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Original Article

Effect of Bisphenol A on invasion ability of human trophoblastic cell line BeWo

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Abstract: Bisphenol A (BPA) is a kind of environmental endocrine disruptors (EEDs) that interfere embryo implantation. Trophoblast invasion plays a crucial role during embryo implantation. In this study, the effects of BPA on invasion ability of human trophoblastic cell line BeWo and its possible mechanism were investigated. BeWo cells were exposed to BPA and co-cultured with human endometrial cells to mimic embryo implantation in transwell model. The proliferation and invasion capability of BeWo cells were detected. The expression of E-cadherin, DNMT1, MMP-2, MMP-9, TIMP-1 and TIMP-2 were also analyzed. The results showed that the invasion capability of BeWo was reduced after daily exposure to BPA. BPA had biphasic effect on E-cadherin expression level in BeWo cells and expression level of DNMT1 was decreased when treated with BPA. Moreover, BPA treatment also changed the balance of MMPs/TIMPs in BeWo cells by down-regulating MMP-2, MMP-9 and up-regulating TIMP-1, TIMP-2 with increasing BPA concentration. Taken together, these results showed that BPA treatment could reduce the invasion ability of BeWo cells and alter the expression level of E-cadherin, DNMT1, TIMP-1, TIMP-2, MMP-2, and MMP-9. Our study would help us to understand the possible mechanism of BPA effect on invasion ability of human trophoblastic cell line BeWo.

Keywords: Bisphenol A, BeWo cells, cell invasion, E-cadherin, DNMT1, MMPs/TIMPs

Introduction

Environmental Endocrine Disruptors (EEDs) are exogenous, natural and synthetic chemicals which disrupt endocrine function through mimicking or blocking endogenous hormones [1]. EEDs have numerous adverse effects to humans such as obesity, metabolic disturbances, reproductive dysfunctions, and cancers of breast, prostate or ovary [2-4]. BPA is a kind of EEDs and used to synthesize epoxy resins and polycarbonate plastic which is the common raw material of bottles, food packaging, dental materials, and thermal papers and so on. BPA widely appears in the environment, from atmosphere to water and dust. BPA could be absorbed from dermal contact, respiration and digestive system. In a review, concentrations of BPA on human tissues and fluids were ranging from 0.5 nM to 40 nM [5, 6]. In human body, BPA was metabolized through glucuronidation, it has a half-time of 6 h and excretes within 24 h. However recent study showed that BPA might stay in the body for more than 24 h [7].

Clinical research showed that in women who received in vitro fertilization (IVF) treatment, there was a positive linear dose-dependent association between BPA urinary concentrations and implantation failure [8]. At the same time, exposure to BPA might be associated with unexplained recurrent spontaneous abortion [9]. BPA may impair embryo implantation which includes embryo apposition, attachment and invasion [10]. Embryo implantation is one of the crucial processes of successful pregnancy. After epithelial to mesenchymal transition (EMT), cytotrophoblasts differentiate into extravillous trophoblasts (EVTs) which gain the capability to invade [11]. Optimal trophoblast

cell invasion into the uterine deciduas is the basis of successful pregnancy. Any alterations in the process of invasion of EVTs may relate to a series of pregnancy associated diseases such as preeclampsia, intrauterine growth restriction, spontaneous abortion and trophoblastic disease [12]. Although BPA have negative effects on embryo implantation, the underlying mechanisms remain unclear.

