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Bisphenol Analogs Downregulate the Self-Renewal Potential of Spermatogonial Stem Cells

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Purpose: In this study, we investigated the effect of bisphenol-A (BPA) and its major analogs, bisphenol-F (BPF), and bisphenol-S (BPS), on spermatogonial stem cells (SSCs) populations using *in vitro* SSC culture and *in vivo* transplantation models.

Materials and Methods: SSCs enriched from 6- to 8-day-old C57BL/6-eGFP^{+} male mice testes were treated with varying concentrations of bisphenols for 7 days to examine bisphenol-derived cytotoxicity and changes in SSC characteristics. We utilized flow cytometry, immunocytochemistry, real-time quantitative reverse transcription-PCR, and western blot analysis. The functional alteration of SSCs was further investigated by examining donor SSC-derived spermatogenesis evaluation through *in vivo* transplantation and subsequent testis analysis.

Results: BPF exhibited a similar inhibitory effect on SSCs as BPA, demonstrating a significant decrease in SSC survival, inhibition of proliferation, and induction of apoptosis. On the other hand, while BPS was comparatively weaker than BPA and BPF, it still showed significant SSC cytotoxicity. Importantly, SSCs exposed to BPA, BPF, and BPS exhibited a significant reduction in donor SSC-derived germ cell colonies per total number of cultured cells, indicating that, like BPA, BPF, and BPS can induce a comparable reduction in functional SSCs in the recipient animals. However, the progress of spermatogenesis, as evidenced by histochemistry and the expressions of PCNA and SSC specific markers, collectively indicates that BPA, BPF, and BPS may not adversely affect the spermatogenesis.

Conclusions: Our findings indicate that the major BPA substitutes, BPF and BPS, have significant cytotoxic effects on SSCs, similar to BPA. These effects may lead to a reduction in the functional self-renewal stem cell population and potential impacts on male fertility.

Keywords: Bisphenol A; Bisphenol F; Bisphenol S; Male fertility; Self-renewal; Spermatogonial stem cells

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INTRODUCTION

Spermatogonial stem cells (SSCs) are the only male germ cells with the potential for self-renewal and differentiation. Similar to other tissue-specific stem cells, SSCs are very rare, comprising only 0.03% of mouse testes [1]. The biological activities of SSCs, including their ability to regulate self-renewing divisions and differentiating divisions, are essential for spermatogenesis and male fertility throughout a lifetime [2]. Of

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note, in SSC biology, the development of spermatogonial transplantation has allowed the study of SSC as an unequivocal functional assay of stem cells, enabling the quantification of SSCs in a cell population transplanted into a recipient testis [3].

Bisphenols are chemical compounds used in various plastic product manufacturing. Bisphenol-A (BPA) is the most common representative of bisphenols and is known as an endocrine disruptor. BPA's adverse health effects include developmental, reproductive, immune, metabolic, respiratory, and neural systems [4]. BPA-induced reduction in reproductive hormones may disrupt meiosis and trigger apoptosis, impairing spermatogenesis [5]. BPA has been reported to induce apoptosis in SSCs [6] and spermatogonia [7]. Additional damages attributed to BPA include diminished semen quality and DNA damage in spermatozoa [8], as well as the induction of oxidative stress and apoptosis in the testis of male offspring [9].

Because of health concerns and imposed restrictions on consumer products containing BPA [10], other bisphenol analogs, such as bisphenol-F (BPF) and bisphenol-S (BPS), have been used as substitutes for BPA [11]. However, according to National Toxicology Program report, their structural similarity can lead to similar endocrine-disrupting effects [12]. Furthermore, recent studies on BPA, BPF, and BPS have demonstrated similar endocrine-disrupting effects [13]. Therefore, this study aims to investigate the effects of BPF and BPS in comparison to BPA on the self-renewal potential alterations of SSCs and spermatogenesis in the testes.

MATERIALS AND METHODS

1. Ethics statement

The procedures and the care of animals were approved by the Institutional Animal Care and Use Committee (IACUC) in Chung-Ang University (IACUC Number: 201800105).

2. Germline stem cell establishment

Germline stem cells were isolated from 6- to 8-day-old C57BL/6-TG-EGFP mice (Jackson Laboratory) as previously described [14]. SSCs were maintained in mouse serum-free medium (mSFM) supplemented with glial cell line-derived neurotrophic factor (GDNF) (10 ng/mL, 212-GD-50; R&D Systems), GDNF family receptor α1 (GFRα1) (75 ng/mL, 560-GR-100; R&D Systems), and basic fibroblast growth factor (1 ng/mL, 354060; BD Biosciences), as previously reported [15]. Established SSCs were cryopreserved and stored at -80 ℃ and used for this study.

3. Bisphenol preparation and treatment

All reagents were purchased from Sigma-Aldrich unless otherwise specified. Bisphenols (BPA, Sigma-239658; BPS, Sigma-43034; and BPF, Sigma-51453) were dissolved in dimethyl sulfoxide (DMSO). Through serial dilution, working stocks were prepared to store at room temperature (RT) and used for the duration of a single 7-day experiment. An equal amount of DMSO (v/v) was used as DMSO control. Since the previously reported doubling time for mouse SSCs was 5.6 days [15], and the most common general SSC culture duration was 7 days [16], a bisphenol treatment of 7 days was determined.

4. Survival and proliferation assay

Trypan blue exclusion assay was conducted as previously reported [17]. Unstained (viable) and stained (nonviable) cells were visualized under a microscope (T1-SM; Nikon) and counted (n=5). The survival rate was calculated as follows:

$$\text{Survival rate (\%)}=\frac{\text{The number of live cells}}{\text{the number of live cells+the number of dead cells}}\times 100$$

The proliferation rate was also calculated as follows:

$$\text{Proliferation rate (\%)}=\frac{\text{The number of harvested bisphenol-treated cells}}{\text{The number of seeded cells}}\times 100$$

5. Western blot

SSCs total proteins were lysed in radioimmunoprecipitation assay buffer with a protease inhibitor cocktail for 30 minutes at 4 ℃. After centrifugation at 18,000×*g* for 20 minutes at 4 ℃, soluble proteins from the supernatant were quantified using BCA protein assay (Thermo Fisher Scientific). Five micrograms of proteins were loaded onto a 15% SDS-polyacrylamide gel with a protein marker (PJM-0605N; T&I). Separated proteins were transferred onto a methanol-activated polyvinylidene difluoride membrane (Millipore). After blocking with 5% w/v skim milk in 1X phosphate-buffered saline (PBS)-T for 1 hour at RT, primary antibody staining was performed overnight at 4 ℃. Secondary antibody staining was performed for 1 hour at RT. Proteins were detected by electrochemiluminescence (Clarity™ Western ECL Substrate; Bio-Rad). Antibod-

ies used include anti-proliferating cell nuclear antigen (PCNA) (1:1,000, ab18197; Abcam), anti-α-tubulin (1:1,000, ab7291; Abcam), anti-rabbit IgG (1:10,000, 7074S; CST), and anti-mouse IgG (1:10,000, 7076S; CST). Image analysis was conducted using ImageJ software (version 1.8.0) from National Institutes of Health to quantify protein intensity.

