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Effect of tetrabromobisphenol A (TBBPA) on early implantation using the three-dimensional spheroid model with human endometrial cell line, Ishikawa

Myoungjoo Koo[1†], Inyoung Kang[1†], Jin Hyun Jun[1] and Jaewang Lee[1*]

Abstract

Background Tetrabromobisphenol A (TBBPA) can be characterized as an endocrine-disrupting chemical (EDCs). It has been widely used as a brominated flame retardant in industrial products. EDCs have effects on female reproduction leading to issues, such as infertility, hormone imbalance, and endometriosis. In Korea, the problems of infertility and decreasing birth rate are of significant concern. Exposure to EDCs might have a harmful effect on female fertility by mediating a decrease endometrial receptivity. This study aimed to investigate the effects of TBBPA on infertility, particularly on early implantation events in the uterine endometrium. Human endometrial adenocarcinoma and trophoblastic cell lines were used in this study. The cytotoxicity of TBBPA on Ishikawa cells and Jeg-3 cells was measured using the Cell Counting Kit-8 assay. The mRNA expression was analyzed by reverse transcription-quantitative polymerase chain reaction, and protein levels were measured by western blotting. The attachment rate was analyzed using an attachment assay, and the outgrowth area was measured using an outgrowth assay.

Results The mRNA expression of interleukin (IL)-6, IL-1β, tumor necrosis factor-α, and leukemia inhibitory factor was significantly increased upon treatment of Ishikawa cells by TBBPA. Moreover, the outgrowth area in the TBBPA group was significantly decreased compared to that in the control. In contrast, TBBPA had a minor effect on protein levels and attachment rates.

Conclusions In this study, TBBPA induced an inflammatory *milieu* in mRNA expression. An increase in inflammation-related cytokines in the endometrium can disrupt embryo implantation. TBBPA disrupted the outgrowth of spheroids in the endometrium; however, the protein levels and attachment rate were comparable to those in the control group. The effect of TBBPA on implantation events should be elucidated further.

Keywords Implantation, Endocrine disrupting chemicals (EDCs), Tetrabromobisphenol A, Ishikawa cells, Jeg-3 cells, Infertility

Background

Female infertility refers to the inability of a woman to conceive or carry a pregnancy to full term. Many causes of female infertility are known, including problems with ovulation, fallopian tube or uterus, and hormone imbalance [1, 2]. According to a report published by the Korea Society of Obstetrics and Gynecology, the infertility rate in Korea was estimated to be approximately 10% in 2019,

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which is higher than the global average of 8% [3]. Additionally, over the decades, the number of patients receiving assisted reproductive technology (ART) for infertility has increased in South Korea [4, 5]. Several causes of implantation failure are known, including uterine factors, embryo factors, endometrial receptivity, hormonal factors, and immunological factors. However, the effects of endocrine-disrupting chemicals (EDCs) including tetrabromobisphenol-A (TBBPA) on the uterine endometrium have not been fully investigated [6, 7].

EDCs interfere with the function of the endocrine system in humans and animals [8]. The endocrine system regulates body function, especially reproduction and the hormone cycle [9, 10]. EDCs can block the effects of natural hormones though structures similar to hormones, leading to alterations in hormone levels and disruption of physiological homeostasis [11, 12]. They can bind to sex hormone receptors and inhibit or activate the production of hormones, such as estrogen and progesterone [13]. As a result, the hormone pathway is interfered by EDCs, which might have an impact on cell signaling [14].

Bisphenol A (BPA) is an industrial chemical used in the production of plastics and epoxy resins [15]. It is commonly found in polycarbonate plastics, which are used in water bottles, food containers, and canned foods [16]. BPA is an EDC that binds to estrogen receptors in the human endocrine system [17]. Exposure to BPA leads to reproductive and developmental disorders and impaired immune function [18, 19]. TBBPA is a member of the bisphenol family; it is structurally similar to BPA. Despite being considered a general and perpetual contaminant of the environment, TBBPA is a widely used industrial product [20]. Exposure to TBBPA is common in everyday life as it is included in plastic materials, and it accounts for approximately 60% of the production in the entire brominated flame retardants (BFRs) market, thereby accounting for the highest percentage of BFRs [21, 22]. In addition, highest consumption of TBBPA has been recorded in Asia when compared to global consumption [23] and TBBPA has been detected in serum of Korean population, ranging from 0.05 to 75 ng/g lipid weight [24]. Unfortunately, the function of TBBPA as an EDC has not been fully elucidated. Various studies have its effects on endocrine and reproductive systems [25]. TBBPA promotes the growth of uterine fibroids, which are known to cause recurrent implantation failure (RIF) [26–28]. Nevertheless, TBBPA is not regulated, and the effect of TBBPA on early human implantation has not been studied.

Embryo implantation is also an important part of reproduction, specifically in the process of pregnancy, and a major cause of pregnancy loss [29]. It refers to the attachment and invasion of a fertilized embryo into the uterine endometrium. The synchronized receptive state of the uterus and development of the embryo are crucial for the implantation process [30, 31]. Endometrial receptivity is maintained for only a few days, which is defined as the implantation window. Endometrial receptivity is regulated by ovarian hormones, such as estrogen and progesterone. These hormones influence the expression of attachment and inflammation molecules such as leukemia inhibitory factor (LIF), interleukin (IL)-6, and tumor necrosis factor (TNF)-α. Currently, whether TBBPA leads to implantation failure and decreased endometrial receptivity has not been examined [32, 33]. Therefore, the aim of this study was to investigate the harmful effects of TBBPA on the early implantation and outgrowth processes using a three-dimensional spheroid cell culture model with Jeg-3 and Ishikawa cells.

