Received: 22 October 2017 | Revised: 26 December 2017 | Accepted: 27 December 2017

DOI: 10.1002/tox.22529

RESEARCH ARTICLE

WILEY ENVIRONMENTAL TOXICOLOGY

Butyl paraben promotes apoptosis in human trophoblast cells through increased oxidative stress-induced endoplasmic reticulum stress

Changwon Yang[1] | Whasun Lim[2] | Fuller W. Bazer[3] | Gwonhwa Song[1]

[1]Department of Biotechnology and Institute of Animal Molecular Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea

[2]Department of Biomedical Sciences, Catholic Kwandong University, Gangneung 25601, Republic of Korea

[3]Department of Animal Science and Center for Animal Biotechnology and Genomics, Texas A&M University, College Station, Texas 77843-2471

Correspondence
Gwonhwa Song, Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, Republic of Korea.
Email: ghsong@korea.ac.kr

Funding information
Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), the Ministry of Health & Welfare, Republic of Korea, Grant/Award Number: HI15C0810 and HI17C0929.

Abstract

Butyl paraben (BP) has antimicrobial effects and is widely used as a preservative in cosmetics, foods, and pharmaceuticals. It is also absorbed into various tissues of the human body. It is known that BP is measurable in maternal and fetal tissues during pregnancy, but the effects of BP on placental development, essential for maintaining normal pregnancy, are unclear. Therefore, we investigated the effect of BP on the proliferation, apoptosis, and invasiveness of human trophoblast cells, using an HTR8/SVneo cell line. BP inhibited cell proliferation and induced both apoptosis and endoplasmic reticulum stress. In addition, BP promoted the production of intracellular reactive oxygen species, increased Ca^{2+} concentration in HTR8/SVneo cells, and induced mitochondrial membrane depolarization. BP also inhibited the activation of PI3K/AKT pathways including AKT, ribosomal protein S6, P70 S6 kinase, and glycogen synthase kinase 3β. Furthermore, pretreatment of cells with LY294002 (an AKT inhibitor) and U0126 (ERK1/2 inhibitor) revealed that ERK1/2 activity is also involved in BP-mediated signal transduction in HTR8/SVneo cells. We therefore suggest that exposing human trophoblast cells to BP diminishes normal physiological activity, leading to apoptosis and problems with early placental development.

KEYWORDS
Butyl paraben, trophoblast, ROS, ER stress

1 | INTRODUCTION

Parabens are alkyl ester compounds of parahydroxybenzoic acid, and are widely used as preservatives in cosmetics, foods, beverages, and pharmaceuticals. Parabens exert potent antimicrobial effects through the inhibition of ATPases and phosphotransferases, and through disruption of the membrane transport system.[1] Parabens are mainly synthesized industrially and are known to disrupt endocrine function by mimicking the active form of natural estrogens.[2] Butyl paraben (BP) is more estrogenic than other parabens, including methyl, ethyl, and propyl paraben.[3] BP contained in consumer products has been thought to be safe; however, toxicity studies regarding BP are increasing, as it is easily absorbed and retained in body tissues, including the skin and gastrointestinal tract.[4] BP causes adverse effects, particularly in the development and function of the reproductive organs. Various animal studies revealed that exposure to BP changes histological characteristics of the uterus and hinders steroidogenesis.[5,6] Moreover, prenatal exposure to BP is related to adverse reproductive outcomes, although the mechanism by which maternal exposure to BP affects normal pregnancy maintenance is unclear.[7]

Normal placental development is essential for maintenance of pregnancy as it ensures the supply of adequate nutrients to the fetus. Precise regulation of trophoblast differentiation and growth is important in trophoblast invasion of the maternal endometrium, where these cells modify blood flow dynamics by inducing spiral artery remodeling.[8] Failure of vascular remodeling due to incomplete trophoblast invasion causes pregnancy disorders, such as preeclampsia and intrauterine growth retardation.[9] Various external factors, including diet and environmental pollutants, affect the survival and invasiveness of human trophoblast cells. Typically, bisphenol A (BPA) inhibits the invasion of

Changwon Yang and Whasun Lim authors contributed equally to this study.

human trophoblast cells by modulating MAPK signaling pathways and the membrane estrogen receptor.[10] In addition, endocrinological homeostasis is important for normal placental development in early pregnancy. The apoptotic rate of trophoblast cells is closely related to the level of serum estradiol, which is kept low in early pregnancy and increases as pregnancy progresses.[11] BP increases expression of the estrogen receptor in estrogen receptor-positive breast cancer cells, but not in normal breast epithelial cells.[12] Moreover, recent research found that BP modulates BPA in female and male reproductive organs by exerting estrogenic effects.[13] Furthermore, the level of BP in the urine of pregnant women is associated with oxidative stress.[14] Excessive oxidative stress in human trophoblast cells promotes endoplasmic reticulum (ER) stress and mitochondrial Ca^{2+} overload, leading to apoptosis.[15] However, it is unknown how BP affects cell proliferation or invasiveness in human trophoblast cells.

In this study, we investigated whether BP could alter the properties of human trophoblast cells. First, we verified the anti-proliferative effects of BP on a human trophoblast cell line, HTR8/SVneo. We also investigated whether BP induces reactive oxygen species (ROS)-mediated cell death and ER stress in HTR8/SVneo cells. Moreover, we verified that BP inhibits invasiveness of HTR8/SVneo cells. Finally, we estimated whether phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) pathways are regulated in response to BP in HTR8/SVneo cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals

BP was purchased from Selleckchem (Houston, TX). Antibodies against glucose-related protein 78 (GRP78), growth arrest- and DNA damage-inducible gene 153 (GADD153), activating transcription factor 6α (ATF6α), and tubulin α-chain (TUBA) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibodies against inositol-requiring enzyme 1α (IRE1α), cytochrome C, phosphorylated AKT (Ser^{473}), ribosomal protein S6 (S6, Ser^{235}/Ser^{236}), P70 S6 kinase (P70S6K, Thr^{421}/Ser^{424}), glycogen synthase kinase 3β (GSK3β, Ser^{9}), ERK1/2 (Thr^{202}/Tyr^{204}), P90 S6 kinase (P90RSK, Thr^{573}), and total AKT, P70S6K, GSK3β, S6, ERK1/2, and P90RSK were purchased from Cell Signaling Technology (Beverly, MA). LY294002 was purchased from Cell Signaling Technology, and U0126 was purchased from Enzo Life Science (Farmingdale, NY).

2.2 | Cell culture

HTR8/SVneo cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 with 2.05 mM L-Glutamine (Cat No: SH30027.01, HyClone, Carlsbad, CA) with 5% fetal bovine serum (FBS) at 37°C in a CO_2 incubator. For experiments, monolayer cultures of HTR8/SVneo cells were grown in culture medium to 70% confluence in 100-mm tissue culture dishes. Cells were serum starved for 24 h, and then treated with BP. In each assay, dimethyl sulfoxide (DMSO) was used as a vehicle.