E-cadherin, as a kind of calcium-dependent transmembrane glycoprotein, plays a critical role in the suppression of cells invasion. During the implantation process, trophoblastic E-cadherin expression is temporarily down-regulated and then trophoblast cells possess a potential ability of migration and invasion [13, 14]. High-level E-cadherin gene expression and E-cadherin promoter hypomethylation were observed in BeWo cells [11]. E-cadherin promoter methylation is associated with several tumor cells invasiveness. DNA methylation is a crucial way to down-regulate E-cadherin [15]. Recent studies demonstrate BPA can alter epigenome through changing DNA methylation [16-18]. BPA exposure perturbed placental development after epigenome changes in mouse model [18]. DNA methyltransferase 1 (DNMT1) is an enzyme, characterized for its role in maintaining DNA methylation patterns during replication and involved in transcriptional repression [19]. DNMT1 are also implicated in the transcriptional regulation of E-cadherin [20]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with the ability to degrade the extracellular matrix (ECM). MMP-2 and MMP-9 were members of MMPs family and defined to induce trophoblast invasion [21]. Inhibitor of matrix metalloproteinases (TIMP-1 and TIMP-2) were inhibitors of MMP-9 and MMP-2 respectively [22, 23]. MMP-2, MMP-9, TIMP-1, and TIMP-2 play crucial roles in human embryo implantation and trophoblast invasion [24].

In our study, BeWo cells were co-cultured with primary endometrial cells in a transwell system to mimic the trophoblast invasion during embryo implantation. The effect of BPA on the invasion ability of trophoblast cells and the relevant genes expression was evaluated. Our study would help us to understand the possible mechanism of BPA effect on invasion of trophoblast cells.

Materials and methods

Cell culture

Cell lines: The BeWo cell line was obtained from CHINA CENTER FOR TYPE CULTURE COLLECTION (Wuhan, China) and confirmed to be mycoplasma negative. All cells in this study were cultured in DMEM/F12 1:1 (HyClone, USA) containing 10% fetal calf serum (Gibco, USA) and 1% penicillin/streptomycin (HyClone) in 5% CO_2 at 37°C.

Primary endometrial cells: The endometrial tissue samples were obtained from six fertile women of reproductive age with leiomyoma and regular menstrual cycles. These women underwent hysterectomy in the Gynecology Department of Zhongnan Hospital, Wuhan, China. None of them suffered chronic disease or had taken any hormonal treatment in 3 months before operations. The endometrium was certified to be without endometriosis, endometrial hyperplasia, submucous myoma or malignant diseases by pathological examination. This study was approved by the Hospital Ethical Committee of Zhongnan Hospital.

After the uterus was separated in the operation room, endometrial tissue samples of half uterus were collected, stored in cold sterile DMEM/F12 medium and sent to the laboratory in 30 minutes. Endometrial tissues were rinsed thoroughly in sterile PBS at room temperature and digested 1 hour with 0.1% collagenase I (Gibco) for at 37°C. The final suspension was filtered (150 μmm pores), then centrifuged at 1000 rpm for 3 min. Finally, the endometrial cells were plated onto 25 cm^2 culture flasks in DMEM/F12 medium, with 15% fetal calf serum at 37°C in 5% CO_2 and 95% air.

Cell proliferation assay

BeWo cells in the logarithmic phase were digested by trypsin and plated on 96-well culture plates at a density of 5 × 10^4 cells/well. BPA (99+%, Sinopharm Chemical Reagent, China) was dissolved in DMSO and diluted by complete culture solution. BeWo cells were cultured with BPA (1000 μM, 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, containing 0.1% DMSO) or vehicle (0.1% DMSO). After exposure to BPA for 48 h, MTT solution (20 μl, 5 mg/ml, Sigma, USA) was added to each well and incubated for

Table 1. RT-PCR primer sequences

Gene	Sequence	Length
GAPDH	Sense: 5'-CACCCACTCCTCCACCTTTG-3'	110 bp
NM_001256799.1	Antisense: 5'-CCACCACCCTGTTGCTGTAG-3'	
E-cadherin	Sense: 5'-GAGAACGCATTGCCACATACAC-3'	164 bp
NM_004360.3	Antisense: 5'-AAGAGCACCTTCCATGACAGAC-3'	
DNMT1	Sense: 5'-AGTGAAGCCCGTAGAGTG-3'	108 bp
NM_001130823.1	Antisense: 5'-TGAACGCTTAGCCTCTCC-3'.	

