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# Mixtures of xenoestrogens disrupt estradiol-induced non-genomic signaling and downstream functions in pituitary cells

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## Abstract

**Background:** Our study examines the effects of xenoestrogen mixtures on estradiol-induced non-genomic signaling and associated functional responses. Bisphenol-A, used to manufacture plastic consumer products, and nonylphenol, a surfactant, are estrogenic by a variety of assays, including altering many intracellular signaling pathways; bisphenol-S is now used as a bisphenol-A substitute. All three compounds contaminate the environment globally. We previously showed that bisphenol-S, bisphenol-A, and nonylphenol alone rapidly activated several kinases at very low concentrations in the GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> rat pituitary cell line.

**Methods:** For each assay we compared the response of individual xenoestrogens at environmentally relevant concentrations ( $10^{-15}$ – $10^{-7}$  M), to their mixture effects on  $10^{-9}$  M estradiol-induced responses. We used a medium-throughput plate immunoassay to quantify phosphorylations of extracellular signal-regulated kinases (ERKs) and c-Jun-N-terminal kinases (JNKs). Cell numbers were assessed by crystal violet assay to compare the proliferative effects. Apoptosis was assessed by measuring caspase 8 and 9 activities via the release of the fluorescent product 7-amino-4-trifluoromethylcoumarin. Prolactin release was measured by radio-immunoassay after a 1 min exposure to all individual and combinations of estrogens.

**Results:** Individual xenoestrogens elicited phospho-activation of ERK in a non-monotonic dose- (fM-nM) and mostly oscillating time-dependent (2.5-60 min) manner. When multiple xenoestrogens were combined with nM estradiol, the physiologic estrogen's response was attenuated. Individual bisphenol compounds did not activate JNK, while nonylphenol did; however, the combination of two or three xenoestrogens with estradiol generated an enhanced non-monotonic JNK dose-response. Estradiol and all xenoestrogen compounds induced cell proliferation individually, while the mixtures of these compounds with estradiol suppressed proliferation below that of the vehicle control, suggesting a possible apoptotic response. Extrinsic caspase 8 activity was suppressed by estradiol, elevated by bisphenol S, and unaffected by mixtures. Intrinsic caspase 9 activity was inhibited by estradiol, and by xenoestrogen combinations (at  $10^{-14}$  and  $10^{-8}$  M). Mixtures of xenoestrogens impeded the estradiol-induced release of prolactin.

**Conclusions:** In mixtures expected to be found in contaminated environments, xenoestrogens can have dramatic disrupting effects on hormonal mechanisms of cell regulation and their downstream functional responses, altering cellular responses to physiologic estrogens.

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## Background

Humans and wildlife do not usually experience XEs as single compounds, and in fact, they probably are exposed to dozens of them simultaneously, if low concentration ranges are considered [1,2]. Newer studies demonstrating that many of these compounds are quite active at very low concentrations necessitate examination of this question to determine if these multiple actions pose a greater health hazard. We have chosen several compounds to study relatively simple multiple exposure scenarios: bisphenol A (BPA); its recently introduced alternative, bisphenol S (BPS); and another ubiquitous environmental estrogen, nonylphenol (NP). Our studies are well-based in our knowledge of their performance as single compounds.

Bisphenol A (BPA) is a well-known endocrine disruptor that has been commercially used since 1957 [3] for the production of consumer plastic goods, the inner lining of metal food cans and drums, as well as the coating of thermal receipt paper [4,5]. Humans are typically exposed to BPA by skin contact and consumption of contaminated food and water that have come in contact with packaging containing BPA, particularly during the heating of plastic containers [6]. A survey by the National Health and Nutrition Examination Survey (NHANES) found levels of BPA to range from 0.4 - 149  $\mu\text{g/L}$  (1.8 - 660 nM) in urine samples from 92.6% of U. S. residents  $\geq 6$  years of age [7].

*In-vivo* studies have linked the presence of BPA to developmental disruptions including uterine morphological alterations [8], disruptions in gonadotropin releasing hormone signaling [9,10], and increases in the incidence of ovarian cyst-adenomas when administered prenatally to female rats [11,12]. Human epidemiological studies have also associated BPA exposure with decreased sperm viability and mobility [13], recurrent miscarriages [14], as well as ovarian dysfunction and obesity [15]. Extensive review of the literature by expert panels of the National Institute of Health [16] and the National Toxicology Program [17] also highlighted concern for altered prostate, neurodevelopmental, and behavioral outcomes.

The FDA in mid-July 2012 banned the use of BPA in the manufacturing of baby bottles and drinking cups [18]. Increased global regulations such as these have in turn resulted in the synthesis of alternative bisphenol analogues as replacements for BPA [19]. One such analogue is 4,4'-dihydroxydiphenyl sulphone, also known as BPS. Replacement of the BPA-carbon for the BPS-sulfur atom in the central position allows the structure to have increased stability against high temperatures and increased resistance to sunlight [20], thus resulting in a less leachable compound compared to BPA [21]. However, if XEs are present even in very small amounts and are potent, they can have effects that mimic or alter responses to physiologic estrogens.

BPS was recently detected in a variety of paper products, including 87% of paper currencies sampled from 21 different countries (geometric mean of 0.029  $\mu\text{g/g}$ ) [22]. BPS was detected in 97% of urine samples ( $n = 31$ ) from residents of Albany, NY in appreciable amounts [geometric mean of 0.299 ng/ml; 1.2 nM] [23]. The structural similarity of BPS to physiologic estrogens and to the known endocrine disruptor BPA originally raised questions over its safety and its endocrine-disrupting potential. Several *in vitro* studies testing the effects of BPS via genomic mechanisms have shown BPS to mimic estrogenic responses [20,24-27]; however, such studies were done at high concentrations unlikely to be leached from BPS-containing products [21]. We very recently demonstrated that BPS is potently estrogenic via non-genomic signaling pathways in the GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> rat pituitary cell line, including at the low-dose ranges likely to be present in food items and human fluids [28]. This study also demonstrated that BPS can strongly interfere with the signaling actions of the endogenous estrogen, estradiol ( $E_2$ ), at *picomolar*- and *sub-picomolar* concentrations, predominantly via membrane-bound estrogen receptor- $\alpha$  (mER $\alpha$ ), leading to alterations in functional responses – cell proliferation and prolactin (PRL) release. Previous studies from our group also examined the estrogenic actions of BPA and NP via this rapid signaling pathway with similar consequences on functional endpoints [29-32].

