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Bisphenol S Increases Cell Number and Stimulates Migration of Endometrial Epithelial Cells

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Abstract

Objective. To determine whether bisphenol S (BPS), a common substitute for bisphenol A (BPA), induces cell proliferation and migration in human endometrial epithelial cells (Ishikawa) and adult mouse uterine tissues.

Methodology. Human endometrial Ishikawa cells were exposed to low doses of BPS (1 nM and 100 nM) for 72 hours. Cell proliferation was assessed through the viability assays MTT and CellTiter-Glo®. Wound healing assays were also used to evaluate the migration potential of the cell line. The expression of genes related to proliferation and migration was also determined. Similarly, adult mice were exposed to BPS at a dose of 30 mg/kg body weight/day for 21 days, after which, the uterus was sent for histopathologic assessment.

Results. BPS increased cell number and stimulated migration in Ishikawa cells, in association with the upregulation of estrogen receptor beta *(ESR2)* and vimentin *(VIM)*. In addition, mice exposed to BPS showed a significantly higher mean number of endometrial glands within the endometrium.

Conclusion. Overall, *in vitro* and *in vivo* results obtained in this study showed that BPS could significantly promote endometrial epithelial cell proliferation and migration, a phenotype also observed with BPA exposure. Hence, the use of BPS in BPA-free products must be reassessed, as it may pose adverse reproductive health effects to humans.

Key words: BPS, endocrine-disrupting chemicals, Ishikawa cells, uterus, hyperplasia

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are examples of environmental contaminants that alter the normal function of hormones in the body.[1] Their ability to disrupt the synthesis, secretion, transport, and binding of hormones essential in maintaining homeostasis, and in reproductive and developmental processes can lead to adverse health effects in intact organisms and their offspring.[1,2] One of the most common EDCs is bisphenol A (BPA), a chemical that is extensively used in manufacturing plastic consumer products.[3] BPA causes deleterious effects on human health by mimicking the action of estrogen and disrupting the normal endocrine pathways.[3–8] For instance, exposure to environmentally relevant doses of BPA increases susceptibility to prostate cancer[6] and promotes estrogen-related diseases, such as breast cancer and endometriosis, as observed in animal models.[9–11]

Rising concerns about the negative impact of BPA on human health have led to restrictions regarding its production. This has also resulted in the search for safer alternatives to this chemical. For example, bisphenol S (BPS) has been used as a common chemical substitute for BPA and is found in many consumer products labeled as "BPA-free." BPS was originally thought to be a safer alternative to BPA, but studies now show that BPS also exhibits endocrine disrupting activities similar to that of BPA. Indeed, subcutaneous injection of BPS in rat neonates delayed their onset of puberty and altered their estrous cycle.[12] BPS is obesogenic, cardiotoxic, neurotoxic and immunotoxic,[13–16] but its impact on cell proliferation and migration in adult endometrial cells has not yet been examined. Thus, this study determined the proliferative and migratory effects of BPS on adult human endometrial epithelial cell lines and adult mouse uteri.

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METHODOLOGY

Chemicals

BPS (Supelco 43034) reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the *in vitro* model, stock solutions of BPS were dissolved in dimethyl sulfoxide (DMSO). For the *in vivo* model, BPS was diluted at the desired concentration using distilled water (vehicle). The solution was sonicated for 10 minutes at room temperature and was fed to mice through oral gavage.

Cell culture maintenance

Ishikawa (ECACC 99040201) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and maintained in a growth medium containing Minimum Essential Medium (MEM) with 2 mM glutamine (Gibco™ 11095080), 5% fetal bovine serum (FBS) (Gibco™ 10500064), 1% non-essential amino acid (NEAA) (Gibco™ 11140050), and 1 μg/ml of gentamicin (Gibco™ 15750060). Cells were kept in a 25 cm^2 tissue culture flask at 37°C with 5% CO_2. Steroid-depleted medium containing phenol-free DMEM/F12 (Gibco 21041025) with or without charcoal-stripped FBS (CS-FBS) was used for all assays involving BPS.

Cell viability assay

Cell viability was assessed through MTT and CellTiter-Glo® assays. For the MTT assay, cells were seeded at 10,000 cells/well in 96-well plates (Corning 3596) and incubated in 100 μl steroid-depleted medium containing 2% CS-FBS at 37°C under 5% CO_2 for 48 hours. Cells were then treated with BPS (1 nM or 100 nM) or vehicle control (DMSO) in a fresh steroid-depleted medium with 0% or 2% CS-FBS for 72 hours. The spent medium was aspirated and processed for MTT assay as previously described.[17] For the CellTiter-Glo® assay, cells were also seeded and treated as above and processed following the manufacturer's instructions. Plates were read using a luminometer to obtain the relative luminescence units (RLU). Relative viability was calculated by normalizing the RLUs with the vehicle control. Experiments were performed in quadruplicates per treatment.

Wound healing assay

Cell migration was evaluated by wound healing assay. Cells were seeded at 300,000 cells/well into 6-well plates containing a growth medium. After 24 hours, wells were replaced with a steroid-depleted medium supplemented with 2% CS-FBS, and cells were allowed to grow to 80% confluency. The cell monolayer was carefully scraped using a 200 μl pipette tip to form a cell-free area. The wounded monolayer was washed thrice with the same medium to remove cell debris. Before treatment, cells were exposed to 10 μg/ml mitomycin C (MMC) for 2 hours to ensure that wound closure was due to migration and not proliferation. After exposure to MMC, cells were washed twice with 1x phosphate-buffered saline (PBS) and incubated with a freshly prepared treatment medium containing 1 or 100 nM BPS for 72 hours. Photomicrographs of cells were captured using an inverted microscope (Nikon ECLIPSE Ts2-FL). Denuded areas at 0 and 72 hours post-induction of injury were measured using the Image J Software (version 1.50i, National Institute of Health, Bethesda, MD, USA). Percent closure was determined by subtracting the final gap area from the initial gap area and dividing the difference with the initial gap area, multiplied by one hundred. Experimental results were shown as mean ± SEM percent closure. A wound healing assay was performed thrice in triplicates per trial.

