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The chick embryo: an animal model for detection of the effects of hormonal compounds

Sandrine Biau · Sandrine Bayle ·
Pascal de Santa Barbara · Benoit Roig

Abstract Hormonal compounds are a class of pharmaceutical product that disrupt the endocrine system of animals and humans. Exposure to these molecules, even at low concentrations, can have severely damaging effects on the environment, to organisms, and to humans. The cumulative presence of these compounds is also characterized by synergistic effects which are difficult to estimate. They are an underestimated danger to the environment and to the human population. This paper presents an in-vivo model enabling to assessment of the real impact of exposure to hormonal compounds and the synergistic effect which can be involved. The anatomical effects of in-ovo exposure to two natural estrogen compounds (estrone and estriol, at 600 ng g^{-1}) and a synthetic estrogen (ethynylestradiol, at 20 ng g^{-1}) have been investigated. Estrone and estriol lead to morphological defects, mainly in the urogenital system of the developing chick embryo, whereas ethynylestradiol has fewer effects. Estriol caused persistence of Müllerian ducts in 50% of male embryos and hypertrophic oviducts in 71% of females. Estrone had the same effects but at the percentages were lower. Kidney dysfunction was also observed, but only with estrone, in both males and females. We also tested estrogenic compounds in two types of cell

line which are estrogen sensitive (BG1 and MCF7) then completed and confirmed our previous in-vivo results. Seven pharmaceutical-like compounds—estrone (E1), estradiol (E2), estriol (E3), ethynylestradiol (EE₂), carbamazepine (C), genistein (G), and bisphenol-A (BPA)—were tested alone or in mixtures. Different effects on the two cell lines were observed, indicating that endocrine compounds can act differently on this organism. Experiments also showed that these molecules have synergistic action and induce more severe effects when they are in mixtures.

Keywords Estrogenic compound · Synergy ·
Animal model · Chick embryo · Human

Introduction

Pharmaceutical products are a large group of chemical molecules which have not always been well studied or regulated [1]. They can, however, be found at a large range of concentrations (from ng L^{-1} to mg L^{-1}) in environmental compartments such as municipal wastewater [2, 3], surface water [3–5] and groundwater [6].

Hormonal compounds were among the first pharmaceutical-like products investigated in the environment, because of their endocrine-disrupting potential. Exposure to hormonal compounds may affect sexual differentiation, for example by inducing vitellogenesis feminization in fish [7–9] and may also affect human reproduction [10]. It has recently been suggested that these hormonal compounds could affect the human immune system [11, 12].

The estrogenic potential of hormonal compounds has been demonstrated by several methods, for example competitive estrogen receptor-binding assay, reporter gene

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assays, bioassays by use of biomarkers (for example vitellogenin), in male oviparous vertebrates [13, 14]. Male fish (rainbow trout, zebra fish) and male amphibians have also been used to detect estrogenic activity, by using the whole animals or primary cultured hepatocytes [15–18]. Several mammalian models have also been used to assess the impact of estrogenic compounds. The uterotrophic assay in juvenile mice or rats is one of the best-known in-vivo tests [19–23]. Other tests enable measurement of variation of hormones activity in serum [24], gene activation (transgenic mouse luciferase reporter) [25, 26], biosynthesis of other proteins (e.g. lactoferrins, decarboxylase ornithine) [27], ex-vivo gonad culture [28, 29], behavior [30, 31], developmental growth [32–34], and the characteristics of pregnancy [35].