2.3 | Proliferation assay

Proliferation assays were conducted using Cell Proliferation ELISA, BrdU kit (Cat No: 11647229001, Roche, Indianapolis, IN) according to the manufacturer's recommendations as described previously.[16] HTR8/SVneo cells were treated with a range of doses of BP (0, 50, 100, 200, and 400 μM) for 48 h. The absorbances of the reaction product were measured at 370 and 492 nm using an ELISA reader.

2.4 | Immunofluorescence microscopy

The effects of BP on proliferating cell nuclear antigen (PCNA) expression in HTR8/SVneo cells were determined by immunofluorescence microscopy, as described previously in.[16] Cells were treated with BP (200 μM) for 24 h. Images were captured using an LSM710 (Carl Zeiss, Thornwood, NY) confocal microscope, fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured as the green/blue ratio using MetaMorph software (Molecular Devices).

2.5 | Determination of apoptosis by annexin V and propidium iodide staining

BP-induced apoptosis of HTR8/SVneo cells was analyzed using a fluorescein isothiocyanate annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ) as described previously in.[16] Cells were treated with a range of doses of BP (0, 50, 100, and 200 μM) for 48 h. Fluorescence intensity was analyzed using a flow cytometer (BD Biosciences).

2.6 | Western blot analysis

Western blot analysis of BP-treated HTR8/SVneo cells was performed as described previously in.[16]

2.7 | Determination of intracellular ROS

Intracellular ROS production was estimated using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma), which is converted to fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides. Cells were detached with trypsin-EDTA, collected by centrifugation, and washed with PBS. The cells were treated with 10 μM DCFH-DA for 30 min at 37°C. Cells were then washed twice with PBS, and treated with a range of doses of BP (0, 50, 100, and 200 μM) for 1 h at 37°C in a CO_2 incubator. The treated cells were again washed with PBS, and then fluorescent DCF intensity was analyzed using a flow cytometer (BD Bioscience).

2.8 | Measurement of intracellular Ca^{2+} concentration

HTR8/SVneo cells (4×10^5 cells) were seeded onto 6-well plates, grown to 70%-80% confluency, and incubated for 24 h in serum-free medium. Cells were then treated with BP (0, 50, 100, and 200 μM) for 48 h at 37°C in a CO_2 incubator. Vehicle was added up to the highest treated dose. Supernatant was removed from culture dishes and

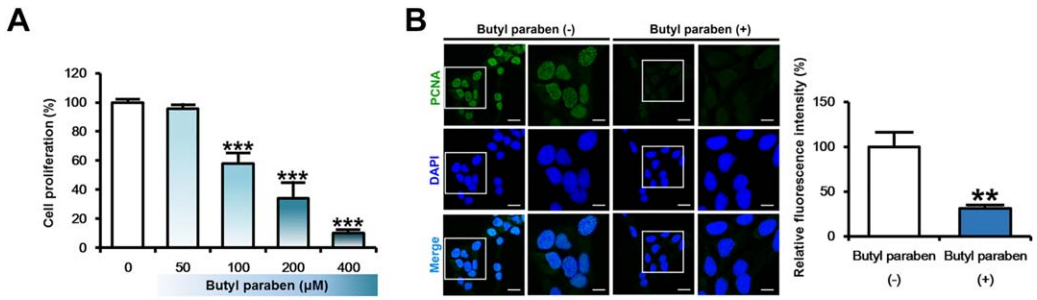


FIGURE 1 Effects of BP on the proliferation of HTR8/SVneo cells. A, Dose-dependent effects of BP on the proliferation of HTR8/SVneo cells were determined, and data are presented as a percentage relative to vehicle (100%). B, PCNA protein was detected (green) and nuclei were counterstained with DAPI (blue) in HTR8/SVneo cells. Cells were treated with 200 μM BP. The asterisks indicate a significant effect of treatment compared with that of control treatment (***$P < 0.001$ and **$P < 0.01$). The *scale bar* represents 40 μm (the first and third vertical panels) and 20 μm (the second and fourth vertical panels) [Color figure can be viewed at wileyonlinelibrary.com]

adherent cells detached with trypsin-EDTA. Cells were collected by centrifugation. For intracellular Ca^{2+} analysis, collected cells were resuspended using 3 μM Fluo-4 AM (Cat No: F14201, Invitrogen) and incubated at 37°C in a CO_2 incubator for 20 min. The stained cells were washed with PBS. Fluorescent intensity was analyzed using a flow cytometer (BD Bioscience).b

2.9 | JC-1 mitochondrial membrane potential assay

The JC-1 mitochondrial membrane potential (MMP) was determined using a mitochondria staining kit (Cat No: CS0390, Sigma-Aldrich) as described previously in.[16] Cells were treated with a range of doses of BP (0, 50, 100, and 200 μM) for 48 h. Fluorescence intensity was analyzed using FACSCalibur (BD Biosciences).

2.10 | Matrigel invasion assay

The cell invasion assay was performed using 8-μm-pore Transwell inserts (Cat No: 3422, Corning, Inc., Corning, NY) coated with Matrigel for 2 h at 37°C. HTR8/SVneo cells (1×10^5 cells per 200 μL) in serum-free medium containing BP (200 μM) were plated onto the upper chamber, whereas medium containing 5% FBS was added to the lower wells. After cells were incubated for 24 h at 37°C in a CO_2 incubator, non-invading cells were removed with a cotton swab. For the evaluation of cells that invaded onto the lower surface, inserts were fixed in methanol for 10 min. The Transwell membranes were then air-dried and stained using hematoxylin (Sigma) for 30 min. After washing gently with tap water, the cells on the upper side of the inserts were removed with a cotton swab. The Transwell membranes were removed and placed on a glass slide with the side containing cells facing upward, and the slide was covered with Permount solution. Invading cells were counted in five non-overlapping locations, which covered approximately 70% of the insert membrane growth area, using a DM3000 (Leica, Wetzlar, Germany) microscope.

2.11 | Migration assay

The cell migration was evaluated using Ibidi migration culture dish inserts according to the manufacturer's instructions (Ibidi, Germany). A 70 μL suspension of HTR8/SVneo cells (2×10^5 cells/mL) was seeded into each well of the culture inserts and grown overnight to full confluence. The cells were then treated with BP (200 μM). After 24 h of incubation at 37°C in a CO_2 incubator, the migration of cells into the defined cell-free gap (500 μm) was observed, and light microscopy images of the gap field were acquired using the DM3000 microscope. For assay analyses, gap closure was computed.

2.12 | Statistical analysis

The data were subjected to analysis of variance according to the general linear model of the SAS program (SAS Institute, Cary, NC) to determine whether there were significant differences in response to BP. Differences with a probability value of $P < 0.05$ were considered statistically significant. Data are presented as mean ± SEM unless otherwise stated.