6. Flow cytometry

After a 7-day bisphenol treatment, flow cytometry was performed according to the manufacturer's instructions. Briefly, SSCs were washed twice in cold PBS, and 1.0×10^6 cells/mL were resuspended in 1× binding buffer (BD Biosciences). The aliquoted 100 μL cell suspensions were incubated with 5 μL Annexin V-APC (BD Biosciences) and propidium iodide (PI) for 15 minutes at RT in the dark for the apoptosis analysis. Subsequently, 400 μL of 1x binding buffer was added. A FACSAria II cells sorter (BD Biosciences) equipped with BD CellsQuest™ Pro software (Becton Dickinson, Oxford, UK) was used for flow cytometry analysis.

7. Histochemistry

To evaluate SSC-specific protein expressions, immunocytochemistry was performed as previously described [18]. Briefly, after bisphenol treatments, SSCs were fixed using 4% paraformaldehyde for 30 minutes at 37 ℃ followed by permeabilizations using 0.1% Triton X-100 in PBS (v/v) treatment for 10 minutes at RT. After blocking with 5% bovine serum albumin in PBS (w/v) for 1 hour, primary antibodies in 5% bovine serum albumin in PBS (w/v) were incubated at 4 ℃ overnight. The primary antibodies used were as follows: anti-GFRα1 (1:200, ab8026; Abcam), anti-promyelocytic leukemia zinc finger (PLZF) (1:200, NBP1-80894; Novus Biologicals), anti-DEAD-box polypeptide 4 (DDX4) (1:200, ab13840; Abcam), and anti-KIT, a receptor tyrosine kinase (c-Kit) (1:200, sc-365504; Santa Cruz Biotechnology). Secondary antibody incubation was performed in darkness. Briefly, cells were washed three times with PBS and incubated with secondary antibodies prepared in 5% bovine serum albumin in PBS (w/v) for 1 hour at RT. Alexa Fluor 568-conjugated anti-rabbit IgG (1:1,000, A11011; Invitrogen) and Alexa Fluor 568-conjugated anti-mouse IgG (1:1,000, A11004; Invitrogen) were used. The cellular nuclei staining with 4',6-diamidino-2-phenylindole (DAPI) was conducted using VectaShield® Mounting Medium (LS-J1032; LSBio). To analyze the marker expression of GFP^+ germ cells (%), a Y-TV55 microscope interfaced with the NIS Elements imaging software (Nikon) was used. The number of cells expressing markers and GFP expressing germ cells was counted and then presented as a ratio of marker-positive expression to GFP^+ germ cells. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) are described in detail in Supplement Data.

8. Real-time quantitative polymerase chain reaction (RT-qPCR)

For RNA extraction, SSCs were separated from Sandos inbred mouse-derived 6-thioguanine-and ouabain-resistant (STO) feeder cells after bisphenol treatments. Briefly, trypsinized SSCs and STO cells were resuspended in mSFM and incubated on 0.1% gelatin-coated culture plate at 37 ℃, 5% CO_2 incubator for 1 hour to separate SSCs from adherent STO cells [19]. TRIzol reagent (Thermo Fischer Scientific) and the PureLink™ RNA Mini Kit (Invitrogen) were used for total RNA extraction according to the manufacturer's instructions. cDNA was synthesized using TOPscript™ RT DryMIX (dT18 plus; Enzynomics). RT-qPCR was performed with 2X SYBR Green PCR Master Mix with primers shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a reference gene. A 7500 Real-Time PCR System (Applied Biosystems) with the following PCR conditions are used. Holding at 95 ℃ for 10 minutes, followed by a two-step cycling stage of 95 ℃ for 15 seconds and 60 ℃ for 1 minutes for 40 cycles, and a melting curve analysis. Quantification was performed using the $2^{(-\Delta\Delta CT)}$ method.

9. Transplantation of bisphenol-treated SSCs into C57BL/6 recipient mice

C57BL/6 wild-type male mice (Samtako Bio Korea) were used as the recipient mice for transplantation. All mice were maintained at a controlled temperature (22±2 ℃) and humidity (50%±10%) with a light cycle of 12-hour light/12 dark settings. Food and water were supplied ad libitum. Transplantation methods were conducted as previously described [3]. The recipient 6-week-old C57BL/6 mice were injected with 45 mg/kg body weight of busulfan six weeks before transplantation by intraperitoneal injection to exclude endogenous germ cells. For transplantation, 5.0×10^6 cells/mL bisphenol-treated SSCs were prepared in mSFM containing 10% (v/v) fetal bovine serum and 10% (v/v) DNase I. Ket-

Table 1. Primer sequence for real-time quantitative PCR

Gene		Forward (F)/ Reverse (R)	Sequence	GenBank accession
Bcl6b	B-cells CLL/lymphoma 6 member B protein	F (5′-3′)	TACTTCAAGGCTTCGCCTCTCT	NM_007528
		R (5′-3′)	CTACGTGTTCCATCTGCAAATAGG	
Bmi1	B cells-specific Moloney murine leukemia virus integration site 1	F (5′-3′)	TGTCCAGGTTCACAAAACCA	NM_007552
		R (5′-3′)	TTGAAAAGCCCTGGGACTAA	
Ddx4 (VASA)	DEAD-box helicase 4	F (5′-3′)	GCTTCATCAGATATTGGCGAGT	NM_010029
		R (5′-3′)	GCTTGGAAAACCCTCTGCTT	
Id4	DNA-binding protein inhibitor ID-4	F (5′-3′)	CAGTGCGATATGAACGACTGC	NM_031166
		R (5′-3′)	GACTTTCTTGTTGGGCGGGAT	
Gfrα1	GDNF family receptor alpha 1	F (5′-3′)	CACTCCTGGATTTGCTGATGT	NM_010279
		R (5′-3′)	AGTGTGCGGTACTTGGTGC	
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	F (5′-3′)	CTGACGTGCCGCCTGGAGAA	NM_001289726
		R (5′-3′)	CCCCGGCATCGAAGGTGGAA	
Kit	KIT proto-oncogene receptor tyrosine kinase	F (5′-3′)	GCCACGTCTCAGCCATCTG	NM_001122733
		R (5′-3′)	GTCGCCAGCTTCAACTATTAACT	
Lhx1	LIM homeobox 1	F (5′-3′)	CCCAGCTTTCCCGAATCCT	NM_008498
		R (5′-3′)	GCGGGACGTAAATAAATAAAATGG	
Pcna	Proliferating cell nuclear antigen	F (5′-3′)	TGTTTGAGGCACGCCTGATCC	NM_011045
		R (5′-3′)	GGAGACGTGAGACGAGTCCAT	
Zbtb16 (Plzf)	Zinc finger and BTB domain containing 16	F (5′-3′)	CTGGGACTT TGTGCGATGTG	NM_001033324
		R (5′-3′)	CGGTGGAAGAGGATCTCAAACA	