In paper we describe use of the embryonated avian egg as a readily accessible, fast, complete, and reliable in vivo test system for assessing the effects of endocrine compounds. There is not yet an established test system for estrogen-like effects in chick embryos. Sexual differentiation occurs in opposite manners in birds and mammals. In mammals, the presence of testosterone and other testicular hormones are a preliminary condition for development of male characteristics. In the absence of testicular hormones development takes the feminine course. In contrast, in reproductive structure and behavior birds develop masculine characteristics in the absence of estrogens [36, 37]. If the synthesis of estrogens is inhibited during gonadal differentiation, genetic females will develop as a male phenotype in avians [38]. The importance of estrogens in the organization of behavioral, neurochemical, and neuro-anatomical features is well established [36, 39–41]. Early differentiation of the urogenital tract is similar for all vertebrates, however, consistent with the unique bipotential system that will later become differentiated as either male or female [42]. Estrogen-dependent sexual differentiation should make the avian embryo a suitable organism for testing potential estrogen-like chemicals.

Assessment of the hazard of potential endocrine-disrupting chemicals is hampered by lack of scientific knowledge enabling extrapolation of in-vitro data to the intact organism. There is also a real need to establish validated in-vivo and in-vitro test systems for examination of the hormonal activity of these chemicals, especially for species with reproductive physiology features which lack mammalian correlates [43].

The objective of this study was to test the effect of some pharmaceutical-like compounds (identified in the effluents of municipal sewage-treatment plants) by use of a chick embryo model (more particularly development of its urogenital tract). The effect of three steroid compounds (E1, EE₂, and E3) was tested as a first stage. E1 and E3 seemed to induce very severe malformations whereas EE₂

induced very few morphologic defects. As a result of these initial observations experiments were performed using the same compounds at the same concentrations on a cellular model (BG1, MCF7) based on the cellular proliferation, and the first results were confirmed.

In a second stage, the effects of different mixtures of E1, E2, E3, EE₂, genistein, bisphenol A, and carbamazepine were tested in experiments on cell lines (BG1 and MCF7), as a preliminary to future transposition to the chick model.

Materials and methods

Reagents

Estrone (E1), estradiol (E2), estriol (E3), ethynylestradiol (EE₂), carbamazepine (C), genistein (G), and bisphenol A (BPA) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).

Chick embryos and treatment of eggs

Timed-fertilized white Leghorn eggs were obtained from a commercial supplier (Haas Farm, France) and incubated at 38 °C in a humidified incubator (Coudelou, France). On the fourth day of embryonic development a solution of an estrogenic compound in phosphate-buffered saline (PBS) was injected into the yolk of each egg through a small needle hole in the shell. The eggs were then incubated for 14 days. The doses were 600 ng g⁻¹ estrone, 600 ng g⁻¹ estriol, and 20 ng g⁻¹ ethynylestradiol. Control eggs received PBS only. Embryonic development was stopped at 18 days (three days before hatching) and the embryos were dissected. The urogenital tract were harvested, washed with fresh PBS, and then fixed in fresh 4% paraformaldehyde (Fermentas) in PBS for 2 h. Digestive tract samples were taken for genetic sex determination.

Determination of sexual genotype

All 18-day-old embryos were typed for their sexual ZZ or ZW genotype, using the method of Fridolfsson and Ellengren [42] based on PCR with a single set of primers. PCR reactions were performed using the primers 2550F “5'-GTT ACT GAT TCG TCT ACG AGA-3'” and 2718R “5'-ATT GAA ATG ATC CAG TGC TTG-3'”. PCR products were separated by 2% agarose-gel electrophoresis, stained with ethidium bromide, detected with UV light, and photographed. All the females had a 450-bp CHD1-W female-specific fragment. The embryos of both sexes had a 600-bp CHD1-Z specific fragment.

A total of 208 embryos were examined.

Photographs

The images were collected in whole-mount under a Nikon SMZ1000 scope and in section under Zeiss Axiophot microscope, both using a Nikon DXM1200 camera.

Cell culture and synergism assay

Human ovarian carcinoma cell line BG-1 derived from the primary tumor tissue of a stage-II ovarian epithelial adenocarcinoma was a kind gift from Pr Pascal Pujol (Inserm U540, Montpellier). Cells were grown in McCoy's medium (Invitrogen Life Technologies, Cergy Pontoise, France) supplemented with 10% fetal calf serum, glutamine and penicillin/streptomycin.

