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Potential effect of combined xenoestrogens during gestation stages on mouse offspring

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Abstract

The synergistic effect of numerous environmental endocrine disrupting chemicals (EDCs) has raised research concern among researchers. To extend previous studies, the measured additional potential interactions among bisphenol A (BPA), 4-nonylphenol (NP), 4-tert octylphenol (OP) and isobutylparaben (IBP) in mouse model were observed. Pregnant Swiss- albino mice were treated with binary combined chemicals (5, 50 or 500 mg kg^{-1} b.wt. day^{-1}) from gestation day (GD) 1 to 21. Interestingly, maternal exposure to these EDCs caused fluctuation in GD time, live ratio, female/ male ratio, body and organ weights of mouse offspring at postnatal day (PND) 1, 21, and 41 days. At most doses early reduced 0.85 to 1.87 GD compared to controls. Besides females / males ratio showed a significant difference in BPA + OP, BPA + IBP groups. Female body weight at PND 21 and 41, showed a significant reduction at all combined levels, whereas male offspring showed reduction in weight at dose 50 mg kg^{-1} b.wt. day^{-1}. The potential effects of synergic estrogenicity detected histopathological abnormities, such as ovary analysis revealed increase of corpora lutea, cystic follicles and an endometrial hypertrophy and morphometric changes in uteri measurement. Taken together, these results provided an additional insight into synergistic effects of EDCs toxicology on reproductive tracts.

Key words

Endocrine disruptors, Gestation stage, Mixed xenoestrogens, Mouse offspring

Introduction

Evaluating the toxicity of chemical mixtures is one of the most concerned issues in the modern society. Some of these toxicities show immediate impact; others can result in subtle alterations that are delayed in their expression (Betancourt *et al.*, 2010). Like other EDCs, bisphenol A, 4-nonylphenol, 4-tert octylphenol and isobutylparaben compounds are among the highest concentrated chemicals produced in the world and originally developed for use as synthetic estrogen (Welshons *et al.*, 2006). However, limited data are available on the additive or synergistic estrogenic effects of these compounds in intrauterine life. Bisphenol A is widely used for manufacturing polycarbonate bottles and resins that are used as linings for food and beverage containers, and as dental sealants (Kang *et al.*, 2006). Alkylphenolic compounds, including 4-nonylphenol and 4-tert octylphenol, are components of soaps, paints, herbicides and pesticides, and are also used as additives in plastics (Fernandez *et al.*, 2007). And parabens are widely utilized as preservatives in food, cosmetics and pharmaceutical products (Soni *et al.*, 2005). Although the estrogenic activities of these compounds alone are weak, the combined exposure to EDCs (*e.g.*, bisphenol A, phenylphenol, nonylphenol and diethylstilbestrol) can induce additional burden to humans and wildlife (Roy *et al.*, 1998).

Previous study have revealed that exposure of developing fetus to environmental estrogens may increase the adverse risk to health at birth or later in life (Unuvar and Buyukgebiz, 2012). Pregnant women's exposed to EDCs can disrupt the hormone for fetal gonadal development, risk of fertility problems, and affect reproductive hormones in adulthood (Nordkap *et al.*, 2012). Failure of human male reproductive tracts (e.g., cryptochidism and hypospadias) may be related to *in utero* exposure to estrogens/ androgens (Fernandez *et al.*, 2007). A developmental toxicity of mixture of 13 EDCs, such as phthlates, pesticides, bisphenol A, parabens during gestation significantly

increased nipple retention, reduced prostate weight, and affected on the hormonal milieu for male sexual differentiation (Christiansen *et al.*, 2012). Pregnant Wistar rats exposed to diisobutyl phthalate, butylparaben, perfluorooctanoate, rosiglitazone from GD 7-21 showed reduction in anogenital distance, testosterone production and genes related to steroidogenesis in male, whereas in females increase in anogenital distance and ovarian aromatase mRNA levels were observed (Boberg *et al.*, 2008).