Gene expression

Cells were seeded at 250,000 cells/well on a 6-well plate using a steroid-depleted medium containing 2% CS-FBS and were incubated for 48 hours. Cells were then treated with BPS (1 nM, 100 nM) or vehicle control for 72 hours with the same steroid-depleted medium. RNA was extracted from each treatment group using the phenol-chloroform extraction protocol with TRIzol reagent (Invitrogen 15596026). Complementary DNA (cDNA) synthesis from a total of 1000 ng of RNA was performed using the SensiFAST cDNA synthesis kit (BIO-65053) with a PCR condition of 25°C for 10 minutes, 42°C for 15 minutes, 48°C for 15 minutes, and 85°C for 5 minutes. Quantitative PCR (qPCR) was done using the SensiFAST SYBR Hi-ROX kit (BIO-92020) containing 400 nM forward and reverse primers and 2 μL of cDNA in RNAse/DNAse-free water. Samples were run in a StepOne Plus qPCR machine with the following PCR conditions: 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and 60°C for 15 seconds, and a melt curve step of 95°C for 15 seconds and 60°C for 1 minute. Primers for genes involved in estrogen signaling (*ESR1, ESR2*), cell proliferation (*CCND1, CCNB1, GREB1A, CMYC*), and epithelial-mesenchymal transition (EMT) (*CDH1, CDH2, ERBB2, VIM*) were used (Table 1). The housekeeping gene, beta-actin (*ACTB*), was used for normalization.

Animals

All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals, following the protocols approved by the Institutional Animal Care and Use Committee, UP Diliman. Inbred wild-type C57BL/6J mice were used in this study to ensure that all mice will have a similar genetic background across treatment groups. Mice were housed in intact polysulphonate cages and maintained in a facility with a 12-hr light/dark cycle, a temperature of 21 ± 1°C, and relative humidity of 60%. A phytoestrogen-free diet (1814P; Altromin, Germany) and ultra-pure water (in glass bottles changed twice per week) were provided *ad libitum*.

Mouse treatment

The sample size for the mouse experiment was determined based on a previous study of BPA, which showed a statis-

Table 1. Primer sequences for qPCR

Gene name	Forward (5'-3')	Reverse (5'-3')
ESR1	CGACTATATGTGTCCAGCCAC	CCTCTTCGGTCTTTTCGTATCC
ESR2	CAGGTCTGGGGTTGGAGAAT	AGTAACTCAAGGGGCCAGTC
CCNB1	CATGGTGCACTTTCCTCCTT	AGGTAATGTTGTAGAGTTGGTGTCC
CCND1	GCTGTGCATCTACACCGACA	TTGAGCTTGTTCACCAGGAG
GREB1A (Greb1 Isoform a)	CTGAAGCTAGACACGGAGGC	AGAGGTTATGAACAGTGCTACTCAC
MYC (c-Myc)	GTCCTCGGATTCTCTGCTCTC	CCAGACTCTGACCTTTTGCCA
CDH1	GTAACGACGTTGCACCAACC	GGGTCAGTATCAGCCGCTTT
CDH2	GCGTCTGTAGAGGCTTCTGG	GCCACTTGCCACTTTTCCTG
ERBB2	TTTGCTGTCCTGTTCACCAC	TCATCCTCATCATCTTCACATTG
VIM	CAGATGCGTGAAATGGAAGA	TGGAAGAGGCAGAGAAATCC
ACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

tically significant increase of approximately 100% in the average number of endometrial glands after BPA exposure at a sample size of four per treatment.[18] From their study, mice exposed to BPA had a mean endometrial glands/stroma ratio of 8 mm^2 and a standard deviation of 2. A minimum sample size of 5 mice per treatment group was needed to achieve 90% power using the sample size formula for endometrial gland proliferation. Factors used to compute the sample size were as follows: 1) significance level of 5% and 2) assumed effect size of 58.7%.[19] In this study, 7-month-old C57BL/6J female mice were randomly assigned to two experimental groups with five animals per group treated with either BPS (30 mg/kg body weight per day) or vehicle (distilled water). BPS was administered daily for 21 days through oral gavage. During the 21-day treatment, mice were subjected to daily vaginal smears to monitor the estrous cycle. Mice were sacrificed at the estrus stage following the 21-day dosing periods. Mouse uteri were then collected and fixed in 10% neutral buffered formalin (NBF) for histopathological analysis.

Histopathological Analysis

Formalin fixed-left uteri were sent to the Pathology Division of Providence Hospital Quezon City for histological processing. Uteri from mice (n=5 per group) were assessed based on the mean number of endometrial glands and thickness of the uterine walls per field of view at the end of the 21-day BPS or vehicle treatment. Each uterine section was cut into ten slices at 5 μm thickness and was divided into ten fields of view with a number assigned to each field of view. Each field of view has an approximate area of 2 x 2 mm under a 10x objective. Five fields of view per slice were randomly selected using a random number generator before observation and quantification of endometrial glands. The thickness of the uterine walls (endometrium, myometrium, perimetrium) was measured in the same field of view where endometrial glands were counted. Uterine walls were also examined for the presence of edema, hyperplasia, hypertrophy and other histopathological changes.