The aim of our present study was to determine if mixtures of XE compounds could cause signaling alterations (i.e. antagonistic or additionally agonistic) on  $E_2$ -induced non-genomic signaling correlated to functional cellular endpoints. To recreate likely real-world scenarios for exposure to mixtures, we compared each compound alone to a tertiary mixture (BPS + BPA +  $E_2$ ), and a quaternary mixture (BPS + BPA + NP +  $E_2$ ) over wide concentration ranges of the XEs. We examined XE mixtures in combination with a physiologic level of  $E_2$ , as that is the way most organisms will experience them. In addition, we evaluated the effects of these mixtures on MAPK-associated downstream functional endpoints: cell proliferation; apoptosis (caspase 8 and 9 activity); and PRL secretion. Our medium-throughput quantitative plate assays make possible within-assay comparisons between responses to different compounds and their mixtures at a wide range of concentrations.

## Methods

### Cells and culture conditions

The GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> rat prolactinoma cell line was originally selected on the basis of its naturally high expression of mER $\alpha$  [33-35]. Cells were routinely sub-cultured with phenol red-free Dulbecco's Modification of Eagle's Medium (DMEM, high glucose; Mediatech, Herndon,VA) containing

12.5% horse serum (Gibco BRL, Grand Island, NY) and defined supplemented calf and fetal serum (Thermo Fisher, Waltham, MA) at 2.5% and 1.5%, respectively. Cells of passages 10–20 were used for these experiments.

#### Quantitative ERK and JNK Phosphorylation assays

A fixed cell-based immunoassay was employed to quantify phospho-activation of ERK (pERK) and JNK (pJNK), as previously developed and described in detail [36]. Cells ( $10^4$ /well) were plated in 96-well plates (Corning Incorporated, Corning, NY) and allowed to attach for 24 hrs. The cells were then cultured in DMEM containing 1% charcoal-stripped (4X) serum for 48 hrs to deprive the cells of serum hormones. Medium was then removed and the cells exposed to individual XEs alone or as mixtures with a physiologic level of  $E_2$  ( $10^{-9}$  M; Sigma-Aldrich, St. Louis, Mo) to assess time- (0–60 min) and concentration-dependent ( $10^{-15}$ – $10^{-7}$  M) changes (at 5 min). Both the short time points and range in concentrations chosen were based upon recently published studies from our group [30,31,37,38]. The short time points ensure that we are indeed observing a rapid non-genomic activation of ERK and JNK without genomic interferences, while the concentrations are reflective of levels found in the environment. Test compounds were dissolved in ethanol then diluted in DMEM containing 1% charcoal-stripped serum. Vehicle control (V) was 0.001% ethanol in DMEM. To stop mER-initiated signaling, cells were fixed with a 2% paraformaldehyde/0.2% picric acid solution (Fisher Scientific, Pittsburgh, PA) and incubated at 4°C for 48 hrs. The cells were then incubated with phosphate-buffered saline (PBS) containing 0.2% fish gelatin and 0.1% Triton X-100 (Sigma-Aldrich) for 1 hr at room temperature (RT), followed by overnight incubation at 4°C with primary antibodies (Abs) against pERK or pJNK (Cell Signaling Technology, Beverly, MA; 1:500 in PBS/0.2% fish gelatin/0.1% TritonX-100). The cells were then washed with PBS (3X) before biotin-conjugated Ab (Vector Labs, Burlingame, CA) was applied and incubated (1 hr) at RT (1:500 in PBS/0.2% fish gelatin). The cells were again washed in PBS (3X) and incubated with Vectastain ABC-AP solution (Vector Labs; 50  $\mu$ L/well) for 1 hr at RT, followed by alkaline phosphatase substrate (pNpp solution; 50  $\mu$ L/well). The plates were incubated in the dark for 30 min at 37°C and the signal for the product of *para*-nitrophenol phosphate (pNpp) (Thermo Scientific, Rockford, IL) breakdown to pNp was read at  $A_{405}$  in a model 1420 Wallac microplate reader (Perkin Elmer, Boston, MA). The pNp signal was normalized to cell number, determined by the crystal violet (CV) assay at  $A_{590}$ , as described in [39].

#### Effects on cell numbers

We have previously described this method for estimating cell numbers in detail [40]. Briefly, sub-confluent cells were seeded into 96-well plates that had been coated with poly-D-lysine (5000 cells/well) and allowed to attach overnight. Plating medium was then replaced with DMEM containing 1% 4X charcoal-stripped serum for 48 hrs, then treated with media containing increasing concentrations of individual XEs ( $10^{-15}$ – $10^{-7}$  M) or mixtures of BPS + BPA +  $10^{-9}$  M  $E_2$  or BPS + BPA + NP +  $10^{-9}$  M  $E_2$ . After 3 days, cells were fixed (2% paraformaldehyde/0.1% glutaraldehyde in PBS; 50  $\mu$ L/well) and cell numbers were assessed by CV assay to compare the proliferative effects of XE-mixtures at different concentrations.