3 | RESULTS

3.1 | BP inhibits proliferation of human trophoblast cells

We investigated whether BP could regulate the proliferative activity of human trophoblast cells using BrdU corporation analysis. HTR8/SVneo cells were treated with 0, 50, 100, 200, and 400 μM BP for 48 h. BP reduced the proliferation of HTR8/SVneo cells in a dose-dependent manner (Figure 1A). Proliferation of HTR8/SVneo cells was decreased by 42% ($P < 0.001$) in response to 100 μM BP and by more than 50% in response to 200 μM BP (66%, $P < 0.001$). We treated HTR8/SVneo cells with BP up to 200 μM for further assays. DMSO was used as a vehicle in all assays. The expression of PCNA, a DNA clamp that plays an essential role in cell proliferation, was observed by fluorescence microscopy after treatment with BP (200 μM) for 24 h in HTR8/SVneo cells. PCNA (expressed as green fluorescence) was only faintly visible in the nuclei of treated cells (Figure 1B). When quantified, BP reduced PCNA expression by 69% compared with that of normal cells. These results indicate that BP inhibits the proliferation of human trophoblast cells.

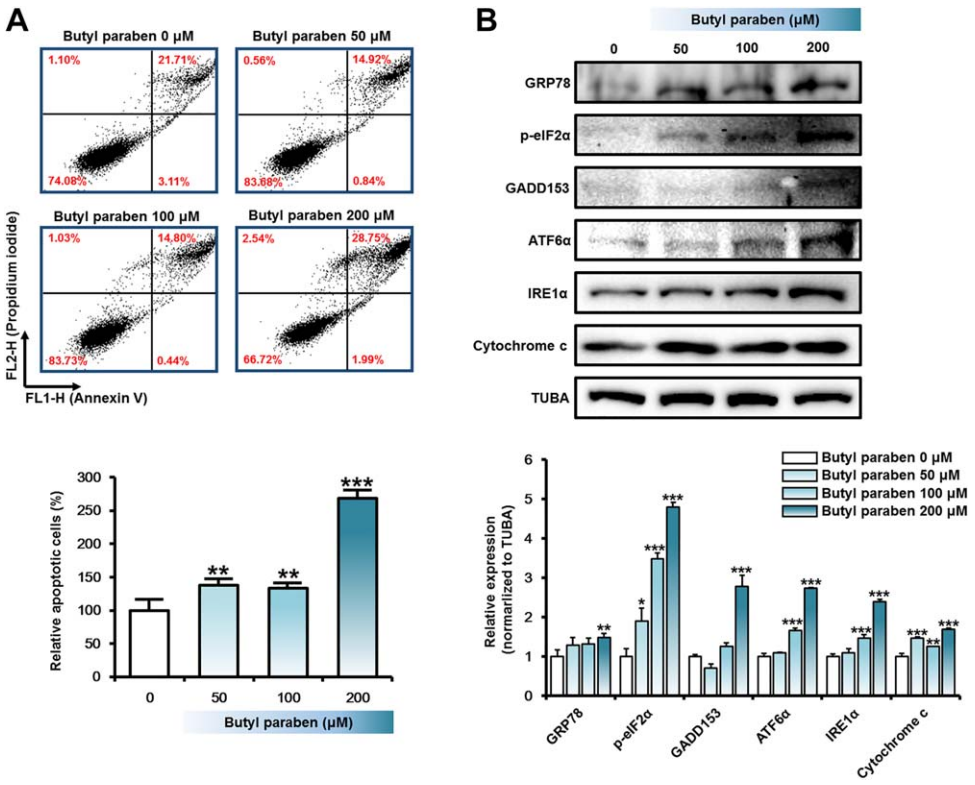


FIGURE 2 BP induces apoptosis and ER stress in HTR8/SVneo cells. A, Flow cytometric detection of apoptosis in HTR8/SVneo cells in response to BP. Annexin V and PI fluorescence values were estimated by flow cytometry. The percentage of apoptotic cells (upper right and lower right quadrants) was analyzed compared with that of controls. B, Dose-dependent effects of BP on the expression of ER stress-related proteins in HTR8/SVneo cells. The asterisks indicate a significant effect of treatment compared with that of control treatment (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | BP induces apoptosis and ER stress in human trophoblast cells

We also estimated whether BP induces cell death in human trophoblast cells. Annexin V and propidium iodide (PI) staining analysis indicated that 48 h of BP treatment elevated the distribution of annexin V-positive apoptotic cells (upper right and lower right of quadrant) in HTR8/SVneo cells (Figure 2A). In response to 200 μM BP, 30.74% of HTR8/SVneo cells were observed to be in an apoptotic state, a 2.7-fold increase compared with cells treated with the same quantity of DMSO ($P < 0.001$). We also identified the expression of proteins related to ER stress after 6 h of BP treatment in HTR8/SVneo cells (Figure 2B). The expression of GRP78, GADD153, ATF6α, and IRE1α was elevated in response to BP. Cytochrome C, which is released by increasing Ca^{2+} stimulation from mitochondria, also showed elevated expression with BP treatment. These results indicate that BP-induced apoptotic cell death correlated with elevated ER stress.

3.3 | BP stimulates oxidative stress and disrupts MMP in human trophoblast cells

We further investigated whether oxidative stress was induced by BP treatment by estimating intracellular ROS levels in human trophoblast cells. Estimated DCF fluorescence, which increases with hydrogen peroxide generation, increased with BP treatment in a dose-dependent manner (0, 50, 100, and 200 μM), with a 5.0-fold increase ($P < 0.001$) at the maximum concentration compared with that of the control (Figure 3A). Intracellular ROS induces cell death by promoting the release of Ca^{2+} from the ER. Therefore, we measured the amount of Ca^{2+} in the cytoplasm through fluo-4 staining in HTR8/SVneo cells (Figure 3B). Cells treated with 200 μM BP showed a 2.1-fold increase ($P < 0.001$) in cytoplasmic Ca^{2+} concentration compared with that of untreated cells. These results indicate that BP induced oxidative stress, followed by elevated Ca^{2+}, in human trophoblast cells. Moreover, we measured MMP to determine whether BP-induced increased oxidative stress could affect mitochondrial morphology and apoptosis. MMP loss increased in response to 48 h of BP treatment in a dose-dependent manner (Figure 4). A 17.1-fold increase ($P < 0.001$) was observed with 200 μM BP, compared to the same quantity of vehicle. These results suggest that elevated ER stress and cytosolic Ca^{2+} levels affected mitochondrial dysfunction, leading to cell death in human trophoblast cells.

