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The impact of bisphenol S (BPS) on bovine granulosa and theca cells

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Abstract

Bisphenol S (BPS) is an endocrine disrupting chemical with multiple potential mechanisms of action, including as an estrogen receptor agonist. BPS is increasingly used in plastics and thermal receipts as a substitute for bisphenol A, which has been phased out due to concerns about human health implications. The ability of BPS to alter female reproductive function in mammals has not been widely studied, despite the importance of normal hormone signaling for female reproduction. The aim of this study was to investigate how BPS (in a wide range of doses, including very low doses) affects granulosa cell and theca cell steroid hormone production and cell viability in the bovine. Granulosa cell estradiol production was stimulated when cells were exposed to 100 μM BPS under basal conditions, but there was no effect of BPS when cells were stimulated with follicle stimulating hormone (FSH). Additionally, there was no effect of BPS on granulosa cell progesterone production or cell viability under basal or FSH-stimulated conditions. BPS did not affect theca cell androstenedione or progesterone production, or theca cell viability under basal or luteinizing hormone-stimulated conditions. This study suggests for the first time that BPS may alter estradiol production by bovine granulosa cells, albeit at a concentration that is unlikely to be physiologically relevant. Further studies are needed to determine the effects of BPS on the bovine oocyte and on other functions of follicular cells.

Keywords

granulosa cells; theca cells; bisphenol S; endocrine disruptors; steroid hormones; ovarian follicle

Introduction

Endocrine disrupting chemicals (EDCs) are compounds that have the ability to alter steroid hormone signaling (Kavlock et al., 1996), thereby disrupting normal endocrine function and often resulting in deleterious health outcomes. Although the primary focus of EDC research has been on effects on humans and aquatic species, agricultural species also have the

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Conflict of interest statement

The authors do not have an conflicts of interest to declare.

potential to be negatively affected. Fertility in dairy cattle has been in decline in recent decades (Lucy, 2001), and although the exact cause is unknown some have suggested that exposure to EDCs may be at least partially responsible (Sweeney, 2002). There are multiple potential routes of EDC exposure for farm animals (Rhind, 2005). Contamination of water sources, including drinking water and standing water, appears to be the most likely source of exposure of farm animals to EDCs (Fromme et al., 2002), although soil and sediment contamination are also known to contribute to overall exposure levels (Ingelido et al., 2009).

Arguably the best studied EDC is bisphenol A (BPA) which is used in polycarbonate plastics, epoxy lined tin cans, and in thermal receipts (Vandenberg et al., 2007). Due to increasing regulation surrounding the use of BPA amid concern about the human health implications, manufacturers have begun using substitute chemicals, such as BPS, which have been found to be present in food and personal care products (Liao and Kannan, 2013, 2014; Ye et al., 2015). Liao et al (2012) reported that, at least in humans, daily exposure levels of BPS is similar or higher than BPA exposure levels. Like BPA, BPS has been reported to interact with the estrogen receptor (Kuruto-Niwa et al., 2005; Molina-Molina et al., 2013), albeit with 3–10 fold lower potency than BPA (Chen et al., 2002; Teng et al., 2013). Further, BPS has been reported to have additional modes of action not reported for BPA, including alteration of progestogen production in human adrenal cortico-carcinoma cells (Rosenmai et al., 2014). Therefore, the biological effects of the rising EDC BPS should be investigated independently of its structural relationship with BPA.

Although a recent study found that BPS was not measurable in the follicular fluid of abattoir derived pigs (Žalmanová et al., 2017), it is possible that the BPS burden of farm animals will increase over time, reflecting the increasing human burden (Ye et al., 2015). Due to the herbivorous diet of cattle, it is likely that they would be exposed to relatively low levels of endocrine disruptors, as these tend to bioaccumulate in animals at higher trophic levels (Magnusson and Persson, 2015). Little is known about the levels of BPS that cattle may be exposed to, although these are likely to be similar to BPA concentrations, which were 229–305 ng/L (1–1.3 nM) in urine from replacement cows (Zhang et al., 2014). Although EDC levels may be low in cattle, low dose effects are well documented to occur in the absence of effects at higher doses (Vandenberg and Prins, 2016), and thus endocrine disruption in cattle may still be of concern.

