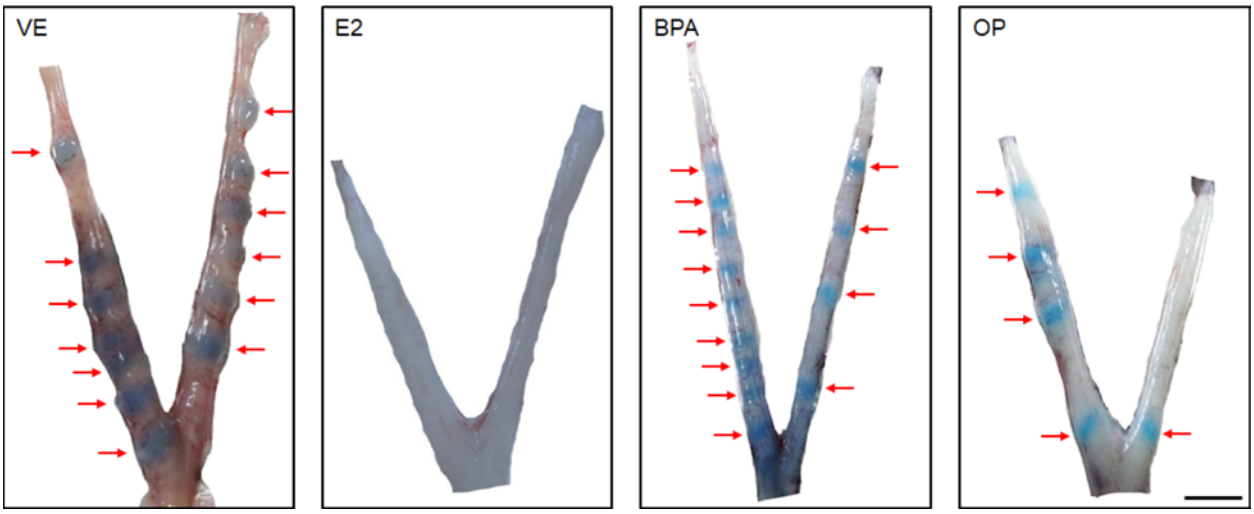

Supplementary Figure 1. Pregnant mice at gestation day (GD) 5.5 were sacrificed 48 h after injection. Implantation sites in uteri were detected by administration of Chicago Sky Blue 5 min before sacrifice. All implantation sites in the control group were detected as distinct blue bands. Images are representative of implanted blastocysts in uteri. The implantation sites in the uterus are indicated by the arrow. N = 3 mice per group.

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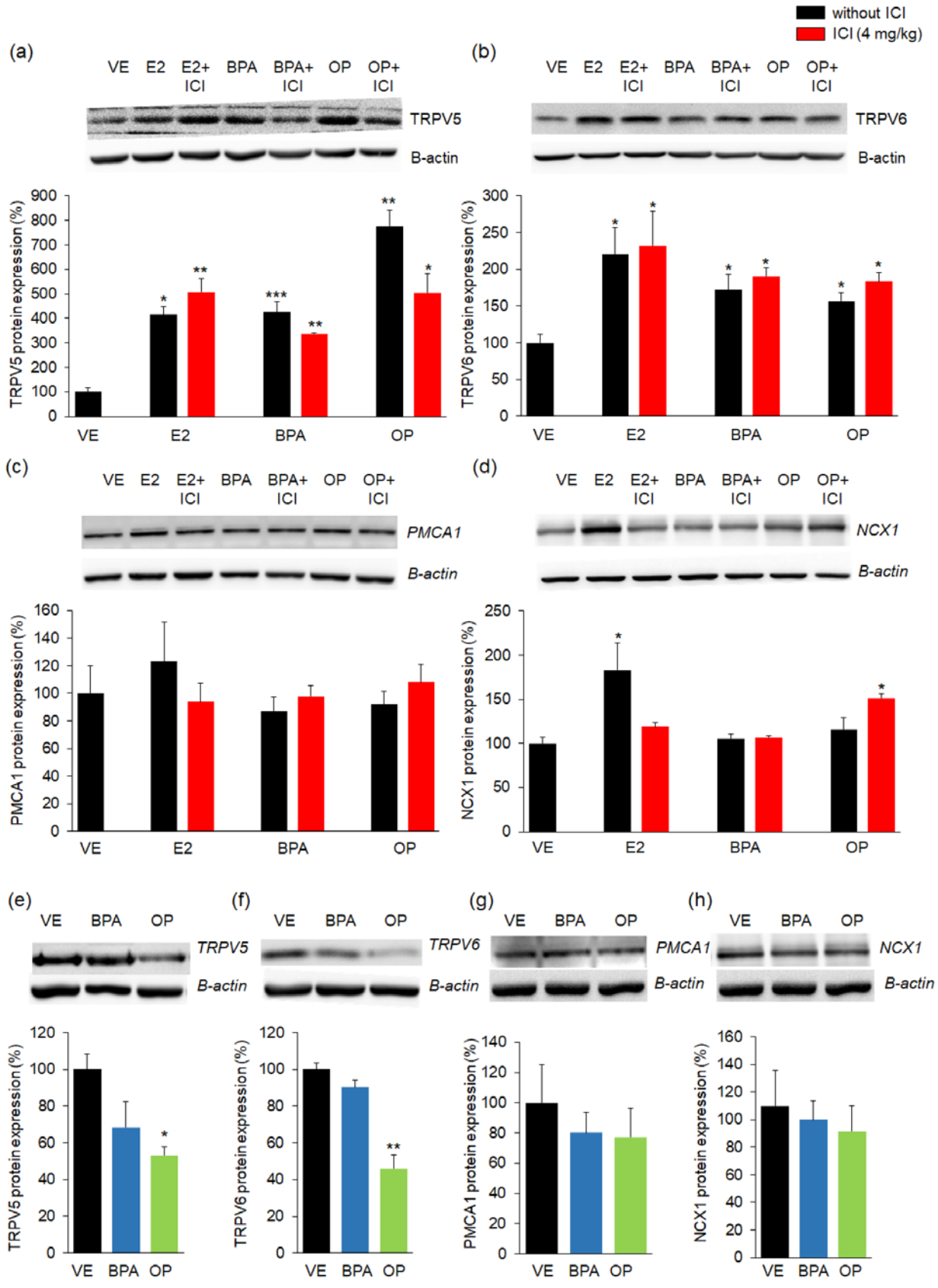

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Supplementary Figure 2. Mice were sacrificed at GD 4.5 (24 h after final injection) to collect uterus tissues and at GD 5.5 (48 h after final injection) to collect implantation sites. Protein expression of calcium transporter channel genes in uterus and implantation sites were assessed. Expressions of TRPV5, TRPV6, PMCA1, and NCX1 proteins were investigated by western blotting and results were normalized to ß-actin. Histograms show quantification of blots. In uterus, (a and b) protein levels of TRPV5 and TRPV6 were markedly high in all groups. (c) There were no changes in PMCA1 expression. (d) Protein levels of NCX1 were significantly increased by E2 and OP+ICI treatment. In implantation sites, (e and f) TRPV5 and TRPV6 protein levels were significantly decreased by OP treatment. (g and h) PMCA1 and NCX1 protein levels were decreased but not significantly. N = 5 mice per group for uterus, N = 3 mice per group for implantation sites. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. $^{*}p < 0.05$ *vs*. VE, $^{\#}p < 0.05$ *vs*. EDs.