Materials and methods

Cell culture

The endometrial epithelial-like Ishikawa cell line and human trophoblastic Jeg-3 cell line were cultured in Dulbecco's modified Eagle medium (DMEM, Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA) and 1% penicillin and streptomycin (P/S, Lonza, Morristown, NJ, USA). Cells were cultured under standard conditions (37 °C, 5% CO_2) [34]. Ishikawa cells were used to represent the human endometrium, which is the implantation site. Jeg-3 cells were used to represent the human embryo because of the inner cell mass surrounded by trophoblastic cells. TBBPA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and used to treat Ishikawa cells to determine its effect of on endometrial cells.

Cell viability assay

A cell viability assay was performed to determine the cytotoxic dose of TBBPA. Ishikawa cells and Jeg-3 cells were detached using 0.25% Trypsin/EDTA (Gibco) and suspended to seed in 96-well plates at a density of 1×10^3/well. The cells were exposed to 0.01–100 μM TBBPA for 24 or 48 h. The viability of the cells was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). After incubating with different concentrations of TBBPA, 10 μL of CCK-8 reagent was added to each well, followed by incubation for 1 h at 37 °C. Cell viability was detected at 450 nm using MultiskanGO (ThermoFisher, Waltham, MA, USA) [35, 36]. The cells were divided into control and TBBPA groups. The control group was cultured with 1% FBS containing 0.1% DMSO in DMEM.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Ishikawa and Jeg-3 cells were detached using 0.25% Trypsin/EDTA and seeded in a 6-well plate (SPL, Pocheon, Korea) at a density of 1×10^5 /well. Ishikawa cells were treated with TBBPA at concentrations of 0.1, 1, and 10 μM for 48 h. For the comparison of 2D versus 3D spheroids of Jeg-3 cells, 1×10^5 cells per well were seeded for the 2D group, and 100 Jeg-3 spheroids were used for 3D groups. The mRNA expression of IL-1α, IL-1β, integrin alpha V (ITGαV), and LIF was detected in 2D and 3D spheroids and compared. Total RNA from cells and spheroids was extracted using TRIzol (Invitrogen, Waltham, MA, USA). For RT-qPCR analysis, 500 ng of mRNA was converted to complementary DNA (cDNA) using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Kusatsu, Japan). RT-qPCR was performed using SYBR green reagent (Meridian Bioscience, Cincinnati, OH, USA) and primers (BIONEER, Daejeon, Korea). Each experiment was performed at least in triplicate and was repeated more than three times. For inflammation targets, IL-6 (Bio-Rad, Hercules, CA, USA), IL-1β, and TNF- α were examined. Furthermore, ITGαV and LIF were examined as attachment targets. All data were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used in this study are summarized in Table 1. The PCR method was performed as follows: hold stage at 95 °C for 15 min and PCR cycle of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 30 s [37].

Western blot analysis

To quantify the level of protein, western blot analysis was performed using cell lysates. Ishikawa cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Thermo Fisher) with proteinase inhibitor (Thermo Fisher). Total protein concentration was determined by bicinchoninic Acid assay (Thermo Fisher). A total of 15 μg of protein was used for the experiments. Each sample was separated using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibody against IL-6 (1:800) (Abcam, Cambridge, UK), TNF-α (1:500) (Abcam), LIF (1:200) (Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (1:5000) (Santa Cruz Biotechnology) overnight at 4 °C on an orbital shaker. The membranes were washed thrice using Tris-buffered saline with 1% Tween 20. For IL-6, TNF-α, and LIF, incubation was performed with anti-rabbit horseradish peroxidase-conjugated immunoglobulin (IgG) secondary antibody for 2 h at room temperature. For GAPDH, incubation was performed with anti-mouse horseradish peroxidase-conjugated IgG secondary antibody for 2 h at room temperature. The proteins on the membranes were analyzed using the iBright CL750 imaging system (Applied Biosystems, Waltham, MA, USA). The data were evaluated using ImageJ software (NIH, Bethesda, MD, USA).

Attachment assay

Ishikawa cells were cultured for 48 h in a T25 flask with or without TBBPA treatment. Then, the cells were detached using 0.25% Trypsin/EDTA and seeded in a 12-well plate at a density of 1×10^5 and cultured with DMEM containing 10% FBS. After 8 h, the medium was changed for each experimental group. DMEM with 1% FBS was used for the control and TBBPA groups. Formation of Jeg-3

Table 1 Primer sequence

Genes	Primer sequence	Product size (bp)	GenBank Accession number	Annealing Temperature (℃)
IL-1α	F: CTG AAG GAG ATG CCT GAG ATA C	383	NM_000575.5	61
	R: GAA CTG TCA ACA CTG CAC AAG			
IL-1β	F: TAA AGA GAG CTG TAC CCA GAG A	217	NM_000576.3	
	R: AAG TGA GTA GGA GAG GTG AGA G			
TNF-α	F: CTC CTC ACC CAC ACC ATC AG	134	NM000594.4	
	R: ATA GAT GGG CTC ATA CCA GGG			
ITGαV	F: AAT CTT CCA ATT GAG GAT ATC AC	140	NM_002210.5	
	R: AAA ACA GCC AGT AGC AAC AAT			
LIF	F: CCA ACG TGA CGG ACT TCC C	82	NM000981.4	
	R: TAC ACG ACT ATG CGG TAC AG			
GAPDH	F: GGA GCG AGA TCC CTC CAA AA	197	NM_002046.7	
	R: GGC TGT TGT CAT ACT TCT CA			

spheroid was performed using hanging drop method at a density of 500 cells per spheroid for 48 h in DMEM supplemented with 10% FBS and rotation at 45 RPM in a shaking incubator, as shown in Fig. 5A. Spheroids with sizes ranging from 150 to 300 were used for this experiment. After harvesting the spheroids, they were seeded on Ishikawa cells treated with TBBPA. The attachment rates were observed at 5, 10, 20, and 30 min and 1, 2, 4, and 24 h [38]. The attachment assay performed in this study is illustrated in Fig. 1A.