3.4 | BP inhibits invasiveness of human trophoblast cells

The proper invasion of trophoblast cells is crucial for normal placental development. The effect of BP on invasiveness of HTR8/SVneo cells

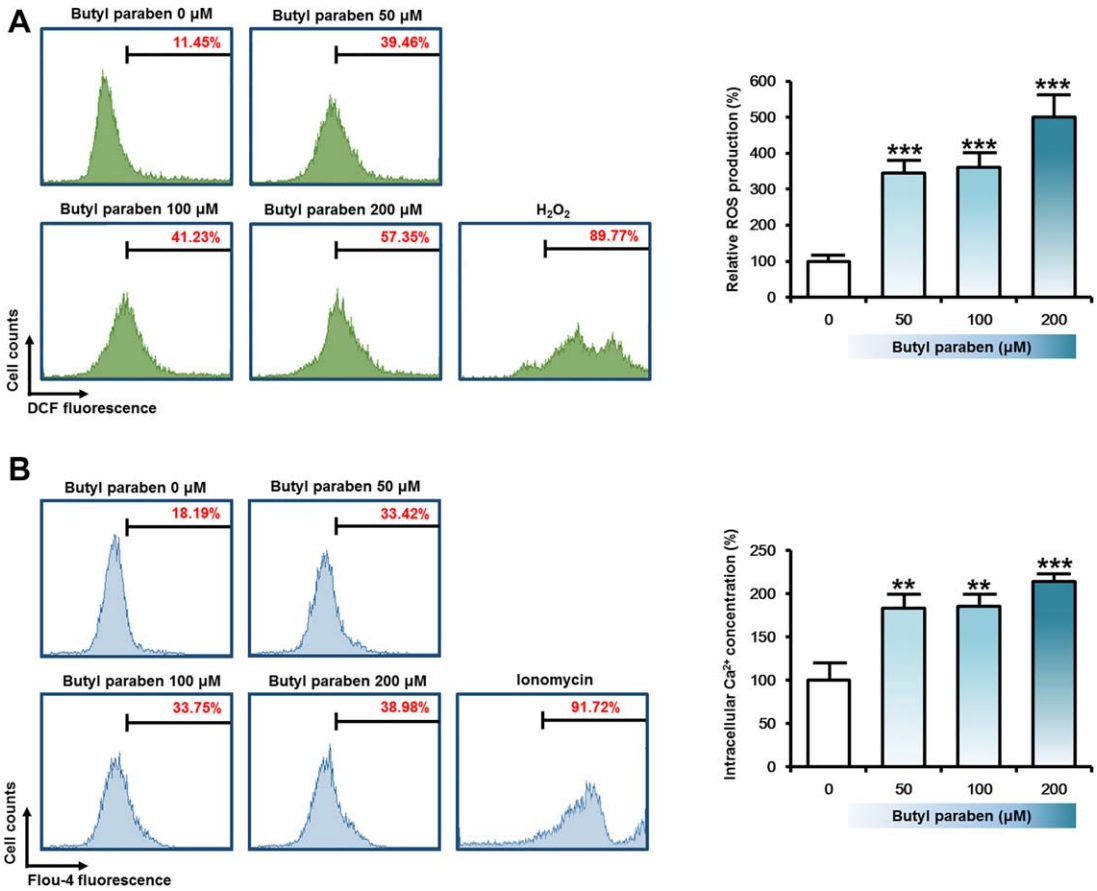
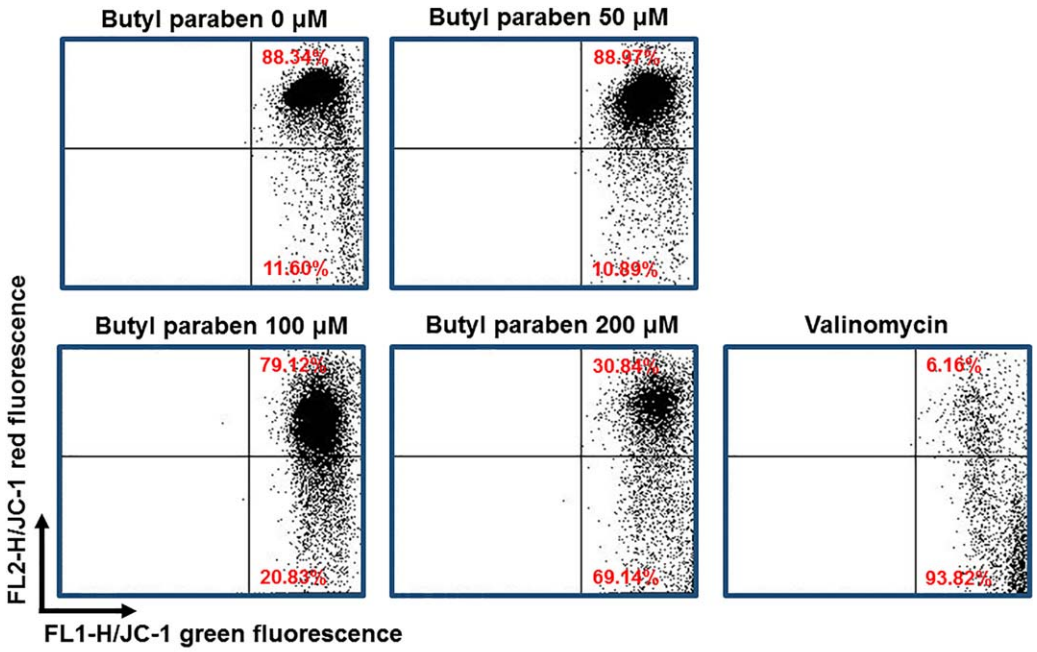


FIGURE 3 Effects of BP on intracellular ROS production and Ca^{2+} levels in HTR8/SVneo cells. A, Dose-dependent intracellular ROS production in response to BP was estimated by flowcytometric detection. The number of DCF green fluorescence-labeled cells represents the relative quantities of intracellular hydrogen peroxide in BP-treated HTR8/SVneo cells. 0.03% hydrogen peroxide was used as a positive control. B, Dose-dependent flowcytometric detection of intracellular Ca^{2+} concentration in response to BP using fluo-4 staining analysis. Ionomycin (1 μM) was used as a positive control. The asterisks indicate a significant effect of treatment compared with that of control treatment (***$P < 0.001$ and **$P < 0.01$) [Color figure can be viewed at wileyonlinelibrary.com]

was determined using Matrigel-coated Transwell inserts. As shown in Figure 5A, cells treated with BP (200 μM) failed to invade the Transwell membrane. The number of cells that passed through the membrane decreased by 51% ($P < 0.05$) in response to 24 h of BP treatment. In addition, BP-treated cells (200 μM) were 1.4-fold ($P < 0.01$) further from the two areas compared with normal cells (Figure 5B). These results indicate that BP influenced motility of human trophoblast cells, which would cause poor invasion of trophoblasts into the maternal endometrium.