amine (75 mg/kg) and medetomidine (1 mg/kg) were used to anesthetize animals intraperitoneally. The GFP^+ donor cells were transplanted into the recipient mice's testes through the efferent ducts. After two months, the recipient testes were collected and decapsulated by removing the tunica albuginea to analyze stem cell colony reformation. Established colonies are reconstructed from the transplanted SSCs with intact stemness. The number of GFP^+ colonies (> 1 mm in length) was counted using a fluorescence microscope (AZ100, Nikon) with the NIS Elements imaging software (Nikon). The colonies per 10^5 transplanted cells were calculated as follows:

$$\text{Colonies per } 10^5 \text{ cells transplanted} = \frac{\text{The number of colonies}}{\text{The number of transplanted cells}}$$

The colonies per total number of cultured bisphenol-treated SSCs were calculated as follows:

$$\text{Colonies per total number of cultured bisphenol-treated SSCs} = \frac{\text{The number of colonies} \times \text{The total number of harvested cells}}{\text{The number of transplanted cells}}$$

10. Statistical analysis

Data are presented as mean±standard error of the mean. The number of replications is presented in the figure legends. Data were analyzed by one-way analysis of variance and multiple comparisons method by Tukey's honestly significant difference test or Dunnett's test. The value of $p<0.05$ was indicated as a statistically significant difference. GraphPad Prism version 8.0.1 (GraphPad Software Inc.) was used to present the data.

RESULTS

1. BPA, BPF, and BPS inhibit SSC proliferation

Mouse SSCs cultured *in vitro* form densely packed clumps of cells as shown in control in Fig. 1A. However, BPA-, BPF-, and BPS-exposed SSCs showed reduced cell clumps in a dose-dependent manner compared to DMSO control (Fig. 1A). The survival rates of BPA- and BPF-treated cells were significantly reduced to 68.7%±4.6% for BPA and 59.7%±7.0% for BPF at 100 μM concentration, respectively, with no significant difference observed in BPS-treated groups (Fig. 1B). Moreover, BPA, BPF, and BPS significantly reduced SSC proliferation (Fig. 1C). Compared to control, SSC proliferation was significantly reduced from 12.5 μM for BPA and BPF (285.0%±16.5% and 287.3%±21.5%, respectively) and from 25 μM for BPS (268.8%±21.9%, Fig. 1C), indicating that BPF can similarly prohibit SSC

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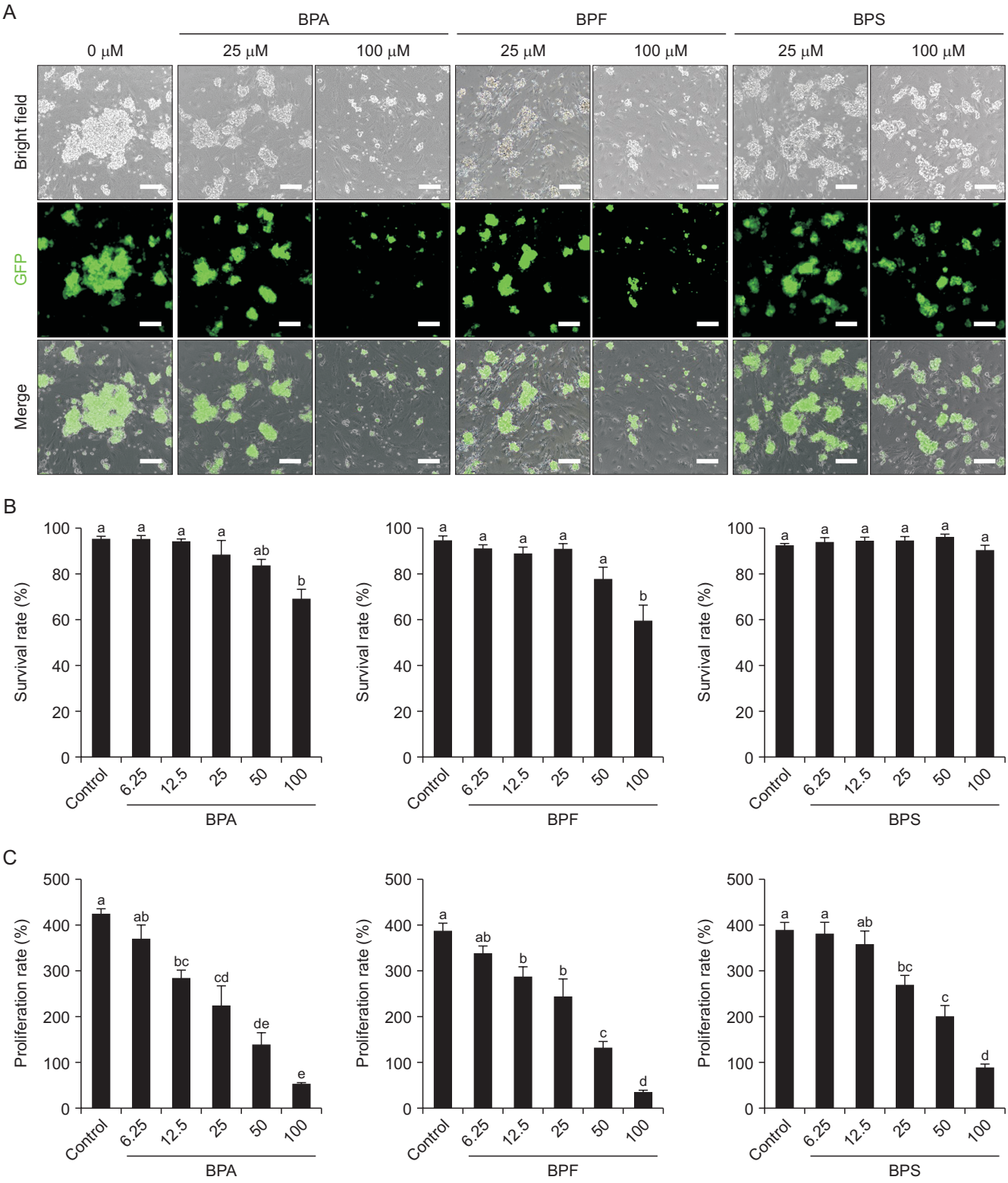


Fig. 1. BPA, BPF, and BPS are cytotoxic to spermatogonial stem cells (SSCs). Bright-field and dark-field fluorescence images of bisphenol-treated SSCs taken using a light and fluorescent microscope (10X magnification, TE2000-U; Nikon), and shown as indicated, scale bar=200 μm (A). Survival rate (B) and proliferation rate (C) of bisphenol-treated SSCs evaluated by trypan blue staining presented as indicated. Results are presented as mean±standard error of the mean of five independent experiments. The values were analyzed by one-way ANOVA and multiple comparisons by Tukey's honestly significant difference. The significant difference was indicated by different letters (a, b, c, d, and e) between treatments when $p<0.05$. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S.

survival and proliferation as BPA, whereas BPS is relatively less toxic but significantly inhibiting SSC proliferation. PCNA protein expressions, however, showed no significant change. However, BPA and BPS with a 100 μM concentration treatment induced significant increase of *Pcna* mRNA expression (Fig. 2).