Although, there are a number of studies describing the potent estrogenic effects of BPA, NP, OP and IBP *in vitro* and *in vivo* (An *et al.*, 2002; Vo *et al.*, 2011), very few studies addressed the estrogenic effects of combined exposure to two/ or three groups of BPA, NP, OP, and IBP chemicals *in vivo* models. Besides, these chemicals have the potential to disrupt or interfere with reproductive functions, the mechanism remains to be clarified. The present study focused on examining the relationship between the combined estrogenic effects of chemicals and malformations of mouse offspring on maternal exposure to these factors.

Materials and Methods

17α-ethynylestradiol bisphenol A and 4-nonylphenol were purchased from Sigma-Aldrich (St. Louis, MO). 4-tert octylphenol was purchased from Fluka Chemie (Seoul, Korea). Isobutylparaben was obtained from Tokyo Kasei Co., Ltd (Tokyo, Japan). All chemicals were dissolved in ethanol and stored at 4 ºC to avoid contamination.

Animals and treatment : Swiss Albino 8 week-old female and 10 week-old male mice were purchased from National Institute of Veterinary Research (Hanoi, Vietnam). Animals were housed in polycarbonate cages in a controlled environment under an illumination schedule of 12 hr light/12 hr dark, fed with soy-free pellet food (CIMADE Center, National Institute of Hygiene and Epidemiology- NIHE, Vietnam) and water provided *ad libitum*, at a temperature of 20–24 ºC with relative humidity of 40–50%. Female and male mice were mated and evidence of vaginal plugs and/or sperm presence in vaginal smear was considered as GD 0. The Ethics Committee at NIHE (Vietnam) approved of all experimental procedures and animals used. All pregnant mice were divided into eleven groups (n = 4/group) using randomized body weight (b.wt.) to ensure equal distribution of weight among groups (Vo *et al.*, 2011).

From GD 1 to 21, timed pregnant mice were given a daily oral gavage with 17α-ethynylestradiol (1 mg kg^{-1} b.wt. day^{-1}) used as positive control; 5, 50 or 500 mg kg^{-1} b.wt. day^{-1} in various combinations with equivalent concentrations of each compound (e.g., BPA + NP, BPA + OP, and BPA + IBP); or corn oil (5 ml kg^{-1} b.wt. day^{-1}) used as a vehicle. On the day of delivery, which was considered as PND 1, the following endpoints were recorded: viability and development (little pup size, numbers of live and dead pups, female/male ratio), changes in pup weights, and clinical signs of toxicity and/or abnormal behaviour throughout the experimental period. Pup female or male mice were weaned on PND 21. All F1 pup mice were euthanized by diethyl ether 99% on PND 41. Gross of tissue was examined, and weight of body, pituitary, thyroid, adrenal, liver, spleen, kidneys, uterus, ovaries and testis were recorded. Especially, female reproductive organs (*e.g.*, uteri and ovaries) were taken for histopathological tests.

Histological examination : The reproductive organs (*e.g.,* uteri, ovaries) were removed under ether anesthesia, stripped of fat, and weighed. All organ tissues were fixed in 10% neutral buffered formalin immediately after weighing, then dehydrated at room temperature for 24 hr, and embedded in paraffin. The tissues were then sectioned to a thickness of 5μm across the mid-region of each tissue, and mounted on slides. These sections were stained with hematoxylin and eosin (H&E), and the histopathological changes in tissues were examined under light microscope.

Statistical analysis : Litters are experimental units used in statistical analysis. Gestation duration, live rate, absolute body weight (PND 1, 21, and 41), and organ weights at the time of necropsy were analyzed by one-way ANOVA. Significant differences between treatment groups and their respective controls were tested using Tukey's multiple regression tests at $p < 0.05$. Incidence data (*i.e.*, female and male ratio) were analyzed by row × column Chi square test, followed by Fisher's exact probability test. Results were provided as mean ± SE.

Results and Discussion

Chemicals used in the present study are environmental endocrine disrupting chemicals, which are considered of weak estrogenicity (Song *et al.*, 2006), however, combination of these EDCs at low concentrations used in consumer products, have still shown estrogenic activities, as endocrine modulators, hormone active agents, endocrine active agents, endocrine toxins or xenohormones (Foster, 2008).