3.5 | BP modulates activation of PI3K/AKT pathways in human trophoblast cells

We investigated whether BP regulates cell signaling pathways involved in the proliferative and invasive properties of human trophoblast cells, including PI3K/AKT and ERK1/2 pathways. Phosphorylation of proteins involved in the PI3K/AKT pathways including AKT, P70S6K, GSK3β, and S6 decreased in response to 2 h of BP treatment (200 μM) in HTR8/SVneo cells (Figure 6A-D). Conversely, ERK1/2 activity increased in response to BP, whereas P90RSK was not influenced by BP treatment (Figure 6E,F). Inhibition of AKT using LY294002 (20 μM) further reduced phosphorylation of P70S6K and S6 with BP treatment, whereas inhibition of ERK1/2 using U0126 (20 μM) activated AKT and P70S6K, which were inhibited by BP. These results indicated that antiproliferative and antiinvasive properties of BP on human trophoblast cells are mediated by PI3K/AKT signaling pathways, which are affected by ERK1/2 activity. In addition, HTR8/SVneo cells were treated with LY294002 (20 μM) and U0126 (20 μM) to determine the effect of BP-mediated AKT and ERK1/2 activity on the properties of human trophoblast cells. Cell proliferation was decreased by 26% ($P < 0.001$) by BP and LY294002 treatment and 39% ($P < 0.001$) by BP and U0126 treatment (Figure 7A). In addition, the proportion of apoptotic cells was increased 5.1-fold ($P < 0.001$) in response to LY294002 and 7.1-fold ($P < 0.001$) in response to U0126 compared with treatment with BP alone (Figure 7B). Also, LY294002 and U0126 increased the loss of MMP by 3.8- ($P < 0.001$) and 221.3-fold ($P < 0.001$), respectively, compared with BP alone (Figure 7C). Compared with LY294002 alone, the addition of BP further reduced cell proliferation and increased apoptosis and loss of MMP. However, there was no significant difference in cell proliferation and apoptosis for cells treated with U0126 alone or



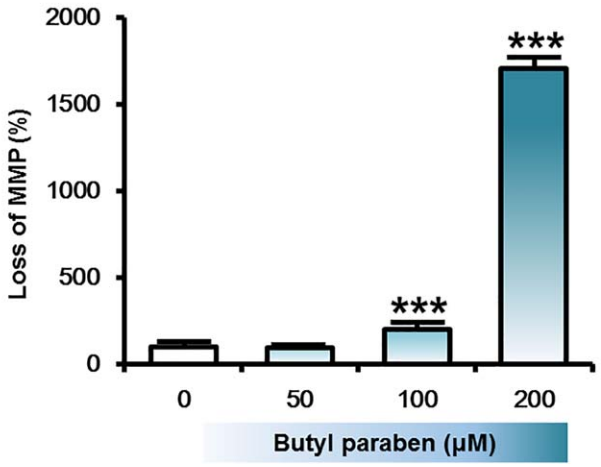


FIGURE 4 The alteration of MMP in response to BP in HTR8/SVneo cells was detected by flow cytometry, and JC-1 staining levels were quantified as the relative ratio of lower right/upper right of quadrant. Valinomycin (1 μM) was used as a positive control. The asterisks indicate a significant effect of treatment compared with that of control treatment (***$P < 0.001$) [Color figure can be viewed at wileyonlinelibrary.com]

co-treated with U0126 and BP, and BP alleviated the effect of BP on loss of MMP in human trophoblast cells. These results suggest that the PI3K/AKT signaling pathway regulated by BP plays an important role in regulating properties of human trophoblast cells.

4 | DISCUSSION

The results of the present study verified that BP alters the physiological characteristics of human trophoblast cells, followed by cell death. We suggest that the underlying mechanisms of BP in human trophoblast cells include elevated ER stress and mitochondrial damage initiated by intracellular ROS production. In particular, BP inhibits the invasion of human trophoblast cells, which is fatal to the development of the placenta in early pregnancy. PI3K/AKT and ERK1/2 signaling pathways, which are important for human trophoblast growth and invasiveness, are also involved in the biological activity of BP, as shown in Figure 8.

Although parabens are naturally contained in many fruits and vegetables, the main route through which humans are exposed is via personal care products such as deodorants, shampoos, and sunscreens. Parabens are industrially synthesized and have been used in various products for decades as a preservative. Naturally, human exposure to parabens has been reported to be higher in women than in men or children.[17] Although the EU and the FDA have acknowledged the safety of parabens, there has been a recent increase in the study of their adverse health effects.[18] Generally, parabens exert estrogenic activity

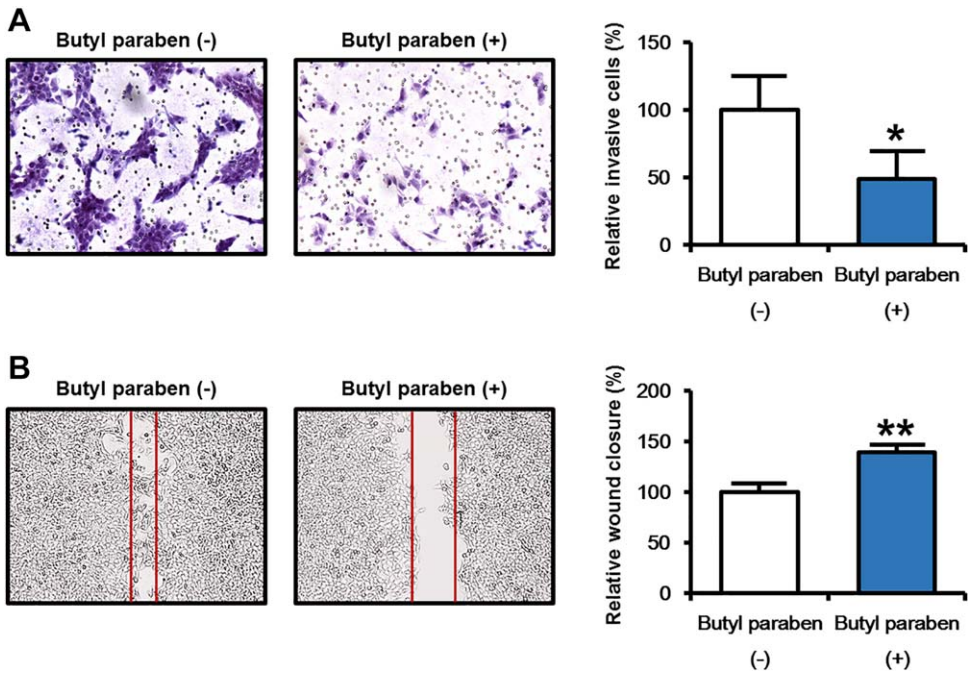


FIGURE 5 BP inhibits invasive properties of HTR8/SVneo cells. A, Invasive capacities were measured using the Transwell chamber assay. A significant decrease in invasion was observed for BP-treated HTR8/SVneo cells. B, Migration of HTR8/SVneo cells in response to BP was calculated based on gap distance and presented as a percentage relative to control (100%). Images of migrated cells were captured at 10× magnification. The asterisks indicate a significant effect of treatment compared with that of control treatment (**$P < 0.01$ and *$P < 0.05$) [Color figure can be viewed at wileyonlinelibrary.com]