2. BPF significantly induces SSC apoptosis as BPA

Next, flow cytometry analysis was conducted to analyze bisphenol-induced apoptosis in SSCs. Early apoptotic cells were determined as Annexin V-positive and PI-negative (APC Annexin V^+/PI^-), whereas late apoptotic and necrotic cells are determined as Annexin V/PI-double-positive (APC Annexin V^+/PI^+) [20]. The results showed that BPF and BPA significantly increased SSC apoptosis in a dose-dependent manner, while BPS showed no significant difference. At the highest concentration of 100 μM, BPA, BPF, and BPS induced 6.4%±0.9%, 7.8%±1.1%, and 4.1%±0.4% of early apoptosis, respectively (Fig. 3).

3. BPA, BPF, and BPS do not induce specific biomarker expression change

Undifferentiated spermatogonia specific GFRα1 (cellular membrane), PLZF (nucleus), DDX4/VASA (cytoplasm) for germ cells, and c-Kit (cellular membrane) for differentiated spermatogonia were investigated by immunocytochemistry (Fig. 4A, 4B and Supplement Fig. 1) and RT-qPCR (Supplement Fig. 2). Compared to control, all bisphenol-treated SSCs displayed comparable biomarker expressions (Fig. 4A, Supplement Fig. 1). The ratio of GFP^+ germ cells per marker-expressing cells showed no significant difference compared to the control for all tested groups, suggesting that BPA, BPF, and BPS do not affect specific biomarkers (Fig. 4B, Supplement Fig. 1). However, bisphenol treatments induced changes of mRNA levels of *Gfrα1, Plzf, Vasa,* and *c-Kit*. In specific, BPS 100 μM treatment induced significant upregulation of mRNA expressions of *GFRα1* and *Vasa*. BPA 25 μM treatment induced a more than 2-fold increase in *Plzf* and *c-Kit* mRNA expression; BPF showed a concentration-dependent increase of *c-Kit* mRNA expression. BPA, BPF, and BPS seem to positively influence the mRNA expressions of specific markers, but each bisphenol has a different impact on markers (Supplement Fig. 2). For other undifferentiated spermatogonia genes, namely *Id4*, *Bmi1*, *Bcl6b*, and *Lhx1* bisphenol treatment did not show significant changes (Fig. 4C).

4. BPA, BPF, and BPS inhibit SSC self-renewal without affecting spermatogenesis

To investigate the effect on the functional SSCs of bisphenols, transplantation was conducted in the busulfan-treated infertile recipient mice. Through spermatogenesis, transplanted GFP^+ SSCs regenerated the germ cell colonies via self-renewal and differentiation, as shown for the control in Fig. 5A. However, the number of GFP^+ colonies per 10^5 cells transplanted significantly decreased in BPA and BPF groups. The BPS-treated group showed no significant dose-dependent

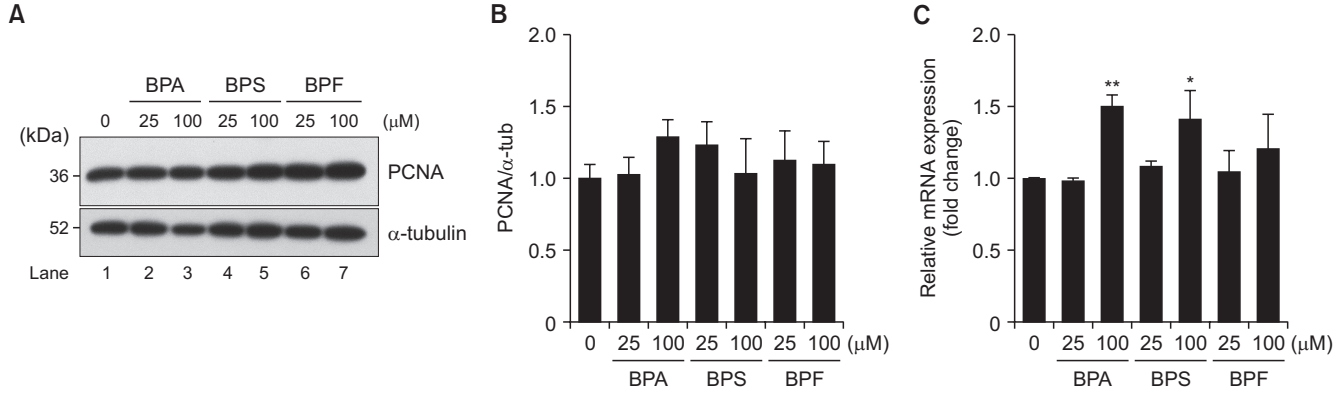


Fig. 2. BPA, BPF, and BPS do not alter PCNA expression in spermatogonial stem cells (SSCs). A representative western blot for PCNA protein levels with α-tubulin as a loading reference is presented (n=6). Control and bisphenol treatments are indicated (A). The graphical representation of (A) displays ratios of optical density of bands (PCNA/α-tubulin) as indicated (B). One-way ANOVA and multiple comparisons by Dunnett's test were conducted, revealing no significant differences between treatments. (C) Relative gene expression of *Pcna* is presented as mean±standard error of the mean. One-way ANOVA and multiple comparisons by Dunnett's test were conducted. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S. Significant difference was indicated with asterisks (*p<0.05, **p<0.01), n=3.