Statistical Analysis

Quantitative data collected were described using the mean ± SEM. The comparison of two mouse groups was performed using unpaired student's t-test, while the comparison of three groups of Ishikawa cells used one-way analysis of variance (ANOVA). Two-way ANOVA was used to determine the effects of serum concentration and BPS treatment on cell viability. Tukey's test was used as a post hoc comparisons test. Fisher's exact test was used to determine the association between BPS treatment and the occurrence of uterine lesions. A 5% level of significance was used for the hypothesis testing conducted. All statistical analyses were performed using GraphPad Prism version 6.0.

RESULTS

BPS increased cell number and migration in human endometrial Ishikawa cells

Previous studies have shown that BPA promoted endometrial cell proliferation.[4,20] This study tested whether the BPA analog BPS can increase the total number of cells in human endometrial epithelial cells, such as Ishikawa cells. In human sera, bisphenol concentrations were previously detected in the picomolar to nanomolar ranges.[21–25] This study used 1 and 100 nM BPS to reflect the physiologically relevant concentration of bisphenols found in human sera. At these concentrations, BPS significantly enhanced the total number of Ishikawa cells in the absence of serum using either the metabolism-based MTT assay (Figure 1A, Appendix 1A) or the ATP-based CellTiter Glo® assay (Figure 1B, Appendix 1B). A 3- to 8-fold increase in cell proliferation was observed after BPS treatment using the MTT assay ($p<0.005$), while a slightly lower but significant increase in fold change was seen in the CTG assay ($P<0.029$). However, while adding 2% serum significantly increased the total number of viable endometrial epithelial cells, this was not enhanced further with BPS treatment ($p>0.999$) (Figure 1A-B, Appendix 1A-B). The proliferative effects of serum appear to blunt the ability of BPS to increase the total cell count of endometrial epithelial cells. It is interesting that the ability of BPS to increase the viability of endometrial Ishikawa cells was comparable to BPA both in 0% and 2% FBS, as indicated by a similar fold change using MTT and CTG assays (Figure 1A-D, Appendix 1A-D).

The wound healing assay examined the effect of BPS on *in vitro* migration in human endometrial Ishikawa cells. Before treatment, cells were exposed to 10 μg/ml MMC to ensure that gap closure was due to migration and not

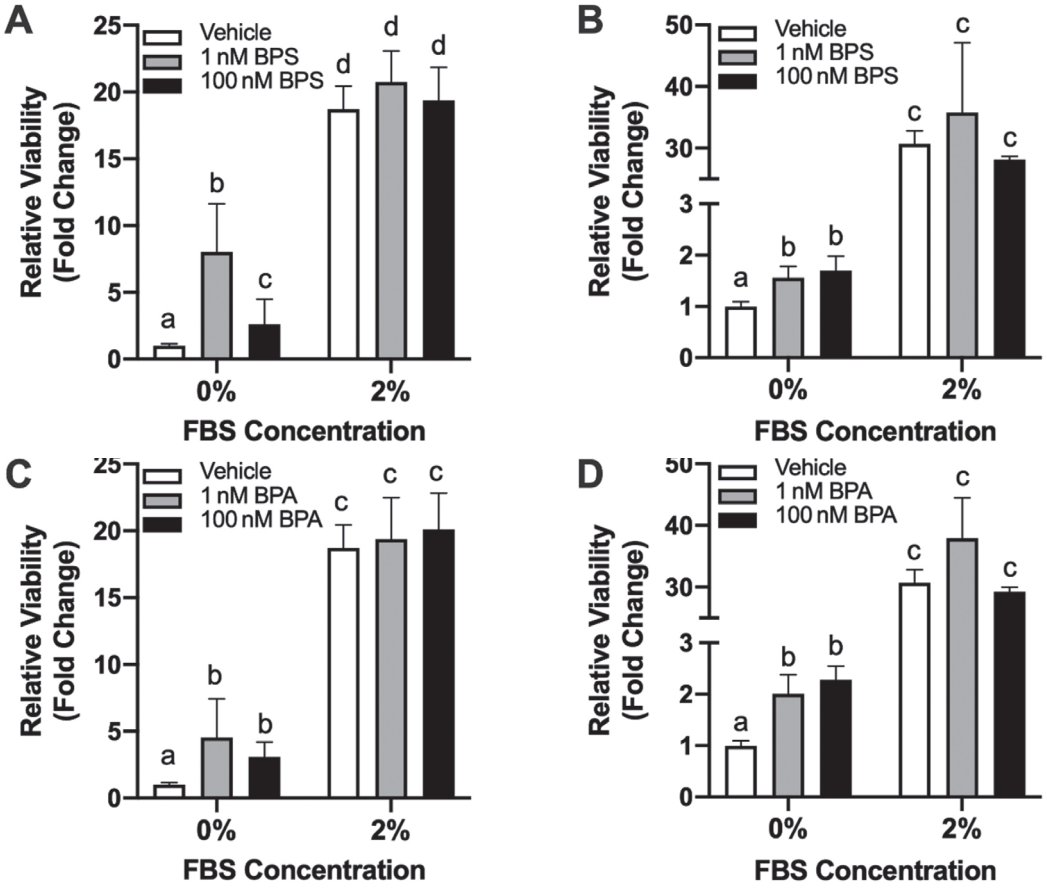

Figure 1. ***BPS increases the mean relative viability of human endometrial Ishikawa cells at low but not at high serum.*** Ishikawa cells were cultured in steroid-free media and then exposed to 1 nM and 100 nM **(A, B)** BPS and **(C, D)** BPA with 0% or 2% CS-FBS for 72 hrs before assessment of proliferation using **(A, C)** MTT and **(B, D)** CellTiter-Glo® assay. Numerical data were analyzed as mean relative viability ± SEM (n=3 and n=6 per treatment, respectively). Different letters denote significant differences at $p<0.05$ using two-way ANOVA followed by Tukey's post hoc test.

proliferation. Results showed that low dose (1 nM) but not high dose (100 nM) of BPS significantly increased the migration of human endometrial Ishikawa cells (Figure 2). A gap closure of 40% was noted in 1 nM BPS as compared to 15% gap closure in the vehicle and 19% gap closure in the 100 nM BPS groups (Figure 2B). Similarly, only 1 nM of BPA was able to significantly enhance the migration of endometrial Ishikawa cells albeit with values that are slightly lower compared to BPS. These data suggest that BPS at 1 nM concentration can promote migration of endometrial Ishikawa cells similar to BPA.