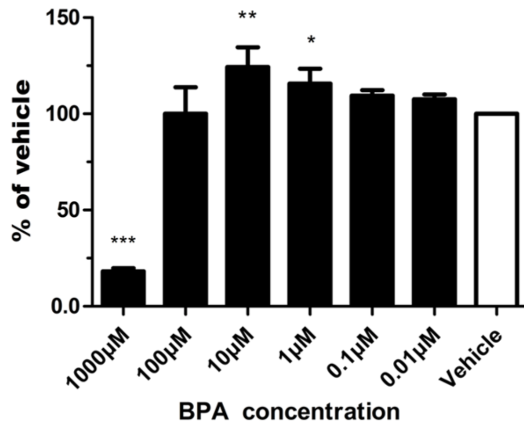

Figure 1. MTT assay. BeWo cells exposure to BPA (1000 μM, 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM respectively) for 48 h. Then cells were incubated for 4 h with MTT reagent and the absorbance was read at 490 nm. The values of the treated cells were compared with the values generated from the untreated control cells and reported as the percentage viability (*P<0.05, **P<0.01, ***P<0.001 compared with vehicle). Three independent assays were performed in at least quadruplicate.

4 h at 37°C. DMSO (200 μl) was added and culture plate was shaken for 10 min to dissolve the purple formazan product. Absorbance was measured at λ=490 nm in the Microplate Reader (BioTek Elx808). The cell viability was expressed as the following equation: Viability (%)=A(test)/A(control) × 100%.

Cell invasion assay-transwell

Endometrial cells were digested and seeded on 24-well culture plates with 5 × 10^5 cells/well. To secure concordance of endometrial cells, cell pooling is not adopted and primary endometrial cells were divided into four groups by first passage. After the endometrial cells stuck wall and reached to 80% in density in the lower chambers, transwell insert with a pore size of 8 μm (BD, Biosciences, Germany) was coated with 50 μl ECM (Sigma, USA) which was diluted by 1:9 with serum-free DMEM-F12. After incubation at 37°C, 4 h for gelling, BeWo cells suspension (200 μl) was seeded at a density of 1 × 10^6/ml in the upper chamber of the insert in DMEM-F12 containing 5% FBS. The medium of lower chambers was removed and filled with 600 μl DMEM-F12 containing 15% FBS and BPA (100 μM, 1 μM, 0.01 μM) or vehicle respectively. The endometrial cells and BeWo cells were co-cultured at 37°C and 5% CO_2 for 48 h. Then filters were washed twice by PBS, methanol was used to fix for 20 min and cells on the upper surface were gently removed by a cotton swab. The invade cells attached to the bottom face of the insert were stained with 0.1% crystal violet for 15 min and washed three times in the pure water. The number of stained BeWo cells was measured in five random non-overlapping fields under a light microscope (Olympus IX73) at a magnification of × 200.

Real time quantitative polymerase chain reaction (RT-qPCR)

BeWo cells were exposed to BPA (100 μM, 1 μM or 0.01 μM) or vehicle for 24 h, RNA was extracted by TRIzol reagent (Invitrogen, USA) according to the manufacturers' protocol, and the quality of RNA was measured by the ultraviolet spectrophotometer. cDNA was synthesized from mRNA by using the Aid First Strand cDNA Synthesis Kit (Thermo, USA). RT-qPCR was used to examine corresponding gene expression level in the samples. GAPDH was chosen as the housekeeping gene for internal control and the primers were listed in **Table 1**. The condition of qPCR was 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 45 s. qPCR was ran on the ABI-7300 PCR system and the datum was analyzed by ABI Prism 7300 SDS Software. The formula of $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of mRNA. To make the result more intuitive, all the data were amplified 100 times.

Western blot

BeWo cells were exposed to BPA (100 μM, 1 μM or 0.01 μM) or vehicle for 48 h. Total protein was extracted and separated by 10% SDS-

