A PRELIMINARY CHARACTERISATION OF STEROID SECRETION FOLLOWING EXPOSURE OF OVARIAN FOLLICULAR CELLS TO A REAL-LIFE MIXTURE OF POPs EXTRACTED FROM ATLANTIC COD LIVER OIL.

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Introduction

Persistent organic pollutants (POPs) such as dioxins, polychlorinated biphenyls (PCBs), dichlorodiphenyldichloroethylenes (DDTs) and brominated flame retardants (BDEs) are distributed globally [1]. Exposure to POPs is an important subject because of the potential hazards to human and animal development and health. Particular attention has been given to xenobiotic compounds that are capable of modulating or disrupting the endocrine system [2,3]. It is critically important that we determine whether environmental concentrations of POPs, such as PCBs and organochlorine pesticides, may elicit adverse effects in animals and ultimately humans, especially during sensitive stages of development, in, for example, gonadal cells.

Contaminants usually occur in mixtures and it is difficult to predict biological effects directly from the composition of such mixtures. In a real-life situation, the combined effects of particular congeners may be additive, synergistic or antagonistic [4,5,6.7]; additive effects are most common. Ramamoorthy et al.[4] demonstrated that for several estrogen-responsive assays in the mouse uterus, and in a yeast based-reporter gene assay, both HO-PCB3 and HO-PCB4 exhibited estrogenic activity. The estrogenic activity of an equimolar mixture of these compounds was additive at high and low levels of estrogen receptor expression. Arnold et al.[5] showed that the effects of a mixture of two weak estrogens increased 160-1600 fold compared to the effects of single compounds. An example of antagonistic action has been demonstrated by Donnelly et al. [7].

There are gaps in our knowledge on which agents occur in real-life mixtures, and at which levels they exert toxicity. Moreover, there is also considerable uncertainty about the possible mechanisms of action.

In the present study we describe the *in vitro* effects of a real-life mixture of POPs on steroidogenesis in porcine ovarian follicular cells. Porcine cells are a valuable tool for the study of potential human toxicants as pigs exhibit a wide range of physiological similarities to humans, notably with respect to metabolism. We used a co-culture model of theca and granulosa cells, thereby mimicking the situation in intact ovarian follicles. In this co-culture model, both cell types retain their developmental and follicular stage-specific ability to secrete steroids.

Materials and methods

Contaminants were extracted from liver oil from Atlantic cod (*Gadus morhua*). The production of pharmaceutical cod liver oil includes several steps to remove pollutants. In a preliminary step, dioxins and planar PCBs are removed with charcoal. This is followed by a distillation process, which removes fractions of the oil and most of the lipid soluble pollutants. The waste from this distillation was used in our study and enabled us to conduct the study without the presence of components contributing to dioxin-like toxicity. In the first step, following removal of dioxins and planar PCBs with charcoal, approximately 20 ml of cyclohexane (CHX) and 50 ml of concentrated sulphuric acid (SA) was added to 1 g of fish oil and left overnight after shaking. The next day the organic phase was removed from the SA and the volume reduced under N_2 to 5 ml. Then 20 ml of SA was added and the mixture was again kept overnight. This procedure was repeated (1 ml CHX and 5 ml SA) to give one ml CHX with contaminants. The second step was to replace the CHX with ethanol. To the CHX solutions 10 ml of ethanol was added, and the volumes were reduced under a N_2 gas stream to one ml, and this procedure was repeated 4 times. The final concentration was adjusted to 3 ml. From this stock solution aliquots were diluted with CHX for chemical characterisation. Concentrations of contaminants were determined by GC-MS and GC-ECD. The detection limits for HCHs, chlordanes, HCB and DDTs were 0.5 to 3 ng/g. The detection limits for PCBs were 0.4 to 4 ng/ml, for toxaphenes 1 ng/ml, for BDEs 2 ng/ml, and for HBCD 3

ng/ml. For exposure of ovarian follicular cells, dose levels were used representing 1, 5, 10, 50, 100, 500, 1000 ng /ml culture medium of the stock solution which contained POPs extracted from 0.33 g of waste from the distillation of cod liver oil.

Theca interna and granulosa cells were isolated from prepubertal porcine follicles. For co-culture experiments, granulosa and theca cells were inoculated at concentrations of 4.0 x 10^4 and 1.0 x 10^4 viable cells/well. Cells were initially cultured in M199/CS without test compounds for 24h to allow for cell attachment to the wells. After 24 h, the medium was discarded and 0.3 ml of fresh M199 alone was added to the control culture.To the test cultures, marine mix was added in the concenrations: 1, 5,10, 50, 100, 500, 1000 ng/ml. For analysis of steroid levels, the media were collected after 24 h of exposure and frozen at – 20°C. Estradiol and testosterone levels were determined using the Dia.Metra (Segrate Milano, Italy). Cell viability and apoptosis were assessed with the lactate dehydrogenase (LDH) cytotoxicity assay (Roche Applied Science, Germany) and by measurement of caspase-3 activity, respectively.

Data were analyzed by 1-way analysis of variance (ANOVA) followed by the Tukey (HSD) multiple range test. P-values below 0.05 were considered statistically significant.

Results and Discussion

The measured total concentrations of various groups of POPs extracted (Marine mix) from the stock solution was: ΣPCBs: 21589 ng; ΣBDEs: 1949 ng; ΣDDTs: 11160 ng; ΣChlordanes: 3460 ng and ΣToxaphenes: 6990 ng.