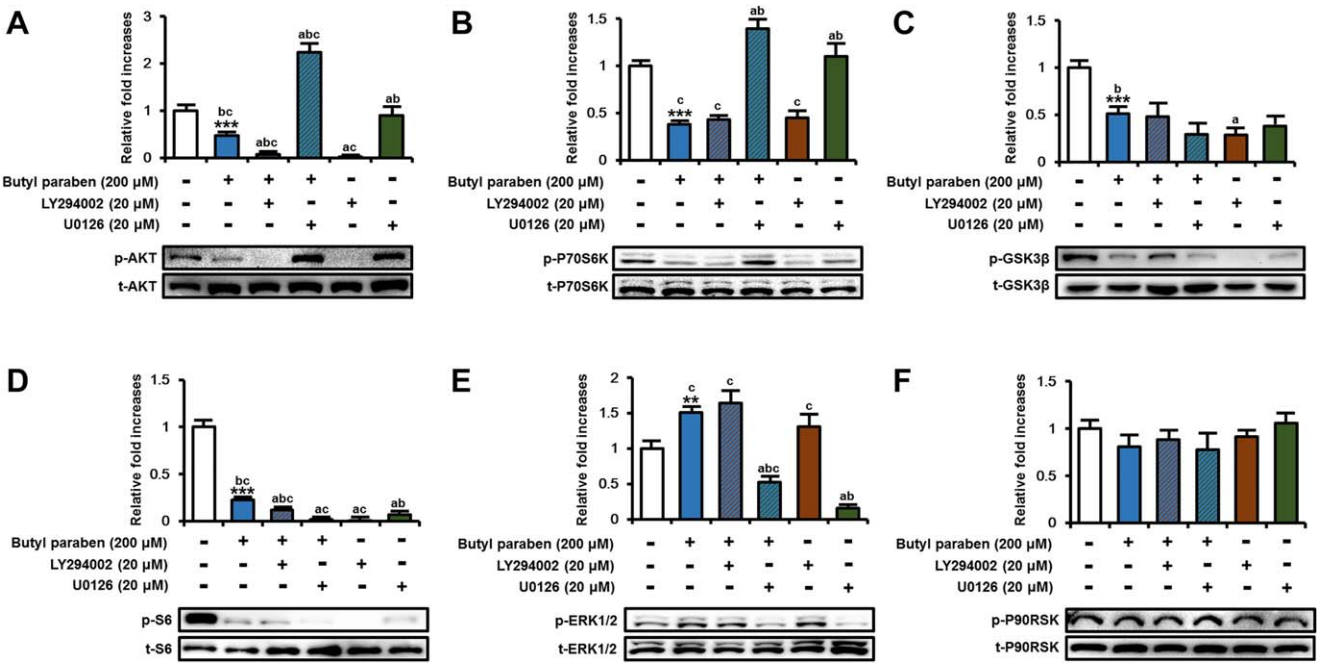


FIGURE 6 The effects of BP alone or with selective inhibitors on phosphorylation of AKT (LY294002, 20 μM) and ERK1/2 (U0126, 20 μM) in HTR8/SVneo cells. Phosphorylated AKT (A), P70S6K (B), GSK3β (C), S6 (D), ERK1/2 (E), and P90RSK (F) were analyzed in response to pretreatment with inhibitors for 1 h before treatment with BP for 2 h. Immunoblots were captured to calculate the normalized values by estimation of expressed levels from phosphorylated proteins relative to total proteins. The asterisks indicate a significant effect of treatment compared with that of control treatment (***$P < 0.001$ and **$P < 0.01$). Different lowercase letters indicate statistically significant changes ($P < 0.05$) among treatments: a, compared with BP alone; b, compared with LY294002 alone; c, compared with U0126 alone [Color figure can be viewed at wileyonlinelibrary.com]

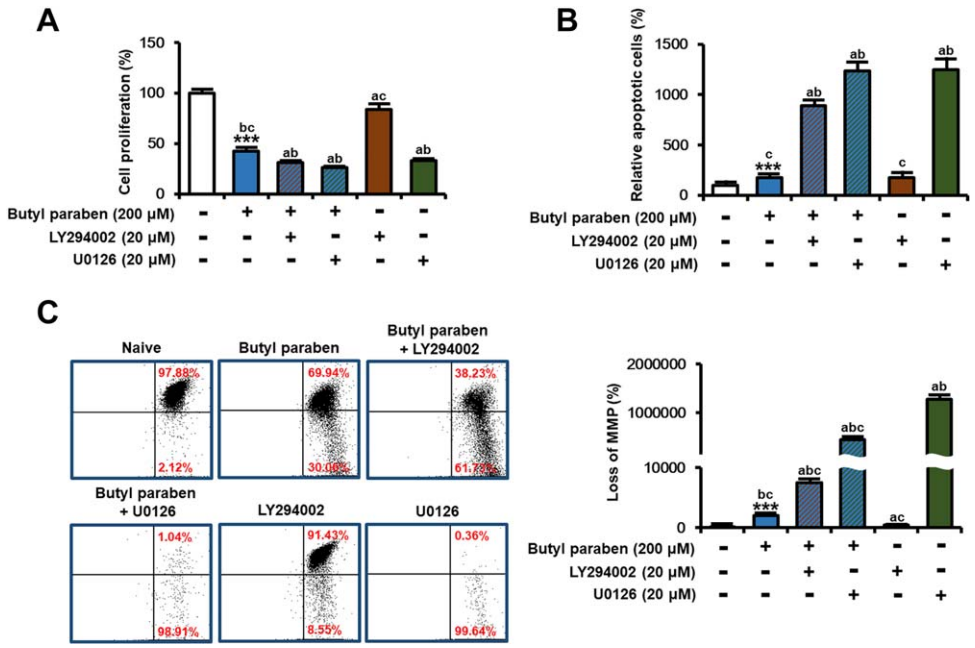


FIGURE 7 The effects of BP alone or with selective inhibitors of cell signaling on properties of HTR8/SVneo cells. A, Effects of BP and selective inhibitors on the proliferation of HTR8/SVneo cells were determined, and data are presented as a percentage relative to control (100%). B, Flow cytometric detection of apoptosis in HTR8/SVneo cells in response to BP and selective inhibitors. Annexin V and PI fluorescence values were estimated by flow cytometry. The percentages of apoptotic cells (upper right and lower right quadrants) were analyzed compared with that of control cells. C, The alteration of MMP in response to BP and selective inhibitors in HTR8/SVneo cells was detected by flow cytometry, and JC-1 staining levels were quantified as the relative ratio of cells in the lower right/upper right quadrants. The asterisks indicate a significant effect of treatment compared with the control treatment (***$P < 0.001$). Different lowercase letters indicate statistically significant changes ($P < 0.05$) among treatments: a, compared with BP alone; b, compared with LY294002 alone; and c, compared with U0126 alone [Color figure can be viewed at wileyonlinelibrary.com]

in vitro and in vivo, and it has been reported that the potency of this effect depends on the length of the chain.[19] Parabens are rapidly absorbed into human tissues and are found not only in human breast milk, but also in cord blood and amniotic fluid.[20] Among parabens, BP is considered to be the most likely to disrupt endocrine function. According to a U.S. National Health and Nutrition Survey, urinary BP was detected at a concentration of 0.2–1240 μg/L in 47% of the population.[21] Maternal exposure to BP causes problems with the development of reproductive organs in male offspring in rats.[22] In addition, prenatal exposure to BP causes impaired neurodevelopment.[23] Moreover, as urinary BP increases with the progress of pregnancy, it is presumed that the metabolism of BP is also influenced by physiological alteration during pregnancy.[24] It is also known that BP is closely related to the metabolism of endocrine disruptors such as BPA, which is known to adversely affect placental development.[13] To our knowledge, this is the first report identifying the effects of BP on the trophoblast cells that develop into the placenta.