The effects of BPS on the ovary are largely unknown. In zebrafish, BPS altered plasma estradiol levels and reduced the number of eggs produced (Ji et al., 2013; Naderi et al., 2014). In the pig, BPS disrupted oocyte meiotic progression and spindle formation, and altered cumulus cell gene expression and hyaluronic acid production (Žalmanová et al., 2017). However, to the best of our knowledge, there is no information about whether BPS affects follicular cell function in mammals. BPA is well established to affect female fertility (Ziv-Gal and Flaws, 2016), including altering the function of granulosa cells (Mansur et al., 2016; Mlynarčíková et al., 2005; Xu et al., 2002), and theca cells (Zhou et al., 2008) in a variety of species. Although BPS and BPA are structurally related, the two compounds exhibit different potencies for steroid hormone receptors (Rochester and Bolden, 2015) demonstrating that the effects of BPS on female reproduction cannot be inferred from what is known about BPA.

The aim of this study was to determine how BPS influences bovine granulosa and theca cell steroidogenesis and cell viability. To address this aim, the effects of a wide range of BPS doses on granulosa and theca cell were tested in the presence or absence of gonadotropin hormones. The inclusion of basal and gonadotropin-stimulated culture conditions allowed determination of potential interactions with follicle stimulating hormone (FSH) and luteinizing hormone (LH).

Materials and Methods

All reagents were purchased from Sigma Aldrich (St Louis, MO) unless otherwise stated.

Isolation of granulosa and theca cells

Ovaries were sourced from a local slaughterhouse (Champlain Beef; Whitehall, NY) and transported back to the laboratory in 0.9% NaCl, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Ethical approval was not required as this study used slaughterhouse discard material. Antral follicles (3–7 mm in diameter) were dissected from ovaries with an active corpus luteum into Minimum Essential Medium, Hanks' Balanced Salts (HMEM (Lonza; Walkersville, MD) supplemented with 25 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.4% w/v bovine serum albumin, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml heparin). Each replicate usually pooled cells from 2–3 animals to generate enough material. To collect the granulosa cells, the follicles were aspirated, bisected and the halves were scraped with an inoculating loop, releasing the granulosa cells into the medium.

To collect the theca cells, the follicle halves were vortexed in dissection medium, and the dislodged granulosa cells were discarded. This was repeated three times. The theca interna layer was carefully peeled from the basement membrane using fine forceps under a dissecting microscope. The theca layers were pooled and incubated for with 1 mg/ml collagenase type IV and 100 μg/ml trypsin inhibitor for 1 h at 37°C with shaking. The cells were triturated for 3 min with a Pasteur pipet four times during the incubation period.

In order to lyse the red blood cells, the granulosa or theca cells were resuspended in one part 1× phosphate buffered saline (PBS), followed by two parts double distilled water. The cells were inverted for 10 seconds to lyse any red blood cells before two parts 2× PBS was added to restore isotonicity. The cells were resuspended in either: granulosa cell culture medium (Phenol red-free McCoy's 5A medium (GE Healthcare Life Sciences; Logan, UT) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM L-glutamine, 20 mM HEPES, 0.1% w/v bovine serum albumin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 10 ng/ml insulin, and 100 nM androstenedione (Steraloids; Newport, RI)) or theca cell culture medium (phenol red-free McCoy's 5A medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM L-glutamine, 20 mM HEPES, 0.1% w/v bovine serum albumin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 10 μg/ml insulin).