#### Determination of caspase activities

Caspase-8 and -9 activities were assessed as previously described [40]. Sub-confluent GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> cells were seeded into 96-well plates ( $5 \times 10^3$ /well) and allowed to attach overnight. Treatments began the next day; cells were exposed for 8 hrs to 1 nM  $E_2$ ,  $10^{-14}$  M and  $10^{-8}$  M individual XEs, and mixtures in DMEM-1% 4X charcoal-stripped serum; treatment medium was suctioned off and the cells lysed with 50  $\mu$ L lysis buffer (10 mM HEPES; 2 mM EDTA; 0.1% CHAPS; pH 7.4) to which 1 mM DTT (1:2000, freshly prepared, Sigma-Aldrich) had been added. Plates were then stored at  $-70^\circ\text{C}$  until assay. Staurosporine [500 nM] (Sigma-Aldrich) dissolved in DMSO was used as a positive control for activation of caspase-8 and -9. The released fluorescent product 7-amino-4-trifluoromethylcoumarin (AFC) was read using a Flexstation 3 spectrofluorometer (Molecular Devices, Sunnyvale, CA) at 400 nm excitation, and 505 nm emission wavelengths.

#### Prolactin release

These assay conditions were based on our previous studies [32,38]. Cells ( $0.5$ – $0.7 \times 10^6$ ) were plated into poly-D-lysine-coated 6-well plates overnight and hormone-deprived in DMEM-1% 4X charcoal-stripped serum for 48 hrs. Cells were then pre-incubated for 30 min in DMEM/0.1% BSA and exposed for 1 min to different concentrations of individual XEs alone ( $10^{-15}$ – $10^{-7}$  M), or as mixtures with  $10^{-9}$  M  $E_2$ , then centrifuged at 4°C,  $350 \times g$  for 5 min. The supernatant was collected and stored at  $-20^\circ\text{C}$  until radioimmunoassay (RIA) for PRL. Cells were then fixed with 1 ml of 2% paraformaldehyde/0.1% glutaraldehyde in PBS, and cell numbers determined via the CV assay. PRL RIA concentrations were determined with a Wizard 1470 Gamma Counter (Perkin Elmer) and normalized to CV values.

#### Statistical analysis

Statistical analyses were performed using Sigmaplot version 12.3 (Systat Software Inc). One-way analysis of variance

(ANOVA) was applied to the dose- and time-dependent studies to assess the statistical significance of mean values produced by varying exposures. A Holm-Sidak comparison against vehicle control or against  $E_2$  treatment was used to evaluate significance. The overall  $\alpha$  level selected for the statistical analysis was 0.05.

## Results

### Temporal changes in phospho-activation of MAPKs by BPS, BPA, and NP, and their combinations, during a 60 min exposure

The time dependence of these responses was examined at optimal response concentrations (see Figure 1).  $E_2$  produced a typical oscillating two-peak ERK response, with the first peak within 5 min, followed by a second peak at 30 min as we have observed previously [31,35-37]. During the same 60 min time frame XEs generated temporal profiles different from  $E_2$  (Figure 1A). The combination of  $10^{-14}$  M XEs and  $10^{-9}$  M  $E_2$  (Figure 1B) caused a deviation from the  $E_2$ -induced temporal pattern, as well a decrease of the overall ERK response, as was also seen in the dose-dependent studies (see below). Similar deviations due to other XE combinations with  $E_2$  have been previously documented [30,31,37,38]. Therefore, even at this very low concentration ( $10^{-14}$  M), XEs are capable of disrupting the timing of the response to a physiologic estrogen.

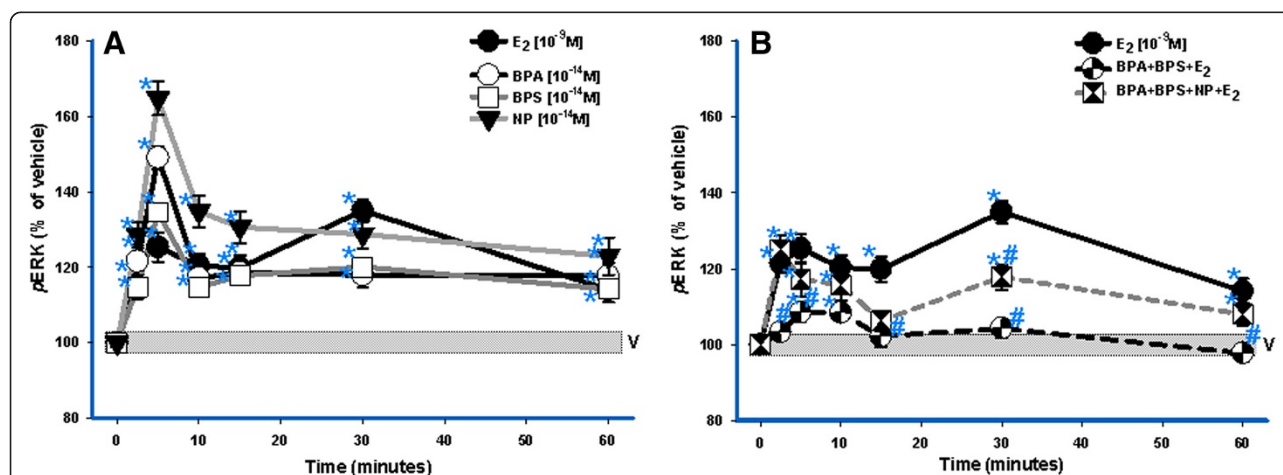
Neither BPA nor BPS could maintain activation of JNK by themselves during the 60-min time course (Figure 2A); however, their combination with  $E_2$  (3-compound mixture) did activate JNK with a significant 60-min response, by which time the response to  $E_2$  had declined to control values (Figure 2B). As a 4-compound

mixture,  $E_2$  plus all XEs inactivated JNK to below vehicle control values early in the time course, but then activated and sustained pJNK after 30 min. Overall, these combinations with XEs markedly attenuated the  $E_2$ -induced JNK response.

