REPRODUCTION
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Bisphenol A and S impaired ovine granulosa cell steroidogenesis

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Abstract

Bisphenols, plasticisers used in food containers, can transfer to food. Bisphenol A (BPA) has been described as an endocrine disruptor and consequently banned from the food industry in several countries. It was replaced by a structural analogue, Bisphenol S (BPS). BPA action on the steroidogenesis is one of the mechanisms underlying its adverse effects on the efficiency of female reproduction. This study aimed to determine whether BPS is a safe alternative to BPA regarding GC functions. Antral follicles (2–6 mm), of approximatively 1000 adult ewe ovaries, were aspired and GC purified. For 48 h, ovine GC were treated with BPA or BPS (from 1 nM to 200 µM) and the effects on cell viability, proliferation, steroid production, steroidogenic enzyme expression and signalling pathways were investigated. Dosages at and greater than 100 µM BPA and 10 µM BPS decreased progesterone secretion by 39% ($P < 0.001$) and 22% ($P = 0.040$), respectively. BPA and BPS 10 µM and previously mentioned concentrations increased oestradiol secretion two-fold ($P < 0.001$ and $P = 0.082$, respectively). Only 100 µM BPA induced a decrease ($P < 0.001$) in gene expression of the enzymes of steroidogenesis involved in the production of progesterone. BPA reduced MAPK3/1 phosphorylation and *ESR1* and *ESR2* gene expression, effects that were not observed with BPS. BPA and BPS altered steroidogenesis of ovine GC. Thus, BPS does not appear to be a safe alternative for BPA. Further investigations are required to elucidate BPA and BPS mechanisms of action.
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Introduction

The ovarian follicle is composed of an oocyte; it includes granulosa cells (GC) that border the antrum and is filled with follicular fluid. GC have two essential functions: cell proliferation and steroidogenesis. Due to their secretory function, these cells play a fundamental role in oocyte growth and maturation (Monniaux *et al*. 2019). Nevertheless, these cells can be affected by environmental factors such as diets or pollutants (Hernandez-Medrano *et al*. 2012, Bloom *et al*. 2016).

Bisphenol A (BPA) has been used in the plastic industry since 1940 (Usman & Ahmad 2016). It is found in food containers, water pipes, cosmetics, dental sealants and medical equipment (Vandenberg *et al*. 2007). Bisphenols can transfer from container to content even under normal conditions of use and therefore contaminate food, the main source of exposure (Vandenberg *et al*. 2007, Wu *et al*. 2018*b*). BPA is found in many human tissues and fluids: breast milk and colostrum (3 and 15 nM, respectively), amniotic fluid (4–39 nM), placental tissue (11.2 ng/g), blood (0.9–90 nM), urine (11 nM) and follicular fluid (9 nM) (Vandenberg *et al*. 2007). BPA exhibits adverse effects on human health. Indeed, high BPA levels have been associated with obesity, type 2 diabetes, cardiovascular diseases, disruption of bone metabolism and alterations in both male and female reproductive functions (Ma *et al*. 2019). BPA shares part of its structure (phenol ring) with that of the hormone 17β-oestradiol and exhibits moderate oestrogenic activity, despite a 10,000-fold lower binding affinity to oestrogen receptors (ER) α (*ESR1*) and ERβ (*ESR2*) compared to oestradiol (Grignard *et al*. 2012, Molina-Molina *et al*. 2013). Previous studies demonstrated that BPA alters ERα and ERβ expression in rats (Tomza-Marciniak *et al*. 2018). Moreover, the interference of BPA on GC steroidogenesis is one of the mechanisms that explain the negative impact of BPA on reproduction (Bloom *et al*. 2016). In porcine and luteinised human GC, 100 µM BPA decreases oestradiol and progesterone secretion, after 48 h or 72 h of culture, an effect that is associated with reduced steroidogenic enzyme (STAR, CYP19A1, CYP11A1 and HSD3B1) expression (Mlynarcikova *et al*. 2005, Bloom *et al*. 2016, Mansur *et al*. 2016, Bujnakova Mlynarcikova *et al*. 2018, Pogrmic-Majkic *et al*. 2019). Nevertheless, other studies

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showed an increase in oestradiol secretion with porcine GC with 10 µM BPA, after 24 h or 48 h of treatment (Wu *et al*. 2018*a*, Song *et al*. 2019). Such modifications in GC steroid production can lead to ovarian dysfunction. Moreover, 100 µM BPA exposure for 30 min increases MAPK3/1 phosphorylation in human GC (Pogrmic-Majkic *et al*. 2019); this pathway is a key regulator of cell survival and proliferation (Fan *et al*. 2009). Furthermore, urine BPA concentration above 1.6 µg/L (9 nM) in women who are undergoing assisted reproductive technology are potentially associated with a reduced number of pre-ovulatory follicles, compared to women exhibiting <0.4–0.9 µg/L urine BPA concentration (Souter *et al*. 2013). BPA has therefore been classified as an endocrine disruptor, and its use has been banned in the food industry in several countries (Canada, Belgium and France) (European-Food-Safety-Authority 2015, Usman & Ahmad 2016). New BPA structural analogues emerged, particularly Bisphenol S (BPS), which is still unregulated even though it is increasingly present in the environment. Thus, BPS is now found in most urine samples at average concentrations similar to BPA (0.08–84 nM; Wu *et al*. 2018*b*). The BPS studies in animal models (zebrafish *Danio rerio*) and cell lines (mouse 3T3-L1 adipocytes) also report harmful health effects, including obesity and disruption of reproductive functions (Chen *et al*. 2016). Only a few studies focused on the impact of BPS on mammalian GC. Nevertheless, 100 µM BPS increases oestradiol secretion in bovine GC after 6 days of exposure (Campen *et al*. 2018), and mice injected with BPS (10 mg/kg from birth to postnatal days 60 by s.c. injection every three days) exhibit increased serum 17β-oestradiol (Shi *et al*. 2017). Conversely, 10 µM BPS decreases oestradiol secretion in porcine GC and cell proliferation (Berni *et al*. 2019).

The discrepancies among studies may be due to differences in sensitivity between mono- (cow) vs poly-ovulating species (rodents and pig). Indeed, rodents are less sensitive (100 to 1000-fold) to bisphenol effects on testosterone secretion by testicular foetal cells compared to human cells (Eladak *et al*. 2015). It is therefore necessary to assess the effects of BPA and BPS in a species relevant to the human female reproduction, as the ewe (Scaramuzzi *et al*. 2011, Monniaux *et al*. 2019). Indeed, ewes and humans have ovaries of similar size and shape (Lunardi *et al*. 2015). They also have similar follicle kinetic (180 days and 200 days from the exit of the follicular reserve to the pre-ovulatory stage for ewe and women, respectively) (Monniaux *et al*. 2014). Moreover, sheep have already been described as a relevant model for toxicology studies, including BPA studies (Rivera *et al*. 2011, Corbel *et al*. 2013, Lunardi *et al*. 2015, Grandin *et al*. 2018, Gingrich *et al*. 2019).

In this study, we investigated whether BPS is a safe alternative to BPA. Indeed, we hypothesized that BPS would have similar deleterious effect as BPA on GC functions, including its steroidogenesis. Our objective was to compare the effects of these two compounds on sheep GC and to study their mechanisms of action. We analysed GC viability and proliferation, secretion of oestradiol and progesterone, expression of steroidogenic enzymes, hormonal receptor gene expression and signalling pathways.