It is well known that conditions experienced *in utero* can have lifelong effect on health (Alonso-Magdalena *et al.*, 2010). *In vivo* experiments, uterotropic assays were also used to assess the sensitive estrogens through the timing of gestation day, number of pups and proportion of female/male, and weekly individual pup's liter weights. At the dose levels of binary compounds tested (i.e., BPA + NP, BPA + OP, and BPA + IBP) had statistically significant effect on dams GD time (early 0.85 to 1.87 day compared to control groups), except for low-dose (5 mg kg^{-1} b.wt. day^{-1}) of BPA + NP, and BPA + OP groups. The proportion of pups born alive and surviving to weaning was not significantly affected by exposure to binary combined compounds, except for BPA + OP 5 mg kg^{-1} b.wt. day^{-1} group that pup survival rate was

highly decreased (reduced 2.08 fold compared to vehicle group) (Table 1). The available experimental evidence showed that combined effects may result in generation of endocrine disrupters and biological perturbation. In previous studies scientist have reported that combined effect of estrogens and xenoestrogens shown to be active in genetic responses (Jeng *et al.*, 2010). Moreover, EDCs have been detected in amniotic fluid, neonatal blood, placenta, cord blood, and breast milk after maternal exposure, demonstrating the potential of EDCs compound to pass from mother to fetus (Vandenberg *et al.*, 2009). These effects, observed in the offspring, may be due to the direct effect of EDCs on the fetus or due to fetal exposure to an altered maternal metabolism, or combination of both factors (Alonso-Magdalena *et al.*, 2010). *In utero* exposure to combined compounds BPA + OP (50 mg kg^{-1} b.wt. day^{-1}) and BPA + IBP (500, 5 mg kg^{-1} b.wt. day^{-1}) significantly decreased the female/male offspring ratio on PND 1 (Table 1). These results are in confirmation with the previous studies (Kundakovic *et al*., 2013; Miller *et al*., 2012). For instance exposure, of female mice during pregnancy to environmentally relevant doses of BPA also induces sex specific alteration, and affects the sexual differentiation and behavior (Kundakovic *et al.*, 2013). Another rodent experiment, co-exposure to EDCs also determined the sex effects on male and female offspring (Miller *et al*., 2012). Estrogens play a pivotal role in regulating the rate of blastocyst passage through the fallopian tubes (Paria *et al.*, 1998). It is also possible that estradiol ended pregnancy *via* direct embryo toxicity during the cleavage stage of embryonic development (Valbuena *et al*., 2001). Besides, the changes in proportion of female/ male may be due to EDCs metabolic fate being under hormonal control in both species, but male rats exhibited greater than female rats (Takeuchi *et al.*, 2002).

Pregnant mice treated with binary compounds at different concentrations from GD 1 to 21 displayed small body weight changes in male offspring in a dose dependent manner, whereas a significant decrease in body weight on PND 21 and 41 in all female offspring treatments as compared to control animals was observed (data not shown). The basic mechanism of these alterations is not yet known, although it is plausible that epigenetic modifications of maternal tissues may influence metabolism later in life and perhaps during subsequent pregnancies (Alonso-Magdalena *et al.*, 2010). Moreover, the effect of EDCs on body weight may be due to its regulation on postnatal food intake and metabolism change (Rubin and Soto, 2009). Alonso- Magdalena (2010) also reported that long-term exposure to BPA in mice has deleterious effect on glucose metabolism during pregnancy and postpartum, as well as in their adult offspring, because this EDC crosses placenta and affects glucose tolerance, insulin, and leptin signaling during gestation (Alonso-Magdalena *et al.*, 2010). Another reason is that estrogens also regulate production of growth hormone (Leung, 2004) and activation of insulin like growth factor 1 pathway, which induces cellular proliferation in tissues (Kahlert *et al.*, 2000).