The human breast adenocarcinoma derived from a metastatic site of pleural effusion cell line MCF7 was obtained from Sigma (Saint Quentin Fallavier, France; ECCAC #860012803). Cells were grown in DMEM medium (Invitrogen Life Technologies), also supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. The cells were cultured at 37 °C in a humidified 5% CO₂ incubator.

Three days before the experiments, the cell lines were transferred into phenol red-free medium supplemented with 6% dextran-coated charcoal (DCC)-treated FCS and 1% antibiotic (penicillin/streptomycin) (6% DCC-FCS), DMEM/F12 for BG-1 and DMEM for MCF7. Induction experiments were performed by seeding BG1 and MCF7 cells lines at a density of 5000 cells/well in 96-well white opaque tissue-culture plates and maintained in 6% DCC-FCS. They were exposed twelve hours later and incubated for 72 h at 37 °C. Experiments were performed in quadruplicate and repeated twice. The cells were quantified indirectly by use of the CellTiter-Glo luminescent cell viability assay (Promega) and a microplate luminometer (Centro LB 960; Berthold Technologies).

The cells were exposed to different pharmaceutical-like compounds at concentrations of 11 pmol L⁻¹ (3 ng L⁻¹) for estrone (E1), 11.7 pmol L⁻¹ (3.2 ng L⁻¹) for estradiol (E2), 9.8 pmol L⁻¹ (2.9 ng L⁻¹) for ethynylestradiol (EE₂), 8.7 pmol L⁻¹ (2.5 ng L⁻¹) for estriol (E3), 50.7 pmol L⁻¹ (0.87 ng L⁻¹) for carbamazepine (C), 1.13 nmol L⁻¹

(258 ng L⁻¹) for bisphenol-A (BPA), and 88 nmol L⁻¹ (20 μg L⁻¹) for genistein (G), which correspond to levels observed in environmental water samples [43–45]. The two cell lines were exposed to all the different combinations of the seven compounds. The effects of G, BPA, and C on the proliferation effect induced by the combination of E1, E2, E3 and EE₂ were particularly investigated. Thus, a series of tests was performed by using solutions of these compounds (alone or in combination) to which G or BPA or C (alone or in combination) were added. Table 1 gives some examples of the mixtures tested.

All possible combinations (127) were studied.

Results

Hormonal compounds were tested in the chicken embryo model before exposure of cell lines.

Chick embryos

The chick embryo is a highly suitable model for organogenesis studies, and enables rapid, qualitative, and quantitative assessment. Avian eggs have frequently been used as a bioindicator in environmental pollutant-monitoring programs, and contaminated eggs are useful for risk assessment of lipophilic environmental contaminants [46–50].