BPS significantly upregulated the expression of *ESR2* and *VIM* genes

BPS is an endocrine-disrupting chemical that is known to mimic estrogen.[12] We therefore measured whether BPS could influence the expression of estrogen receptors, *ESR1* and *ESR2*. We found that BPS increased the expression of *ESR2* but not *ESR1* (Figure 3A). We then examined the effects of BPS on the expression of genes involved in cell proliferation and migration. BPS affected the expression of proliferation -related genes (Figure 3A), but only low-dose BPS significantly increased the expression of *VIM* (Figure 3B), suggesting that BPS may induce a mesenchymal-like transition in these cells, consistent with their increased migratory phenotype at this dose. Although no gene expression analysis was carried out in cells exposed to BPA, several studies have already demonstrated that BPA can enhance the expression of genes involved in proliferation and migration.[26–28]

BPS increased the mean number of endometrial glands in the mouse uterus

To confirm whether the results in cell culture will also manifest *in vivo*, mice were fed orally with 30 mg/kg body weight per day BPS for 21 days, as this concentration is lower than the no adverse effect limit (NOAEL) (60 mg/kg body weight per day) set by the European Chemical Agency (ECHA) for BPS[29] and is the concentration of BPA which rendered significant histological effects in tissues of mice and other vertebrate species.[11,30]

We then performed a microscopic examination of the uterus from exposed and unexposed mice. Mouse uteri from the control group exhibited benign proliferative endometrium characterized by the presence of small, tubular glands

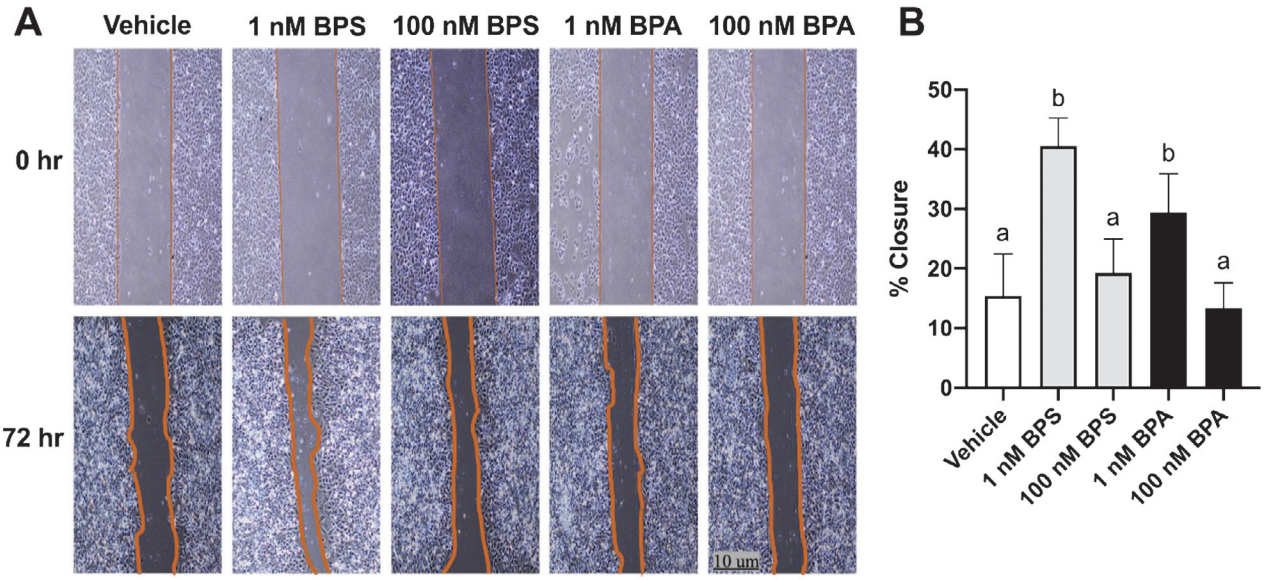

Figure 2. ***A low-dose of BPS promotes migration in human endometrial Ishikawa cells.*** **(A)** Representative photomicrographs of Ishikawa cells exposed to vehicle, 1 nM, or 100 nM BPS or BPA in steroid-depleted medium supplemented with 2% FBS taken at 0 hr and 72 hrs post induction of gap. Cells were exposed to 10 µg/mL MMC before treatment with BPS. **(B)** Percent (%) gap closure of Ishikawa cells after 72 hr exposure to BPS or vehicle. Data were analyzed as mean % closure ± SEM (n=9). Different letters denote significant differences at $p<0.05$ using one-way ANOVA followed by Tukey's post hoc test.