Unexposed follicular cells in culture secreted 5.4 ng/ml progesterone, 0.5 ng/ml testosterone and 42 pg/ml estradiol into the medium. Marine mix (50 ng/ml dose) increased progesterone secretion to 120% of control, while a 1000 ng/ml dose significantly decreased progesterone levels to about 45% of control. Testosterone secretion was significantly increased by doses ranging between 1 and 500 ng/ml, and estradiol by doses ranging between 1 and 100 ng/ml (Fig. 1). None of the exposed groups demonstrated increased cell death as determined by LDH levels in the culture medium, or increased apoptosis as determined by caspase-3 activity (Fig. 2). The dose-response relationship for testosterone and estradiol indicated that exposure of porcine ovarian follicular cells to low doses of the Marine mix caused a high steroidogenic response, whereas high dose levels had no effect on steroidogenesis.

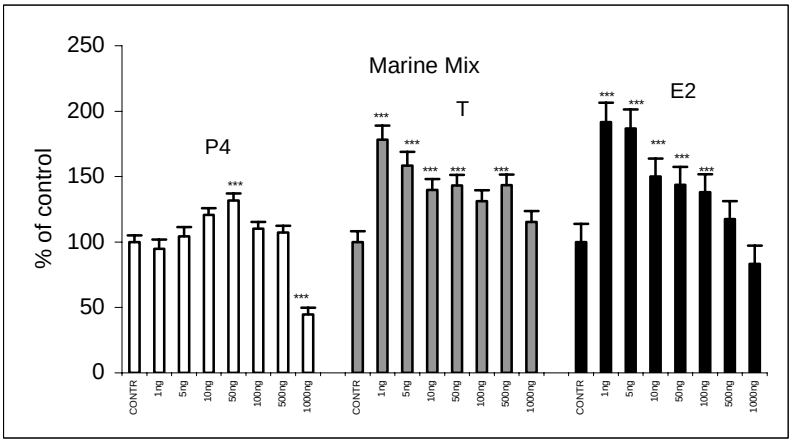

Fig. 1. Mean progesterone (P4), testosterone (T) and estradiol (E2) concentrations relative to control (%; +SE) in a co-culture of porcine theca and granulosa cells. Cells were exposed to different doses of a real-life mixture harvested from Atlantic cod liver oil (Marine mix). Dose levels are given in ng and represent ng/ml culture medium of an extract from the stock solution. CONTR= Unexposed group. *** Exposed group significantly different from control (P< 0.001).

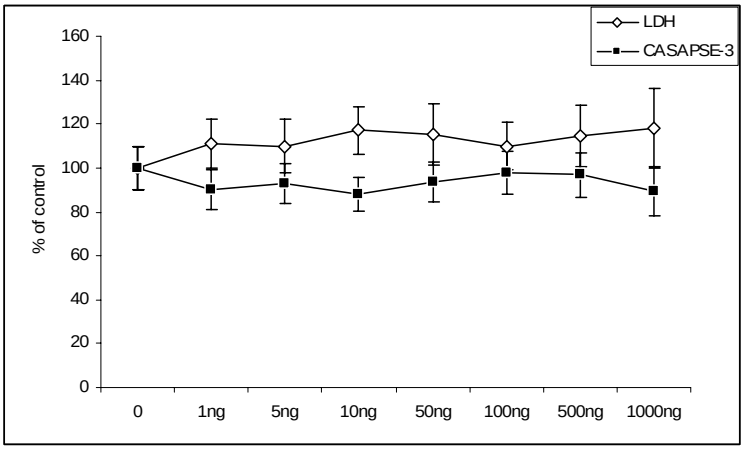

Fig. 2. Cell viability (LDH) and cell apoptosis (caspase-3 activity) in cells exposed to different doses of a real life mixture harvested from Atlantic cod liver oil (Marine mix). Dose levels are given in ng and represent ng/ml culture medium of an extract from the stock solution.

The data suggested a stimulatory action by the marine mix on testosterone and estradiol secretion. The changes in steroid secretion could not be explained by effects on cell viability and apoptosis, since LDH and caspase-3 activity were not affected at any of the doses used. . Moreover, the data indirectly suggested an effect on the conversion of progesterone to testosterone and then to estradiol. Inhibition of 3ß-hydroxysteroid dehydrogenase, 17α-hydroxylase/lyase, and 17ß-hydroxysteroid dehydrogenase activities as a mechanism for Acoclor1248-action (technical mixture) in the testis have been previously postulated by Andric et al. [8].

The non-linear dose-response relationship between POP contamination in the culture medium and the steroidogenic response by ovarian follicular cells is indicative of a hormesis-like effect caused by the Marine Mix. A similar effect has also been reported after exposure of the same type of cells to the single congeners, PCB 126 and PCB 153 [9].

A fundamental concept in toxicology, is the use of an expected dose-response relationship as a basis for risk assessment. The threshold model or the linear non-threshold (LNT) model is commonly used. However, a curve-linear dose-response relationship may be common, as indicated by Calabrese and Calabrese [10]. Hence, it is possible that current models provide unreliable estimates of low-dose risks. The curve-linear or hormesis–like response is characterised by a modest stimulatory effect at low doses, and an inhibition or no response at high concentrations [11]. As also indicated by Renn [12], the present data suggest that further attention should be directed towards dose-response relationships in environmental toxicology. Such studies can provide useful information that might have a significant impact on the strategies for risk assessment of toxic substances.

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