Outgrowth assay

To determine the effect of TBBPA on spheroid outgrowth, Ishikawa cells were treated with TBBPA for 48 h in a T25 flask. The cells were then detached using 0.25% Trypsin/EDTA and seeded on a 12-well plate at a density of 5×10^4 in DMEM with 10% FBS. After 8 h, the medium was replaced with DMEM supplemented with 1% FBS for the control and TBBPA groups. The cells in the negative control groups were replaced with DMEM without FBS. Spheroids were seeded on Ishikawa cells after the medium was changed, followed by incubation for 72 h under standard conditions (37 °C, 5% CO_2) [39, 40]. The EVOS M500 imaging system (ThermoFisher) was used for capturing photos of outgrowth areas with a magnification of 40X. The outgrowth area was measured using the ImageJ software (NIH). The outgrowth assay in this study is illustrated in Fig. 1A.

Statistical analysis

All experiments were performed at least in triplicates. All values are represented as ± standard error of the mean (SEM). Attachment rate data were analyzed using the chi-square test. The outgrowth area data were analyzed using the *t*-test, and a difference between groups with a *P*-value of less than 0.001 was considered statistically significant. Other results were analyzed using one-way analysis of variance (one-way ANOVA) with Tukey's post-hoc multiple comparison tests. Statistical significance was set at *P* value of less than 0.05. The Jeg-3 2D vs. 3D mRNA expression was analyzed by t-test and a difference with a *P* value of less than 0.05 was considered statistically significant.

Results

The expression of Jeg-3 cells 2D vs Jeg-3 spheroids

To investigate the difference between 2 and 3D cultures, the expression of mRNA markers related to attachment

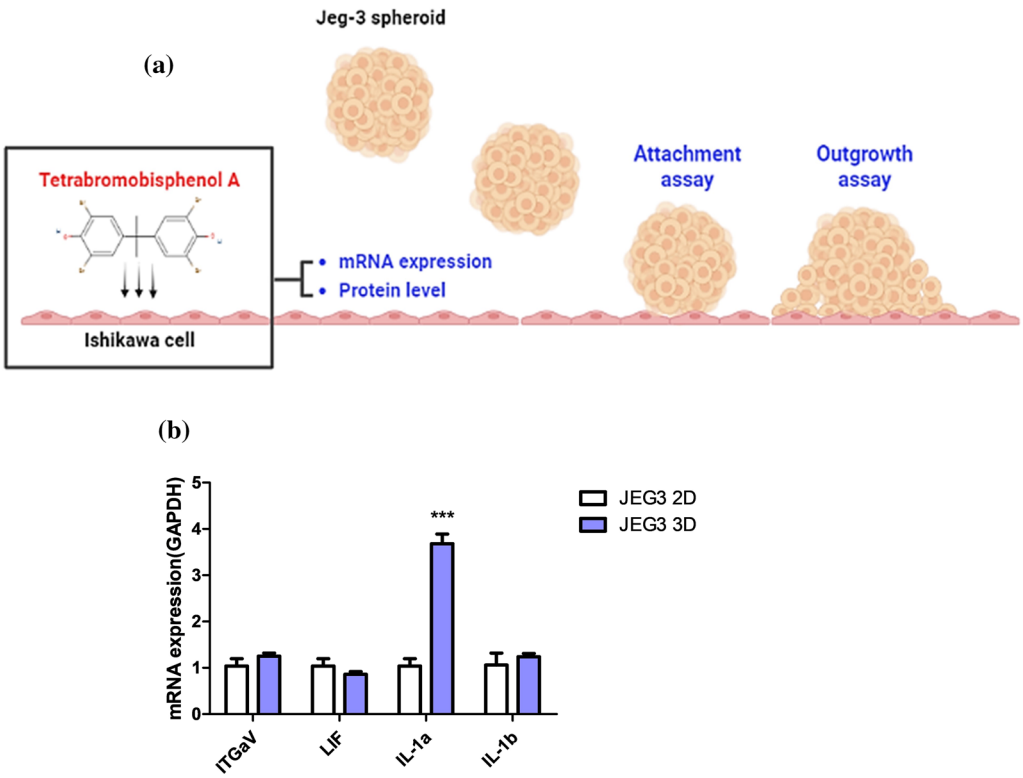

Fig. 1 Experimental design and difference of mRNA expression between 2D culture and 3D culture of Jeg-3. The mRNA expression of Jeg-3 2D culture and 3D culture using qRT-PCR (*ITGaV, LIF, IL-1α and IL-1β*) (n > 3) One-way analysis of variance; Tukey's multiple comparison test ($p < 0.05$). **A** Experimental design for this study. **B** Jeg-3 2D cultured and 3D culture mRNA expression. ***P < 0.0001

(ITGαV and LIF) and inflammation (IL-1α and IL-1β) was evaluated using qRT-PCR. The mRNA expression of IL-1α was increased in the 3D culture group than in the 2D culture group. (IL-1α, 2D 1.04 ± 0.16 vs 3D 3.68 ± 0.21) (Fig. 1B). The mRNA expression levels of ITGαV, LIF, and IL-1β in the 2D culture group were comparable to those in the 3D culture group. Based on these results, we confirmed the difference in mRNA expression between 2D models and 3D models, and 3D models were more suitable for recapitulating the physiological state. Therefore, in this study, we applied 3D spheroid model to the following experiments performed under in vivo conditions.