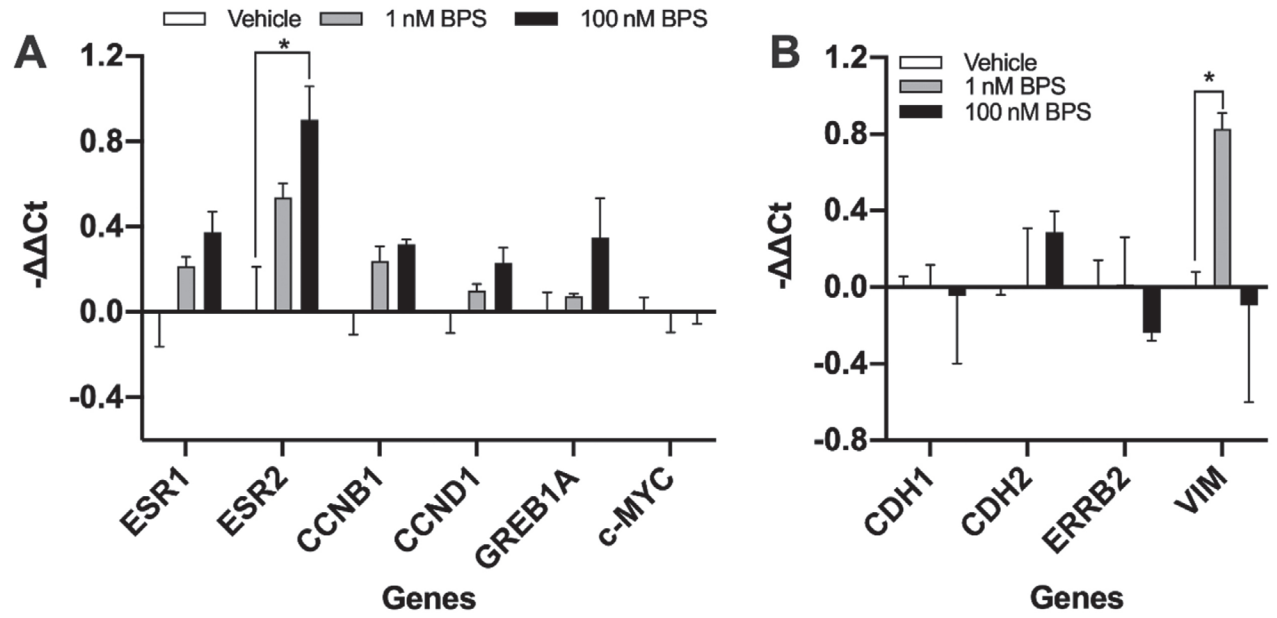

Figure 3. ***BPS significantly upregulates the expression of ESR2 and VIM genes.*** Relative $\log_2$ mRNA expression (-ΔΔCt) of **(A)** ESR and cell proliferation-related genes and **(B)** migration-associated genes in Ishikawa cells treated with BPS (1 nM, 100 nM) or vehicle (DMSO) for 72 hrs using qRT-PCR. All data were expressed as mean ± SEM (n=3 per treatment). Asterisks denote significant differences at $p<0.05$ relative to vehicle control using Kruskal–Wallis one-way ANOVA with Tukey post hoc test.

which were regularly spaced in the abundant stroma (Figure 4A). On the other hand, the uterus of mice exposed to BPS developed non-atypical hyperplasia, which is characterized by the presence of hyperproliferative, irregularly-shaped glands (Figure 4A). In addition, the mean number of glands per field of view in the BPS-treated group was 55 ± 0.42 which was significantly higher than the vehicle-treated mice which has a mean value of 22 ± 0.25 glands per field of view (Figure 4B). The incidence of non-atypical hyperplasia was also more evident in 80% of mice exposed to BPS than in the vehicle, as supported by a significant p-value of less than 0.05 using Fisher's exact test (Table 2). Hence, this provides further evidence for the proliferative effect of BPS on endometrial epithelial cells.

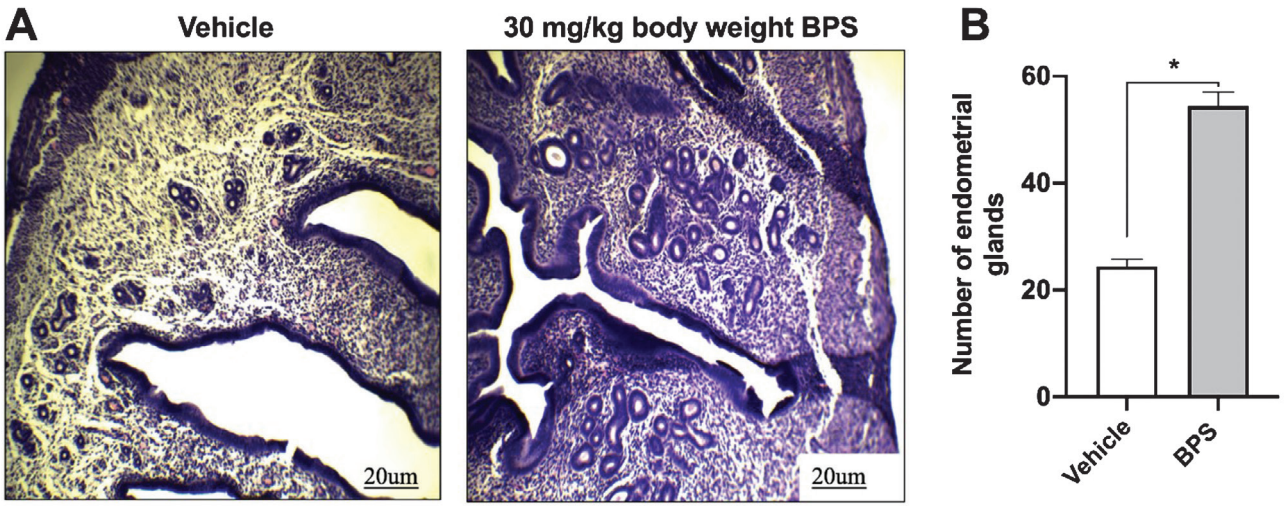

Figure 4. ***BPS increases the number of endometrial glands in the mouse uterus.*** **(A)** Representative photomicrographs of H&E-stained uteri from mice exposed orally to 30 mg/kg body weight BPS or vehicle for 21 days. **(B)** Quantification of the number of endometrial glands. Numerical data were analyzed as the mean number of glands ± SEM (n=5). Differences in letters denote significance at p<0.05 using Student's t-test.