Culture of granulosa and theca cells

Cells were counted using a hemocytometer, and viability was determined using the trypan blue exclusion method. Average granulosa cell viability was 56% and average theca cell viability was 92%. Cells were seeded into wells of Primaria™ coated 96-well tissue culture plates (Corning; Corning, NY) at a density of 75×10^3 cells/well. The appropriate pre-warmed culture medium with or without treatments was added to each well so that the final volume in each well was 250 μl. Cells were treated with the vehicle only control (0.05% DMSO) or BPS (Sigma Aldrich catalog number 103039) at concentrations from 1 fM increasing in 10-fold increments up to 100 μM (12 concentrations total). A control in which the cells were incubated with medium alone (i.e. without treatment or vehicle) was also included. Treatment of the granulosa cells with BPS was performed in the presence or absence of 0.33 ng/mL FSH (oFSH-19-SIAFP; National Hormone & Peptide Program, Torrance, CA). This concentration of FSH was chosen to approximate the plasma levels of FSH during the final 6 days of follicle growth (Butler et al., 2008). Treatment of the theca cells with BPS was performed in the presence or absence of 100 pg/ml LH (oLH-26; National Hormone & Peptide Program, Torrance, CA). This concentration of LH was chosen as it resulted in maximal androstenedione production by theca cells (Glister et al., 2005) and approximated the follicular fluid concentration in the cow at certain times of the estrous cycle (Dieleman et al., 1983). The cells were cultured at 38.5°C with 5% CO_2 in air for 6 days. Every 48 h, 80% of conditioned medium was removed, and replaced with fresh medium and treatments.

Determination of cell viability

After 6 days culture, 175 μl of conditioned medium was removed and stored at −80°C for measurement of steroid hormones. Cells were incubated with 200 μl neutral red dye (0.05 μg/ml) in the appropriate culture medium for 3 h at 38.5°C. Following centrifugation and removal of the dye, the cells were fixed with 4% paraformaldehyde. The paraformaldehyde was replaced with acidified ethanol (1% acetic acid, 50% ethanol, 49% water). The plate was incubated at 4°C overnight prior to the absorbance being measured at 540 nm.

Enzyme-linked immunosorbent assays

Concentrations of estradiol were measured in the conditioned medium collected from granulosa cell cultures using an ELISA kit (Cayman Chemicals; Ann Arbor, MI) according to the manufacturers' instructions. The interassay coefficient of variation was 14% and the intraassay coefficient of variation was 7%.

Concentrations of androstenedione were measured in conditioned medium collected from theca cell cultures using an ELISA kit (Eagle Biosciences; Nashua, NH). The interassay coefficient of variation was 8% and the intraassay coefficient of variation was 4%.

Concentrations of progesterone were measured in conditioned medium collected from either granulosa or theca cell cultures using an ELISA kit (Cayman Chemicals; Ann Arbor, MI). The interassay coefficient of variation was 9% and the intraassay coefficient of variation was 5%.

Statistical analysis

Data are expressed relative to the untreated control. Data represent the mean ± standard error of the mean. Each experiment was independently repeated 5–9 times. The data were analyzed by a Kruskal-Wallis test with a Dunn-Bonferroni post-hoc test. Differences were considered significant when $P<0.05$.

Results

Granulosa cells

Estradiol concentrations in the untreated controls were 0.9 ± 0.3 and 2.0 ± 0.6 ng/ml under basal and FSH-stimulated conditions respectively. Basal secretion of estradiol was increased by BPS at a concentration of 100 μM ($P<0.0001$), as depicted in Fig 1A (unfilled bars). None of the lower concentrations of BPS had an effect on granulosa cell estradiol production under basal conditions. FSH induced a 2-fold increase in estradiol production in untreated cells. Under FSH-stimulated conditions, there was no effect of BPS on granulosa cell estradiol production (Fig 1A, filled bars). Progesterone concentrations in the untreated controls were 36.2 ± 7.3 and 103.1 ± 34.0 ng/ml under basal and FSH-stimulated conditions respectively. Secretion of progesterone was not affected by any concentration of BPS under basal or FSH-stimulated conditions (Fig 1B). FSH induced a 3-fold increase in progesterone production in untreated granulosa cells. There was no effect of BPS on granulosa cell viability under basal or FSH-stimulated conditions (Fig 1C).