The precise regulation of the proliferating cell-to-invasive cell ratio in trophoblast differentiation in early pregnancy is important for subsequent normal vascular remodeling in the endometrium.[8] BP exerts both antiproliferative and anti-invasive effects, which suggests cytotoxicity of BP in human trophoblast cells. If trophoblast cells do not grow adequately or become unable to invade the endometrium, they will lead to various pregnancy disorders such as preeclampsia and intrauterine growth retardation.[25] Many endocrine disruptors are known to be involved in this process. Dioxin causes oxidative stress-mediated apoptosis of human trophoblast cells, evidenced by ROS production, mitochondrial damage, and the preceding release of cytochrome C.[26] Parabens induce oxidative stress and toxicity in various types of cells, and the most potent mechanism involved in BP toxicity in trophoblast cells is elevated oxidative stress.[14] Exposure to BP reduces nonenzymatic antioxidants, including glutathione, and enzymatic antioxidants, including superoxide dismutase and glutathione transferase, in mouse liver, leading to hepatoxicity.[4] Our flowcytometric analysis reveals that an increased apoptotic rate in human trophoblast cells is accompanied by ROS production, followed by ER stress. GRP78, also known as binding immunoglobulin protein, is a chaperone protein located in the ER, which is induced in response to the accumulation of unfolded proteins in the ER. ER stress causes GRP78 to separate from PKR-like ER kinase (PERK), IRE1α, and ATF6α. In the placenta of patients with preeclampsia or intrauterine growth retardation, phosphorylation of eIF2α, the primary target of PERK, is increased, and GADD153 expression is also highly elevated.[27] Although the exact molecular mechanism has not been elucidated, it is suspected that excessive ER stress is closely related to the onset of the pregnancy disorders.

Oxidative stress based on ROS production is well known to be the cause of ER stress. When the ER is stimulated by ROS, excessive Ca^{2+} is released into the cytoplasm. To maintain Ca^{2+} homeostasis, mitochondria introduce increased Ca^{2+}.[28] Excessive Ca^{2+} uptake into

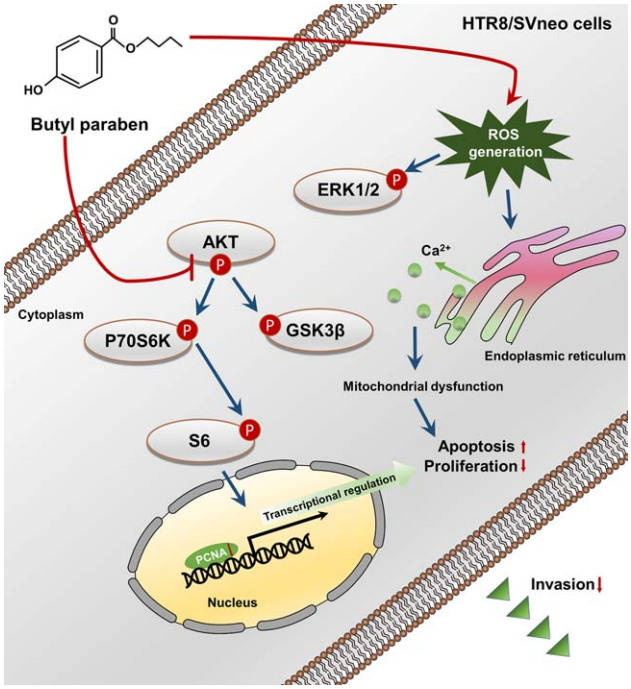


FIGURE 8 A schematic diagram illustrating the current working hypothesis regarding effects of BP on human trophoblast cells. BP stimulates intracellular ROS production followed by Ca^{2+} release from ER. Excessive influx of Ca^{2+} into mitochondria disrupts mitochondrial membrane integrity, leading to apoptosis of human trophoblast cells. BP inhibits phosphorylation of AKT and its downstream kinases, including P70S6K, GSK3β, and S6. Inhibition of signal transduction regulates transcriptional activity related to cell survival and invasion. Overall, survival of human trophoblast cells exposed to BP is difficult and the development of placenta is impeded [Color figure can be viewed at wileyonlinelibrary.com]

mitochondria leads to mitochondrial dysfunction, which eventually leads to apoptosis. In the present study, BP not only increased intracellular ROS levels but also significantly induced cytosolic Ca^{2+} elevation. Oxidative stress induced by BP in human trophoblast cells would lead to mitochondrial membrane collapse. The effects of BP on cytoplasmic oxidation and cell organelles such as the ER and mitochondria suggests the need to identify the biological activity of BP in various cell types, as well as in trophoblast cells, as cell organelle dysfunction correlates with the pathology of metabolic, neurological, and reproductive diseases.[29] We reported that increases in intracellular ROS are positively correlated with the activity of ERK1/2 in human trophoblast cells. BP not only increases the activity of ERK1/2 and associated increase in ROS, but also inhibits the activity of the PI3K/AKT cell signaling cascade that plays a key role in the proliferation and invasiveness of trophoblast cells.[16,30] These results suggest that BP is not only a substance that disrupts the endocrine system, but also regulates protein phosphorylation and transcriptional activity. Furthermore, AKT activity increases when ERK1/2, a signal transduction protein involved in the growth of human trophoblast, is increased, and some cross-control effect would be expected between these two signaling transduction pathways. Moreover, proliferation, apoptosis, and loss of MMP were measured in cells after treatment with the inhibitors of AKT and ERK1/2 and BP. Based on results of those experiments, we propose that AKT regulated by BP plays an important role in the changes in characteristics of human trophoblast cells.

As BP is widely contained in various beauty products, women of childbearing age are easily exposed to it, but the risk is not reported due to its relatively low content and toxicity. However, a recent study of BP-induced histopathological or proteomic changes in the female reproductive organs of animal models suggested that the accumulation of BP may affect offspring.[13,24] Based on this study, it is necessary to identify the adverse effects of BP, which accumulates in the placenta during pregnancy, and to study its stability. Collectively, we have verified that BP is toxic to human trophoblast cells through oxidative stress-induced ER stress and mitochondrial dysfunction. These physiological changes also appear to be due to the inhibition of AKT-mediated signaling transduction pathways. These results present an opportunity to raise awareness of the risk of BP exposure from women's personal care products in early pregnancy, and to clarify the specific molecular mechanism of BP in human cells.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.