Materials and methods

Chemicals and antibodies

All chemicals were obtained from Sigma-Aldrich, unless otherwise stated in the text. The rabbit polyclonal antibody to human cytochrome P450 family 19 subfamily A member 1 (CYP19A1) and mouse monoclonal antibody to human vinculin and horseradish-peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat antibodies were obtained from Sigma-Aldrich. The goat polyclonal antibody to human cytochrome P450 family 11 subfamily A member 1 (CYP11A1) was purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody to human hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) was obtained from Abgent (San Diego, CA, USA). Rabbit polyclonal antibodies to human protein kinase B (Akt), 5′ AMP-activated protein kinase alpha (AMPKα), phospho-AMPKα (Thr172), p44/42 mitogen-activated protein kinase 3/1 (MAPK3/1) and rabbit monoclonal antibodies to human phospho-Akt (Ser473), phospho-p44/42 MAPK (MAPK3/1; Thr202/Tyr204), p38 MAPK (MAPK14) and phospho-p38 MAPK (MAPK14, Thr180/Tyr182) were purchased from Cell signalling Technology. Ethanol was used as a solvent for BPA and BPS at concentrations that do not negatively impact the cells. Absence of ethanol effect on GC viability and progesterone secretion was confirmed for all ethanol concentrations used, ranging from 1/100,000,000 to 1/500 (corresponding to the BP concentrations used, ranging from 1 nM to 200 µM) (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

Isolation and culture of GC

Approximately 1000 ovaries of adult ewes were collected from local slaughterhouses to perform the experiments described in this paper. Antral follicles (2 to 6 mm) were punctured with an 20G needle connected to a vacuum pump and a collection tube to receive follicular fluid that contained cumulus-oocyte complex (COC) and GC. COC were removed under binocular observation. Negligible presence of cumulus cells (CC) compared to GC was confirmed by measuring expression of CC and GC specific genes by qPCR (Supplementary Fig. 2). GC were washed in complemented serum-free McCoy's 5A medium: 3 mM L-glutamine, 0.1% BSA (Cell Signalling Technology), 100,000 UI/L penicillin, 100 mg/L streptomycin, 20 mM HEPES (pH 7.6), 100 nM 4-androsten-11β-ol-3,17-dione, 5 mg/L bovine apo-transferrin, 250 nM selenium and 1.72 nM insulin. After centrifugation and washing in medium, GC were purified with a Percoll gradient (50% Percoll and 50% medium). Recovered cells were counted in a haemocytometer. GC were plated overnight in 96-well

plates (Thermo Scientific Biolite) at 1×10^5 viable cells/150 µL medium/well in complemented serum-free McCoy's 5A medium. Then, GC were cultured for 48 h in the presence or absence (control) of BPA or BPS at eight concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM. This concentration range extends from doses found in human biological fluids during chronic exposure (Vandenberg *et al*. 2007), including people exposed professionally (Hines *et al*. 2017), to the supraenvironmental dose of 200 µM. Cultures were performed at 38.5°C in a humidified atmosphere that contained 5% CO_2 and 20% O_2 in air. There was no foetal bovine serum in medium, and the relatively short culture time was chosen in order to limit GC differentiation and retain the round shape. GC were still able to proliferate and exhibit steroidogenic activity. For the assessment of cell proliferation and steroid secretion, a positive control was carried out during cell cultures (10 nM Insulin Growth Factor-1), as well as a negative control for the measurement of cell viability (1% saponin and 70% methanol).

Cell viability

After a 48-h treatment in the presence or absence of BPA or BPS (10 µM, 50 µM, 100 µM or 200 µM, doses impacting cell proliferation and steroidogenesis), cell viability was determined according to two complementary methods: Live/Dead staining and spent media adenylate kinase activity assay. Concerning Live/Dead staining, GC (250×10^3 viable cells/250 µL media/well) were cultured in an eight-chamber slide in complemented serum-free McCoy's 5A media. Live/Dead staining was applied (green fluorescence corresponds to living cells and red fluorescence corresponds to dead cells) according to the manufacturer's instructions (Thermo Fisher Scientific). The number of living and dead cells was counted in four fields/chamber (an average of 800 cells per field was counted) using ImageJ software (ImageJ-win64). The results are expressed as percentage of dead cells of three independent cultures. For the adenylate kinase assay, GC (1×10^5 viable cells/100 µL medium/well) were cultured in 96-well dishes in complemented serum-free McCoy's 5A media. After a 48-h treatment, the activity of adenylate kinase released into the culture supernatant by dead cells was assessed by bioluminescence according to the manufacturer's instructions (MBL International Corporation, Nanterre, France). The results are expressed as the mean ± S.E.M. of six independent cultures and normalised to the mean of the control condition.

Cell proliferation

GC (1×10^5 viable cells/150 µL medium/well) were cultured in 96-well plates in complemented serum-free McCoy's 5A medium. After a 48-h treatment with 10 µM bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) in the presence or absence of BPA or BPS (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM), the supernatants were removed. Cell proliferation was measured with an enzyme immunoassay according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU (colourimetric), Roche Applied Science, Germany). The results are expressed as the mean ± S.E.M. of 13 independent cultures, with each condition performed in duplicate and normalised to the control condition.

Progesterone assay

GC (1×10^5 viable cells/150 µL medium/well) were cultured in 96-well dishes in complemented serum-free McCoy's 5A medium. After 48-h treatment in the presence or absence of BPA or BPS (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM), the supernatants were collected to measure the progesterone and oestradiol secretion. The progesterone concentration was measured using an enzyme immunoassay protocol, as described previously (Canépa *et al*. 2008). The cell layers were used to quantify the proteins in each corresponding well (BCA protein quantification kit; Interchim, Montluçon, France). For progesterone concentrations that ranged from 0.2 to 10 ng/mL, the intra-assay coefficient of variation (CVs) averaged less than 10%. Progesterone concentration was normalised by the protein concentration of each well. The results are expressed as the amount of progesterone (ng/mL) secreted per 48 h per protein amount (µg/mL) per well as the mean ± S.E.M. of 12 independent cultures, with each condition performed in duplicate and normalised to the control condition.

Oestradiol assay

The oestradiol concentration was measured using an enzyme immunoassay (E2-EASIA-kit, DIAsource, Louvain-La-Neuve, Belgium), according to the manufacturer's instructions. Cell layers were used to quantify the proteins in each corresponding well (BCA protein quantification kit). For oestradiol concentrations that ranged from 1.56 to 50 pg/mL, the intra-assay CVs averaged 15%. The oestradiol concentration was normalised by the protein concentration of each well. The results are expressed as the amount of oestradiol (pg/mL) secreted per 48 h per protein amount (µg/mL) per well as mean ± S.E.M. of five independent cultures, with each condition performed in duplicate and normalised to the control condition.

Protein extraction and immunoblotting

GC were cultured in 96-well plates (1×10^5 viable cells/150 µL medium/well) in complemented serum-free McCoy's 5A medium. After a 48-h treatment in presence or absence of 10 µM BPA or BPS (the lowest concentration of BPS affecting GC steroidogenesis), protein was extracted. Twelve micrograms of protein were subjected to Western blotting as previously described (Maillard *et al*. 2018). The lysates were used to measure steroidogenic enzymes (CYP19A1, CYP11A1 and HSD3B1) and cell signalling pathway proteins (Akt, MAPK3/1, AMPKα and MAPK14). The blots were incubated overnight at 4°C with appropriate primary antibodies (Table 1) in Tris-buffered saline with 0.1% Tween 20 (TBST) with 5% non-fat dry milk powder (NFDMP). After several washes in TBST, the membranes were incubated for 90 min at room temperature with horseradish-peroxidase-conjugated secondary anti-rabbit,

Table 1 Antibodies used in this study.

Protein	Source	Primary Ab dilutions	Secondary Ab dilutions	Molecular weight (kDa)
Vinculin	Mouse	1/1000	1/7000	117
CYP19A1	Rabbit	1/500	1/5000	58
CYP11A1	Goat	1/500	1/5000	55
HSD3B1	Rabbit	1/500	1/5000	42
p-Akt (Ser473)	Rabbit	1/2000	1/5000	60
Akt	Rabbit	1/1000	1/5000	60
p-AMPKα (Thr172)	Rabbit	1/1000	1/5000	62
AMPKα	Rabbit	1/1000	1/5000	62
p-p44–42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	1/2000	1/5000	42–44
p44/42 MAPK (Erk1/2)	Rabbit	1/1000	1/5000	42–44
p-p38 MAPK (Thr180/Tyr182)	Rabbit	1/1000	1/5000	40
p38 MAPK	Rabbit	1/1000	1/5000	40

anti-goat or anti-mouse IgG (according to primary antibody; Table 1) in TBST with 5% NFDMP. After washing again in TBST, the signal of specific bands was visualised by ECL (West Dura; Thermo-Fisher Scientific) and quantified using a GeneGnome charge-coupled device camera (Syngene, Cambridge, United Kingdom) and Genesys 1.5.5 software (Syngene). Signal intensity was analysed using GeneTools 4.01 software (Syngene). For steroidogenic enzymes, protein expression is reported relative to the expression of vinculin (a housekeeping protein) and normalised to the control condition. The results of cell signalling pathway proteins are expressed as the ratio of phosphorylated protein to total protein, normalised to the control before treatment. Protein lysates from six independent cultures were used for each protein.