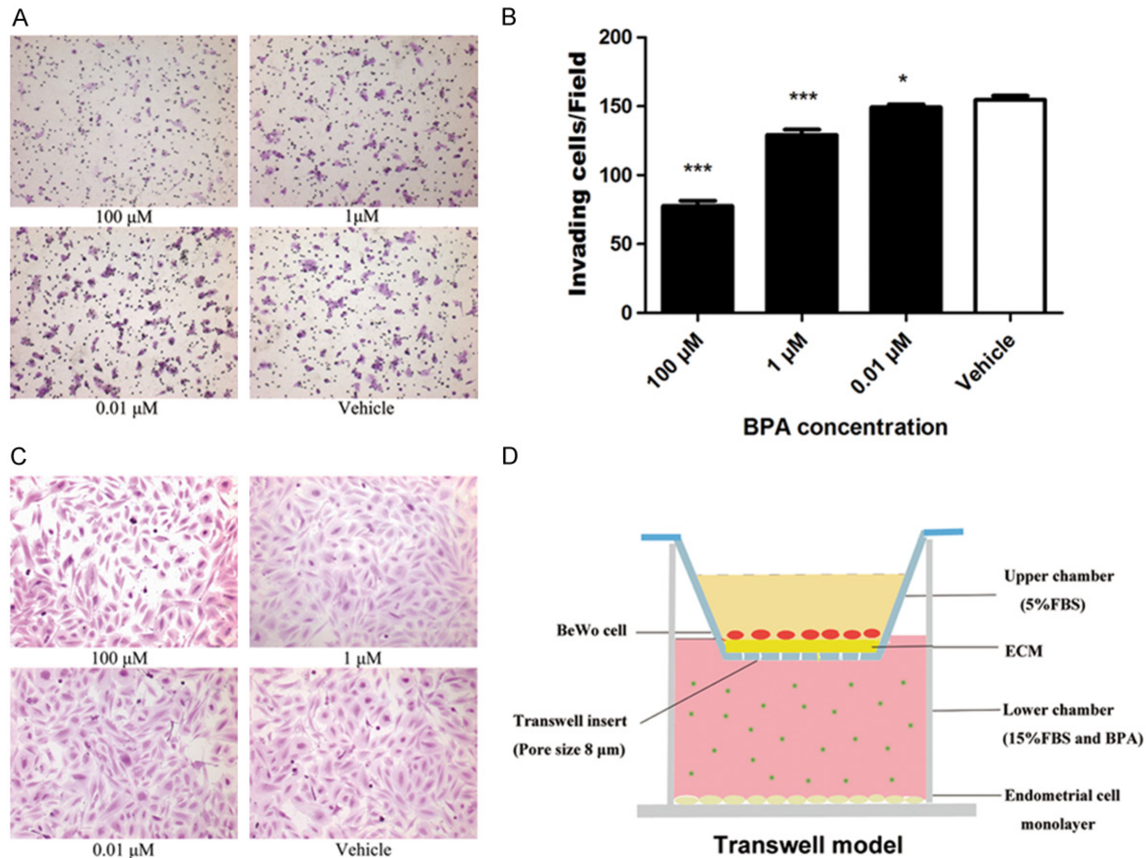

Figure 2. BeWo cells were exposed to BPA and co-cultured with human endometrial cells to mimic embryo implantation in transwell model. The invasion capability of BeWo cells were detected by transwell assay. A. Representative image of invade BeWo cells on the bottom face of transwell insert. B. The numbers of invasive cells treated with BPA (100 μM, 1 μM, 0.01 μM) and vehicle (*P<0.05, ***P<0.001 compared with vehicle). C. The endometrial cells in the lower chambers. D. The schematic diagram of transwell model.

polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk power and 0.05% Tween 20 for 1 h. Then the membranes were respectively incubated overnight at 4°C with primary antibodies at dilution of 1:1000 (anti-E-Caherin and GAPDH, Santa Cruz; anti-MMP-2, anti-MMP-9, anti-TIMP-1, anti-TIMP-2, and anti-DNMT1, Abcam, UK). After that the membranes were incubated with secondary antibodies at room temperature for 1 h. Then the immunoreactive signals were detected with ECL detection system according to manufacturers' protocol. All the experiments were repeated for at least three times and the results were analyzed by Image J software.

Statistical analysis

The statistical analysis was performed by using SPSS17.0. The data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) with LSD test was used for multiple-group and Student's t test was used for two-group comparison. The differences were considered statistically significant at P<0.05. Figures were made by GraphPad Prism 5.0 and Adobe Photoshop CS5.