ORCID

Gwonhwa Song http://orcid.org/0000-0003-2817-5323

REFERENCES

[1] Ma Y, Marquis RE. Irreversible paraben inhibition of glycolysis by Streptococcus mutans GS-5. *Lett Appl Microbiol*. 1996;23(5):329–333.

[2] Zhang Z, Sun L, Hu Y, Jiao J, Hu J. Inverse antagonist activities of parabens on human oestrogen-related receptor gamma (ERRgamma): in vitro and in silico studies. *Toxicol Appl Pharmacol*. 2013;270(1):16–22.

[3] Daughton CG, Ternes TA. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect*. 1999;107(Suppl 6):907–938.

[4] Shah KH, Verma RJ. Butyl p-hydroxybenzoic acid induces oxidative stress in mice liver–an in vivo study. *Acta Polon Pharm*. 2011;68(6):875–879.

[5] Taxvig C, Vinggaard AM, Hass U. Do parabens have the ability to interfere with steroidogenesis? *Toxicol Sci*. 2008;106(1):206–213.

[6] Lemini C, Hernandez A, Jaimez R, Franco Y, Avila ME, Castell A. Morphometric analysis of mice uteri treated with the preservatives methyl, ethyl, propyl, and butylparaben. *Toxicol Indust Health*. 2004;20(6–10):123–132.

[7] Philippat C, Botton J, Calafat AM. Prenatal exposure to phenols and growth in boys. *Epidemiology*. 2014;25(5):625–635.

[8] Carter AM, Enders AC, Pijnenborg R. The role of invasive trophoblast in implantation and placentation of primates. *Philos Trans Roy Soc Lond Ser B Biol Sci*. 2015;370(1663):20140070

[9] Albrecht ED, Bonagura TW, Burleigh DW, Enders AC, Aberdeen GW, Pepe GJ. Suppression of extravillous trophoblast invasion of uterine spiral arteries by estrogen during early baboon pregnancy. *Placenta*. 2006;27(4–5):483–490.

[10] Li X, Wang Y, Wei P, et al. Bisphenol A affects trophoblast invasion by inhibiting CXCL8 expression in decidual stromal cells. *Mol Cell Endocrinol*. 2017; doi: 10.1016/j.mce.2017.07.016

[11] Bukovsky A, Caudle MR, Cekanova M, et al. Placental expression of estrogen receptor beta and its hormone binding variant–comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. *Reprod Biol Endocrinol*. 2003;1:36.

[12] Wrobel AM, Gregoraszczuk EL. Actions of methyl-, propyl- and butylparaben on estrogen receptor-alpha and -beta and the progesterone receptor in MCF-7 cancer cells and non-cancerous MCF-10A cells. *Toxicol Lett*. 2014;230(3):375–381.

[13] Pollock T, Weaver RE, Ghasemi R, deCatanzaro D. Butyl paraben and propyl paraben modulate bisphenol A and estradiol concentrations in female and male mice. *Toxicol Appl Pharmacol*. 2017;325:18–24.

[14] Watkins DJ, Ferguson KK, Anzalota Del Toro LV, Alshawabkeh AN, Cordero JF, Meeker JD. Associations between urinary phenol and paraben concentrations and markers of oxidative stress and inflammation among pregnant women in Puerto Rico. *Int J Hyg Environ Health*. 2015;218(2):212–219.

[15] Burton GJ, Yung HW, Murray AJ. Mitochondrial - Endoplasmic reticulum interactions in the trophoblast: Stress and senescence. *Placenta*. 2017;52:146–155.

[16] Lim W, Yang C, Bazer FW, Song G. Chrysophanol induces apoptosis of choriocarcinoma through regulation of ROS and the AKT and ERK1/2 pathways. *J Cell Physiol*. 2017;232(2):331–339.

[17] Ye X, Zhou X, Wong LY, Calafat AM. Concentrations of bisphenol A and seven other phenols in pooled sera from 3–11 year old children: 2001–2002 National Health and Nutrition Examination Survey. *Environ Sci Technol*. 2012;46(22):12664–12671.

[18] Błędzka D, Gromadzińska J, Wąsowicz W. Parabens. From environmental studies to human health. *Environ Int*. 2014;67:27–42.

[19] Darbre PD, Harvey PW. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. *J Appl Toxicol*. 2008;28(5):561–578.

[20] Pycke BF, Geer LA, Dalloul M, Abulafia O, Halden RU. Maternal and fetal exposure to parabens in a multiethnic urban U.S. population. *Environ Int*. 2015;84:193–200.

[21] Calafat AM, Ye X, Wong LY, Bishop AM, Needham LL. Urinary concentrations of four parabens in the U.S. population: NHANES 2005–2006. *Environ Health Perspect*. 2010;118(5):679–685.

[22] Guerra MT, Sanabria M, Leite GAA, et al. Maternal exposure to butyl paraben impairs testicular structure and sperm quality on male rats. *Environmental Toxicology*. 2017;32(4):1273–1289.

[23] Ali EH, Elgoly AH. Combined prenatal and postnatal butyl paraben exposure produces autism-like symptoms in offspring: comparison with valproic acid autistic model. *Pharmacol Biochem Behav*. 2013;111:102–110.

[24] Karwacka A, Zamkowska D, Radwan M, Jurewicz J. Exposure to modern, widespread environmental endocrine disrupting chemicals and their effect on the reproductive potential of women: an overview of current epidemiological evidence. *Hum Fertil*. 2017;1–24.

[25] Lyall F, Bulmer JN, Kelly H, Duffie E, Robson SC. Human trophoblast invasion and spiral artery transformation: the role of nitric oxide. *The Am J Pathol*. 1999;154(4):1105–1114.

[26] Chen SC, Liao TL, Wei YH, Tzeng CR, Kao SH. Endocrine disruptor, dioxin (TCDD)-induced mitochondrial dysfunction and apoptosis in human trophoblast-like JAR cells. *Mol Hum Reprod*. 2010;16(5):361–372.

[27] Yung HW, Calabrese S, Hynx D, et al. Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. *Am J Pathol*. 2008;173(2):451–462.

[28] Yuan Y, Xu X, Zhao C, et al. The roles of oxidative stress, endoplasmic reticulum stress, and autophagy in aldosterone/mineralocorticoid receptor-induced podocyte injury. *Lab Invest* . 2015;95(12):1374–1386.

[29] Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. *Annu Rev Pathol*. 2015;10:173–194.

[30] Knfler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. *Int J Dev Biol*. 2010;54(2–3):269–280.

How to cite this article: Yang C, Lim W, Bazer FW, Song G. Butyl paraben promotes apoptosis in human trophoblast cells through increased oxidative stress-induced endoplasmic reticulum stress. *Environmental Toxicology*. 2018;00:1–10. https://doi.org/10.1002/tox.22529