The effects of TBBPA on the viability of Ishikawa and Jeg-3 cells

To examine the concentration of TBBPA that demonstrated cytotoxic effects on cells for 24 h and 48 h, the cells were treated with TBBPA at concentrations of 0.1, 1, 10, and 100 μM. The highest dose of TBBPA decreased viability of both Ishikawa and Jeg-3 cells at 24 h and 48 h ($P < 0.0001$). The concentrations of 0.1, 1, and 10 μM of TBBPA did not affect viability of either Ishikawa cells or Jeg-3 cells for 24 h (Fig. 2A, C) or 48 h (Fig. 2B, D). (Ishikawa 24 h, control, 1.00 vs TBBPA 0.1 μM, 1.23 vs TBBPA 1 μM, 1.12 vs TBBPA 10 μM, 1.04 vs TBBPA 100 μM, 0.19), (Ishikawa 48 h, control, 1.00 vs TBBPA 0.1 μM, 0.96 vs TBBPA 1 μM, 0.99 vs TBBPA 10 μM, 0.97 vs TBBPA 100 μM, 0.14), (Jeg-3 24 h control, 1.00 vs TBBPA 0.1 μM, 1.04 vs TBBPA 1 μM, 1.04 vs TBBPA 10 μM, 1.04 vs TBBPA 100 μM, 0.21), (Jeg-3 48 h, control, 1.00 vs TBBPA 0.1 μM, 1.04 vs TBBPA 1 μM, 1.01 vs TBBPA 10 μM, 1.00 vs TBBPA 100 μM, 0.16). Since no statistical differences were observed among TBBPA groups except among cells treated with a concentration of 100 μM, the cells were treated with 0.1, 1, and 10 μM during the next steps. A cytotoxicity test was performed to confirm the absence of cytotoxic effects.

Gene expression in Ishikawa cells treated with TBBPA

To investigate the effect of TBBPA on Ishikawa cells, mRNA expression was detected using qPT-PCR. The mRNA expression of inflammatory markers was increased in Ishikawa cells treated with TBBPA. Among inflammation-related genes, the mRNA expression of

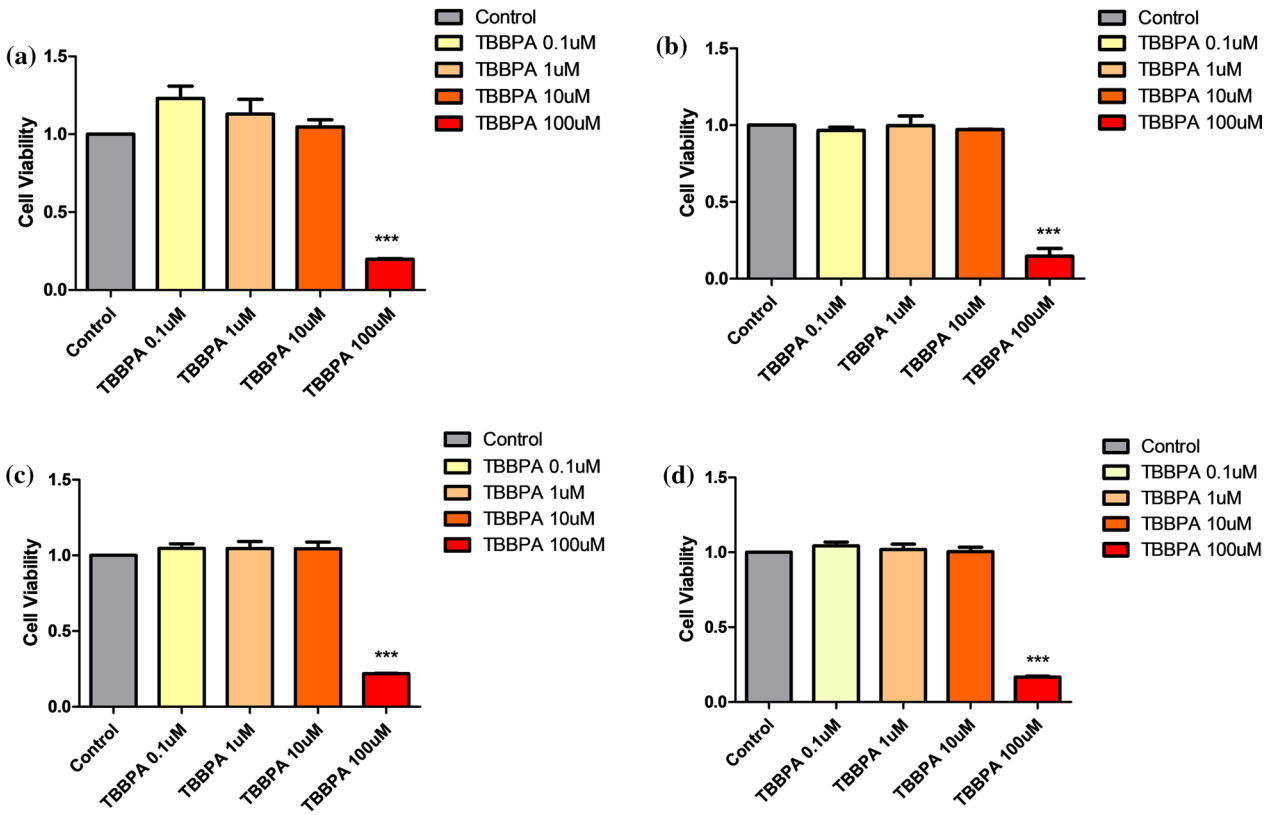

Fig. 2 The cell viability of Ishikawa and Jeg-3 by Cell Counting Kit (CCK8) assay. Cell count kit 8 (CCK-8) assay was conducted to determine cell cytotoxicity of TBBPA. Ishikawa cells and Jeg-3 cells were treated with TBBPA for 24/48 h ($n > 3$). One-way analysis of variance; Tukey's multiple comparison tests, a vs b vs c vs d vs e ($p < 0.05$). **A** Ishikawa cell line TBBPA 0.1–100 μM treated for 24 h. **B** Ishikawa cell line TBBPA 0.1–100 μM treated for 48 h. **C** Jeg3 cell line TBBPA 0.1 μM ~ 100 μM treated 24 h. **D** Jeg3 cell line TBBPA 0.1 μM ~ 100 μM treated 48 h. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$