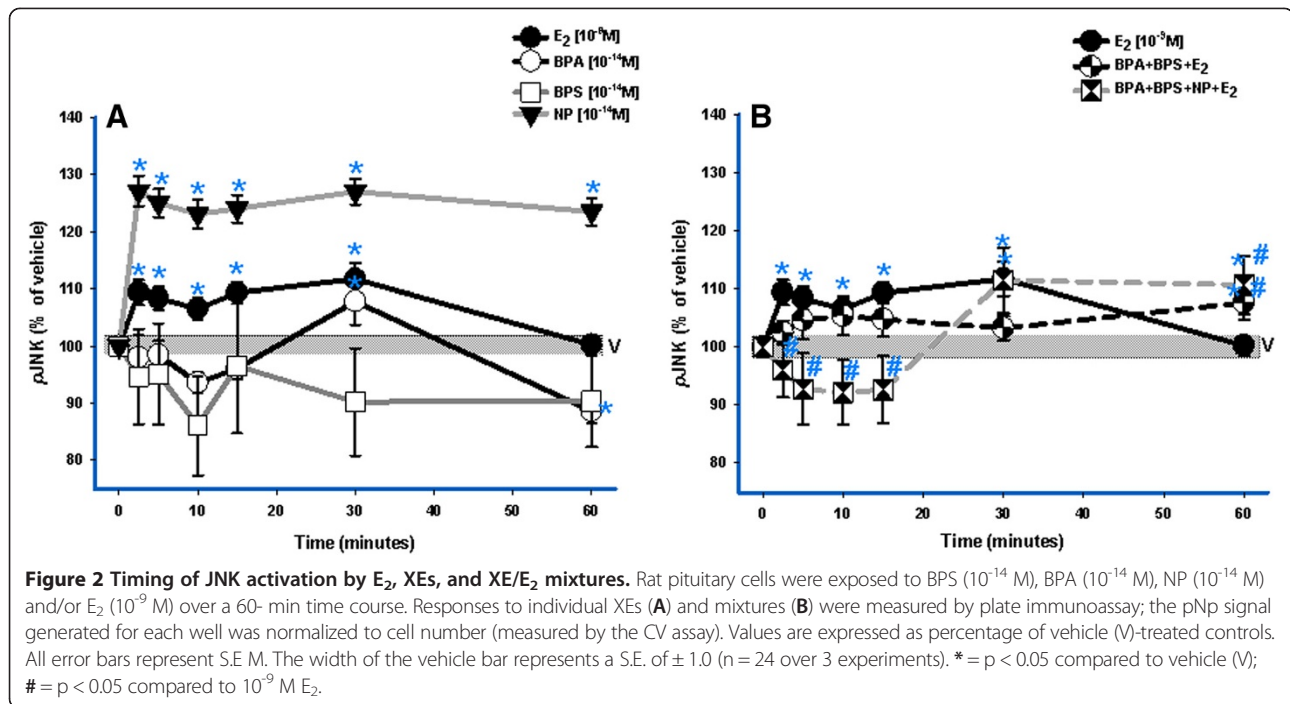
### Concentration-dependent changes in phospho-activation of MAPKs by a short exposure to BPS, BPA, and NP, and their combinations

We have previously determined dose-response profiles for BPS [28], and they are described here for comparison. Short exposures (5 min) to individual XEs (Figure 3A) caused ERK activation in GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> cells at concentrations similar to those elicited by  $E_2$  [30,31]. The lowest tested XE concentrations evoked a higher pERK response than did  $10^{-9}$  M  $E_2$ . The responses steadily decreased with increasing XE concentrations. Responses to femtomolar concentrations of individual XEs were statistically different (by one-way ANOVA) from those in the nanomolar range and from the zero concentration point, indicating a non-monotonic dose-response [41]. The combination of XEs of increasing concentrations with constant  $10^{-9}$  M  $E_2$  (Figure 3B) reduced ERK activity below that of either  $E_2$  or XEs alone, reaching vehicle control levels at the highest concentrations.

Individual bisphenol compounds deactivated pJNK below vehicle levels (Figure 4A), unlike  $E_2$  and NP that both activated JNK. However, when  $E_2$  was administered together with both bisphenol compounds (Figure 4B), JNK was strongly activated, featuring a non-monotonic dose-response curve with the lowest concentrations evoking the largest responses; the 4-compound mixture evoked no activation of JNK and was consistently,



**Figure 1** Timing of ERK activation by  $E_2$ , XEs, and XE/ $E_2$  mixtures. Rat pituitary cells were exposed to BPS ( $10^{-14}$  M), BPA ( $10^{-14}$  M), NP ( $10^{-14}$  M) and/or  $E_2$  ( $10^{-9}$  M) over a 60-min time course. Responses to individual XEs (A) and mixtures (B) were measured by plate immunoassay; the pNp signal generated for each well was normalized to cell number (measured by the CV assay). Values are expressed as percentage of vehicle (V)-treated controls. All error bars represent S.E.M. The width of the vehicle bar represents a S.E. of  $\pm 1.2$  ( $n = 24$  over 3 experiments). \* =  $p < 0.05$  compared to vehicle (V); # =  $p < 0.05$  compared to  $10^{-9}$  M  $E_2$ .