Theca cells

Androstenedione concentrations in the untreated controls were 2.7 ± 1.2 and 84.1 ± 17.9 ng/ml under basal and LH-stimulated conditions respectively. Secretion of androstenedione by theca cells was unaffected by BPS under basal or LH-stimulated conditions (Fig 2A). LH induced a ~30-fold increase in androstenedione production in untreated theca cells. Progesterone concentrations in the untreated controls were 7.1 ± 1.0 and 88.5 ± 20.5 ng/ml under basal and FSH-stimulated conditions respectively. Secretion of progesterone was also unaffected by BPS under basal or LH-stimulated conditions (Fig 2B). LH induced a 12-fold increase in progesterone production in untreated theca cells. There was no effect of BPS on theca cell viability under basal or LH-stimulated conditions (Fig 2C).

Discussion

The current study revealed that 100 μM BPS induced an increase in basal estradiol secretion by bovine granulosa cells. There was no effect of BPS on granulosa cell function under FSH-stimulated conditions, nor on theca cells under basal or LH-stimulated conditions. These results are the first to examine the effects of BPS on granulosa and theca cells in a mammalian species.

Estradiol produced by the granulosa cells is a modulator of oocyte developmental competence and is also an important component of the hypothalamic-pituitary-gonadal (HPG) axis. Therefore alterations in granulosa cell estradiol production by BPS may disrupt the HPG axis, as previously described with regards to BPA (Xi et al., 2011). The

intraovarian roles of estradiol are less defined. For example, estradiol caused reductions in oocyte maturation and increased spindle abnormalities (Beker et al., 2002; Woudenberg et al., 2004) but also enhanced oocyte cytoplasmic maturation and improved fertilization and cleavage rates (Tesarik and Mendoza, 1995). A recent study demonstrated that BPS induced abnormalities in porcine oocytes, including *in vitro* maturation rates, spindle morphology, and cumulus expansion (Žalmanová et al., 2017). Investigation into the effects of BPS in the whole follicle is necessary to elucidate the modulating impact that intrafollicular interactions between cell types may have.

Our finding that BPS increased estradiol production by bovine granulosa cells is consistent with previous reports in the zebrafish and mice where BPS upregulated plasma estradiol levels (Ji et al., 2013; Naderi et al., 2014; Shi et al., 2017). Although beyond the scope of this study, further investigation will reveal whether BPS alters estradiol production by affecting aromatase expression, such as in porcine cumulus cells and oocytes (Žalmanová et al., 2017), or via an alternate mechanism, such as changes in the aromatase inhibitor PPARγ, as is the case in murine preadipocytes (Ahmed and Atlas, 2016). In addition to altering aromatase expression in porcine cumulus cells and oocytes, BPS also altered estrogen receptor α and β expression (Žalmanová et al., 2017). In prepubertal mice, BPS also prevented follicle activation in response to an estrogen challenge (Hill et al., 2017). These data suggest that BPS causes dysregulation of the estrogen hormone synthesis and signaling pathway at multiple levels.

Interestingly, our finding that BPS stimulates granulosa cell estradiol production is in contrast to the reported effects of BPA. These disparities demonstrate that the effects of BPA cannot be extrapolated to make conclusions about BPS or other structurally related substitutes, and that thorough testing is required for each individual chemical. Previous studies have reported that μM concentrations of BPA reduce estradiol production in rat and human granulosa cells (Mansur et al., 2016; Zhou et al., 2008). All of these studies also detected BPA-mediated alterations in granulosa cell progesterone production, whereas no effects of BPS on progesterone production were found in the current study. Further testing to provide a direct comparison within species is needed.

There was no effect of BPS on theca cell steroid hormone production or on granulosa cell progesterone production in our study. This suggests that BPS specifically targets the estradiol pathway as theca cells have little to no capacity for aromatizing androgens to produce estrogens, in contrast to granulosa cells (Fortune and Armstrong, 1978). With regards to the effects of BPS on progesterone production, a previous study by Feng and colleagues (2016) reported that BPS altered progesterone secretion, but had no effect on estradiol production. However, that study was undertaken in the human adrenocortical carcinoma cell line H295R whereas the current study utilized primary granulosa and theca cells, suggesting that the effects of BPS are cell type specific.