In utero and/ or lactation assays, the fetal stage might be the critical window of susceptibility to EDCs exposure (Liu *et al.*, 2013). In the present study, these alterations makes the animal adapted to metabolic regulation in adult stage. At the time of necropsy (PND 41), regulatory roles of combined xenoestrogen during pregnancy have been well established in female and male offsprings. A few treatment related to clinical signs of toxicity were observed on thyroids, spleen, uteri, and ovaries weight of F1 female mice, but showed in significant difference. However, pituitary, adrenal, liver and kidney weights in female offspring were pronounced more significantly ($p < 0.05$) than control, when treated with BPA + OP (500 mg kg^{-1} b.wt. day^{-1}), BPA + OP (5 mg kg^{-1} b.wt. day^{-1}), and BPA + IBP (50, 5 mg kg^{-1} b.wt. day^{-1}) respectively (Table 2). Besides, the effect of binary combined EDCs have also been implicated in the developmental process of male offsprings. In Table 3, no difference in male pituitary, liver, spleen, and testis weights were seen between treatment and control groups. Especially, thyroid weight was significantly reduced by 1.6 to 2.4 fold in all BPA + NP treatments, BPA + OP (50 mg kg^{-1} b.wt. day^{-1}), and BPA + IBP (50, 5 mg kg^{-1} b.wt. day^{-1}) as compared to the control groups. Exposure to dose of 500, 5 mg kg^{-1} b.wt. day^{-1} of BPA + OP groups showed a statistically significant increase (3 fold higher than controls) in adrenals, whereas BPA + IBP at dose of 500, 5 mg kg^{-1} b.wt. day^{-1} significantly decreased kidney weight (1.4 fold lower than controls) in male offsprings (Table 3). However, no significant effects on reproductive organ weights (e.g., uteri, ovaries, and testis) in both female and male offsprings were detected for any dose of binary combined compounds, although the present study observed many of the histopathological changes in these organs (these are mentioned in the next section). These compounds caused reduction in organ weight in the present experiment, implying that it could be related to the changes in the neuroendocrine system involved in controlling puberty/ or feeding and energy homeostasis during pregnancy (Laws *et al.*, 2007). In animal models, Jordan *et al.* (2012) examined combined exposure to BPA, Di (2-ethylhexyl) phthalate, NP and indicated changes in energy and lipid metabolism in liver due to AMPK and cAMP signaling disruption (Jordan *et al.*, 2012). Wade *et al.* (2002) reported that mixture of persistent contaminant exposure to rats induced alterations in the liver, kidney, and general metabolism that caused by changes in reproduction hormone levels, or general reproductive function indicators. Moreover, effects of estrogen on various genes that produce mediate cell growth, differentiation and general homeostasis of reproductive and other systems were reported (Nilsson *et al*., 2001). On the contrary, some distinct works reported no treatment related or converse alterations of body, liver, kidney, adrenal, ovary, or uterus weights in pups when exposed to EDCs (Nikaido *et al*., 2004). The cause of such discrepancies is unclear, but it might be related to dose, time, sex and species. Here, this experiment detected a statistically significant change in the risk of malformation of mouse offsprings on xenoestrogenic exposure

during pregnancy. Thus, results revealed significant relationship between female/ male developmental malformation and estrogenicity of the combined compounds (BPA, OP, NP, and IBP) due to bio-accumulated xenoestrogens.

In the present study a significant association was observed between maternal involvements in xenoestrogenic exposure and increased histopathological abnormalities of uterus and ovary organs in the offsprings. *In utero* combined exposure to BPA, NP, OP and IBP resulted in disorganization of endometrial gland, and muscular layers in female offsprings in PND 41. A dose-dependent binary combined compound might lead to endometrial hypertrophy and morphometric changes in the thickness of uteri (Fig. 1). Also, almost ovaries performed a significant increase of corpora lutea in dose dependent manner; especially histopathological examination of all BPA + IBP combined groups also revealed hyperplasia changes of cystic follicles (Fig. 2). It is well known that *in utero* assays, hypertrophy of luminal epithelium is highly sensitive marker of estrogenic effects on mouse and rat uterus, and a modification of the