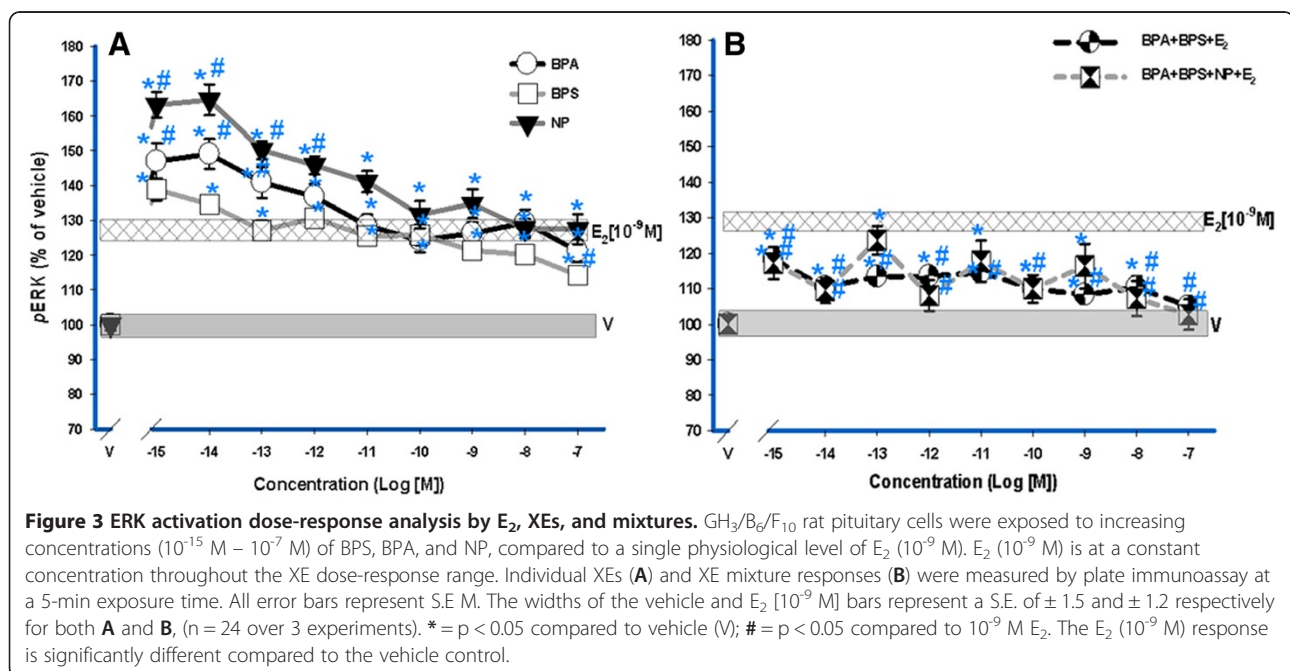


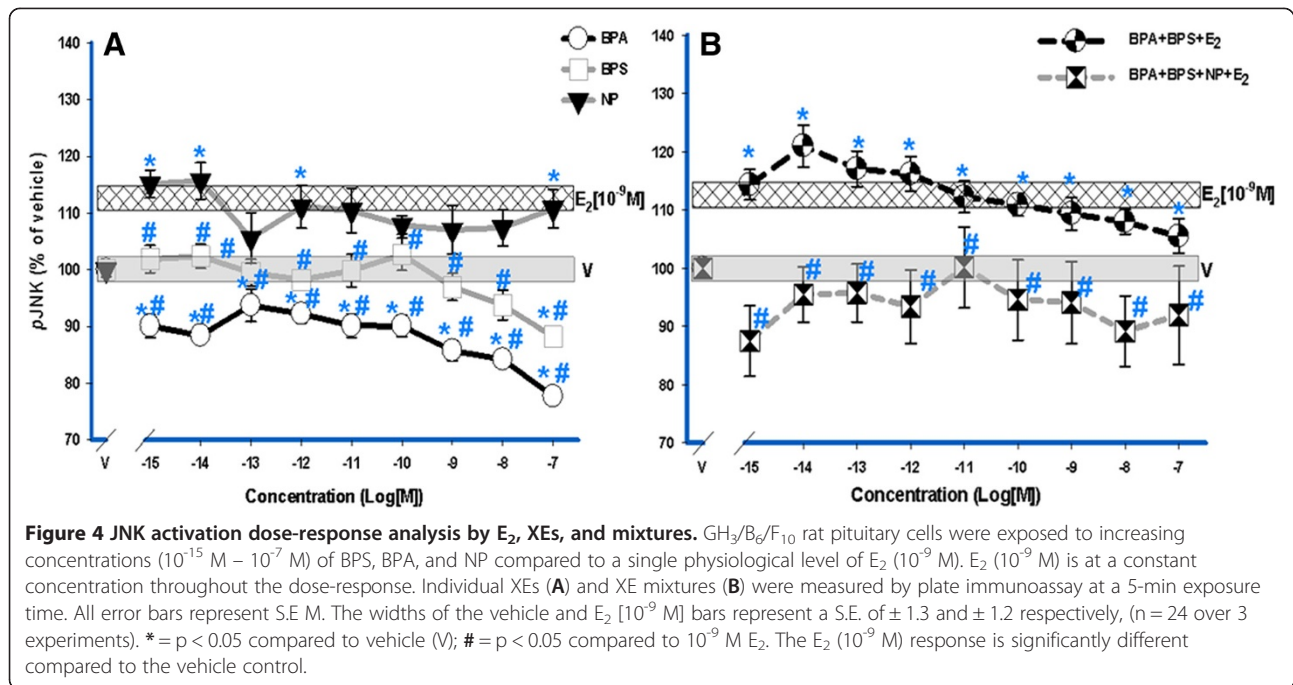
though not statistically, below the level of the response to vehicle, thus erasing the response to 1 nM  $E_2$ .

### XEs and mixtures affect cell proliferation

After a 3-day exposure,  $10^{-9}$  M  $E_2$  and BPS had similar effects on cell proliferation [28]. We now looked at the dose responsiveness at this 3-day time point, demonstrating non-monotonic stimulations (Figure 5A), as we

observed previously with  $E_2$  and other XEs [38,40]. NP did not increase cell numbers significantly compared to vehicle until it reached  $10^{-11}$  M, and BPA until it reached  $10^{-7}$  M. Both XE mixtures with  $E_2$  (Figure 5B) failed to stimulate cell proliferation, but instead suppressed cell numbers far below those seen with vehicle, again showing these compounds' ability to disrupt a response to a physiologic estrogen.