RNA extraction, real-time polymerase chain reaction (qPCR) and gene expression

GC were cultured in 96-well plates (1×10^5 viable cells/150 µL medium/well) in complemented serum-free McCoy's 5A medium. After a 48-h treatment in presence or absence of BPA or BPS (10 nM, 100 nM, 1 µM, 10 µM, 50 µM or 100 µM), cells were collected and mRNA was extracted. DNase treatment was performed with NucleoSpin® RNA kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's recommendations. The quantity and quality of RNA was assessed with a Nanodrop ND-1000 Spectrophotometer (Nyxor biotech, Paris, France). RT was performed on 500 ng of total RNA extracted with Maxima Strand cDNA Synthesis Kit (Thermo-Fisher Scientific), according to the manufacturer's instructions. qPCR reactions for androgen receptor (*AR*), progesterone receptor (*PR*), oestrogen receptor 1 (*ESR1*), *ESR2*, *CYP19A1*, *CYP11A1*, *HSD3B1* and StAR protein (*STAR*) gene expression were performed. qPCR were carried out with specific primers pairs (Table 2; at a final concentration of 125 nM of each), RT reaction (diluted 1/150) and qPCR Mastermix Plus for Sybr Green I (Bio Rad), according to the manufacturer's instructions, on a CFX96 (Bio Rad), and analysed as previously described (Maillard *et al*. 2018). The efficiency of the primers (Table 2) and standard curve were determined for each gene. Relative gene expression levels were calculated. The geometric mean of two housekeeping genes (ribosomal protein L19 (*RPL19*) and beta-actin (*ACTB*)) was used to normalise gene expression. The relative amounts of gene transcripts (R) were calculated with six independent cultures using the following equation:

$$R = \frac{\left(E_{\text{gene}}^{-Ct\ \text{gene}}\right)}{\left(\text{geometric mean}\left(E_{\text{ACTINE}\,\beta}^{-Ct\ \text{ACTINE}\,\beta}; E_{RPL19}^{-Ct\ RPL19}\right)\right)}$$

where *Ct* corresponds to the cycle threshold and *E* indicates the PCR efficiency for each primer pair.

Statistical analysis

Statistical analyses were performend with R version 3.5.1 using the R Commander package. All results, except for viability experiments with Live/Dead staining, are presented as the mean ± S.E.M., and non-parametric permutation ANOVA (R package lmperm) was performed. Thus, treatment effect, culture effect and treatment-by-culture interactions were assessed. When the treatment condition was significant, the Tukey post hoc test (R package nparcomp) was executed. Regarding viability experiments with Live/Dead staining, a logistic regression analysis was used, and data are presented as percentage ± S.E.M. Differences were considered significant when P value was <0.05 and as a tendency when $0.05<P<0.1$.

Results

Effects of BPA and BPS on ovine GC viability

The effects of BPA and BPS (10 µM, 50 µM, 100 µM or 200 µM) on cell viability after 48-h treatment were determined according to two complementary methods (Fig. 1): Live/Dead staining (Fig. 1A) and adenylate kinase activity assay in the medium (Fig. 1B). No BPS treatment affected cell viability compared to control. Only the highest BPA concentration (200 µM) increased cell mortality: 99.5% compared to 42.7% in control ($P<0.001$; Fig. 1A). This result was confirmed with adenylate kinase activity assay. Indeed, 200 µM BPA induced a 1.8-fold increase in mortality compared to control ($P<0.001$; Fig. 1B).

Table 2 Oligonucleotide primer sequences used in this study.

Abbrev.	Name	Gene ID	Forward (5′→3′)	Reverse (5′→3′)	Size (bp)	E (%)
ACTB	β-actin	NM_001009784.3	CCAGCACGATGAAGATCAAG	ACATCTGCTGGAAGGTGGAC	102	93.2
AR	Androgen receptor	NM_001308584	CCTTCACCAATGTCAACT	ATCCACTGGAATAATGCTAA	200	81.8
CYP11A1	Cytochrome P450 family 11 subfamily A member 1	NM_001093789.1	CGGACAAGTTTGACCCAACCAG	GCCGGAAGACAAGGAAGATGG	243	90.3
CYP19A1	Cytochrome P450 family 19 subfamily A member 1	NM_001123000.1	GGTCATCCTGGTCACCCTTCTG	GCCGGTCGCTGGTCTCGTCTGG	119	140.8
ESR1	Oestrogen receptor 1	XM_027972563.1	GGTTCCGTATGATGAATCT	CAAGGTGTCTGTGATCTT	158	98.5
ESR2	Oestrogen receptor 2	NM_001009737.1	ACTATGGAGTCTGGTCAT	GTCGGTTCTTATCTATGGTA	114	121.0
HSD3B1	Hydroxyl-δ-5-Steroid Dehydrogenase, 3β- and steroid δ-isomerase 1	NM_001135932.1	GGAGACATTCTGGATGAGCAGTGCC	GCCACCTCTATGGTGCTGGTGTGGA	206	82.1
PR	Progesterone receptor	Z66555.1	AGTCATCATTCTATTCATTATGC	TGGCTTCTTAGTCCTTCT	142	93.8
RPL19	Ribosomal protein L19	AY158223.1	CACAAGCTGAAGGCAGACAA	TGATGATTTCCTCCTTCTTGG	130	83.8
STAR	Steroidogenic acute regulatory protein	NM_001009243.1	GGGCCCTGGGCATCCTCAAAGA	TGACACTGGGGTTCCACTCGCCC	258	97.2

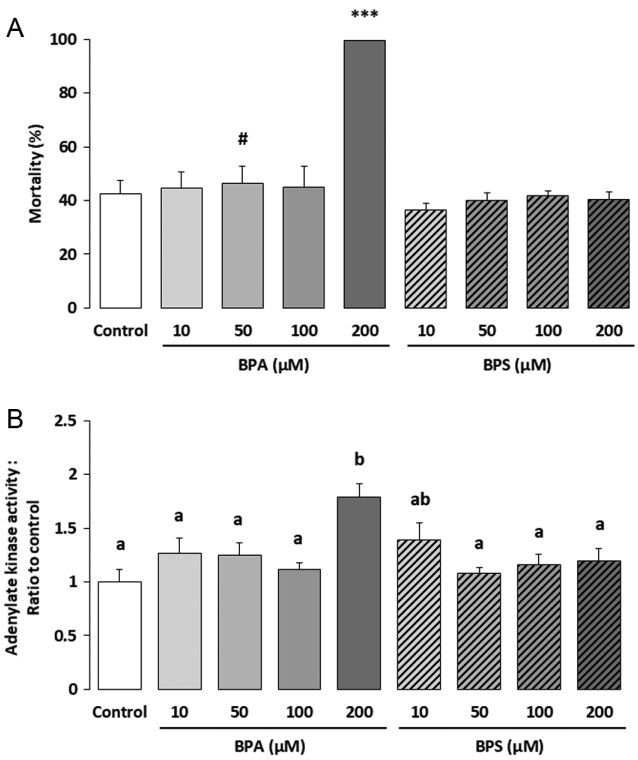

Figure 1 Effects of bisphenol A or S (BPA or BPS) on cell viability. Cell viability was assessed in ovine granulosa cells (GC) after 48-h treatment in the presence or absence of BPA or BPS (10, 50, 100 or 200 µM) according to two complementary methods. Live/Dead staining (A) was performed according to the manufacturer's instructions; green fluorescence corresponds to living cells and red fluorescence corresponds to dead cells. The results are expressed as the percentage of dead cells from three independent cultures. *** indicates a significant difference ($P<0.001$) and # indicates a tendency ($0.05<P<0.1$). The activity of adenylate kinase (B) released into the culture supernatant by dead cells was assessed by bioluminescence (according to the manufacturer's instructions). The results are expressed as the mean ± S.E.M. of six independent cultures and normalised to the mean of control condition. Bars that do not exhibit at least one common letter are significantly different ($P<0.05$).