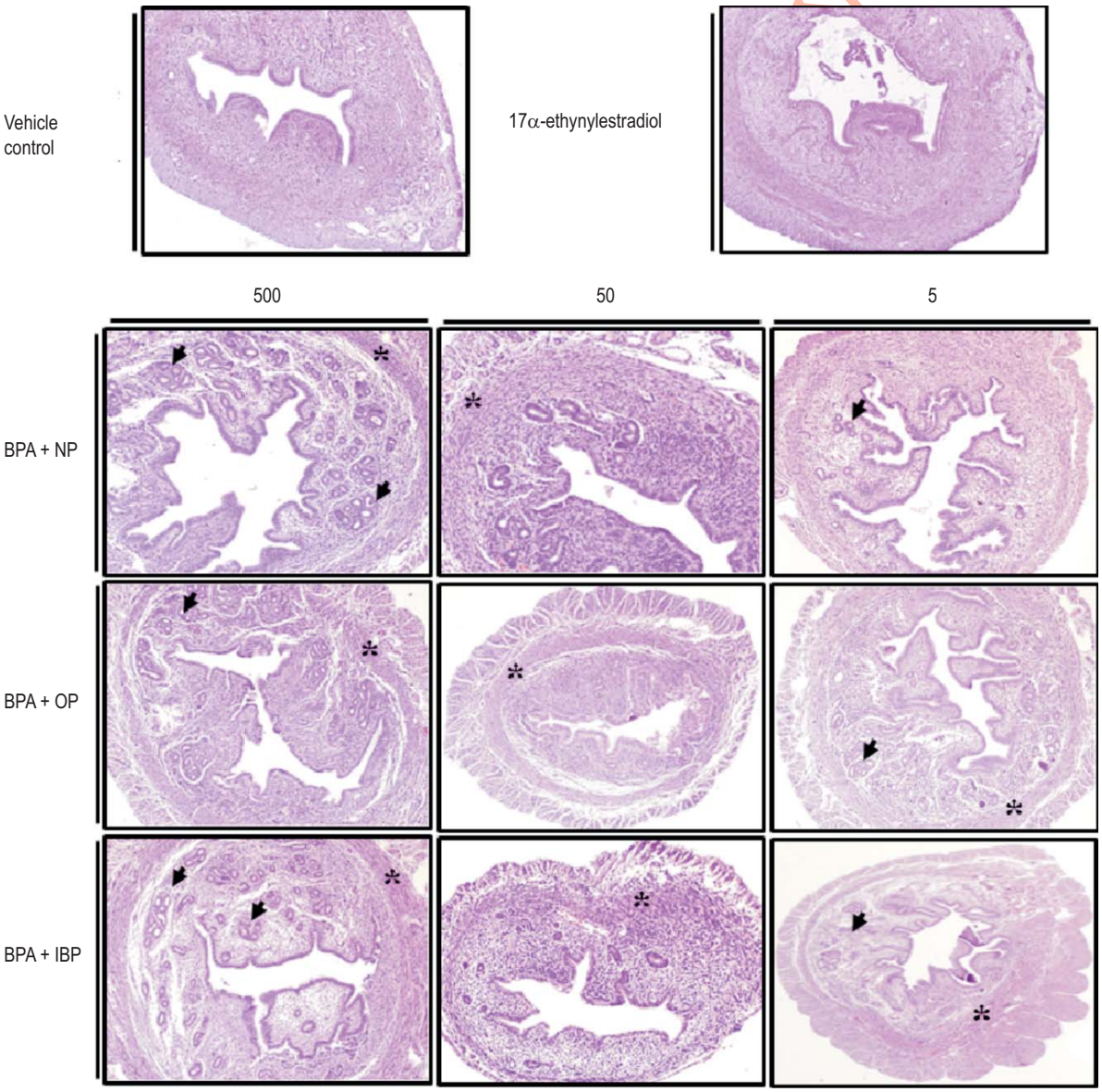

Fig. 1 : Photomicrographs of uterus of mouse exposed to combination of BPA, NP, OP and IBP. H&E x 100. A disorganization of the endometrial gland and muscle layers, including endometrial hypertrophy (arrow signals) and morphometric changes in the thickness of uteri (asterisk signals)