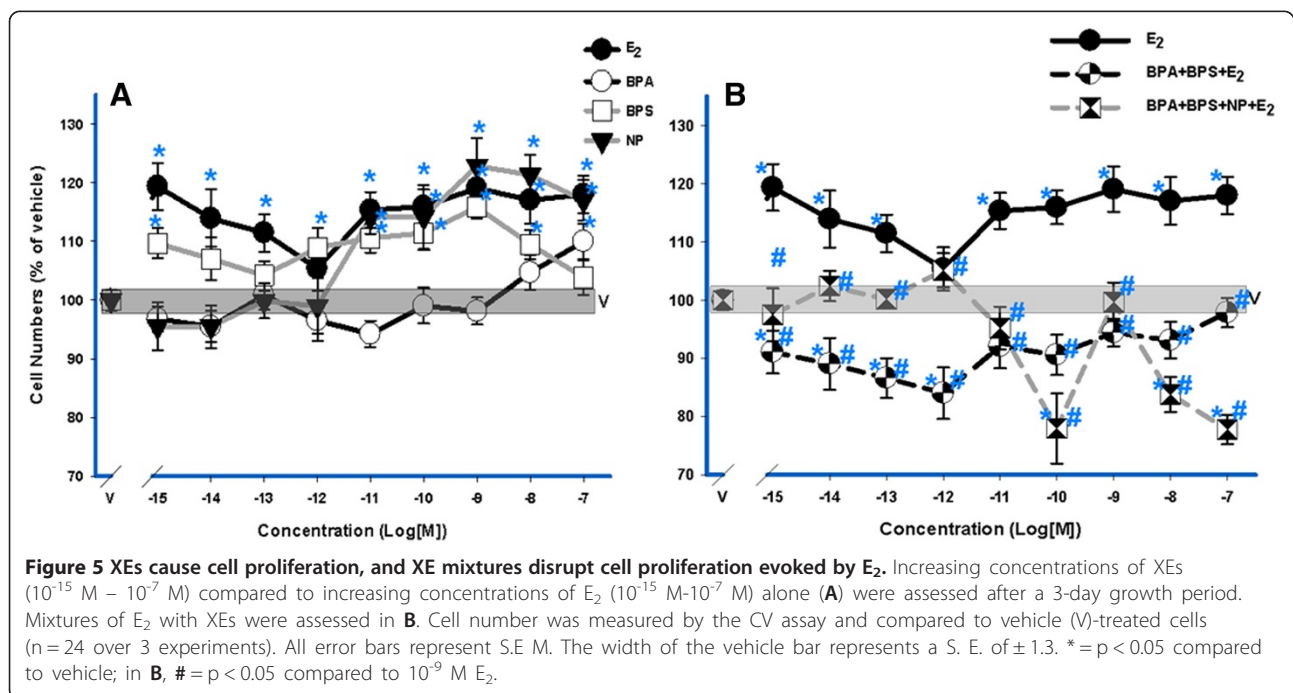


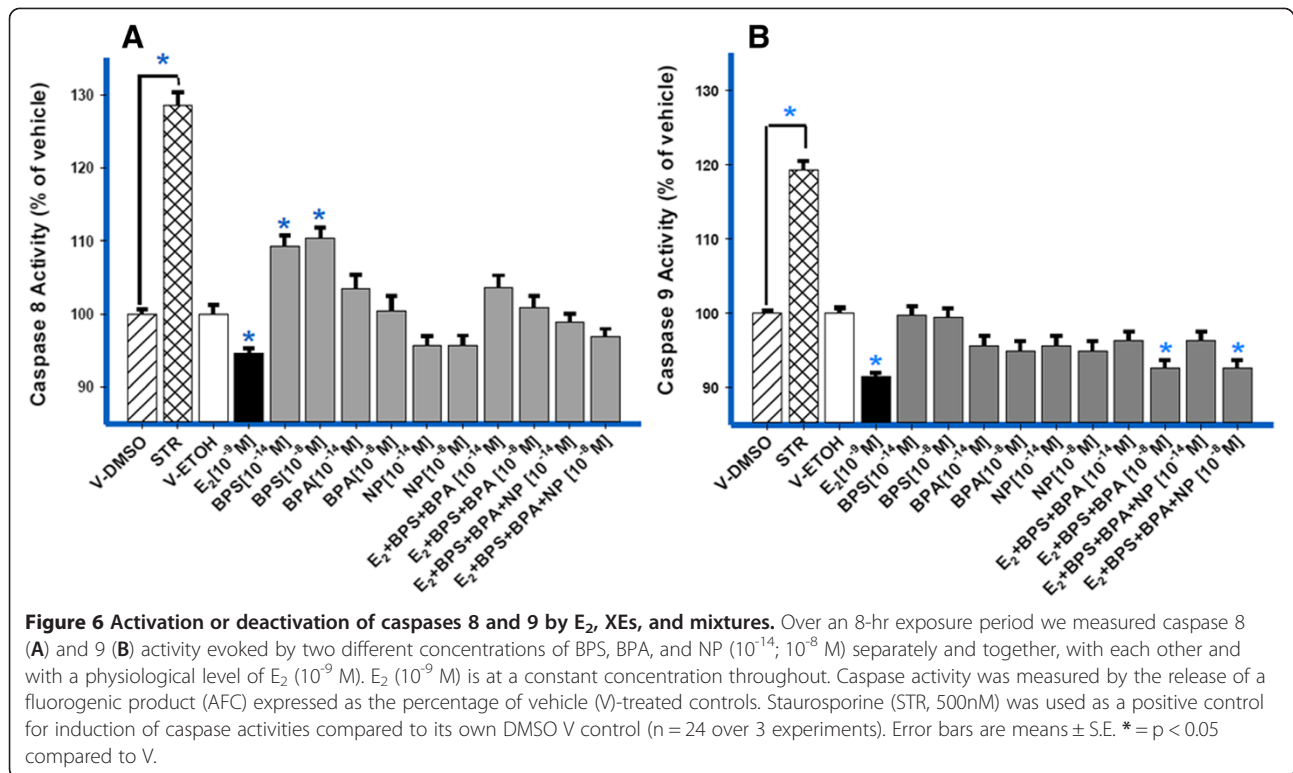


### Caspases activated and deactivated

Initiation of apoptosis is one of several factors that can influence cell numbers; we therefore assayed caspase 8 and 9 activities to determine if the extrinsic or intrinsic apoptotic pathways were activated over an 8-hr exposure period, the optimum time that was determined previously [28]. Caspase 8 was significantly

activated by BPS at both concentrations used ( $10^{-14}$  M and  $10^{-8}$  M), while BPA, NP, and the mixture solutions at their respective concentrations did not result in significant activations (Figure 6A). Activations of caspase 9 were not detected with either individual XEs or mixtures, indicating that the extrinsic pathway (caspase 8) and not the intrinsic pathway