IL-6, IL-1β, and TNF-α was significantly increased in the TBBPA-treated groups compared to that in the control groups (IL-6, control, 1.001 ± 0.028 vs TBBPA 10 μM, 1.38 ± 0.05), (IL-1β, control, 1.00 ± 0.03 vs TBBPA 0.1 μM, 1.73 ± 0.07 vs TBBPA 1 μM, 1.30 ± 0.03) (TNF-α, control 1.00 ± 0.03 vs TBBPA 0.1 μM, 4.09 ± 0.36 vs TBBPA 1 μM, 4.56 ± 0.25 vs TBBPA 10 μM, 4.72 ± 0.56) (Fig. 3A). For attachment-related genes, the expression of LIF in the TBBPA groups was significantly increased compared to that in the control groups (Fig. 3B; LIF, control, 1.00 ± 0.03 vs TBBPA 1 μM, 3.29 ± 0.13 vs TBBPA 10 μM, 4.31 ± 0.15). These findings indicate that TBBPA affects inflammation and attachment of endometrium cells, which was determined based on the effects observed at the mRNA level.

Protein expression of Ishikawa cells treated with TBBPA

To identify the protein levels of TBBPA in Ishikawa cells, the expression of inflammation- and attachment-related proteins, namely, LIF, TNF-α, and IL-6 were detected. In the previous experiment, the mRNA expression of LIF, TNF-α, and IL-6 was elevated in the TBBPA-treated groups. To identify whether mRNA elevation led to an increase in protein levels, western blot assay was used. The levels of the proteins were slightly increased in all TBBPA groups; however, the difference was not statistically significant (Fig. 4A, B).

Attachment rate of Jeg-3 spheroid on Ishikawa cells treated with TBBPA.

As observed in the previous experiment, the expression of attachment-related marker LIF was increased at the mRNA and protein levels, indicating that TBBPA might enhance the attachment rate of spheroids. To determine the attachment rate of spheroids to Ishikawa cells treated with TBBPA, attachment assays were performed (Control vs TBBPA 10 μM, 5 min 0/89 vs 0/93, 10 min 1/89 vs 2/93, 20 min 17/89 vs19/93, 30 min 27/89 vs 32/93, 60 min 80/89 vs 85/93, 90 min 85/89 vs 87/93, 2 h 89/89 vs 92/93, 4 h 89/89 vs 92/93, 24 89/89 vs 93/93; Fig. 5A). Early attachment rates (0 ~ 30 min) were slightly higher in the TBBPA groups (10 μM) than in the control groups. After 90 min, the attachment rate was not significantly different among the experimental groups (Fig. 5B).

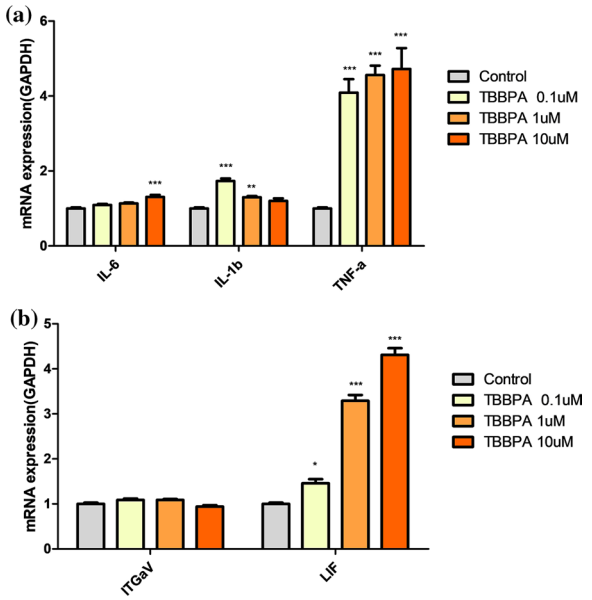

Fig. 3 The mRNA expression related to inflammation and attachment in Ishikawa cells. The mRNA markers related to inflammation were assessed by qRT-PCR. The mRNA expression of Ishikawa cells treated with TBBPA (0.1 μM ~ 10 μM) for 48 h. The mRNA expressions were normalized with *GAPDH*. (n > 3) One-way analysis of variance; Tukey's multiple comparison tests ($p < 0.05$) **A** *IL-6, IL-1β,* and *TNF-α* mRNA expression of Ishikawa cells treated with TBBPA for 48 h. **B** *ITGaV* and *LIF* mRNA expression of Ishikawa cells treated TBBPA for 48 h. *P < 0.05, **P < 0.001, ***P < 0.0001