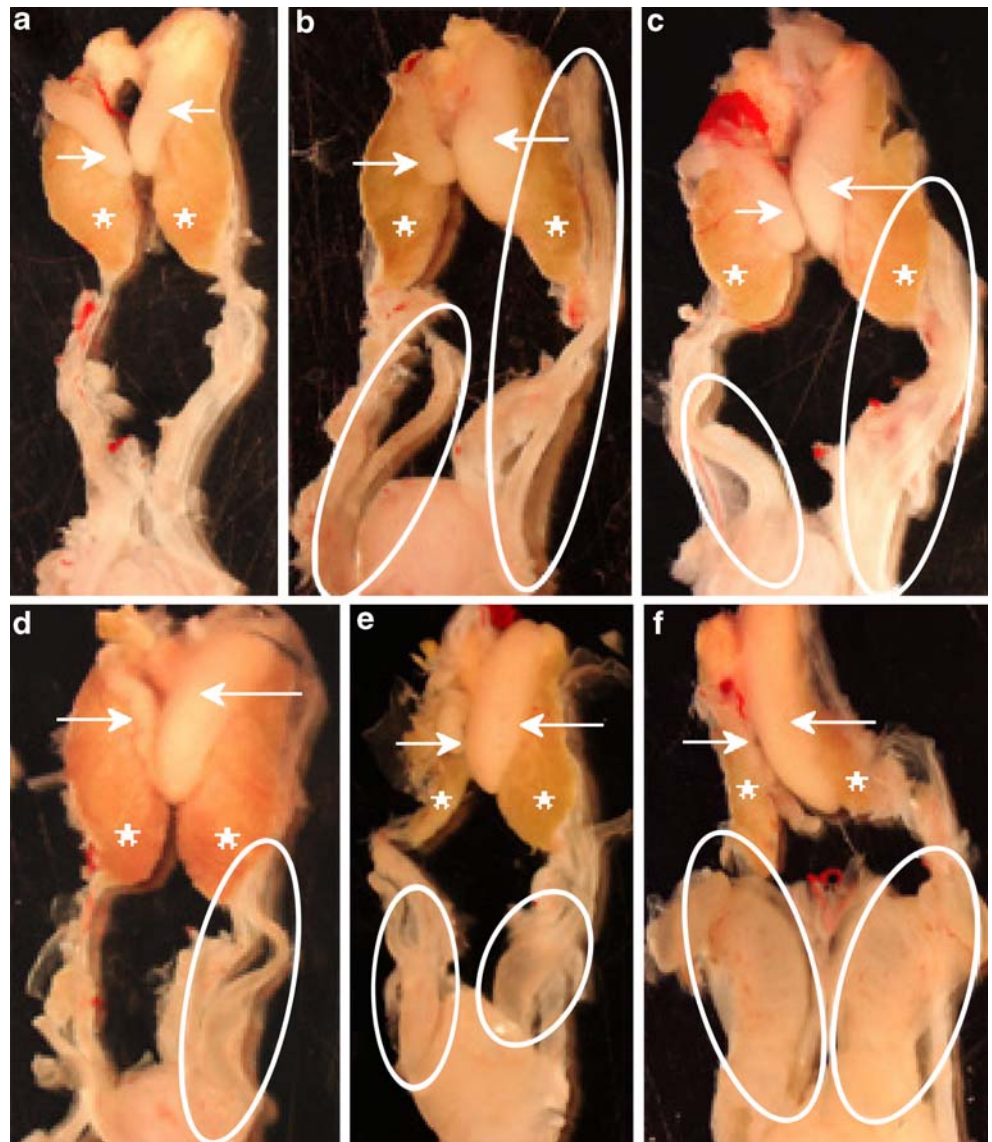
Chick embryos were exposed to estrone, estriol, and ethynylestradiol (commonly used in contraceptive pills) during development of the chick embryo. The concentrations of E1 and E3 were deliberately ten times more than those found in environmental water samples, to take into consideration a bioaccumulation factor described and discussed in the literature [51–55]. EE₂, in contrast, was used at the concentration found in the environment, to serve as a control, as described in the literature [33, 56].

Exposure to E1 and E3 led, mainly, to the development of morphological defects of the urogenital system in chick embryos (Fig. 1). These defects were observed at lower frequencies after exposure to EE₂, as described in the literature. The observations below therefore apply to E1 and E3 experiments only and the impact of these compounds on the development of the urogenital system.

Table 1 Examples of test mixtures prepared from E1 base solution

Base	Base+C	Base+G	Base+BPA	Base+BPA+G	Base+BPA+C	Base+G+C	Base+BPA+G+C
E1+E2+	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +E3+
EE ₂ +E3	E3+C	E3+G	E3+BPA	E3+BPA+G	E3+BPA+C	E3+G+C	BPA+G+ C
E1+E2+	E1+E2+EE ₂ +C	E1+E2+EE ₂ +G	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +	E1+E2+EE ₂ +
EE ₂			BPA	BPA+G	BPA+C	G+C	BPA+G+C
E1+E2	E1+E2+C	E1+E2+G	E1+E2+BPA	E1+E2+BPA+G	E1+E2+BPA+C	E1+E2+G+C	E1+E2+BPA+G+C