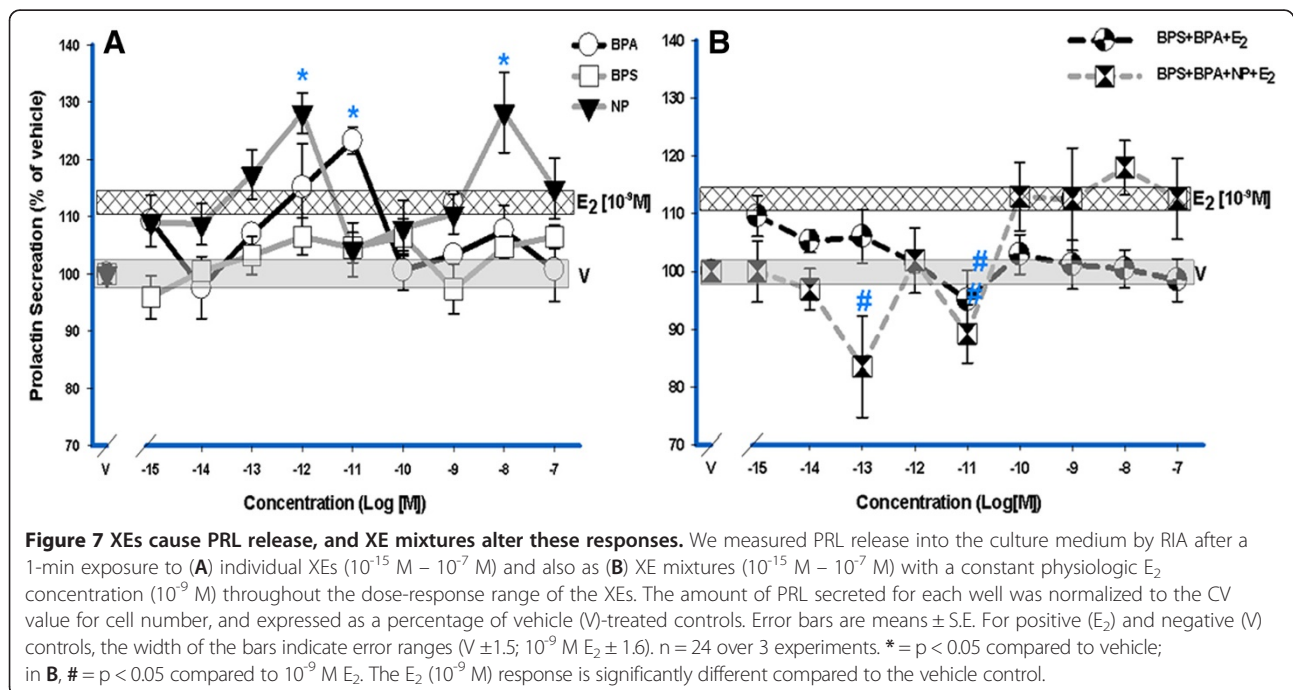


(caspase 9) is the primary apoptotic pathway activated. However, both mixture combinations at the highest concentrations (10<sup>-8</sup> M) resulted in a significant deactivation of caspase 9 activities (Figure 6B). Staurosporine, the positive control for activation, was active on both caspases, as expected. E<sub>2</sub> by itself suppressed caspase

activity below vehicle controls for both apoptotic pathways, as we had seen previously [28,40].

#### Mixtures of XEs disrupt E<sub>2</sub>-induced PRL release

The rapid non-genomic secretion response for PRL caused by estrogenic exposure in GH<sub>3</sub>/B<sub>6</sub>/F<sub>10</sub> cells has



become a standard tool in our lab for evaluating functional endpoints [30-32,38,42,43]. After a 1-min exposure, BPS could not increase PRL secretion as did  $E_2$  (Figure 7A [28]). At certain concentrations, BPA and NP were able to significantly increase PRL release, even above that caused by nM  $E_2$  (Figure 7A); the shape of these dose–response curves are non-monotonic (as confirmed by determining that values at the peaks of activation were statistically different than those at other, usually higher, concentrations). As XE mixtures with  $10^{-9}$  M  $E_2$ , the 3-compound mixture inhibited  $E_2$ -induced PRL release at low concentrations (significantly at  $10^{-11}$  M). The 4-compound mixture caused more extreme inhibitions, even below the vehicle level at the lower concentrations (Figure 7B). Though the 4-compound mixture at  $10^{-8}$  M appears to have resulted in PRL release, the errors in these mixture measurements did not allow this response to be distinguished as statistically different from vehicle, and the mixed signaling patterns caused by the multiple ligands may contribute to this variability.

## Discussion

Our study confirms that the novel BPA substitute, BPS, can initiate rapid non-genomic signaling in pituitary cells at environmentally relevant concentrations (as low as *femtomolar-picomolar*), as do the more thoroughly tested BPA and NP. In combination these compounds altered endocrine responses differently, and more dramatically. Together, these compounds also interfered with the actions of the physiologic estrogen  $E_2$  resulting in alterations to functional endpoints. These results not only highlight the need for stricter regulatory requirements for XEs, but also address the need to identify potentially adverse interactions of new chemicals with already existing chemicals in the environment. Such endocrine-disrupting effects should be identified during the initial phases of product development so that hazardous new combination exposures can be prevented [44].

We previously determined that BPS, BPA, and NP had similar high potencies, compared to  $E_2$ , for initiating the phospho-activation of ERK and JNK across a wide range of concentrations and times [28,31,32,37,38,40,45]. Non-monotonic dose–response curves were seen, as low concentrations of individual XEs produced high MAPK activation, decreasing as concentrations increased. The occurrence of non-monotonic responses is the source of much debate. In regards to our system, such occurrences could be due to: 1) negative feedback regulation of MAPKs as concentrations increase, thereby preventing unnecessary pathway activation; 2) receptor down-regulation or desensitization; 3) the presence of multiple receptor subtypes that bind to the same ligand yet initiate interactions with different signaling partners, thereby eliciting different response patterns (stimulatory or

inhibitory); and/or 4) the activation of multiple pathways from the same receptor, where signaling can be redundant or divergent [41,46-49]. The more detailed mechanisms responsible for these non-monotonic responses are still largely unexplored at the cellular level.