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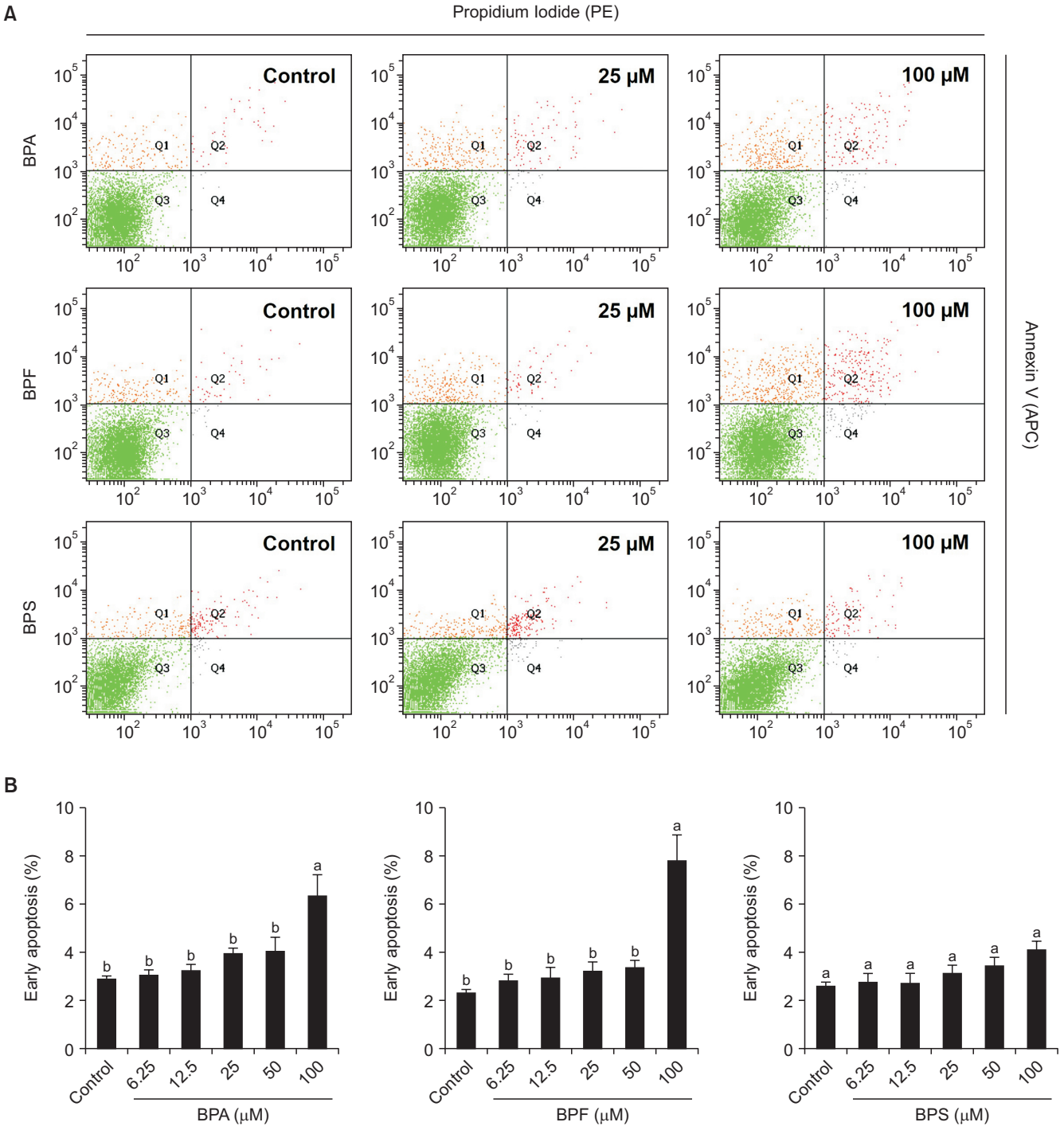


Fig. 3. BPA and BPF induce apoptosis of spermatogonial stem cells (SSCs). Flow cytometry images of APC-Annexin V and PI-stained GFP$^+$ cells as indicated (A). Early apoptosis rates of BPA-, BPF-, and BPS-treated SSCs are presented as mean±standard error of the mean of four independent experiments (B). One-way ANOVA and multiple comparisons by Tukey's honestly significant difference were conducted. The significant difference was indicated by different letters (a, b) between treatments when $p<0.05$. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S.

reduction in colony number per 10^5 cells transplanted. The respective colony numbers at 100 μM concentration treatments were 128.5±35.0 for BPA, 93.8±19.1 for BPF, and 217.1±37.2 for BPS respectively, compared with 313.3±35.9 for DMSO control (Fig. 5B). Furthermore, GFP$^+$ colonies per total number of cultured SSCs were significantly reduced in a dose-dependent manner in BPA, BPF, and BPS-treated SSCs groups relative to the DMSO control (2,322.0±255.7, Fig. 5C). For instance, at 100 μM concentrations, colonies per total number