Although we did see an increase in granulosa cell estradiol production under basal conditions after exposure to 100 μM, this concentration is unlikely to occur naturally in cattle. Many EDCs are generally thought to bioaccumulate, due to their small size and lipophilic nature (Diamanti-Kandarakis et al., 2009), and thus cattle are likely to be exposed

to low levels due to their lower trophic levels (Magnusson and Persson, 2015). To the best of our knowledge, no data are available for the concentrations of BPS in cattle or other agricultural species, with the exception of Žalmanová et al (2017) who reported that BPS was not detectable in porcine follicular fluid. Further studies are needed to determine follicular fluid levels of EDCs which would reveal the local burden for cells of the developing follicle. The current study saw no effects of physiologically relevant low dose concentrations (likely to be in the pM or nM concentration range) of BPS on granulosa or theca cell steroidogenesis or viability. Our study also did not detect a non-monotonic dose response (NMDR) curve, described as when the sign of the curve changes producing a U or inverse-U shaped curve. NMDRs have been reported for many endocrine disrupting chemicals (Lagarde et al., 2015) including BPS (Eladak et al., 2015; Nadal et al., 2017; Žalmanová et al., 2017).

Interestingly, our finding that BPS increased granulosa cell estradiol production under basal but not FSH-stimulated conditions suggests that FSH may inhibit the actions of BPS on estradiol synthesis. Little is known about the potential interactions between gonadotropins and endocrine disruptors (Kwintkiewicz and Giudice, 2009), despite the important role of changing concentrations of FSH and LH throughout the estrous cycle on granulosa and theca cell steroid hormone production (Hillier et al., 1994). BPS is converted into glucuronide and sulfate conjugated forms by a number of zebrafish and human cell lines and these conjugated forms have significantly lower estrogenic activity than the unconjugated form (Le Fol et al., 2015). Levels of glucuronosyltransferase enzymes have been reported to be upregulated in response to gonadotropins in rats (Becedas et al., 1998; Magnanti et al., 2000). Therefore, further investigation into whether FSH alters the ratio of conjugated/unconjugated BPS in bovine granulosa cells is warranted. Furthermore, given the importance of changing concentrations of gonadotropins, it will be pertinent to test a range of FSH and LH concentrations to determine whether ovarian cells are more or less susceptible to BPS at different stages of the estrous cycle.

In summary, we report for the first time that at a high dose, BPS alters estradiol production by bovine granulosa cells. Furthermore, we discovered that FSH had a protective effect, thereby attenuating the effect of BPS on granulosa cell estradiol production. Under our experimental conditions, other measures (cell viability, androstenedione production, and/or progesterone production) were not affected in either granulosa or theca cells. Future investigations will reveal whether BPS alters other granulosa cell and theca cell functions that ultimately may impair oocyte quality.