Table 2. Two-sided Fisher's exact test for BPS and endometrial hyperplasia

	With endometrial hyperplasia	Without endometrial hyperplasia	Total
Vehicle	0	5	5
BPS	4	1	5
Total	4	6	10
p-value <0.05			

Table 3. Two-sided Fisher's exact test for BPS and adenomyosis-like lesion

	With Adenomyosis	Without Adenomyosis	Total
Vehicle	0	5	5
BPS	2	3	5
Total	2	8	10
p-value = 0.17			

BPS stimulated the migration of endometrial glands into the myometrium

Aside from the proliferation of endometrial glands, the migration and invasion of endometrial mucosal cells and stroma into the mouse myometrium were also apparent in two out of five mice exposed to BPS (Figure 5A), but not in any of the mice in the vehicle group, although the difference was not statistically significant (Table 3). These changes were reminiscent of adenomyosis, a gynecologic disorder wherein normal-appearing endometrial mucosa is found within the myometrium of the uterus. Adenomyosis was observed in 40% of mice exposed to BPS. Microscopic features of adenomyosis seen in mice exposed to BPS include the presence of endometrial glands and stroma within a hypertrophic smooth muscle (Figure 5A-B). The presence of histiocytes and neutrophils in the ectopic endometrial tissues was also observed (Figure 5C). In addition, uterine wall thickness of exposed and unexposed mice was measured to determine the presence of myometrial hypertrophy. Results showed a significant increase in the myometrial thickness of mice exposed to BPS relative to the vehicle group (Figure 5D). An increase in myometrial thickness was associated with adenomyosis and leiomyoma in mice exposed to BPA.[31] In humans, myometrial thickening is often brought about by myometrial hyperplasia and hypertrophy which may indicate the presence of uterine lesions such as adenomyosis.[32]

DISCUSSION

Studies on BPA have demonstrated that this chemical can increase the proliferation and migration of malignant cells, such as in colon and endometrial cancer.[3,4] However, the role of BPS on cell proliferation and migration in endometrial epithelial cells has not yet been explored. This study has revealed that BPS increased the total number of viable cells and stimulated migration in endometrial epithelial cells both *in vitro* and *in vivo*, specifically in the Ishikawa cell line.

This increase in cell number is likely associated with cell proliferation, as BPA, an analog of BPS, also induced cell proliferation in endometrial cancer cells.[20] Surprisingly, the proliferative effect of BPS on endometrial cells occurred in the absence but not in the presence of serum. The ability of serum to blunt BPS-induced proliferation is likely due to its stronger growth-stimulatory effect than BPS alone. The serum contains several growth factors, nutrients, and other elements essential for cell growth and survival.[33]

The proliferative effects of BPS were also observed in mouse uteri and were evident in the endometrial glandular epithelium—the primary site of endometrial hyperplasia. This is similar to the disordered proliferation of endometrial epithelial glands without changes in cell morphology when exposed to estradiol.[34] In BPS-treated mice, the following observations were made: the presence of higher gland-to-stroma ratios in the endometrium, irregularly-shaped endometrial glands, and highly variable density of proliferative glands. Because the dose used