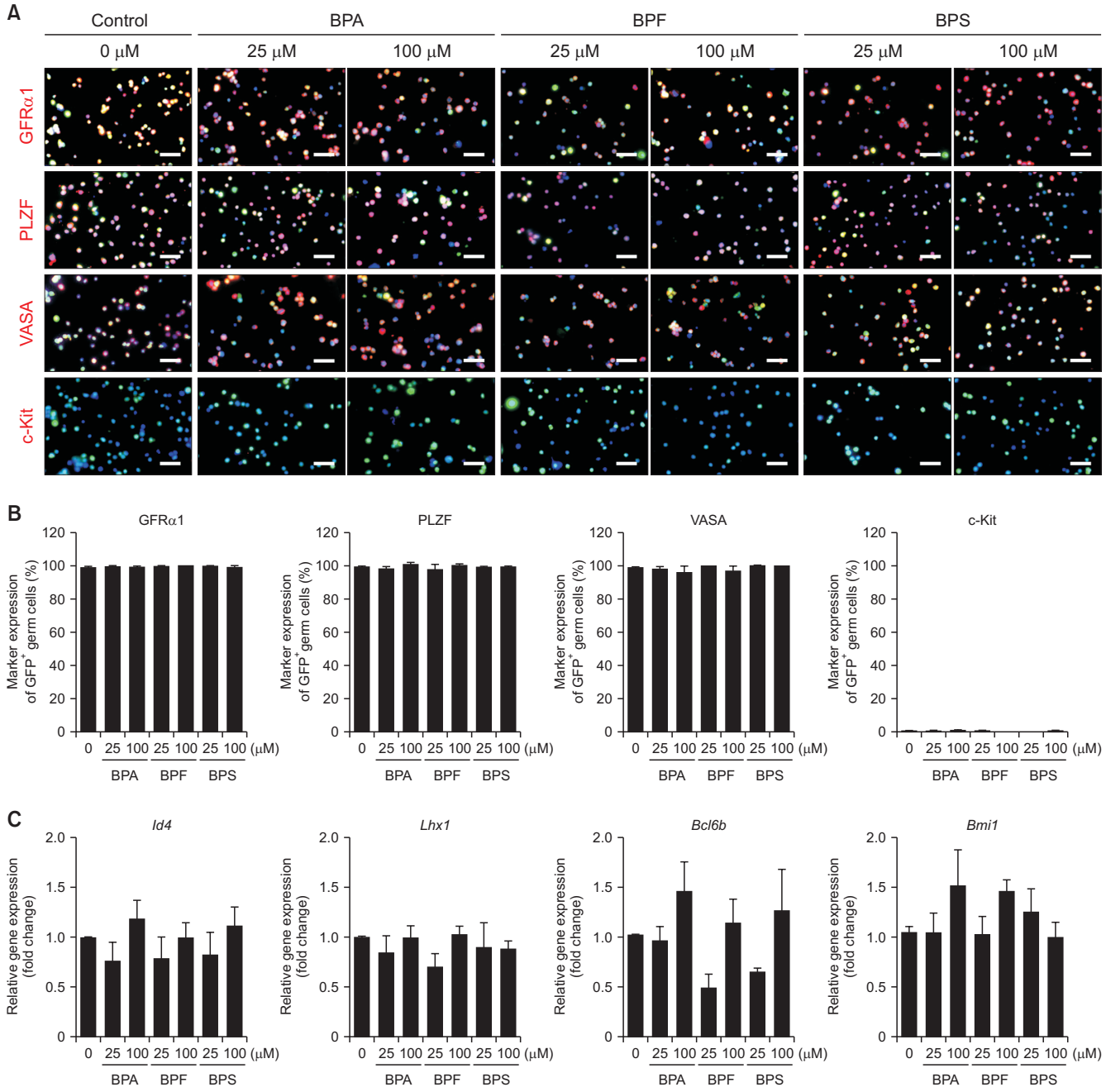


Fig. 4. BPA, BPF, and BPS do not alter specific biomarker expression. Fluorescence microscopic images of immunocytochemistry analysis of BPA-, BPF-, and BPS-treated (0, 25, and 100 μM) spermatogonial stem cells (SSCs) (A), scale bar=50 μm, 40X magnification. Localization of specific proteins GFRα1 and PLZF (undifferentiated spermatogonia, red), VASA (germ cells, red), and c-Kit (differentiated spermatogonia, red), and DAPI (nuclei, blue), GFP (cultured germ cells enriched for SSCs, green). The ratio of marker-expressing cells per GFP^+ germ cells is presented by bar graph as mean±standard error of the mean (SEM) from three independent experiments. (B) Graphical representation of A is shown as the ratio of marker-expressing cells to GFP^+ germ cells presented as mean±SEM from three independent experiments. (C) Relative gene expression of undifferentiation biomarker genes (*Id4*, *Bcl6b*, *Lhx1*, and *Bmi1*) are presented by bar graph as mean fold change±SEM from four independent experiments. Statistical analysis with one-way ANOVA and multiple comparisons by Dunnett's test was conducted. There were no significant differences between treatments. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S.

of cultured SSCs were 179.5±49.9 for BPA, 85.2±21.5 for BPF, and 373.4±67.9 for BPS, respectively (Fig. 5C). However, the functional status of donor SSC-derived spermatogenesis exposed to BPA, BPF and BPS at 100 μM treatment (a more severe condition) exhibited comparable morphological appearance relative to control in H&E staining and GFP IHC (Fig. 6). The composition of developed germ cells in the seminiferous tubules ap-

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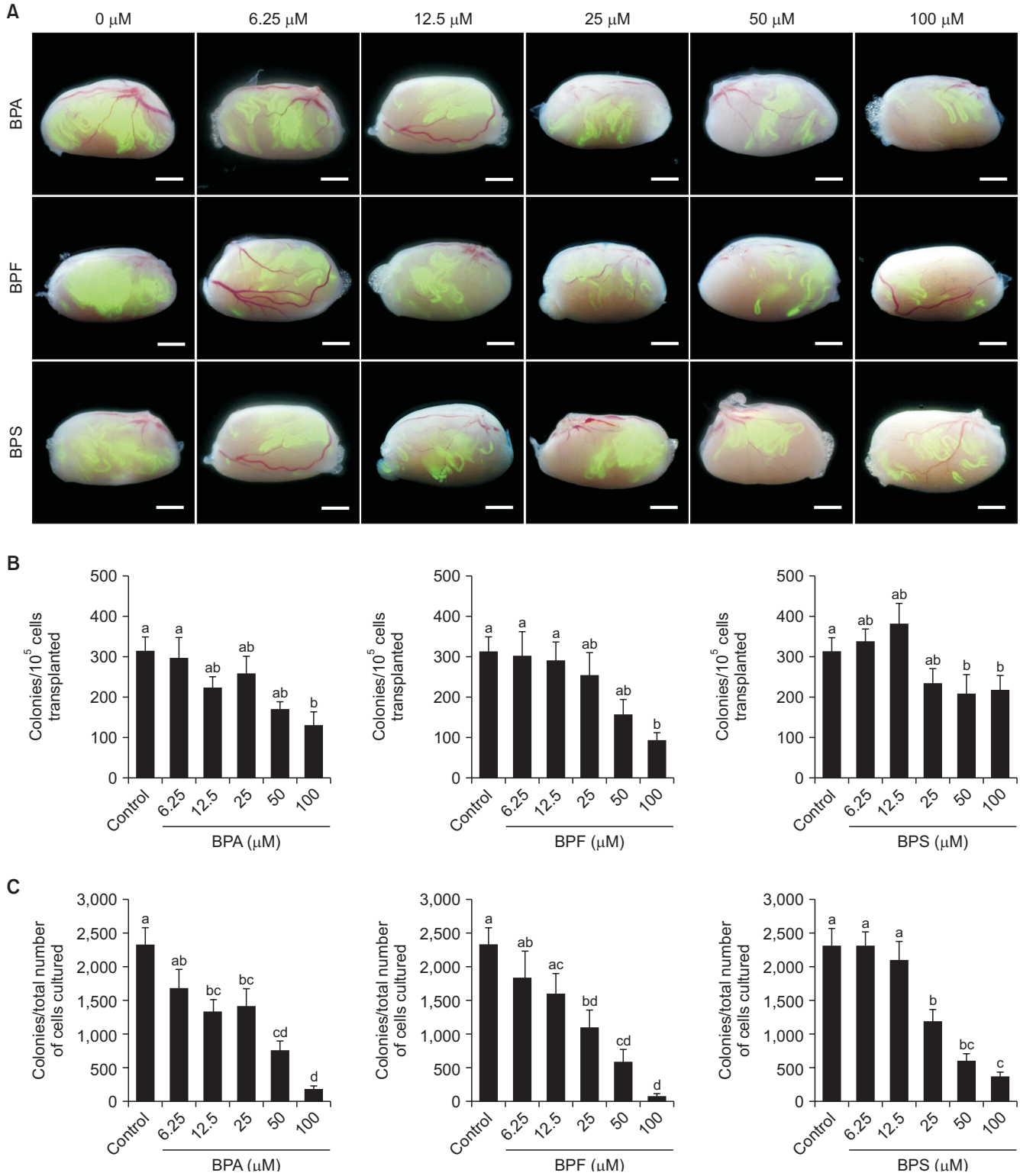