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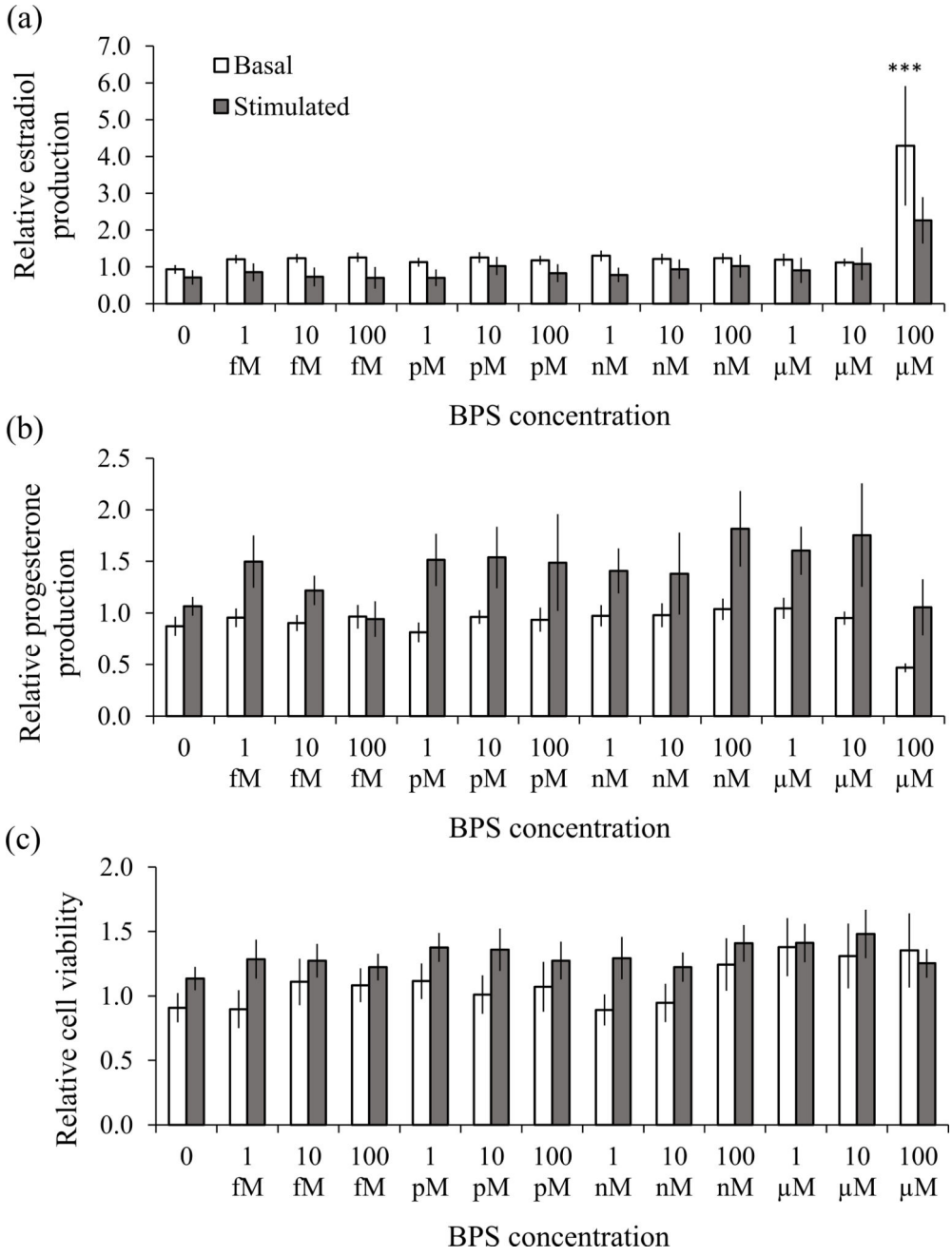

Fig 1. Effect of BPS on granulosa cell estradiol (a) and progesterone (b) production and cell viability (c)

Cells were incubated with various concentrations of BPS or the vehicle alone under basal (unfilled bars) and FSH-stimulated (filled bars) conditions for 6 days. Estradiol and progesterone concentrations were measured in the conditioned medium, and are expressed relative to the untreated control. Values represent the mean ± SEM (9–10 independent experiments for basal conditions, and 5–7 independent experiments for stimulated conditions). Asterisks indicate significant differences compared to the vehicle alone control group (*** $P<0.0001$).