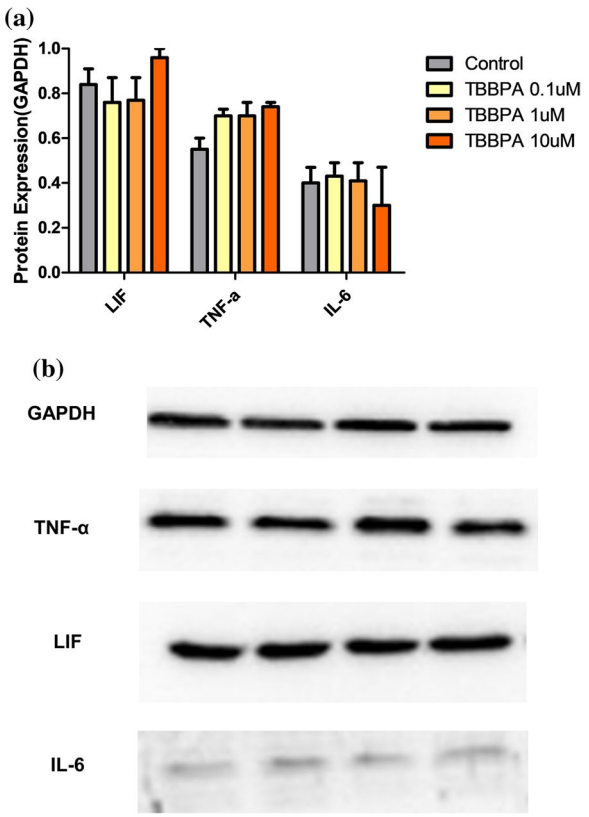

Fig. 4 The protein level of Ishikawa cells treated with TBBPA. The protein level that has increased in the qRT-PCR. All targets normalized with GAPDH. Ishikawa cells were treated with TBBPA for 48 h and 20 μg/mL were used for Western blot assay. **A** The protein level of Ishikawa cell treated with TBBPA (LIF, TNF-a, IL-6) (n > 3) One-way analysis of variance; Tukey's multiple comparison tests ($p < 0.05$) **B** The Western blot band of GAPDH, TNF-a, LIF, and IL-6

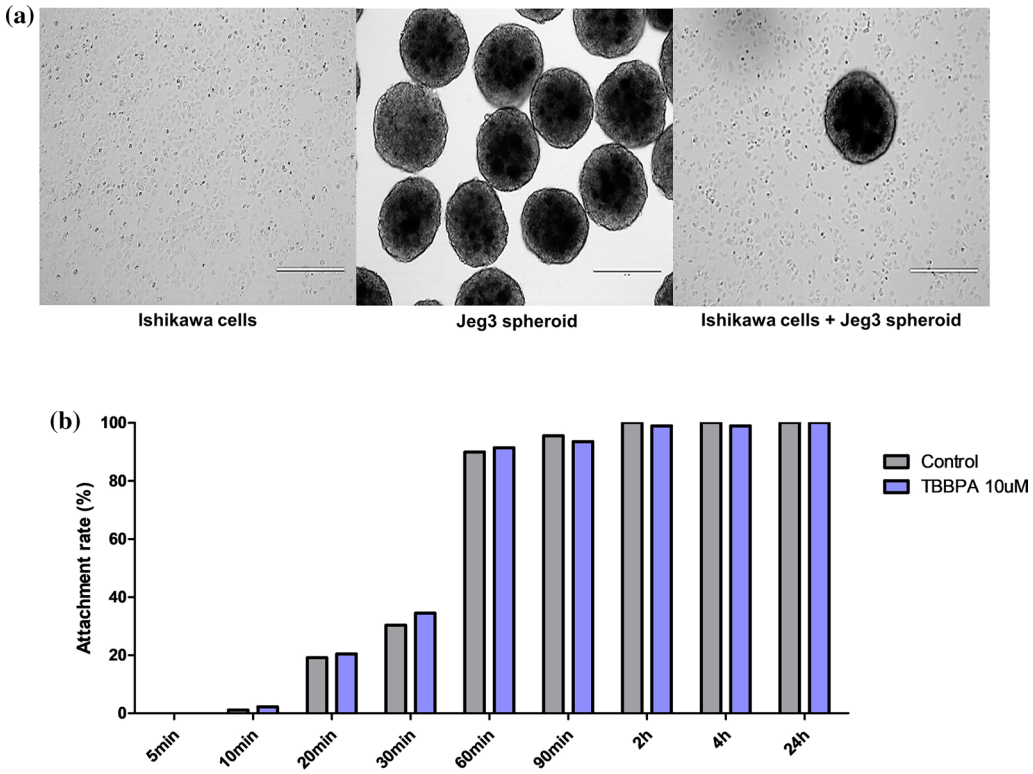

Fig. 5 The attachment assay of Jeg3 spheroid on Tetrabrombisphenol A treated Ishikawa cells. **A** The morphology of Ishikawa cells, Jeg-3 spheroid, and Jeg-3 Spheroid on Ishikawa cells treated with TBBPA. **B** Attachment rate (%) ($n > 65$) T-test; ($p < 0.05$). The morphology of Ishikawa cells and Jeg-3 spheroids were taken by the EVOS imaging system. The attachment assay was performed to detect the Jeg-3 spheroids attachment rate

Outgrowth assay of Jeg-3 spheroids on Ishikawa cells treated with TBBPA.

To determine the effect of TBBPA on outgrowth, an outgrowth assay was conducted by examining Jeg-3 spheroids seeded on Ishikawa cells, as shown in Fig. 6A. Ishikawa cells were treated with TBBPA at a concentration of 10 μM. In the TBBPA groups, the outgrowth area significantly decreased ($P < 0.0001$; control 11.6 ± 0.55 vs TBBPA 10 μM 7.38 ± 0.35 vs negative control 6.77 ± 0.36; Fig. 6B). This result indicates that TBBPA negatively influenced the outgrowth area even though the attachment-related protein levels were comparable.

Discussion

In the present study, we investigated whether TBBPA has deleterious effects on the implantation and invasion processes using the three-dimensional spheroid cell culture method. For examining the potential risks and evidence of the effects of TBBPA on female infertility, it is essential to investigate the toxicity of TBBPA in the endometrium and trophoblastic spheroids, which represented the embryo in this study. TBBPA induced inflammation *milieu*, increased the expression of the IL-6, IL-1β and TNF-a mRNA levels, and reduced the outgrown area, even though the protein levels and attachment rate were comparable to the control groups in this study. However, the potential mechanism of TBBPA has not yet been elucidated.