Fig. 1 Effects of EDC on the development of the urogenital system in chick embryos. **a–f** show the urogenital system of 18-day-old chick embryos treated with different EDC: **(a)** male control, **(b)** E1-exposed male, **(c)** E3-exposed male, **(d)** female control, **(e)** E1-exposed female, **(f)** E3-exposed female. Gonads are indicated by *arrows*, kidneys by *asterisks*, and Müllerian ducts by *ellipses*



On day 18 of development (three days before hatching) the female urogenital tract was characterized by severe disymmetry—only the left ovary was developed and functional. The right gonad was just visible. Similarly, under the stimulation of gonadic estrogens only the left Müllerian duct was able to develop in a functional oviduct. The right Müllerian duct regressed during embryonic life and in adulthood, only the posterior rudiment was present (Fig. 1b).

In male embryos, gonads and Wolffian ducts developed into a bilateral, symmetrical reproductive system. The two testicles and the two deferense ducts—derivatives of the Wolffian ducts were shown to be functional by stimulation with testicular androgens; the right testicle was slightly smaller than the left. The anti-Müllerian hormone (AMH) produced by the testes induced regression of the two Müllerian ducts during embryonic life (Fig. 1d).

We decided to inject our compounds on the 4th day when development of the bipotential and undifferentiated gonad starts. We injected the EDC directly into the yolk of each egg through a small needle hole in the shell and then incubated the eggs until complete sexual differentiation (when the egg was approx. 18 days old).

After injection of E3 regression of the right gonad, an increase in the size of the left gonad, and persistence of the Müllerian ducts were observed in males. These differences (compared with the control) suggest that E3 disturbs male development and leads to the feminization of the urogenital system. In E3-treated females (Fig. 1f), an increase in the size of the left gonad and the presence of one or two hypertrophic oviducts were specifically observed; these correspond to a hyperfeminization phenotype.

The results obtained after injection of E1 were similar to those described above, but with lower frequency

Table 2 Percentage malformation of the reproductive organs observed for 18-day-old chick embryos after injection of E1 or E3 into the yolk on day 4 of incubation

Treatment	Female with hypertrophic Müllerian duct (%)	Male with persistence of Müller's canals (%)	Mortality
Control	0% (0/39)	0% (0/30)	8%
Estriol (E3)	71% (30/42)	48% (10/21)	15%
Estrone (E1)	49% (23/47)	18% (5/27)	10%

(Table 2). Exposure to E1 also effected development of the kidney in both sexes. E1-exposed kidneys were not functional (as observed from the green color and accumulated urine).

As is apparent from Table 2, these experiments were performed on 208 avian eggs. Treatment with E1 and E3 induced a high frequency of malformations of the urogenital tract associated with a moderate death rate. These findings let us hypothesize that the observed malformations are because of the specific action of EDC on development of the urogenital tract.

E3 caused persistence of Müllerian ducts in 50% of male embryos and hypertrophic oviduct in 71% of females (Table 2). E1 caused the same phenomena but to a lesser extent: approximately 49% for persistence of the Müllerian ducts and 18% for hypertrophic oviduct. Kidney dysfunction was observed for 80% of embryos treated with E1, in both males and females. E1 and E3 exposure mainly affect urogenital tract development without causing other macroscopic malformations of the brain, heart, or neural tube.

From these results the chick embryo model can be envisaged as relevant for study of the impact of hormonal compounds during development of the urogenital tract.

Synergistic effects could also be investigated by use of this easy, rapid, and relevant model. Preliminary cell experiments are required in the first stage.