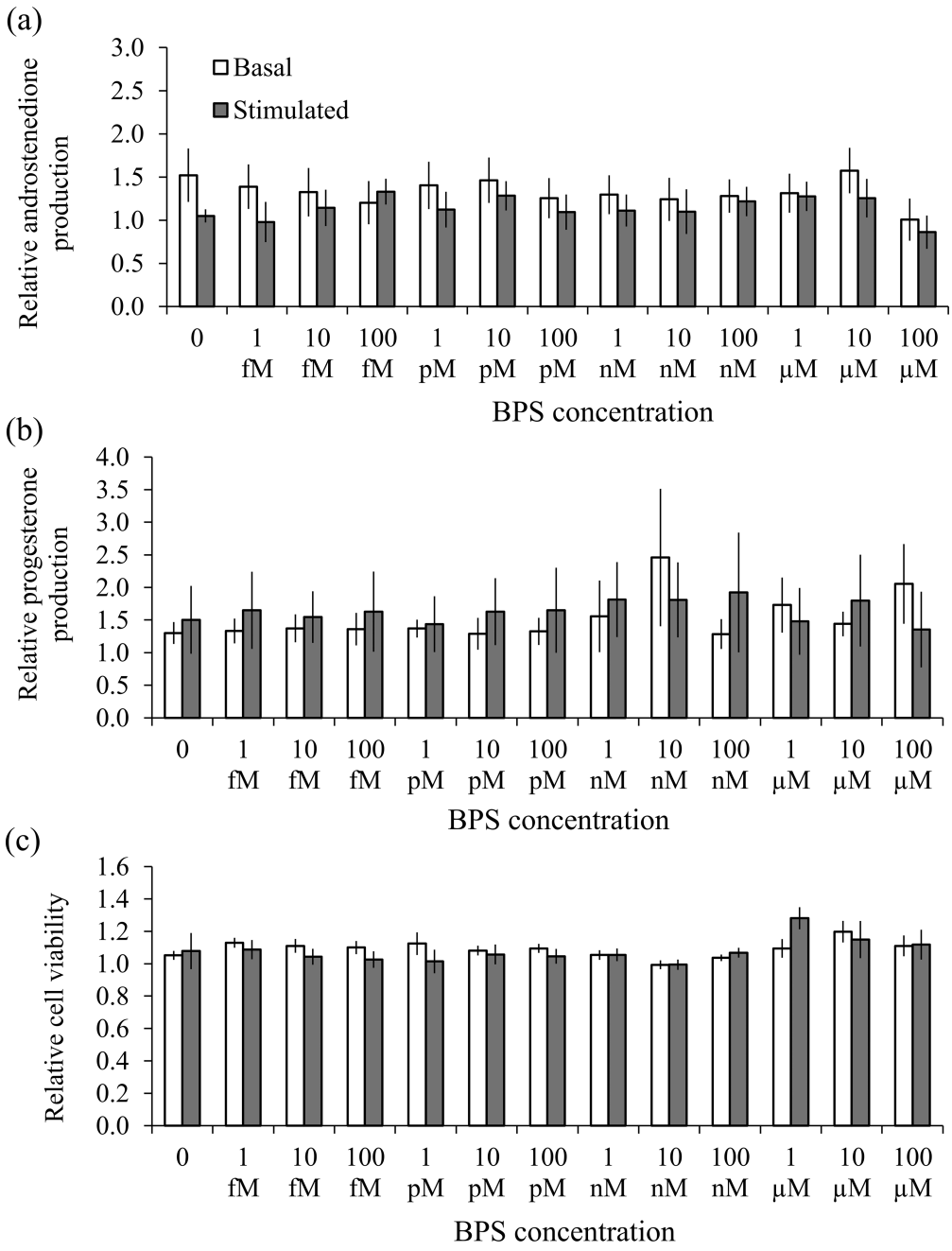

Fig 2. Effect of BPS on theca cell androstenedione (a) and progesterone (b) production and cell viability (c)

Cells were incubated with various concentrations of BPS or the vehicle alone under basal (unfilled bars) and LH-stimulated (filled bars) conditions for 6 days. Androstenedione and progesterone concentrations were measured in the conditioned medium, and are expressed relative to the untreated control. Values represent the mean ± SEM (6–7 independent experiments for basal conditions, and 5 independent experiments for stimulated conditions). There were no significant differences between any groups ($P>0.05$).