Fig. 5. Functional activity of BPA-, BPF-, and BPS-exposed spermatogonial stem cells (SSCs) was determined by *in vivo* transplantation. Dark-field fluorescence images of recipient testes of C57/BL6 mice transplanted with *in vitro* bisphenol-treated GFP$^+$ SSCs (A). Donor SSC-derived GFP$^+$ germ cell colonies are shown as merged images of bright field and green phase, with treatments indicated (scale bar=2 mm). Total numbers of mice and testes (*n*/*n*) per treatment for analysis are as follows: Control (0 μM) 8/13; BPA 6.25 μM 7/14, 12.5 μM 6/11, 25 μM 7/13, 50 μM 7/13, 100 μM 7/13; BPF 6.25 μM 6/11, 12.5 μM 6/11, 25 μM 6/11, 50 μM 6/11, 100 μM 6/10; BPS 6.25 μM 7/12, 12.5 μM 6/12, 25 μM 8/15, 50 μM 7/13, 100 μM 8/13. The number of colonies per 10^5 cells (B) and the number of colonies from total harvested cells (C) for BPA-, BPF-, and BPS-treated SSCs are presented as a bar graph with indicated concentrations of each bisphenol. One-way ANOVA and multiple comparisons by Tukey's honestly significant difference were conducted for statistical analysis. The significant difference was indicated with different letters (a, b, c, and d) when $p<0.05$. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S.

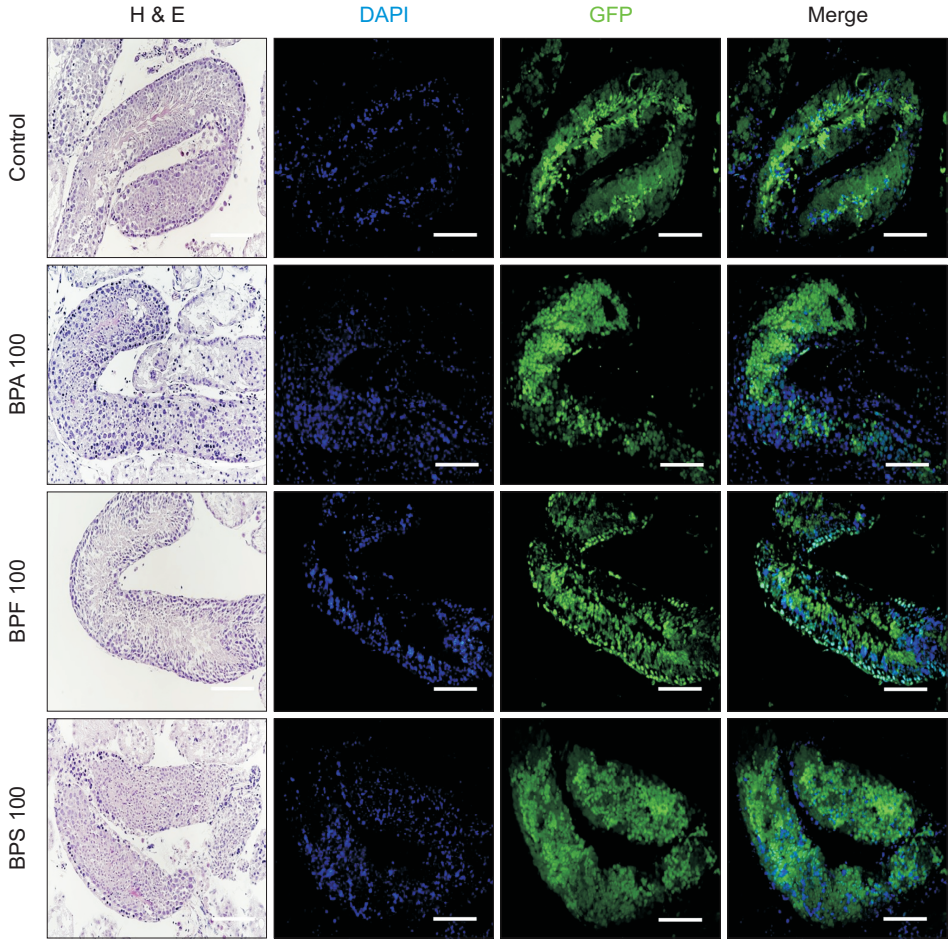


Fig. 6. BPA, BPF, and BPS may not interrupt spermatogenesis. Fluorescence microscopic images of immunocytochemistry and H&E staining of BPA-, BPF-, and BPS-treated (100 μM) spermatogonial stem cells (SSCs)-derived germ cell colonies, 20X magnification. Representative H&E staining, and DAPI (nuclei, blue), GFP (germ cells, green) are presented. The immunohistochemistry tissue sections presented are sequential sections from the same tubule. Donor SSC-derived germ cell reconstitution was observed in the seminiferous tubules of the recipient testis under control condition, as well as after treatment with BPA, BPF, and BPS. GFP indicates donor SSC-derived germ cell colonies. Scale bars=100 μm. BPA: bisphenol-A, BPF: bisphenol-F, BPS: bisphenol-S.

peared very similar across all tested groups, suggesting that bisphenol treatment may not interrupt spermatogenesis in bisphenol-exposed SSCs in the recipient animal (Fig. 6).

DISCUSSION

Endocrine disrupting chemical BPA and its two main analogs BPF and BPS are the most largely used bisphenols to date. However, compared to BPA research, the effect of BPF and BPS on adult male reproductive stem cells was poorly understood. We have demonstrated that, similar to BPA, the exposure of BPF *in vitro* significantly and similarly decreased the SSC survival rate and increased apoptosis. Moreover, both BPF and BPS inhibited SSC proliferation, just like BPA.

Previous studies reported that bisphenols could modulate the differentiation of stem cells, such as in adipocytes [21] and fetal neural stem cells [22]. However, results in this study demonstrated insignificant SSC-specific gene product changes by bisphenols both at the transcriptional and translational levels. To evaluate bisphenols-derived SSC characteristics changes, we examined specific biomarkers; GFRα1 for a cell fate decision modulator of undifferentiated spermatogonia [23], PLZF as a transcription factor inhibiting the differentiation and supporting the self-renewal [24], VASA/DDX4 as a germ cell lineage biomarker [25] and c-Kit as a marker that identifies SSCs in the differentiating stage [26]. We observed no significant biomarker expression changes in protein levels. In addition, BPA, BPF, and BPS positively influenced the mRNA expressions of specific markers: *Gfrα1, Plzf, Vasa,* and *c-Kit* but showed no significant alteration in *Id4*, *Bmi1*, *Bcl6b*, and *Lhx1*. And each bisphenol has a different impact on the transcription of specific markers. This gene regulation pattern further supports that BPA, BPF, and BPS may reduce SSC survival without changing SSC characteristics.