Results

Effects of BPA on the proliferation of BeWo

After incubated with BPA for 48 h, the proliferation of BeWo cells was tested by MTT. As shown in **Figure 1**, the results showed that BPA had no effect on the cell proliferation rate at 100 μM or below 1 μM. However, the proliferation rate was decreased at 1000 μM and increased at 1 μM and 10 μM compared with vehicle. When treated with 10 μM of BPA, the most proliferation rate of BeWo was observed. The above results

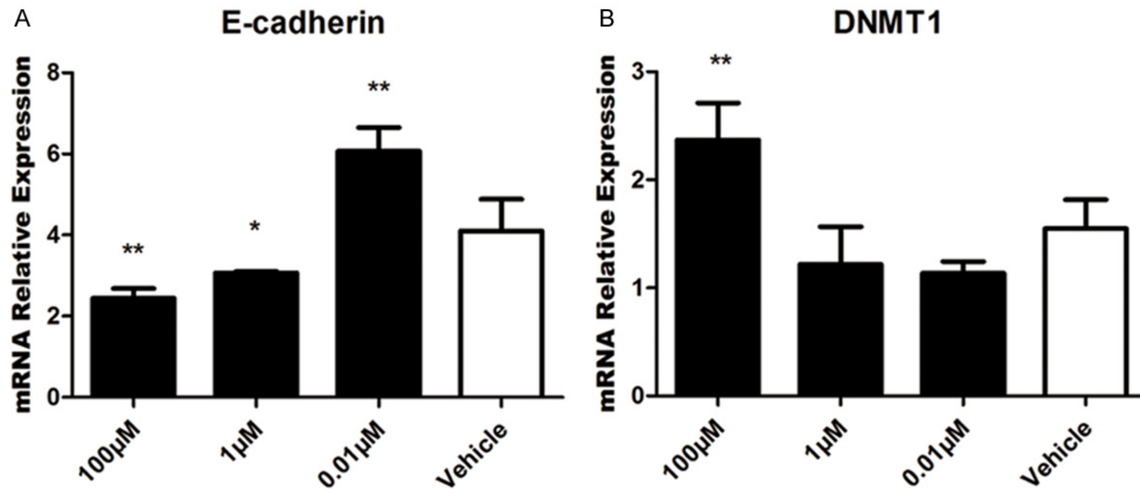

Figure 3. The mRNA expression of E-cadherin (A) and DNMT1 (B) of BeWo cells were exposed to BPA (100 μM, 1 μM or 0.01 μM) or vehicle for 24 h. RT-PCR results showed that the low concentration (0.01 μM) of BPA suppressed DNMT1 expression and promoted E-cadherin expression, whereas high concentration (100 μM) of BPA promoted DNMT1 expression and suppressed E-cadherin expression. Densitometric values of E-cadherin and DNMT1 were normalized by GAPDH. The datum are presented as mean ± SD from three independent experiments (*P<0.05, **P<0.01 compared with vehicle).

suggested that BPA may affect the proliferation of BeWo cells in a non-linear manner.

Effects of BPA on the invasion of BeWo cells in transwell system

The schematic diagram of transwell model was shown in **Figure 2D**. The cells in co-culture model were confirmed as endometrial stromal cells by immunohistochemical staining (**Figure 2C**). The numbers of invasive cells that treated with BPA at concentration of 100 μM, 1 μM, 0.01 μM, and vehicle were 77.50±3.87, 129.25±3.86, 149.25±2.22, and 154.75±2.75, respectively (**Figure 2A** and **2B**). The numbers of invasive cells treated with BPA at 100 μM, 1 μM and 0.01 μM decreased by 49.92%, 16.48%, and 0.04% when compared with vehicle. The result suggested that the capacity of invasiveness was reduced when treated with BPA.