Effects of BPA and BPS on ovine GC proliferation

The proliferation of ovine GC in the presence or absence of BPA or BPS (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM) was assessed by the incorporation of BrdU after 48-h treatment (Fig. 2). BPA significantly reduced ($P<0.001$) cell proliferation by 6%, 20% and 72% at 50 µM, 100 µM and 200 µM, respectively, compared to control (Fig. 2A). While only 200 µM BPS decreased ($P<0.001$) GC proliferation by 8%, compared to control (Fig. 2B).

Effects of BPA and BPS on ovine GC progesterone secretion

Progesterone secretion was measured in spent culture media after 48-h treatment with BPA or BPS (Fig. 3).

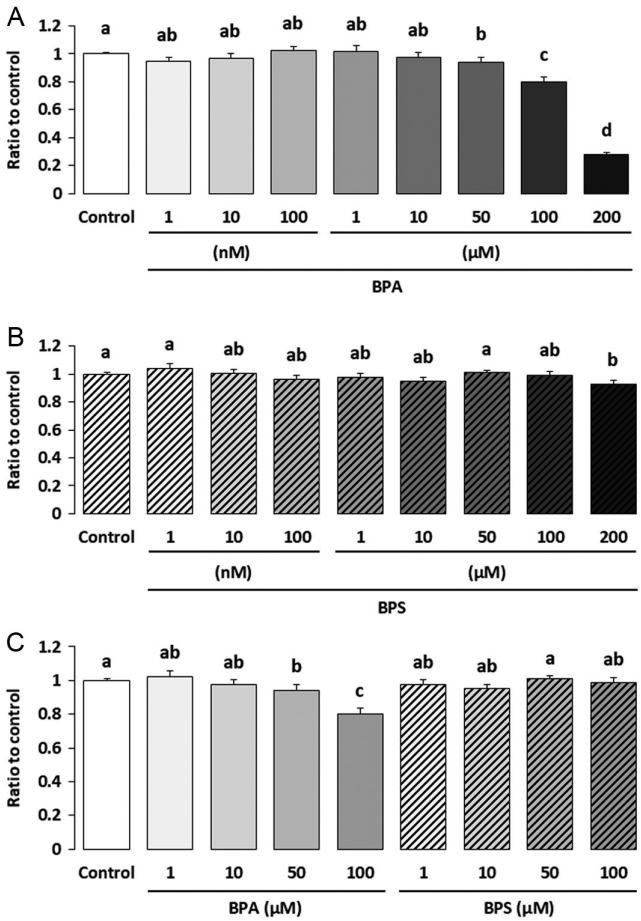

Figure 2 Effects of Bisphenol A or S (BPA or BPS) on cell proliferation. Cell proliferation was assessed in ovine granulosa cells (GC) by bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) incorporation after 48-h culture in complemented serum-free McCoy's 5A media in the presence or absence of various doses of BPA (A) or BPS (B) (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM). Data with BPA and BPS were compared statistically for four relevant concentrations (C) (1 µM, 10 µM, 50 µM and 100 µM). The results are expressed as the mean ± S.E.M. of 13 independent cultures, with each condition in duplicate, and normalised to the control condition of each lot. Bars that do not exhibit at least one common letter are significantly different ($P < 0.05$).

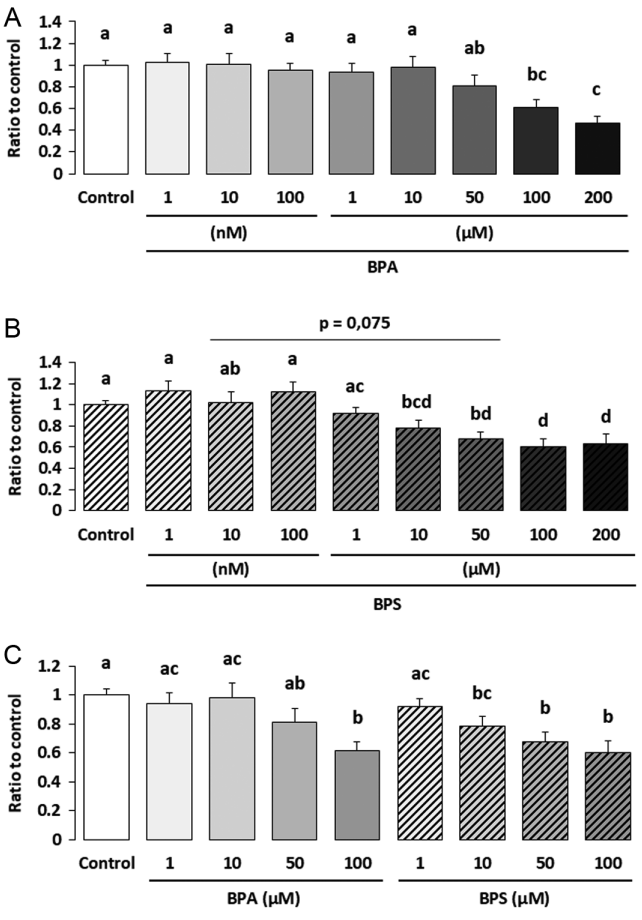

Figure 3 Effects of Bisphenol A or S (BPA or BPS) on progesterone secretion. Progesterone secretion was assessed in ovine granulosa cells (GC). The progesterone concentration was determined in culture medium after 48-h culture in complemented serum-free McCoy's 5A media in the presence or absence of BPA (A) or BPS (B) (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM). Data with BPA and BPS were compared statistically for four relevant concentrations (C) (1 µM, 10 µM, 50 µM and 100 µM). The results are expressed as the mean ± S.E.M. of 12 independent cultures, with each condition performed in duplicate, and normalised to the control condition of each culture experiment. Bars that do not exhibit at least one common letter are significantly different ($P < 0.05$). The actual control value was 36.23 ± 3.88 ng progesterone/mg protein.

Only 100 µM and 200 µM BPA decreased ($P < 0.001$) progesterone secretion by 39% and 54%, respectively (19.09 ± 2.62 and 13.12 ± 1.76 ng progesterone/mg protein, respectively), compared to control (36.23 ± 3.88 ng progesterone/mg protein) (Fig. 3A). Interestingly, 10 µM ($P = 0.038$), 50 µM ($P < 0.001$), 100 µM ($P < 0.001$) and 200 µM BPS ($P < 0.001$) decreased progesterone secretion by 22%, 32% and 40%, respectively (28.91 ± 3.78, 22.31 ± 2.50, 17.13 ± 1.92 and 16.53 ± 1.7 ng progesterone/mg protein, respectively), compared to control (Fig. 3B). Comparatively, 10 µM and 50 µM BPA did not significantly affect progesterone secretion (36.08 ± 5.84 and 24,03 ± 3.02 ng progesterone/mg protein, respectively) (Fig. 3C).

Effects of BPA and BPS on oestradiol secretion

Oestradiol secretion was measured in spent culture media after 48-h treatment with BPA or BPS (Fig. 4). Oestradiol secretion was more than two-fold increased by 10 µM BPA ($P < 0.001$, 76.80 ± 14.96 pg oestradiol/mg protein; Fig. 4A) and 10 µM BPS ($P = 0.007$; 61.97 ± 11.83 pg oestradiol/mg protein; Fig. 4B) compared to control (28.94 ± 6.08 pg oestradiol/mg protein). This increase was even greater ($P < 0.001$) with higher bisphenol concentrations: 5.8- and 4.3-fold for 100 µM BPA and BPS (106.61 ± 12.54 and 78.65 ± 11.28 pg oestradiol/mg protein, respectively) compared to control (Fig. 4C).