morphology and physiology of the uterus has been investigated (Vo *et al*., 2009). For example, a low oral dose of diethylstilbestrol on prenatal stage also showed significant effect on reproductive tract development in F1 female CD-1 mice, such as reduced endometrium thickness and increased polyovular follicles, irregular oocytes with condensed chromatin in ovary at sexual maturity (Maranghi *et al.*, 2008). Although the present result failed to detect change in uterus and, ovary weight however, histological alterations were observed in these organs. The toxic effects of these combined chemicals can be absorbed through placenta and breast milk and can alter the structure of reproductive organs (e.g., uterus and ovary) of mouse offsprings. Hence, it might be speculated that some synergic effects of early exposure to environmental chemicals result in reproductive tissue disruption.

The results of the present study suggests that the combination of endocrine disrupting chemicals should be evaluated as possible risk factors for gestational stage, malformation offspring associated with metabolic syndrome.

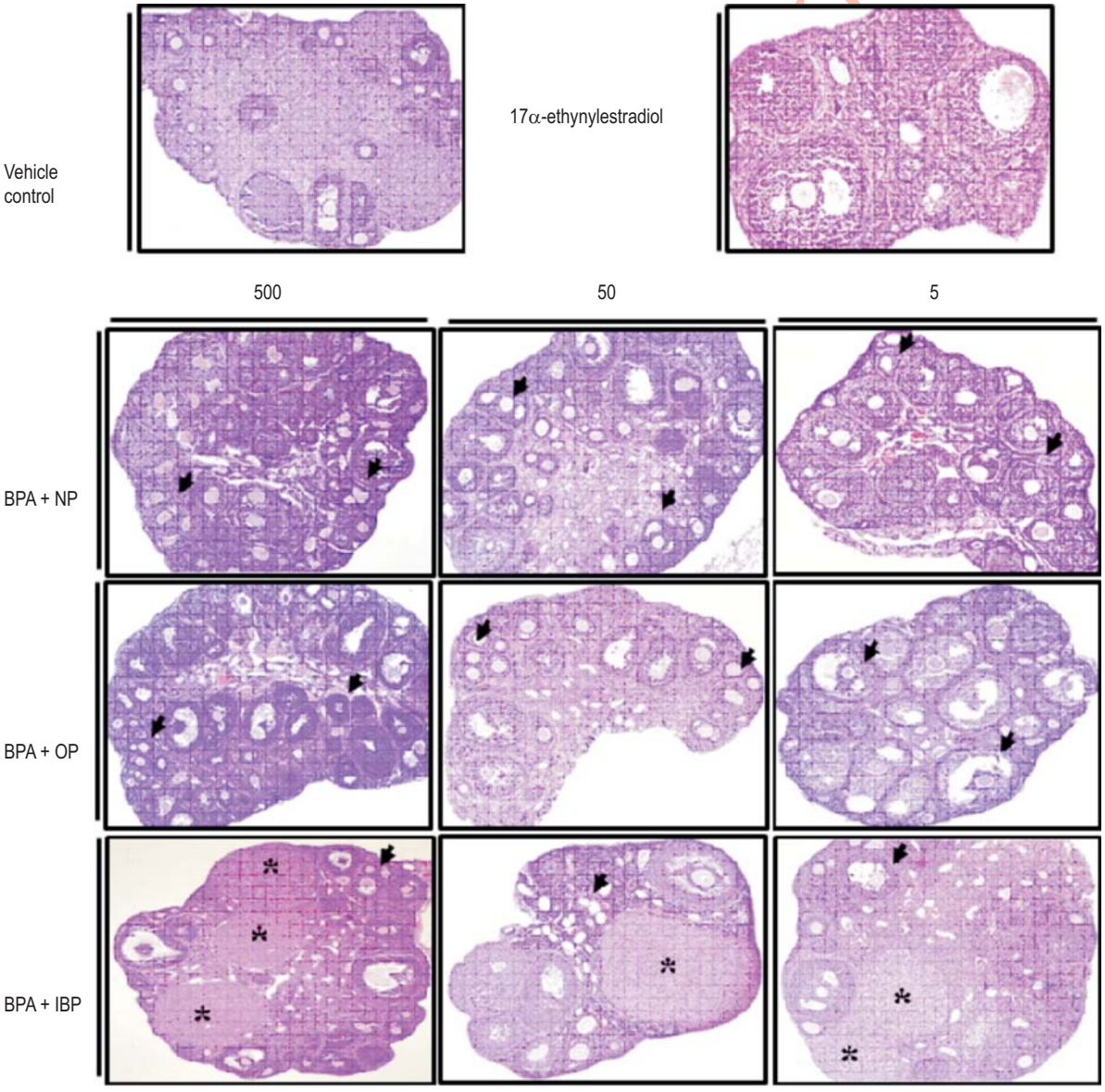

Fig. 2 : Photomicrographs of ovary of mouse exposed to combination of BPA, NP, OP and IBP. H&E x 100. The ovaries revealed an increase of corpora lutea (asterisk signals), cystic follicles (arrow signals) in a dose-dependent manner