Effects of BPA on the mRNA expression level of E-cadherin and DNMT1

The mRNA expression level of E-cadherin and DNMT1 were analyzed by qPCR after BeWo cells treated with BPA for 24 h. As shown in **Figure 3A**, comparing with the control (4.11±0.78), relative mRNA expression level of E-cadherin in experiment groups that treated with 100 μM, 1 μM, and 0.01 μM BPA were 2.44±0.24, 3.07±0.03, and 6.07±0.58, respectively. The result indicated that BPA had biphasic effect on E-cadherin mRNA expression level in BeWo cells. BPA at 100 μM and 1 μM down-regulated the mRNA expression level of E-cadherin, whereas BPA at 0.01 μM up-regulated it. The mRNA expression level of DNMT1 in 100 μM, 1 μM, 0.01 μM BPA, and vehicle groups were 2.37±0.34, 1.22±0.35, 1.14±0.11, and 1.55±0.27, respectively. Only the group that treat with 100 μM BPA showed a significant increase of DNMT1 mRNA level when compared with the vehicle group (**Figure 3B**).

Effects of BPA on the protein expression level of E-cadherin, MMP-2, MMP-9, TIMP-1, TIMP-2 and DNMT1

In order further investigated the effects of BPA on relevant gene expression level, BPA treated BeWo cells were subjected to western blot analysis. As shown in **Figure 4A**, the effect of BPA on E-cadherin expression was dose-dependent and negatively correlated with BPA concentration. The expression of E-cadherin was decreased by half in BeWo cells treated with 100 μM BPA when compared with the vehicle. However, in the groups of BPA at 10 μM, 1 μM, and 0.1 μM, the expression level of E-caherion was increased (**Figure 4A** and **4D**). As to the DNMT1, it showed an expression pattern that opposite to E-caherion when treat with BPA.