Cell proliferation assay

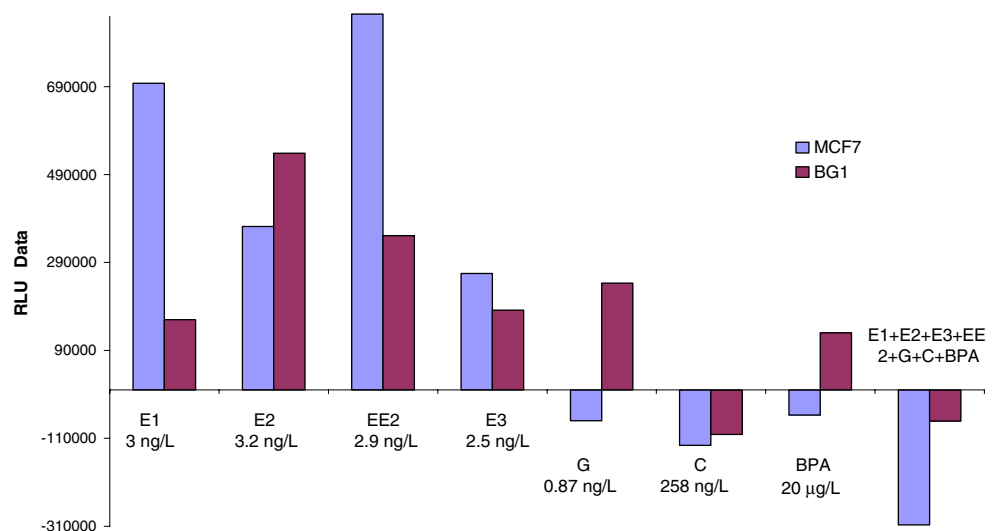
Cellular proliferation assays have been conducted, first in the presence of previously tested compounds and then with other environmental endocrine pollutants (including pharmaceutical compounds) at environmental concentrations. Seven endocrine compounds (E1, E2, E3, EE₂, genistein, carbamazepine, and bisphenol-A) were tested on two cell lines, BG1 and MCF7, chosen because of their sensitivity toward estrogenic and pharmaceutical compounds, because of expression of the estrogen receptor (ER) (activated by these compounds). The first step of this work was to analyze cellular proliferation during the 72 hours after addition of these seven compounds (Fig. 2). The second step was to discover synergistic action of these compounds by using all possible combinations.

Effects of the individual compound on cellular proliferation

Cells were exposed to each compound for 72 h (Fig. 2). A mixture of all the compounds was also used for comparison. In each experiment non-exposed cells were used as control and the value (RLU data) of the effect of the tested combination was deduced from the control.

Responses of the cell lines were different for G and BPA, however. G and BPA increased proliferation of BG1 cells whereas proliferation of MCF7 was inhibited. Inhibition of proliferation also occurred after exposure to C. Finally, the mixture of all the compounds caused inhibition of cell proliferation. This result, first, confirmed the chick embryo results (for E1 and E3) and, second, showed the possibility

Fig. 2 Responses of MCF7 and BG1 cell lines to exposure to each compound and after addition of the mixture of the seven EDC: estrone (E1), estradiol (E2), ethynylestradiol (EE₂), estriol (E3), genistein (G), carbamazepine (C), bisphenol A (BPA), and E1+E2+EE₂+E3+G+C+BPA. E1, E2, EE₂ and E3 induced proliferation of the two types of cell