Table 1: Viability and development of the mouse offspring exposed to combination of BPA, NP, OP, and IBP during gestation days (GD) 1 to 21

Parameters	Vehicle control	17α-ethynyl estradiol	BPA+NP 500	BPA+NP 50	BPA+NP 5	BPA+OP 500	BPA+OP 50	BPA+OP 5	BPA+IBP 500	BPA+IBP 50	BPA+IBP 5
GD time	22.25±0.50	21.67±0.58	20.33±0.58[a]	20.67±0.71[a]	21.00±0.71	20.67±0.52[a]	20.80±0.45[a]	21.40±0.55	20.67±0.58[a]	20.50±0.58[a]	20.75±0.50[a]
Little size (pups), n	50	52	46	52	50	46	60	34	58	58	56
Live ratio (%)	95.45±2.50	88.89±2.89	85.71±2.83	88.46±2.32	100.0±0.58	65.48±1.00	91.67±3.46	45.83±2.83[a]	71.72±3.46	98.46±3.44	100.00±1.63
Female/ Male ratio	64.00/36.00	61.54/38.46 (p=0.8836)	56.25/43.75 (p=0.3123)	54.00/46.00 (p=0.1956)	60.00/40.00 (p=0.6622)	56.52/43.48 (p=0.3855)	43.33/56.67[b] (p=0.0045)	52.94/47.06 (p=0.1511)	34.48/65.52[b] (p=0.0001)	51.72/48.28 (p=0.1148)	39.29/60.71[b] (p=0.0006)

Mean ±SEM; n = 10 female or male mouse offspring for each dose; [a]p<0.05 vs. vehicle control (Tukey's multiple regression test at p<0.05); [b]p<0.01 vs. vehicle control (Fisher's exact probability test. There rejection value for the null hypothesis was p≤0.05)

Table 2 : Effects of combination of compounds (BPA, NP, OP, and IBP) on body weight and organ weights in female offspring at postnatal day (PND) 41