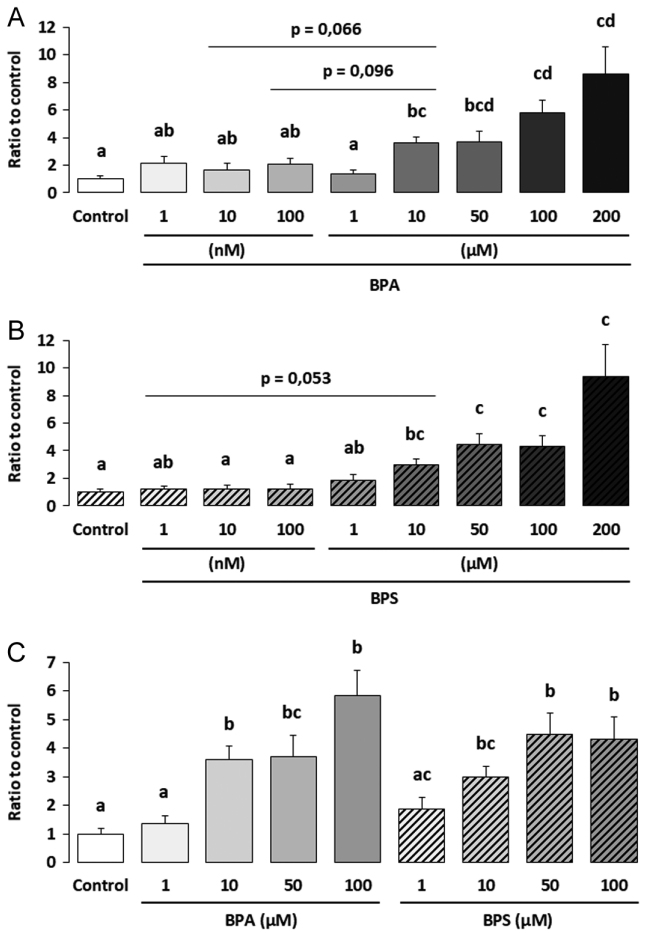

Figure 4 Effects of Bisphenol A or S (BPA or BPS) on oestradiol secretion. Oestradiol secretion were assessed in ovine granulosa cells (GC). The oestradiol concentration was determined in culture medium after 48-h culture in complemented serum-free McCoy's 5A media in presence or absence of BPA (A) or BPS (B) (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM or 200 µM). Data with BPA and BPS were compared statistically for four relevant concentrations (C) (1 µM, 10 µM, 50 µM and 100 µM). The results are expressed as the mean ± S.E.M. of six independent cultures, with each condition performed in duplicate, and normalised to the control condition of each culture experiment. Bars that do not exhibit at least one common letter are significantly different ($P < 0.05$). The actual control value was 28.94 ± 6.08 pg oestradiol/mg protein.

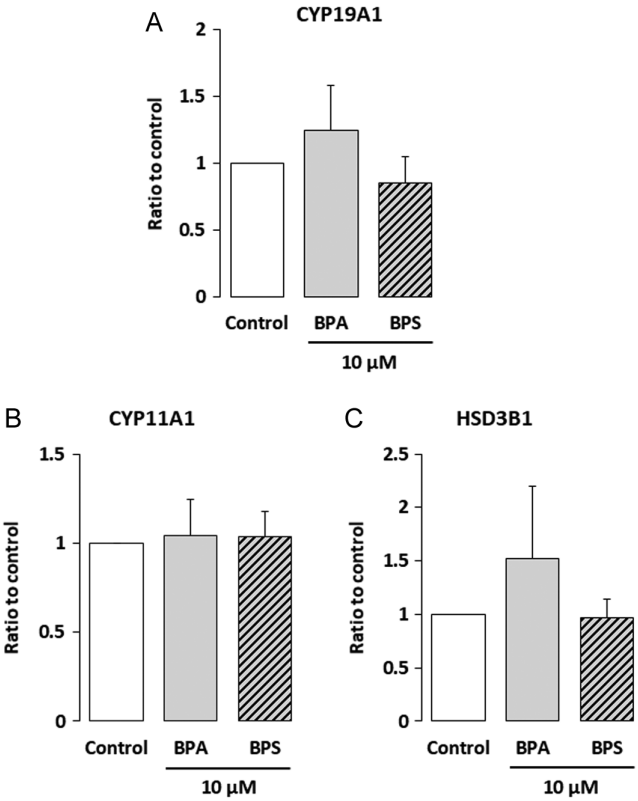

Figure 5 Effects of bisphenol A or S (BPA or BPS) on steroidogenesis enzyme protein expression. The protein expression of three steroidogenesis enzymes, namely cytochrome P450 family 19 subfamily A member 1 (CYP19A1; A), cytochrome P450 family 11 subfamily A member 1 (CYP11A1; B) and hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1; C), was assessed in ovine granulosa cells (GC). Protein levels were determined after 48-h culture in complemented serum-free McCoy's 5A media in presence or absence of BPA or BPS (10 µM). Protein extracts were separated by electrophoresis in a 4-–12% sodium dodecyl sulphate (SDS) polyacrylamide gel. After transfer to nitrocellulose membranes, the proteins were probed with anti-CYP19A1 (A), anti-CYP11A1 (B), anti-HSD3B1 (C) or anti-vinculin. Bands on the blots were quantified, and the protein/vinculin ratio was calculated. The results are expressed as the mean ± S.E.M. of six independent cultures and normalised to the control condition of each culture experiment.

Effects of BPA and BPS on steroidogenic enzymes protein expression

The protein expression of three steroidogenic enzymes was measured in ovine GC after 48-h treatment with 10 µM BPA or BPS (Fig. 5). CYP19A1 (Fig. 5A) is involved in oestradiol production, while CYP11A1 (Fig. 5B) and HSD3B1 (Fig. 5C) are involved in progesterone production. There were no statistical differences in protein levels compared to control for any of these steroidogenic enzymes upon BPA or BPS treatment.

Effects of BPA and BPS on gene expression

The expression of four hormonal receptor genes (*AR*, *PR*, *ESR1* and *ESR2*), three steroidogenic enzyme genes (*CYP19A1*, *CYP11A1* and *HSD3B1*) and the cholesterol transporter gene (*STAR*) was analysed in ovine GC after 48-h treatment with BPA or BPS (Fig. 6 and Table 3). Only 100 µM BPA altered *AR*, namely a 58% decrease ($P < 0.001$) compared to control. BPS did not affect *AR* expression (Fig. 6A). *PR* gene expression did not change significantly compared to control. Nevertheless, there was a two-fold increase ($P = 0.003$) in the expression of this gene with 50 µM BPS compared to 50 µM BPA. This