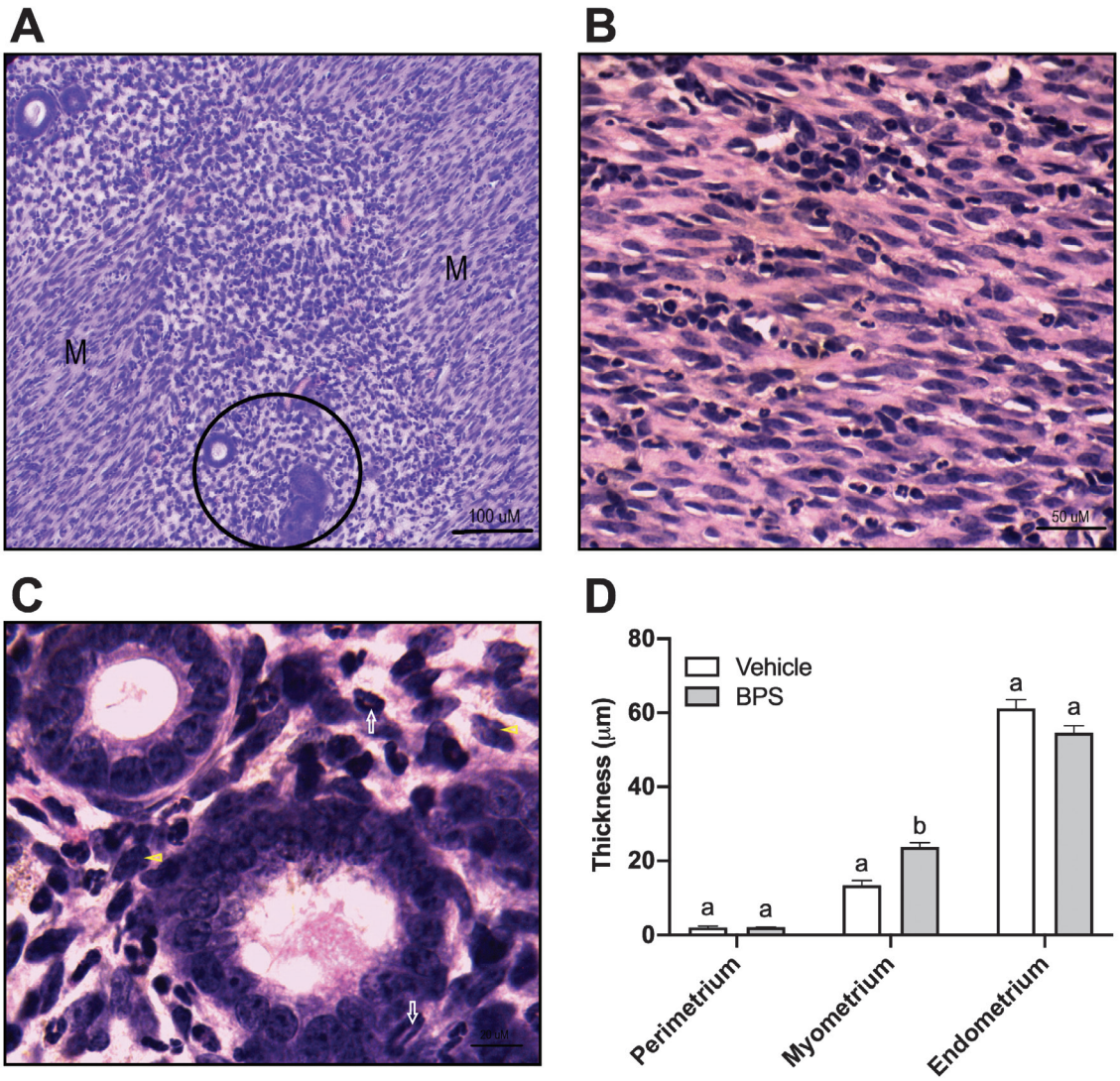

Figure 5. ***BPS promotes the migration of endometrial glands and stroma in the uterus of mice.*** **(A)** Representative photomicrographs of uteri from mice exposed orally to 30 mg/kg body weight per day BPS for 21 days showed the presence of endometrial mucosa and stroma (encircled) within the myometrium (M). **(B)** High power view of the myometrial layer showing hypertrophic smooth muscles. **(C)** High-power view of endometrial glands lined by normal-appearing epithelia within the myometrium. Histiocytes (yellow arrow) and neutrophils (white arrow) were seen in the ectopic endometrial tissues. **(D)** The thickness of uterine walls (i.e., perimetrium, myometrium, and endometrium) in mice treated with BPS or vehicle (n=5 per group). Numerical data were analyzed as the mean thickness of uterine walls ± SEM (n=5 per group). Differences in letters denote significance at $p<0.05$ using Student's t-test.

in this study is lower than the NOAEL set by ECHA for BPS[29], exposure to higher concentrations will likely lead to similar uterotrophic effects.

Previous studies showed that early neonatal exposure of rats to BPA and BPS caused long-term changes in reproductive and endocrine phenotypes, which manifested as delayed onset of the estrous cycle and dysregulated production of hormones.[12] In zebrafish, BPA also caused transgenerational aberrant expression of genes related to reproduction and gonadal differentiation up to the F2 and F3 generations.[35] In this study, we showed that even when exposure took place outside the critical window of development, BPS can still elicit immediate architectural changes in the adult mouse uterus.

Previous publications have shown that BPS has an estrogenic activity comparable to BPA.[36,37] Moreover, BPS promotes cell progression, proliferation, and migration through an ER-dependent pathway in MCF-7 breast cancer cells.[38,39] However, it remains unclear whether the ability of BPS to increase endometrial cell viability in this study is due to its estrogenic activity. Further studies are needed to determine the contribution of ER on BPS response in endometrial cells.

The expression of proliferative genes in Ishikawa cells was assessed to understand the molecular basis of the proliferative potential of BPS on endometrial epithelial cells. Although the expression of proliferative genes was slightly higher in BPS-treated cells, no significant difference was noted between the vehicle and BPS-treated

group. Surprisingly, only the expression of *ESR2* was significantly upregulated by 100 nM BPS. This is relatively new, as previous studies have shown the putative role of ESR2 as a tumor suppressor in endometrial cancer cell lines such as RL95/2 and HEC-1A cells.[40] However, elevated ESR2 has been implicated in other non-malignant proliferative diseases, such as endometriosis.[41–43] Hence, the mechanisms involved in the upregulation of ESR2 after BPS treatment are worth investigating in future studies.

Aside from proliferation, the impact of BPS on migration was also studied. *In vitro* migration of Ishikawa cells exposed to BPS followed a non-monotonous response. Compared to the 100 nM dose, 1 nM BPS significantly increased migration. Similar results were also noted in migration assays involving human non-small cell lung cancer cells (NSCLC), MCF-12A human mammary epithelial cells, and triple-negative breast cancer cells (TNBC).[3,44,45] The ability of 1 nM BPS to promote significant migration may be attributed to the activation of store-operated calcium^{2+} channels (SOCE) similar to the mechanism of action of BPA.[46,47] SOCE plays an important role in regulating cell movement at both the front and rear of migrating cells.[48] SOCE in cancers are dysregulated to promote cancer migration, invasion, and metastasis.[46] Interestingly, a recent study involving human prostate cancer (PCa) showed that only 1 nM and 10 nM BPA can modulate ion channel protein expression involved in calcium entry and cancer cell migration.[47]

Our study observed the presence of atypical endometrial mucosa within the myometrial layer of mice treated with BPS. This condition is similar to adenomyosis, which is associated with an enlarged uterus, heavy menstrual bleeding, pelvic pain and infertility in women.[49] The presence of endometrial epithelial cells and fibroblasts in the myometrium is often associated with hyperplasia of surrounding smooth muscles.[49,50] While the histology of adenomyosis is well-described in humans, the etiology of the disease remains unknown.[50] However, some studies suggest a potential relationship between environmental exposures to toxicants and the risk of adenomyosis.[50,51] Hence, it is tempting to speculate that the BPS-induced migration in endometrial epithelial cells that was observed in this study may also be observed in humans, implicating the role of BPS on the development of adenomyosis. It is important to note that the sample size used in this study was not large enough to show a statistically significant increase in the appearance of adenomyosis-like lesions after BPS versus vehicle treatment. Additional experiments are still needed to ascertain the contribution of BPS to the development of adenomyosis. Nonetheless, one study has already implicated the role of BPA in increasing the incidence of adenomyosis in mice[31], further supporting the idea that BPS-induced adenomyosis is also likely possible.