Parameters	Vehicle control	17α-ethynyl estradiol	BPA+NP 500	BPA+NP 50	BPA+NP 5	BPA+OP 500	BPA+OP 50	BPA+OP 5	BPA+IBP 500	BPA+IBP 50	BPA+IBP 5
Pituitary weight (mg g^{-1} b.wt.)	0.038±0.009	0.060±0.009	0.061±0.007	0.068±0.04	0.057±0.019	0.087±0.014[a]	0.067±0.013	0.053±0.001	0.038±0.003	0.031±0.001	0.038±0.012
Thyroid weight (mg g^{-1} b.wt.)	0.82±0.18	0.93±0.08	1.10±0.37	1.17±0.05	1.18±0.15	1.00±0.11	1.01±0.17	0.88±0.05	0.95±0.10	0.92±0.20	1.02±0.07
Adrenal weight (mg g^{-1} b.wt.)	0.31±0.10	0.51±0.07[a]	0.41±0.08	0.36±0.05	0.45±0.02	0.42±0.17	0.21±0.04	0.50±0.11[a]	0.37±0.05	0.20±0.15	0.23±0.02
Liver weight (mg g^{-1} b.wt.)	23.78±8.30	35.06±5.69	37.79±0.65	46.56±16.62	47.62±11.20	46.76±1.53	49.80±10.58	35.28±6.25	37.16±6.25	12.79±15.12[a]	43.68±12.11
Kidney weight (mg g^{-1} b.wt.)	14.08±1.59	12.99±1.13	12.56±2.51	11.55±2.19	13.03±1.87	11.77±1.16	11.73±0.59	12.11±2.42	11.92±1.60	13.03±1.62	11.35±1.62[a]
Spleen weight (mg g^{-1} b.wt.)	1.81±0.25	2.63±0.77	2.92±1.87	1.29±0.36	1.10±0.64	2.09±0.34	2.33±0.77	1.50±0.30	1.16±1.11	1.58±0.08	1.35±0.53
Uterus weight (mg g^{-1} b.wt.)	1.20±0.29	2.90±0.54[a]	1.57±0.41	1.89±0.48	1.78±0.59	1.67±0.52	1.95±0.67	1.41±0.01	1.78±0.23	1.29±0.04	1.34±0.35
Ovary weight (mg g^{-1} b.wt.)	0.36±0.15	0.63±0.09[a]	0.42±0.01	0.33±0.21	0.33±0.11	0.35±0.09	0.45±0.13	0.49±0.10	0.34±0.17	0.25±0.14	0.37±0.02

Mean±SEM; n=10 female offspring for each dose; [a]p < 0.05 vs. vehicle control (Tukey's multiple regression test at p<0.05)

Table 3 : Effects of combination of BPA, NP, OP, and IBP on body weight and organ weights in male offspring at postnatal day (PND) 41

Parameters	Vehicle control	17α-ethynyl estradiol	BPA+NP 500	BPA+NP 50	BPA+NP 5	BPA+OP 500	BPA+OP 50	BPA+OP 5	BPA+IBP 500	BPA+IBP 50	BPA+IBP 5
Pituitary weight (mg g^{-1} b.wt.)	0.055±0.008	0.039±0.003	0.043±0.010	0.045±0.018	0.35±0.015	0.034±0.014	0.044±0.012	0.033±0.008	0.052±0.011	0.045±0.020	0.037±0.019
Thyroid weight (mg g^{-1} b.wt.)	1.00±0.17	0.64±0.08[a]	0.42±0.10[a]	0.49±0.04[a]	0.45±0.06[a]	0.98±0.08	0.63±0.08[a]	0.95±0.02	1.09±0.01	0.57±0.06[a]	0.50±0.18[a]
Adrenal weight (mg g^{-1} b.wt.)	0.10±0.05	0.18±0.01	0.13±0.05	0.14±0.07	0.16±0.04	0.38±0.04[a]	0.22±0.04	0.40±0.06[a]	0.29±0.05	0.29±0.06	0.17±0.04
Liver weight (mg g^{-1} b.wt.)	32.70±5.99	30.79±6.78	26.61±4.66	28.85±2.89	31.43±1.87	35.66±5.92	40.33±9.67	28.63±1.30	27.24±4.02	23.31±2.79	28.28±0.61
Kidney weight (mg g^{-1} b.wt.)	15.55±2.33	15.27±1.41	12.97±2.30	13.91±2.70	14.60±1.71	13.47±0.94	12.51±0.84	13.44±1.83	11.91±2.34[a]	12.67±1.51	11.02±1.01[a]
Spleen weight (mg g^{-1} b.wt.)	2.62±0.47	1.24±0.35	1.43±0.10	1.22±0.53	1.19±0.09	1.74±0.38	2.06±0.06	3.12±0.93	2.59±0.40	2.52±0.62	1.61±0.16
Testis weight (mg g^{-1} b.wt.)	8.30±0.01	7.78±1.52	7.51±0.27	7.52±1.14	7.20±0.62	7.54±0.66	8.03±0.90	6.13±0.06	6.72±0.76	7.03±0.79	6.04±0.45

Mean±SEM; n=10 male offspring for each dose; [a]p < 0.05 vs. vehicle control (Tukey's multiple regression test at p<0.05)

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