For in vitro spheroid models, 2D cultured systems are generally used, but they do not fully represent physiological human cells. However, 3D spheroids can mimic human in vivo cell conditions much better than 2D culture systems [41]. Differences in data among 2D and 3D models have been observed [42, 43]. The main concern associated with the use 3D culture models in this study was that they were prepared with a cancer cell line. Cancer cells do not represent primary non-cancer cells, which may lead to contrasting results [44]. In this study, the 2D and 3D culture systems showed different mRNA expression patterns of inflammation-related genes, such as IL-1β. Only IL-1β showed a significant difference. These results indicate that our 3D and 2D models are different from those used in other studies. In addition, the differences in mRNA expression showed that the use of the 3D culture system in the study possibly recapitulated the actual physiology of the human body as previously described.

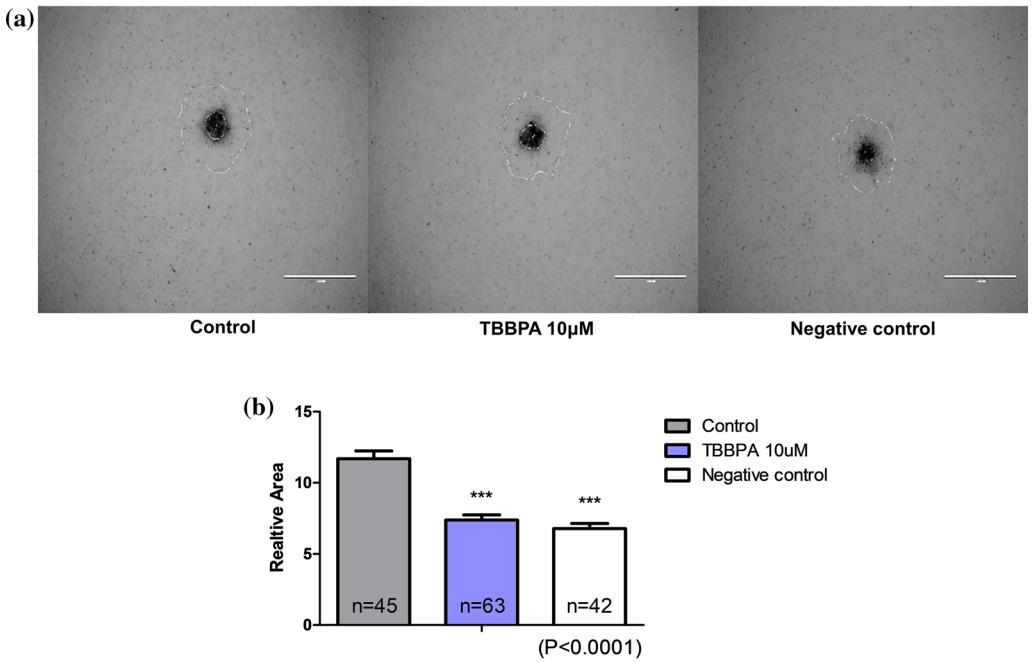

Fig. 6 The outgrowth area of Jeg3 spheroid on TBBPA-treated Ishikawa cells. **A** The morphology and area of Jeg-3 spheroid outgrowth. **B** The outgrowth area. (n = 45 control group, n = 63 TBBPA group, n = 42 negative control group) One-way analysis of variance; Tukey's multiple comparison tests ($p < 0.0001$). The outgrowth area was detected and calculated by the ImageJ system. The relative area was measured compared to the control group. ***$P < 0.0001$

In this study, TBBPA did not affect viability of the human endometrial cell line at concentrations of 0.1, 1, and 10 μM for 24 or 48 h. However, a concentration of 100 μM showed significantly decreased cell viability ($P < 0.0001$). To confirm the effect of TBBPA at the cellular level, the concentration that did not exert cytotoxicity was determined using the CCK-8 assay. With regard to the elements concerning authentic human endocrine systems and the environment, exposure to EDCs occurs over extended periods and at low concentrations. It is preferable to conduct the experiment in an environment identical to the actual setting. However, it is quite difficult to maintain a cell line for a long time, and it cannot be reconstructed using cell line experiments. As a reference, TBBPA treatment was performed at a high dose without short-term cytotoxicity [45, 46].

Quantitative RT-PCR was used to examine the effect of TBBPA on mRNA expression. To determine the effect of TBBPA on the endometrium, the marker associated with inflammation and attachment genes was examined [47]. When the human embryo is attached and invaded in the endometrium, cytokines related to attachment and inflammation are released into the endometrium and embryo. For inflammation, IL-6, TNF-α, and IL-1α were used, and for attachment, LIF and ITGαV, which are released from the embryo, were used [48]. The inflammation marker IL-6 and attachment marker LIF interact with each other [49–51]. The mRNA expression associated with inflammation and attachment can show the effect of EDCs on embryo implantation and invasion. The levels of IL-6, TNF-α, and IL-1β, which are associated with inflammation, were remarkably increased in TBBPA-treated endometrium cells ($P < 0.001$). The findings indicate that TBBPA induced an inflammatory *milieu* in embryos and the endometrium.

The protein levels were evaluated using western blot and primary antibodies against IL-6, TNF-α, and LIF. The results are reflected by an increase at the mRNA level. Western blotting was performed to confirm the increase in mRNA expression at the protein level. In this experiment, TBBPA did not change the protein levels significantly. However, the levels of TNF-α in cells treated with 10 μM of TBBPA were slightly increased ($P = 0.058$), indicating that TBBPA has significant effects on mRNA expression and protein levels, but not on these targets that used in this experiment. We did not detect changes in protein levels in response to TBBPA exposure. Their non-responsiveness to TBBPA may be attributed to the fact that the treatment time might be too short to detect responses in the changes in expression. Further studies should clarify how TBBPA regulates the expression of inflammation and attachment-related genes to evaluate its toxicity on female reproduction.