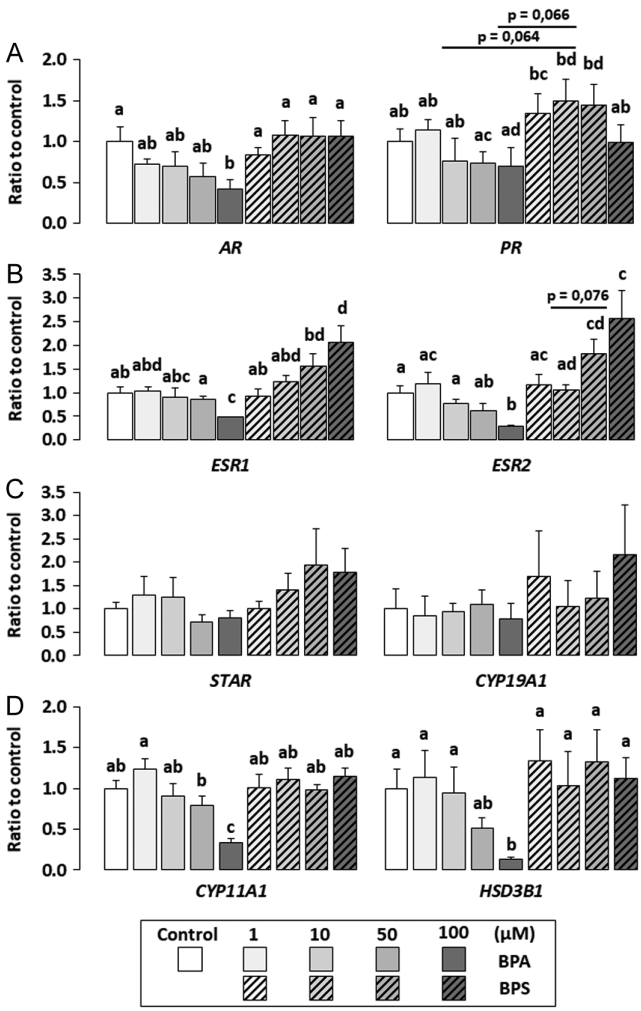

Figure 6 Effects of Bisphenol A or S (BPA or BPS) on gene expression. The gene expression of four hormonal receptors (androgen receptor (*AR*) and progesterone receptor (*PR*); A, oestrogen receptor 1 (*ESR1*) and oestrogen receptor 2 (*ESR2*); B), the cholesterol transporter (steroidogenic acute regulatory protein (*STAR*); C) and three steroidogenic enzyme (*CYP19A1*; C, *CYP11A1* and *HSD3B1*; D) were assessed in ovine granulosa cells (GC). Gene expression was determined after 48-h culture in complemented serum-free McCoy's 5A media in the presence or absence of BPA or BPS (1 µM, 10 µM, 50 µM or 100 µM). Total messenger RNA (mRNA) was extracted from GC and reverse transcribed, and real-time polymerase chain reaction (qPCR) was performed. The geometric mean of two housekeeping genes (beta-actin (*ACTB*) and ribosomal protein L19 (*RPL19*)) was used to normalise gene expression. The results are expressed as mean ± S.E.M. of six independent cultures and normalised to the mean of control condition. Bars that do not exhibit at least one common letter are significantly different ($P<0.05$).

increase occurred as a tendency ($P=0.066$) between 10 µM BPA and BPS (Fig. 6A). Both *ESR1* and *ESR2* showed a similar gene expression pattern. Indeed, there was a two-fold decrease ($P<0.001$) in *ESR1* and *ESR2* expression with 100 µM BPA and a two-fold increase in *ESR1* ($P=0.023$) and *ESR2* ($P<0.001$) for 100 µM BPS compared to control. Moreover, this increase in *ESR2* gene expression already occurred with 50 µM BPS ($P=0.040$). Thus, *ESR1* and *ESR2* gene expression was increased four- and nine-fold ($P<0.001$) with 100 µM BPS compared to 100 µM BPA, respectively. *ESR1* and *ESR2* gene expression was increased two-fold ($P=0.036$) and three-fold ($P<0.001$) with 50 µM BPS compared to 50 µM BPA, respectively (Fig. 6B). Concerning steroidogenic enzyme genes, *CYP19A1* and *STAR* gene expression did not significantly vary with BPA or BPS (Fig. 6C). Only 100 µM BPA caused an effect: a 77% and 87% decrease ($P<0.001$) in *CYP11A1* and *HSD3B1* expression, respectively, compared to control (Fig. 6D), while none of the BPS concentrations altered steroidogenic gene expression.

Effects of BPA and BPS on signalling pathways

Signalling pathways involved in GC functions were investigated after 0, 5, 10, 30 and 60 min treatment in the presence or absence of 10 µM BPA or BPS (Fig. 7). Phosphorylation of Akt, MAPK3/1 and MAPK14, all of which are involved in cell viability and proliferation (Peter & Dhanasekaran 2003, Fan *et al*. 2009, Lapointe & Boerboom 2011), and AMPKα, which is involved in energetic metabolism including lipid metabolism (Scaramuzzi *et al*. 2010), was assessed. For the MAPK3/1 pathway, there was a rapid, transient increase ($P<0.001$) in phosphorylation, reported after 5 min of treatment with control (three-fold), 10 µM BPA (2.5-fold) or 10 µM BPS (two-fold) compared to control before treatment (Fig. 7A). MAPK3/1 phosphorylation was lower ($P<0.001$) after 5, 10 and 30 min of 10 µM BPA treatment compared with control at the same time. There were no reductions after BPS treatment at any time point. For the AMPKα pathway, there was no change in phosphorylation in the control (Fig. 7B). After 60-min treatment, 10 µM BPS increased AMPKα phosphorylation 3.25-fold compared to the control at the same time ($P<0.001$). Ten micromolar BPA did not alter AMPKα phosphorylation compared to either the control or 10 µM BPS. BPS increased ($P<0.001$) AMPKα phosphorylation at 5, 10, 30 and 60 min (6-, 8.5-, 4.6- and 5.2-fold, respectively) compared to control before treatment. Finally, there were no significant differences at any time among control, 10 µM BPA and 10 µM BPS in Akt (Fig. 7C) and MAPK14 (Fig. 7D) phosphorylation.

Discussion

This study aimed to compare the effects of BPA and BPS on ovine GC functions. For the first time, we reported that BPS affected ovine GC steroidogenesis. Concerning progesterone secretion, BPS-induced inhibition occurred at lower concentrations compared to BPA. Moreover, BPS and BPA effects were partially mediated through independent mechanisms because they did not similarly modulate the gene expression of steroidogenic

Table 3 Effects of BPA and BPS on gene expression.

Gene/bisphenol	Control	10 nM	100 nM	1 µM	10 µM	50 µM	100 µM
AR							
BPA	1.000 ± 0.183^{a}	0.958 ± 0.187^{a}	0.898 ± 0.210^{a}	0.721 ± 0.063^{ab}	0.695 ± 0.180^{ab}	0.573 ± 0.167^{ab}	0.420 ± 0.112^{b}
BPS	1.000 ± 0.183^{ab}	1.101 ± 0.119^{a}	0.688 ± 0.140^{b}	0.840 ± 0.089^{ab}	1.082 ± 0.170^{ab}	1.060 ± 0.238^{ab}	1.066 ± 0.185^{ab}
CYP11A1							
BPA	1.000 ± 0.092^{ab}	1.203 ± 0.132^{a}	1.162 ± 0.178^{ab}	1.233 ± 0.135^{a}	0.908 ± 0.151^{ab}	0.788 ± 0.119^{b}	0.329 ± 0.052^{c}
BPS	1.000 ± 0.092	1.123 ± 0.123	1.119 ± 0.200	1.010 ± 0.163	1.111 ± 0.130	0.977 ± 0.074	1.151 ± 0.098
CYP19A1							
BPA	1.000 ± 0.423	1.236 ± 0.360	1.165 ± 0.628	0.855 ± 0.409	0.952 ± 0.162	1.101 ± 0.312	0.789 ± 0.338
BPS	1.000 ± 0.423	0.799 ± 0.252	1.043 ± 0.652	1.698 ± 0.971	1.049 ± 0.556	1.223 ± 0.583	2.157 ± 1.059
ESR1							
BPA	1.000 ± 0.120^{a}	1.121 ± 0.114^{a}	1.198 ± 0.153^{a}	1.037 ± 0.085^{a}	0.912 ± 0.199^{ab}	0.869 ± 0.062^{a}	0.483 ± 0.016^{b}
BPS	1.000 ± 0.120^{ab}	1.052 ± 0.191^{ab}	0.749 ± 0.106^{a}	0.926 ± 0.159^{ab}	1.236 ± 0.123^{bc}	1.557 ± 0.278^{bc}	2.062 ± 0.357^{c}
ESR2							
BPA	1.000 ± 0.151^{a}	0.998 ± 0.211^{a}	1.022 ± 0.207^{a}	1.188 ± 0.239^{a}	0.778 ± 0.084^{a}	0.624 ± 0.153^{ab}	0.290 ± 0.033^{b}
BPS	1.000 ± 0.151^{a}	1.123 ± 0.189^{ac}	0.497 ± 0.110^{b}	1.168 ± 0.217^{ac}	1.058 ± 0.114^{a}	1.817 ± 0.307^{c}	2.574 ± 0.578^{c}
HSD3B1							
BPA	1.000 ± 0.240^{a}	1.444 ± 0.419^{a}	1.329 ± 0.382^{a}	1.129 ± 0.332^{a}	0.937 ± 0.327^{a}	0.510 ± 0.130^{ab}	0.133 ± 0.029^{b}
BPS	1.000 ± 0.240	1.343 ± 0.442	1.247 ± 0.625	1.340 ± 0.380	1.036 ± 0.411	1.325 ± 0.397	1.120 ± 0.256
PR							
BPA	1.000 ± 0.157	0.912 ± 0.152	1.207 ± 0.207	1.136 ± 0.135	0.755 ± 0.287	0.731 ± 0.139	0.701 ± 0.228
BPS	1.000 ± 0.157	1.487 ± 0.228	0.879 ± 0.162	1.338 ± 0.252	1.497 ± 0.267	1.445 ± 0.248	0.982 ± 0.219
STAR							
BPA	1.000 ± 0.138	1.541 ± 0.544	1.693 ± 0.431	1.300 ± 0.394	1.256 ± 0.418	0.717 ± 0.152	0.802 ± 0.151
BPS	1.000 ± 0.138	1.096 ± 0.221	1.190 ± 0.217	1.000 ± 0.163	1.408 ± 0.359	1.950 ± 0.757	1.780 ± 0.516