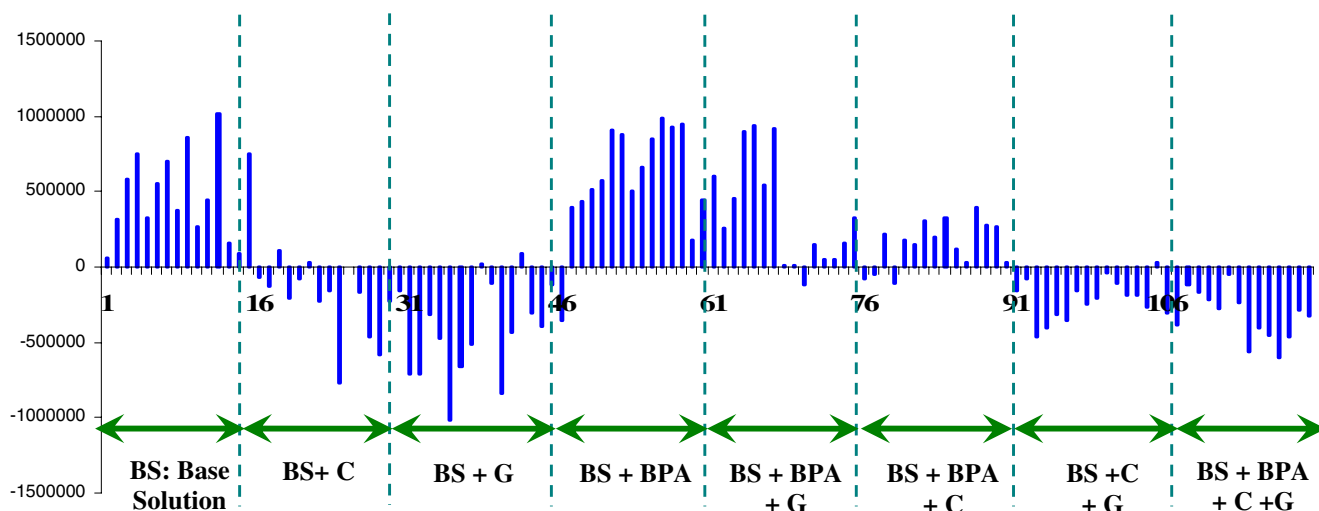


Fig. 3 Action of EDC after addition of different mixtures to MCF7 Cell line (from 2 to 7 compounds)

of differentiation of some endocrine disruptors by use of different cellular models.

Exposure to the mixture of the seven compounds

Numerous mixtures were prepared and added to BG1 and MCF7 cells to study the potential synergy effect on cellular proliferation. The effects of G, BPA, and C were particularly studied. They were added (alone or in combination) to base solution composed of E1, E2, E3, and EE₂ alone or in combination (Fig. 3).

For MCF7 cell line (Fig. 3), clear effects were apparent after exposure to C, G, C+G, and BPA+C+G. All induced inhibition of cellular proliferation. BPA always induced an increase in proliferation but coupled with G or C the response was more moderate.

Addition of C, G, C+G, and BPA+C+G to BG1 cell line always induced inhibition of cellular proliferation (data not shown) whereas BPA induced an increase in proliferation.

These results emphasize the particular effects of combinations of compounds. For example, a dominant effect order seems to appear:

BPA>G and BPA>C but C+G>BPA.

C and G are result in complete inhibition of cellular proliferation and BPA induces a strong cellular proliferation. BPA+G and BPA+C also induce cellular proliferation whereas C+G and BPA+C+G cause inhibition.

These preliminary results must be investigated further, in particular by testing exposure to other concentrations. Estrogenic and pharmaceutical compounds have, however, been shown to have different actions, depending on cell line and biological target. The different combinations had several effects—synergy, inhibition, or additive—which

differed in nature and intensity for the two cell lines. Thus, an index (I_{sr}) characterizing the cumulative effect of different estrogenic and pharmaceutical pollutants could be envisaged.

Conclusion

In this study the effects of environmental hormonal and pharmaceutical products were tested by use of two complementary models—the chick embryo and cell models. Malformations and feminization of males were observed, with high representative statistical percentages, after exposure to the two steroid compounds estrone and estriol. These results were confirmed by cellular proliferation assays on two specific cell lines. These assays were expanded to mixtures of seven pharmaceutical-like compounds. Numerous synergistic effects (positive or negative) were observed enabling a potential synergy index to be envisaged. The chick embryo model must be used to test more relevant combinations, to assess the effect on living organisms. The different pharmaceutical products affecting the steroidogenic pathway show the high sensitivity of bird gonadal development; this enables us to consider use of the avian egg in environmental bioassays.

These in-vivo and in-vitro systems represent a complementary approach to understanding the mechanisms of action of endocrine disruptor compounds and identifying and characterizing their synergistic effects.

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