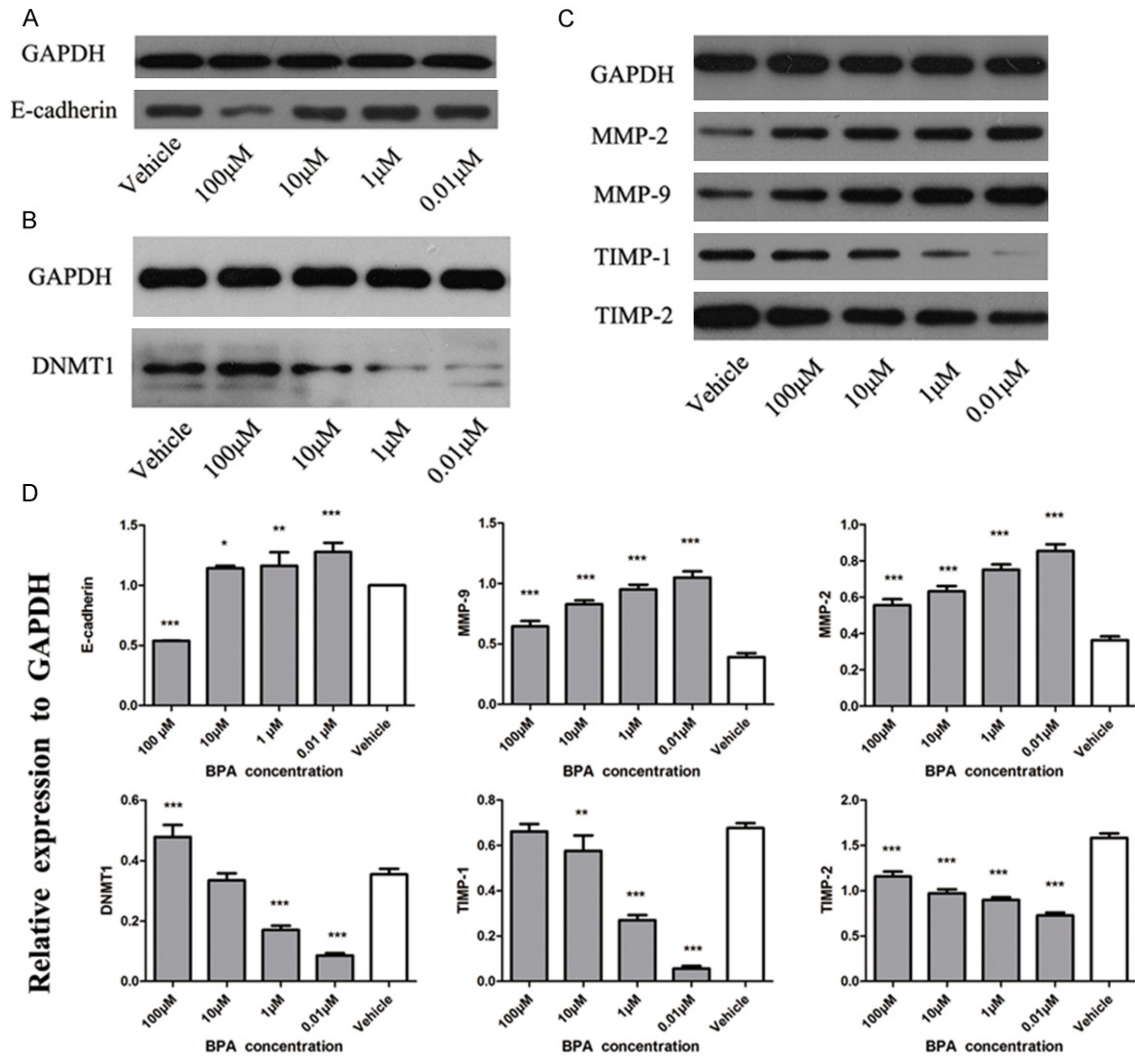

Figure 4. The protein expression of E-cadherin, DNMT1, MMP-2, MMP-9, TIMP-1 and TIMP-2 of BeWo cells were exposed to BPA (100 μM, 1 μM or 0.01 μM) or vehicle for 48 h. The western blot showed a similar trend of E-cadherin and DNMT1 to that in RT-PCR. The protein expression level of MMPs (MMP-9 and MMP-2) decreased and TIMPs (TIMP-1 and TIMP-2) increased with increasing BPA concentration. The ratio of MMPs/TIMPs was negatively correlated with the concentration of BPA. GAPDH was chosen as loading control of the samples (*P<0.05, **P<0.01, ***P<0.001 compared with vehicle). Three independent assays were performed.

BPA at 100 μM up-regulated the expression of DNMT1, whereas BPA under 10 μM, 1 μM, and 0.1 μM down-regulated the expression of DNMT1 (**Figure 4B** and **4D**). High concentration of BPA treated group (100 μM) showed the contrary effect on the expression of E-cadherin and DNMT1 to the low concentration of BPA treated group (concentration of BPA treated group). This trend is similar to RT-PCR. In addition, the expression level of MMPs and TIMPs were also measured. The protein expression level of MMPs (MMP-9 and MMP-2) decreased and TIMPs (TIMP-1 and TIMP-2) increased with increasing BPA concentration. The ratio of MMPs/TIMPs was negatively correlated with the concentration of BPA (**Figure 4C** and **4D**).

Discussion

BPA is widely used and about 3.6×10^9 kg BPA was produced every year all over the world [25]. In 2003-2004 National Health and Nutrition Examination Survey, BPA was detected in the urine of 92.6% of the tested persons in the USA and median concentration was ~3 μg/L [26]. BPA was regarded as a reproductive toxicant since it could impair oocyte quality, interfere with uterine endometrial proliferation, reduce

endometrial receptivity and eventually result in implantation failure in vitro or in vivo experiments as reported in recent years [27].

Many researches focused on the endometrial receptivity during implantation. It was suggested that BPA exposure could decrease the number of implantation sites and the expression of endometrial receptivity associated genes in the pregnant uterus of rat. Clinical research indicated exposure to BPA may be associated with unexplained recurrent spontaneous abortion [28]. Numerous studies indicated that as a kind of EDCs, BPA followed Non-monotonic dose-response curves (NMDRCs) and BPA at low dose could also have significantly pathophysiological influence which is different from high doses. So the daily low exposure dose of BPA is more important in toxicology study [29]. However, in the implantation window which is during day 20 to 24 in normal menstrual cycle of 28 days [30], the interactions between uterus and the implanting embryo are both critical for successful implantation and placentation. Few studies about the effect of BPA on the adhesion and invasion of embryo were performed. In this study, we explore effects of BPA on invasion ability of BeWo cells and the relevant genes expression level under BPA treatment was also evaluated.