No letters in common within a row indicates a significant difference ($P < 0.05$).

enzymes and hormonal receptors or signalling pathway activation.

BPA and BPS disrupted progesterone and oestradiol secretion

In this study, we showed that 100 µM BPA decreased ovine GC progesterone secretion; this inhibition occurred with a ten-fold lower BPS concentration (10 µM). Previous studies also demonstrated a decrease in progesterone secretion in rat GC after 100 µM BPA treatment (Samardzija *et al*. 2018), porcine GC after 100 nM BPA treatment (Grasselli *et al*. 2010) or luteinised human GC after 8.8 µM BPA treatment (Mansur *et al*. 2016). Other studies on rat GC (Zhou *et al*. 2008) and pig GC (Mlynarcikova *et al*. 2005) showed BPA dose-dependently changes progesterone secretion. Indeed, inhibitory effects are reported with high concentrations (100 µM), while the opposite effect is observed with low concentrations (100 nM with rat GC and 10 µM with pig GC). BPS reportedly does not affect progesterone secretion at a concentration lower than 100 µM in porcine or bovine GC (Campen *et al*. 2018, Berni *et al*. 2019). On the contrary, here we reported an inhibitory effect of 10 µM BPS on progesterone secretion of ovine GC. In all these models, GC are recovered from similar follicular stages (late maturation but not pre-ovulatory stage), this finding might therefore suggest a higher sensitivity to BPS effects in ovine cells. Nevertheless, differences in culture methods or duration of exposure (up to 6 days) might also explain these differences between species. Intriguingly, neither *PR, STAR, CYP11A1, HSD3B1* gene expression nor CYP11A1, HSD3B1 protein expression were different between control and BPS treated conditions. Further studies are therefore required to elucidate the BPS mechanisms of action impairing progesterone secretion. Progesterone plays a role in the maturation and developmental capacity of the oocyte (Bujnakova Mlynarcikova *et al*. 2018). In fact, low serum progesterone levels are associated with low ovulation percentages in women (Chou & Chen 2018). These data therefore suggest that the BPS-induced impairment in progesterone secretion may be detrimental for the oocyte quality and, consequently, fertility.

Regarding oestradiol, we found that both BPA and BPS increased oestradiol secretion in sheep GC at 10 µM or higher. This result is consistent with an *in vivo* study in mice where injection of BPA or BPS increases serum oestradiol (Shi *et al*. 2017). Such an increase is also reported in porcine GC after 10 µM BPA treatment (Wu *et al*. 2018*a*, Song *et al*. 2019). Nevertheless, in rat and luteinised human GC, BPA treatment (between 10 µM and 100 µM) decreases oestradiol secretion (Zhou *et al*. 2008, Mansur *et al*. 2016, Banerjee *et al*. 2018, Pogrmic-Majkic *et al*. 2019). There are also discrepancies among species with regards to BPS. Indeed, BPS increases oestradiol secretion in bovine GC at a high

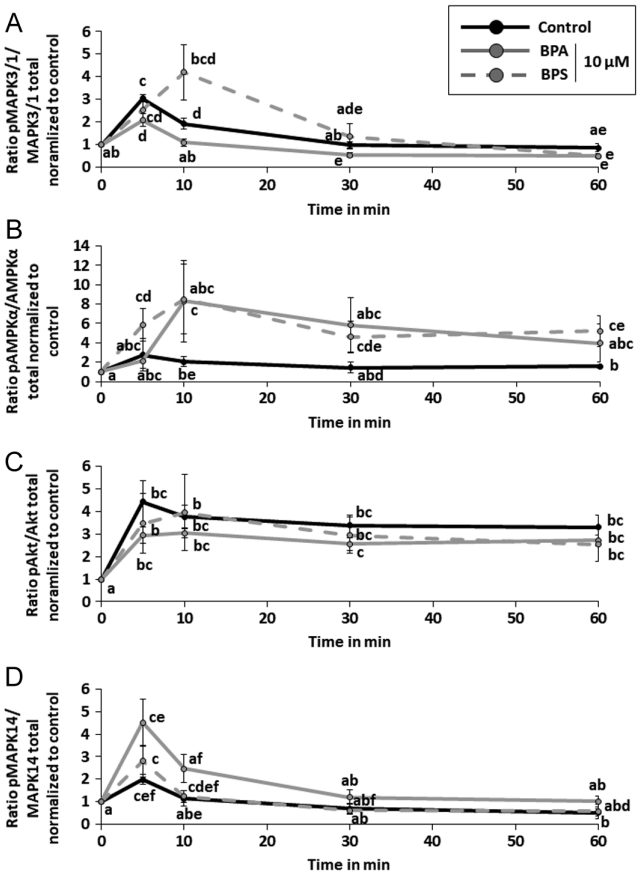

Figure 7 Effects of Bisphenol A or S (BPA or BPS) on protein phosphorylation of signalling pathways. The phosphorylation of signalling pathways proteins, namely mitogen-activated protein kinase 1/3 (MAPK3/1; A), 5′ adenosine monophosphate-activated protein kinase (AMPKα; B), protein kinase B (Akt; C) and mitogen-activated protein kinase 14 (MAPK14; D) were assessed in ovine granulosa cells (GC). Protein levels were determined after 0, 5, 10, 30 or 60 min culture in complemented serum-free McCoy's 5A media in presence or absence (control) of BPA or BPS (10 µM). Protein extracts were separated by electrophoresis in a 4–12% sodium dodecyl sulphate (SDS) polyacrylamide gel. After transfer to nitrocellulose membranes, the proteins were probed with anti-phospho-MAPK3/1 (A) or anti-phospho-AMPKα (B). The blots were stripped and reprobed with antibodies against MAPK3/1 or AMPKα, respectively. Bands on the blots were quantified, and the ratio of phosphorylated protein to total protein was calculated. The results are expressed as the mean ± S.E.M. of four independent cultures and normalised to the control condition before treatment of each culture experiment. No letter in common indicate a significant difference ($P < 0.05$).

concentration (100 µM; Campen *et al*. 2018), while it decreases oestradiol secretion in porcine GC after 1 µM BPS treatment (Berni *et al*. 2019). Oestradiol reportedly exhibits mitogenic and anti-apoptotic effects and is a modulator of the developmental competence of oocytes (Campen *et al*. 2018, Chou & Chen 2018). Moreover, the balance between oestradiol and progesterone secretions is crucial, and its disruption may impact reproductive efficiency (Chou & Chen 2018). These data therefore suggest that both BPA and BPS may affect oocyte competence through an impairment in GC oestradiol and progesterone secretion.