To compare each bisphenol-derived hindrance in spermatogenesis, *in vivo* transplantation was conducted to verify functional SSC in the testis. Donor SSC-

derived spermatogenesis changes revealed significant harmful effects of BPF and BPS comparable to BPA on SSC self-division potential. BPF showed SSC self-division inhibitory effect equivalent to BPA, demonstrated with significant reduction in germ cell colonies derived from BPF treated SSCs, indicated as colony number per 10^5 cells transplanted. In addition, like BPA, both BPF and BPS reduced germ cell colonies per total number of cultured cells in recipient testes to a similar degree. However, despite the significant reduction in germ cell colony numbers, the progress of spermatogenesis, as evidenced by germ cell reconstitution from donor SSCs, and supported by the expressions of PCNA and specific markers, collectively indicates that BPA, BPF and BPS may not adversely affect the spermatogenesis. This result offers a new perspective on previous studies, suggesting that the hindrance of spermatogenesis by bisphenols is primarily attributed to their endocrine-disrupting effects on hormone-producing cell functions in rat and human fetal testes [5,27].

In the comparative analysis of three bisphenol analogs, BPF demonstrated comparable SSC cytotoxicity to BPA, as evidenced by similar outcomes in SSC survival, proliferation, apoptosis induction, and donor SSC-derived germ cell colony formation within the recipient testis. Compared to BPA and BPF, BPS exhibited milder yet significant SSC toxicity in this study. Despite its lower SSC cytotoxicity, BPS similarly reduced functional SSC numbers in this study. Previous research have highlighted its xeno-estrogenic effects, disrupting membrane-initiated estradiol-induced cell signaling, leading to altered cell division and apoptosis [28], and oxidative stress in the testes [29]. Furthermore, further research is required to comprehensively understand the impacts of bisphenol analogs on SSCs and spermatogenesis. This involves establishing donor SSC-derived spermatogenesis in the recipient testis, followed by *in vitro* fertilization to evaluate their influence on embryonic development. Alternatively, conducting mating studies would allow examination on fertility through multigenerational analysis, along with an assessment of spermatogenesis gene expression.

CONCLUSIONS

BPF and BPS, the two major substitutes for BPA, similarly induce a significant decrease in functional SSC survival comparable to BPA. Their inhibitory action on functional SSCs directly results from a reduction in SSC numbers. The significant cytotoxic effects of these primary BPA substitutes on SSCs could potentially diminish the population of functional self-renewal stem cells, thereby impacting male fertility.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: SHK, SEJ, BYR. Data curation: SHK, SHS, BJS. Formal analysis: SHK, SHS, SMK. Investigation: SHK, SHS, SMK, BJS. Funding acquisition: BYR. Methodology: SHK, JSA, DHK. Supervision: KTL, KL, BYR. Validation: KTL, KL, BYR. Writing – original draft: SHK, SHS. Writing – review & editing: all authors.

Supplementary Materials

Supplementary materials can be found via https://doi.org/10.5534/wjmh.230166.

Data Sharing Statement

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

REFERENCES

1. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 1993;290:193-200.

2. Oakberg EF. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 1956;99:391-413.
3. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 1994;91:11298-302.
4. Adegoke EO, Rahman MS, Pang MG. Bisphenols threaten male reproductive health via testicular cells. Front Endocrinol (Lausanne) 2020;11:624.
5. Jin P, Wang X, Chang F, Bai Y, Li Y, Zhou R, et al. Low dose bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult rats. J Biomed Res 2013;27:135-44.
6. Gong X, Xie H, Li X, Wu J, Lin Y. Bisphenol A induced apoptosis and transcriptome differences of spermatogonial stem cells in vitro. Acta Biochim Biophys Sin (Shanghai) 2017;49:780-91.
7. Wang C, Zhang J, Li Q, Zhang T, Deng Z, Lian J, et al. Low concentration of BPA induces mice spermatocytes apoptosis via GPR30. Oncotarget 2017;8:49005-15.
8. Li DK, Zhou Z, Miao M, He Y, Qing D, Wu T, et al. Relationship between urine bisphenol-A level and declining male sexual function. J Androl 2010;31:500-6.
9. Quan C, Wang C, Duan P, Huang W, Yang K. Prenatal bisphenol A exposure leads to reproductive hazards on male offspring via the Akt/mTOR and mitochondrial apoptosis pathways. Environ Toxicol 2017;32:1007-23.
10. Baluka SA, Rumbeiha WK. Bisphenol A and food safety: lessons from developed to developing countries. Food Chem Toxicol 2016;92:58-63.
11. Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, et al. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 2005;84:249-59.
12. Pelch KE, Wignall JA, Goldstone AE, Ross PK, Blain RB, Shapiro AJ, et al. NTP research report on biological activity of bisphenol A (BPA) structural analogues and functional alternatives: research report 4. National Toxicology Program; 2017.
13. Rochester JR, Bolden AL. Bisphenol S and F: a systematic review and comparison of the hormonal activity of bisphenol A substitutes. Environ Health Perspect 2015;123:643-50.
14. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 2006;103:9524-9.
15. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2004;101:16489-94.
16. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 2003;68:2207-14.
17. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol 2015;111:A3.B.1-3.
18. Lee YA, Kim YH, Ha SJ, Kim BJ, Kim KJ, Jung MS, et al. Effect of sugar molecules on the cryopreservation of mouse spermatogonial stem cells. Fertil Steril 2014;101:1165-75.e5.
19. Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A 2005;102:17430-5.
20. Brauchle E, Thude S, Brucker SY, Schenke-Layland K. Cell death stages in single apoptotic and necrotic cells monitored by Raman microspectroscopy. Sci Rep 2014;4:4698.
21. Boucher JG, Boudreau A, Atlas E. Bisphenol A induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor antagonist. Nutr Diabetes 2014;4:e102.
22. Gill S, Kumara VMR. Comparative neurodevelopment effects of bisphenol A and bisphenol F on rat fetal neural stem cell models. Cells 2021;10:793.
23. Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, et al. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction 2012;143:325-32.
24. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, et al. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004;36:647-52.
25. Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, et al. The mouse homolog of Drosophila Vasa is required for the development of male germ cells. Genes Dev 2000;14:841-53.
26. Rossi P, Sette C, Dolci S, Geremia R. Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest 2000;23:609-15.
27. Eladak S, Moison D, Guerquin MJ, Matilionyte G, Kilcoyne K, N'Tumba-Byn T, et al. Effects of environmental Bisphenol A exposures on germ cell development and Leydig cell function in the human fetal testis. PLoS One 2018;13:e0191934.
28. Viñas R, Watson CS. Bisphenol S disrupts estradiol-induced nongenomic signaling in a rat pituitary cell line: effects on cell functions. Environ Health Perspect 2013;121:352-8.
29. Ullah H, Jahan S, Ain QU, Shaheen G, Ahsan N. Effect of bisphenol S exposure on male reproductive system of rats: a histological and biochemical study. Chemosphere 2016;152:383-91.