The BeWo cells have preserved many properties of cytotrophoblastic and are considered an appropriate in vitrosystem to investigate. The co-culture model of BeWo cells and ESCs could be used to mimic the invasion process of embryo implantation.

Firstly, the effects of BPA on the proliferation of BeWo were tested. The results showed that BPA at high concentrations (1000 μM) exerted cytotoxic effects and BPA at 10 μM and 1 μM induced cell proliferation slightly. Other concentrations (100 μM, 0.1 μM, 0.01 μM) showed no significant change. The above results suggested that BPA may affect the proliferation of BeWo cells in a non-linear manner. The results were consistent to what were observed in cell lines of Hela cells [31], MCF-7 cells [32] and OVCAR-3 cells [33]. The mechanism by which BPA induces cytotoxicity may relate to the production of reactive oxygen species and chromosomal aberrations. And cytotoxicity occur at similar 1000 μM BPA concentration, thus this concentration was not included in following test [43]. MTT assay confirmed that effect of BPA on following cell invasion model was not due to an impaired cell proliferation.

In our transewell model, BPA was demonstrated to reduce invasion of BeWo cells in the co-culture system with ESCs. The suppression of invasion was positively correlated with BPA concentrations. BPA reduced cell invasion in both low and high doses, which was positively correlated with concentrations.

DNMT1 play an important role in maintaining DNA methylation patterns and reducing E-cadherin transcription [20, 34]. E-cadherin, as an epithelial gene, was repressed by DNA methylation [35, 36]. Infertile patients with endometriosis were indicated to have a higher E-cadherin expression in the mid-secretory endometrium than healthy controls [37]. Infertile patients with hydrosalpinx showed a lower E-cadherin expression in the endometrium than control groups [38]. Down-regulated expression of E-cadherin in the first trimester trophoblast cells was associated with the recurrent abortion [39]. The mRNA expression of E-cadherin and DNMT1 were analyzed by RT-qPCR. Interestingly, we found that the effect of high concentration (100 μM) of BPA was contrary to low concentration (1 μM or 0.01 μM). Low concentration of BPA suppressed DNMT1 expression and promoted E-cadherin expression, whereas high concentration of BPA promoted DNMT1 expression and suppressed E-cadherin expression. The western blot showed a similar trend of E-cadherin and DNMT1 to that in RT-qPCR. Hereon, we speculated that the mechanism of the effect of high and low doses BPA on invasion ability of trophoblast cells were different.

Optimal trophoblast cell invasion is mediated through numerous promoting and inhibitory factors [40]. Among these factors, MMPs are critical markers of invasion. Down-regulation of MMP-9 and up-regulation of TIMP-1 resulted in a reduction of trophoblast invasiveness, which was associated with preeclampsia [41]. The impaired balance between MMPs and TIMPs could result in the migration and invasion of cancer cells. It was showed that the increased ratio of MMPs/TIMPs promoted the invasion of human kidney cancer cells [42]. In our study, we found that BPA decreased the ratio of MMPs/TIMPs by down-regulating MMP-2,

MMP-9 and up-regulating TIMP-1, TIMP-2 with increasing BPA concentration, which consisted with reduce of cell invasion. Moreover, we also found that daily exposure dose (0.01 μM) of BPA was enough to change the ratio significantly.

In summary, as a kind of EEDs, BPA could affect cells proliferation and reduce invasive capability of BeWo cells. The effect of BPA is bidirectional and dose-dependent. We speculated BPA at low dose probably increased the expression of E-cadherin by decreasing DNMT1 and DNA methylation patterns, changing the balance of MMPs/TIMPs by down-regulating MMP-2, MMP-9 and up-regulating TIMP-1, TIMP-2. DNA methylation patterns should be further proved by Methylation-specific PCR of E-cadherin promoter gene. And the differences in the mechanism of action at different concentrations of BPA need further investigation.

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Disclosure of conflict of interest

None.

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