Our data suggested that BPA and BPS exerted a similar effect on ovine GC steroidogenesis. Interestingly, BPS altered the secretion of progesterone at a ten-fold lower concentration than BPA. Thus, regarding steroidogenesis, BPS does not seem to be a safe alternative to BPA. Additionally, compared to the literature, the ewe model used here appears to be more sensitive to BPS compared to other species. Even if all studies reported alterations in hormone secretions, there are discrepancies among studies and species. These differences may be partially related to species differences, follicular stage of GC recovery, treatment durations, doses and/or culture media composition (serum-free or not). Finally, BPA and BPS affected steroidogenesis at concentrations that are supraenvironmental compared with human female body fluid levels (nanomolar range). However, in this study, cells are exposed to high doses of bisphenols for only 48 h, corresponding to an acute exposure. Of note, effects observed after acute high supraenvironmental doses might not reflect low chronic exposure consequences, which correspond to human exposure. Further experiments are therefore required to investigate impacts of chronic low exposure.

BPA, but not BPS, impaired ovine GC viability and proliferation

We showed that only the highest BPA concentration (200 µM) induced a significant increase in cell mortality. No toxicity was evidenced with BPS, even at high concentrations. Lower concentrations of both BPA and BPS (up to 100 µM) did not exhibit any effect on ovine GC cellular viability. This result is consistent with previous studies on luteinised human GC (Pogrmic-Majkic *et al*. 2019), rat GC (Samardzija *et al*. 2018) and bovine GC (Campen *et al*. 2018).

We also reported a significant decrease in ovine GC proliferation after 50 µM, 100 µM or 200 µM BPA treatment, while there was an effect only after 200 µM BPS treatment. Differences among studies and species have been reported. Indeed, in human luteinised cells, up to 44 µM BPA does not impact proliferation (Dominguez *et al*. 2008), whereas in porcine GC there is a decrease in proliferation after 10 µM BPS treatment (Berni *et al*. 2019). During folliculogenesis, GC exhibit increased mitotic activity and secreted hormones, both of which are crucial for the growth and maturation of oocytes (Monniaux *et al*. 2019). The data obtained here may suggest that, considering cellular proliferation alone, BPS is not as detrimental as BPA.

BPA and BPS effects may be mediated through different mechanisms of action

Regarding steroidogenic enzymes, we showed a decrease in the gene expression of *CYP11A1* and *HSD3B1* after 100 µM BPA treatment. Both enzymes are involved in progesterone synthesis in GC. This result is consistent with previous studies in human and porcine GC (Mansur *et al*. 2016, Bujnakova Mlynarcikova *et al*. 2018) and with the decrease in progesterone secretion reported for this concentration in the present study. Ten micromolar BPA did not affect CYP11A1 and HSD3B1 protein and mRNA expression. It would be interesting to assess these protein expressions at the concentrations that affected gene expression (namely 100 µM BPA). BPA did not affect the protein or gene expression of the enzyme CYP19A1, which is involved in oestradiol synthesis. HSD17B is also involved in oestradiol synthesis, and it would be interesting to investigate its expression. *STAR* gene expression was not modified after BPA treatment, and data are consistent with results in luteinised human GC (Mansur *et al*. 2016). BPS did not modulate protein or gene expression of any steroidogenic enzymes, while a decrease in progesterone and an increase in oestradiol is reported. This result is consistent with previous *in vivo* experiment in mice, where no difference in mRNA expression of steroidogenic enzymes was reported after injections of 50 µg/kg BPS (Shi *et al*. 2017). However, in addition to assessing steroidogenic enzyme expression, it would be interesting to measure their activities, as change in their activities could contribute to hormonal imbalance. Thus, BPA and BPS do not appear to act through similar pathways on steroidogenesis. Because our results in terms of mechanisms of action do not reconcile with the modifications of steroidogenesis observed in terms of both progesterone and oestradiol secretion, we could speculate that only the secretion and not the synthesis of these steroids might be affected. Further studies are needed to elucidate the detailed mechanisms of action.

We also investigated hormonal receptor expressions. Both *ESR1* and *ESR2* expression were decreased by BPA and increased by BPS. These contradictory effects may be related to a difference in affinity towards oestrogen receptors. *PR* gene expression was increased after 10 µM and 50 µM BPS treatment compared with the same BPA concentrations. At these concentrations, BPS (but not BPA) decreased progesterone secretion. Further studies are required to elucidate how BPA and BPS impaired progesterone secretion. The increase in *PR* expression may therefore be a compensatory consequence of the decrease in progesterone secretion (Duffy & Stouffer 1995). Finally, BPA decreased *AR* gene expression. This result is consistent with the BPA-induced inhibition of proliferation. Indeed, there is a positive correlation between *AR* gene expression in GC and proliferation (Chou & Chen 2018).

Concerning signalling pathways, BPA induced less MAPK3/1 phosphorylation compared to the control. This phenomenon was not the same with BPS treatment. In addition to the already described mechanisms that involve binding to oestrogen receptors, new mechanisms emerged that should be investigated to elucidate the BPA and BPS mechanisms of action. Indeed, in HEK293T cells, both bisphenols can associate with a Ras protein, K-Ras4B; the affinity of BPA is much higher compared to BPS (Schopel *et al*. 2016). Ras functions as a molecular switch that leads to the activation of signalling cascade, such as MAPK3/1 or AKT phosphorylation (Schopel *et al*. 2018). Differences between the BPA and BPS affinity towards Ras protein may help explain the difference in signalling pathway activation observed in the present study. Both BPA and BPS increased phosphorylation of AMPKα compared to control. The AMPKα signalling pathway is involved in energy metabolism and, in particular, the metabolism of cholesterol, which is a precursor for steroid production (Scaramuzzi *et al*. 2010). In addition to the study of cholesterol transport, assessed in this study through *STAR* gene expression, it would be interesting to measure cholesterol production in GC. Furthermore, a study in rat GC showed that BPA exposure alters cholesterol homeostasis – rendering it inaccessible to mitochondria – and thus decreases progesterone production (Samardzija *et al*. 2018). The disparities between BPA and BPS on gene expression and signalling pathway activation may suggest that BPA and BPS do not share all their mechanisms of action.

Several limitations occurred in this study, related to the use of ovine ovaries collected in slaughterhouse. Indeed, health and metabolic status of ewes are unknown. Culture of GC was performed in atmospheric air humidified with 5% CO_2 and 20% O_2. This excess of O_2 is not physiological (Thompson *et al*. 2015) and could have potential consequences on cellular functions (i.e. production of reactive oxygen species) (Stuart *et al*. 2018). Also, McCoy medium used for culture contained phenol red which has oestrogenic properties and might not be ideal to evaluate endocrine disrupting properties of compounds such as bisphenols (similar effects on GC progesterone secretion were observed in phenol red free and phenol red containing medium, Supplementary Fig. 3). Nevertheless, the medium used also contained oestradiol, because GC are oestradiol-producing cells. As oestradiol has stronger oestrogenic properties, it renders negligible the oestrogenic effect of phenol red. The effects of BPA and BPS are therefore studied in oestrogenic environment, corresponding to physiological conditions. Here, we described the acute effects (48-h culture) of BPA and BPS on GC steroidogenesis in adult ewe. A large range of BPA and BPS doses were evaluated, ranging from environmental (1 nM to 100 nM) to supraenvironmental concentrations (1 µM to 200 µM). We showed here that BPA and BPS reduced progesterone secretion and

increased oestradiol secretion. These data suggested that BPS might not be a safe alternative for BPA, which could have a high relevance for human health and reproduction. Nevertheless, our findings are related to supraenvironmental concentrations.

Conclusions

The present study compared the effects of BPA and BPS on ovine GC. We demonstrated that BPA and BPS altered steroidogenesis by increasing oestradiol secretion and reducing progesterone secretion. BPS was even more detrimental to progesterone secretion compared to BPA because it induced a reduction at a concentration ten-fold lower than BPA. On the contrary, BPA reduced cell proliferation at a lower concentration compared with BPS. Our results on gene expression and signalling pathways suggest that the mechanisms of action involved by which BPA and BPS exert their effects on GC may not be completely similar. Taken together, BPS does not appear to be a safe alternative to BPA. Further investigations are needed to elucidate the BPA and BPS mechanisms of action involved in ovine GC steroidogenesis alterations.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0575.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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Author contribution statement

O T and M J performed experiments, analysed the data and wrote the paper. A D and P P performed experiments. A B and V M helped write the paper. S E conceived the study, performed experiments, analysed the data and wrote the paper.

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