The phospho-activation of ERK and JNK is often associated with opposing functional endpoints. ERK signaling promotes cell growth and differentiation by activating pro-survival enzymes [50] and inhibiting apoptotic enzymes such as caspases [51-53]. Conversely, JNK signaling is often associated with inflammation or the initiation of cell death, and activating pro-apoptotic proteins (including Bax, caspase-3, Fas, cyclin D1 and under some circumstances, interleukin 1) [54-58]. Our data have shown a correlation between the increase in cell numbers and ERK activation by BPS, as we discussed previously [28]. However, BPA and NP were slightly more efficacious than BPS at activating ERK, yet were unable to increase cell numbers as effectively, suggesting that pro-apoptotic proteins may also be involved in regulating final cell number outcomes. Dramatic decreases in cell numbers, in particular with the 4-compound mixture, could be due to the attenuation of the  $E_2$ -induced ERK activation, as ERK activations are usually linked to cell proliferation responses. Our present data therefore present another example of how the final cell number outcome is dependent on the overall balance between ERK and JNK related activities [59,60].

Caspase activation by XE mixtures was also examined to determine whether activation occurred via an external stimulus (caspase 8) or through an internal stimulus (caspase 9), and to correlate caspase activity to changes in cell numbers. We previously reported that BPS at two concentrations ( $10^{-14}$  M and  $10^{-8}$  M) throughout a 4–24 hr time course resulted in activation of caspase 8, with a delayed and probably secondary activation of caspase 9 [28]. However, here we show that BPA, NP, and their mixtures did not activate, and in some cases (as when combined with  $10^{-9}$  M of  $E_2$ ), deactivated caspases. Deactivation of caspase activity protects cells from death and contributes to  $E_2$ 's well-known proliferative effect on the GH<sub>3</sub> cell lines [38,40,61] and other cancer and normal cells. Caspases also contribute to the inhibition of cell proliferation by XE mixtures, but cell numbers are clearly not controlled by caspases alone. The balance of multiple factors – including the actions of several pro-apoptotic or anti-apoptotic enzymes and other proteins – may contribute, along with numerous other proteins that control the cell cycle. However, it is clear that XEs can alter such responses.

A broad range of BPA and NP concentrations induced significant PRL secretion, with some compounds producing non-monotonic dose responses, agreeing with previous studies from our lab [32,38]. However, mixtures of BPS with BPA, and additionally NP, disrupted  $E_2$ -induced



PRL release, causing an overall attenuation of secretion compared to individual XE compounds. Such inhibitory actions could be part of a negative feedback mechanism protecting against excess stimulation by multiple estrogens causing unnecessary PRL release. Because PRL regulates over 300 biological functions directly and indirectly [62], alteration of its secretion (either enhancement or inhibition) can cause many different physiologic complications, including such medical problems as metabolic dysfunctions, behavioral disturbances, or reproductive and offspring-rearing failures.

We cannot know for sure if effects in cells and even animal models represent what will happen to humans, because humans usually will not manifest these exposure-based adverse health effects for many years. BPS has only been present in products and the environment for a short time, and therefore, the epidemiology results will not be available for some time to come. In real-world situations, environmental and even occupational exposures are rarely due to a single chemical, but instead involve complex chemical mixtures. Potential health hazards from mixtures are a challenge for regulatory agencies to evaluate, due to the difficulty of testing a vast number of chemical interactions that target various mechanisms and that can be tissue-dependent; these evaluations also require the testing of ever-increasing numbers of chemicals. These quantitative approaches should also contribute to the ability of any regulatory agency to systematically study the interactions of any combinations of compounds including contaminants, drug-drug interactions and drug-pollutant interactions in the drug development process [63].

## Conclusions

Adverse actions from chemicals introduced to the environment should be suspected whenever they can disrupt the actions of a physiologic hormone like  $E_2$ . In addition, compounds acting as estrogens on their own and possibly causing estrogenic responses in an organism at inappropriate life stages have been shown in many studies to be unfavorable. As growing concern over the safety of BPA has led to stricter regulatory actions, we are likely to see other chemical replacements offered. The increased presence of BPS in an environment already contaminated with BPA, NP, and a variety of other prevalent and persistent environmental pollutants now requires increasing scrutiny of their potential hazards as chemical mixtures, and especially those that act via ERs [31,36,64]. Our tissue-relevant responses, such as the ones we have demonstrated with these medium-throughput quantitative assays in pituitary cells, offer efficient test systems that could be used to monitor pollutant mixtures at the cellular level. In addition, altered pituitary responses are very likely to have consequences for all other endocrine tissues. However, it is important to establish effective pre-screening of the

endocrine-disruptive potential of any new chemicals whose structures make them candidates for these endocrine-disruptive activities in the future [44].

## Abbreviations

Ab: Antibody; BPA: Bisphenol A; BPS: Bisphenol S; JNK: Jun N terminal kinase; CV: Crystal violet; DMEM: Dulbecco's Modified Eagle Medium; ERK: Extracellular signal regulated kinase;  $E_2$ : Estradiol; ER: Estrogen receptor; mER $\alpha$ : Membrane estrogen receptor  $\alpha$ ; mER $\beta$ : Membrane estrogen receptor  $\beta$ ; MAPKs: Mitogen activated protein kinases; NP: Nonylphenol; pERK: Phosphorylated ERK; pJNK: Phosphorylated JNK; PRL: Prolactin; XE: Xenoestrogen.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

RV carried out the experiments on the effects of chemical mixtures on kinase-activation, proliferation, caspase activity, and PRL release studies. All authors participated in the design and analyses of the studies, and wrote, read, and approved the final manuscript.

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