CONCLUSION

Our study demonstrated that BPS at 1 nM concentration can significantly promote cell proliferation and migration in endometrial Ishikawa cells and the phenotypic effect of BPS *in vitro* was confirmed *in vivo*. Similarly, BPS induced the proliferation of endometrial glands in the uterus and promoted the migration and invasion of endometrial mucosa in the myometrium of mice. Further studies are needed to determine the overall impact of BPS on the organism as a whole. Nonetheless, the results obtained in this study support other studies that BPS might not be an ideal substitute for BPA as it can elicit similar estrogenic effects as BPA.

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Statement of Authorship
All the authors certified fulfillment of ICMJE authorship criteria.

Authors Contribution Statement
KB, CMM, LA, and MV conceived the study. KB, CMM, MM, LA, and MV developed the methodology. KB, CMM, MM, EMR, LA, and MV validated the results and synthesized the data. KB, CMM, MM, and EMR conducted the research and investigation process and prepared the original draft of the manuscript. KB and MV provided the study materials. MV curated the data and acquired financial support for the project. LA and MV reviewed and edited the manuscript and supervised the research activity planning. CMM and MV managed the research activity planning and execution.

Author Disclosure
The authors declared no conflict of interest.

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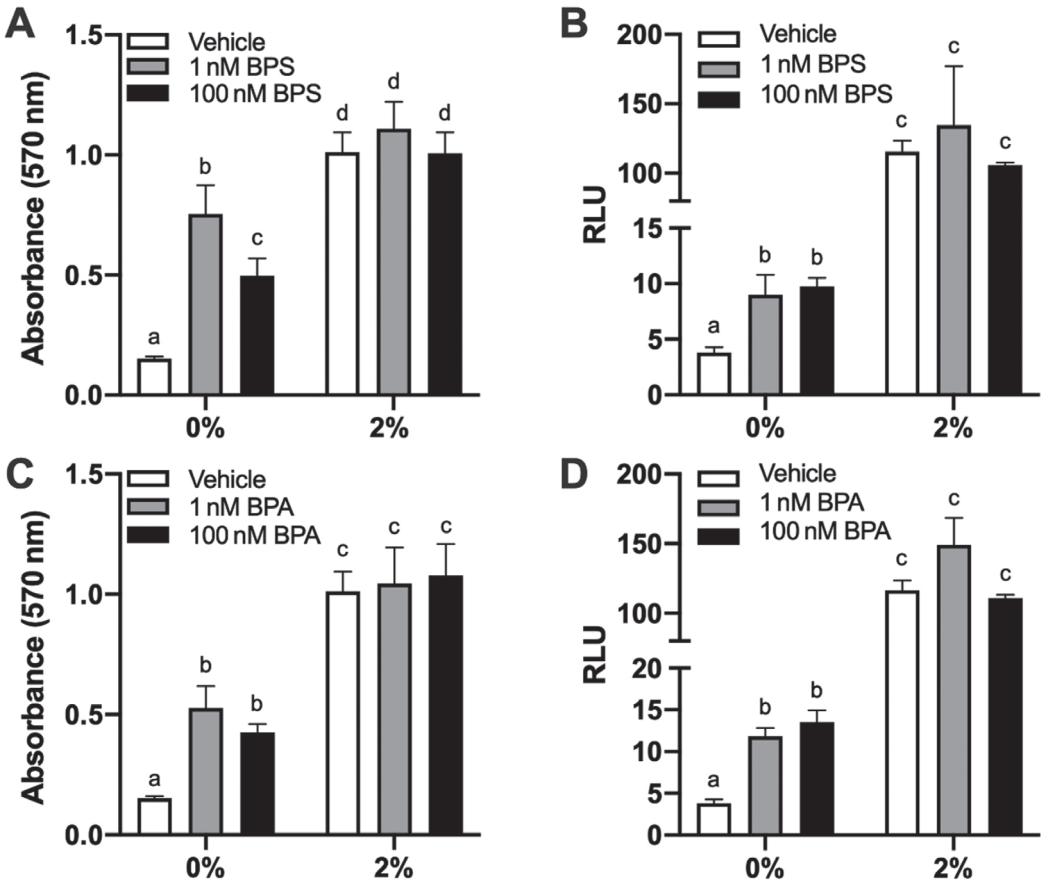
APPENDIX



Appendix 1. ***BPS increases absorbance and luminescence readings in low but not at high serum.*** Ishikawa cells were cultured in steroid-free media and then exposed to 1 nM and 100 nM **(A, B)** BPS and **(C, D)** BPA with 0% or 2% CS-FBS for 72 hrs before assessment of viability using **(A, C)** MTT and **(B, D)** CellTiter-Glo® assay. Numerical data were analyzed as mean absorbance ± SEM (n=3 and n=6 per treatment, respectively). Different letters denote significant differences at $p<0.05$ using two-way ANOVA followed by Tukey's post hoc test.