An attachment assay was used to investigate the effect of TBBPA on implantation, especially on attachment process. We attempted to mimic the EDC-influenced endometrium by treating endometrial cell lines with TBBPA. In the attachment assay, TBBPA-treated group showed a slightly higher rate of early implantation than the control group. However, after 60 min, the TBBPA groups showed a lower attachment rate than the control groups. Moreover, no significant differences were noted between the groups. Because the mRNA expression of LIF was increased in the TBBPA group, it is considered that the rate of TBBPA-treated group increased compared to the control group at the early attachment rate. As mentioned above, a limitation of this assay is that spheroids are made with cancer cells. High proliferation and adhesion are the main characteristics of cancer cell lines and can sufficiently affect the attachment of spheroids to the endometrium [52]. For the next study, we are evaluating the impact of TBBPA on mouse primary uterine cells because cancer cells cannot completely imitate the pathophysiology of primary cells.

The outgrowth assay was conducted to examine the invasion of embryo into the endometrium. This implantation process occurs within 72 h in the human body. Jeg-3 spheroids seeded on TBBPA-treated endometrial cells represent the human endometrium exposed to EDCs and embryo implantation. Hence, the outgrowth assay was conducted for 72 h. This result suggests that the outgrowth area is decreased because TBBPA affects molecules when the embryo attaches to the endometrium. In this experiment, TBBPA might have a negative impact on the implantation process in the human body.

The implantation process follows a series of critical steps, including attachment, invasion, and outgrowth. Each of these steps must occur in a coordinated manner for successful implantation to take place. Disruptions in cytokine levels can affect the delicate balance required during implantation and may lead to implantation failure or decreased outgrowth of the embryo. In our study, we examined inflammatory cytokines, including IL-6, IL-1b, TNF-a, and LIF, which are important cytokines and factors involved in the complex process of implantation [53–55]. LIF plays a crucial role in embryo implantation. It is released from the blastocyst and binds to LIFR on endometrial cells, facilitating the attachment of the blastocyst to the endometrial lining. Moreover, the interaction of IL-6 and IL6-R with LIF is involved in this process of attachment. By studying the mRNA levels of these markers, this study provides valuable insights into how TBBPA may affect the expression and regulation of these important factors during implantation. The fact that these markers interact with each other during the implantation process highlights the importance of examining them in conjunction to understand their combined effects on implantation success. Research in this area may provide crucial information about how EDCs such as TBBPA might impact the process of implantation and potentially lead to recurrent implantation failure. In this study, we found that the outgrowth area was significantly decreased in the TBBPA group, suggesting that TBBPA exposure may have an adverse impact on the outgrowth stage of implantation. Understanding these mechanisms can pave the way for the development of targeted interventions or treatments to counteract negative effects and improve implantation success rates. Overall, we suggest that this is a promising and important study that can contribute significantly to the field of reproductive biology and fertility research.

This study had several limitations. First, the treatment time and concentration of TBBPA. In the natural environment, human bodies are exposed to EDCs for a long time at low concentrations. Cell-based experiments have limitations associated with maintenance of the cell line over the years. Therefore, it is not possible to fully recapitulate the exposure of the human body to EDCs found in the actual environment. It is expected that experiments on EDC can be carried out through animal experiments. Second, the characteristics of the cell line used in this study. The cell lines used in this experiment were human endometrial adenocarcinoma and human choriocarcinoma cell lines. When primary cell lines are compared to cancer cell lines, cancer cell lines demonstrate higher proliferation, adhesion, and differentiation than the primary cell line. Due to these features, the attachment assay might not recapitulate the actual results. For further studies, we strongly suggest using a primary cell line with a real embryo and using an animal model for the attachment assay. Finally, the effects of EDCs on Jeg-3 spheroids are a limitation of this study. In the physiological human body, when implantation occurs, EDCs affect the embryos and endometrium. In this experiment, we only treated Ishikawa cells with TBBPA, which is a representative model of the human endometrium. Examining the effect of EDCs both in the endometrium and spheroids used for mimicking embryos may provide a solution to overcome the limitations of this study.

Conclusion

In this study, the effect of TBBPA on implantation was studied. The the mRNA levels of IL-6, TNF-α, and IL-1β increased significantly. In addition, the outgrowth area decreased significantly in the TBBPA group. Collectively, we confirmed that TBBPA has an effect on implantation, especially on the endometrium.

Abbreviations

ART	Assisted reproductive technology
EDCs	Endocrine disrupting chemicals
BPA	Bisphenol A
TBBPA	Tetrabromobisphenol A
BFRs	Brominated flame retardants
RIF	Recurrent implantation failure
DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
P/S	Penicillin and streptomycin
DMSO	Dimethyl sulfoxide
CCK-8	Cell Counting Kit-8
RT-qPCR	Reverse Transcription-quantitative polymerase chain reaction
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
ITGαV	Integrin alpha V
LIF	Leukemia inhibitory factor
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor α
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
BCA	Bicinchoninic acid assay
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
SEM	Standard error of the mean

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Not applicable.

Author contributions
Conceptualization: MK, KI, LJ; investigation: MK, IK; writing—original draft: MK, IK; writing—review and editing: JL, JHJ; supervision: JL, JHJ; project administration: JL, JHJ; funding acquisition: JL.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agreed to publish the paper.

Competing interests
The authors declare that they have no competing interests.

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