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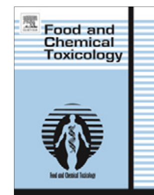

# Effects of mixtures of persistent organic pollutants (POPs) derived from cod liver oil on H295R steroidogenesis

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ABSTRACT

Crude cod liver oil and liver oil supplements are consumed as a source of vitamin A, D and polyunsaturated fatty acids; during winter and early pregnancy. Crude cod liver oil however constitutes a considerable source of persistent organic pollutants (POPs). This paper aimed at characterizing and quantifying the influence of POP mixtures extracted from three different steps in the cod liver oil industrial process on hormone production and the expression of steroidogenesis-related genes in H295R cells. Exposure to extracts from crude cod liver oil and from its industrial waste increased progesterone (P4), cortisol (Cort), testosterone (T) and estradiol (E2) production; and among others, the expression of *MC2R*, *CYP11B1* and *HSD3B2* genes. Observed effects after exposure to pharmaceutical cod liver oil extract were considerably lower. The type of effects on gene expression and hormone production were similar to those induced by forskolin and PCBs, the latter being the major contaminants within the extracts. Additional research is required to further unveil the mechanisms behind the observed steroidogenic effects and to assess whether the potential risk might outweigh the potential benefits of crude and processed cod liver oil consumption.

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## 1. Introduction

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and flame retardants resist degradation, bio-accumulate, are long-range transported and may possess toxic properties (Stockholm Convention Secretariat, 2008). They accumulate along the food chains and are ubiquitous in the environment, especially in temperate and polar areas (Law et al., 2006; Ropstad et al., 2006; de Wit et al., 2006). Trends in sediments and ice cores indicate a reduction in environmental concentrations of restricted or banned POPs since the 1970s (Li et al., 2009; Wang et al., 2008). This reduction however, has levelled off and current concentrations in sediments and food webs still pose a risk for wildlife and humans (Domingo and Bocio, 2007; Foekema et al., 2008). An incremental trend has been observed for recently banned or emerging persistent pollutants like polybrominated diphenylethers (PBDEs) or hexabromocyclododecane (HBCD) (Law et al., 2008). Exposure to POPs has been associated with numerous effects including suppression of the immune system, disturbances of normal development and particularly endocrine disruption (Darnerud, 2008; Hamers et al., 2006; Skaare et al., 2002). While attention has been placed on endocrine modulation through direct interaction with receptors (Hamers et al., 2006), *in vitro* studies suggest that POPs also modulate hormone synthesis through non-receptor mediated mechanisms, such as interference with gene expression, transcript stability and

*Abbreviations:* ACTH, adrenocorticotropic hormone; AhR, aryl hydrocarbon receptor; cAMP, cyclic adenosine monophosphate; Cort, cortisol; DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; DEPC, diethyl-pyrocarbonate; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; E2, estradiol; GC–ECD, gas chromatography–electron capture detector; GC–MS, gas chromatography–mass spectrometry; HBCD, hexabromocyclododecane; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); ITS, insulin–transferrin–selenium; IMARES, Institute for Marine Resources and Ecosystem Studies; mRNA, messenger ribonucleic acid; P4, progesterone; PBDE, polybrominated diphenyl ether; PBFR, polybrominated flame retardant; PCB, polychlorinated biphenyl; POP, persistent organic pollutant; REST, relative expression software tool; RIA, radio-immunoassay; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; T, testosterone; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; TEQ, TCDD equivalents.

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enzyme activity (Lin et al., 2006; Gregoraszczuk et al., 2009; Gracia et al., 2006). Altered steroidogenesis may be responsible for the association between PCBs and endocrine toxicity in humans (Carpenter, 2006). As a tissue with high lipid content, copious blood supply and steroidogenesis capacity, the adrenal cortex is likely to accumulate POPs and be a target of endocrine toxicity (Colby, 1994; Ribelin, 1984).

Exposure to environmental contaminants and particularly to POPs occurs with an extensive range of compounds mixed in various, often low concentrations (Boekelheide, 2007; De Zwart and Posthuma, 2005). Risk assessments of single contaminants provides valuable information for assessment of complex mixtures but fails to consider interactions between the contaminants and their matrix components – e.g. lipids and phospholipids in biological samples (Groten et al., 2001). Assessments of POP binary mixture effects on steroidogenesis have been recently undertaken (Gracia et al., 2006, 2007; Gregoraszczuk et al., 2008a), demonstrating that only some non-additive responses can be explained by mechanistic interactions of known modes of actions (Gracia et al., 2006). Steroidogenic effects on *in vitro* systems, as well as developmental and reproductive effects in male zebrafish has been reported for natural mixtures of POPs extracted from fish liver oil and sediments (Gregoraszczuk et al., 2008b, 2009; Bláha et al., 2006; Nourizadeh-Lillabadi et al., 2009).

Cod liver oil has traditionally been an important source of vitamin D in the Scandinavian countries during the dark winter months and also during early pregnancy. There are still places in Norway were 50% of respondents in a coastal municipality indicated they consumed fish liver and crude fish liver oil 2–3 times per month or more (Sandanger et al., 2006). To produce pharmaceutical cod liver oil, the crude cod liver oil undergoes several cleaning steps which may vary between producers. The pharmaceutical oil used in the present study was industrially processed with clay and charcoal to remove compounds that provide color and dioxin-like substances. After filtration, further cleaning involved pressurized steam drag distillation. The lighter distillation fraction is incinerated as industrial waste and contains most of the non-dioxin-like POPs. The heavier oil fraction then is further enriched with vitamins and sold as the pharmaceutical product to be consumed as a nutritional supplement. Daily intake of the pharmaceutical product is recommended by Norwegian health authorities for pregnant women and others from 4 weeks of age throughout life. It is a source of vitamin D, and also of essential omega-3 fatty acids and vitamin A (VKM, 2006; Minister of Health and Care Services, 2007). In epidemiological studies cod liver oil supplementation has been suggested to reduce the risk for cardiovascular disease, and marine $n-3$ fatty acids are also considered beneficial for neurological, mental development and growth (VKM, 2006). Fifty-nine percent of the pregnant women in Norway reported consumption of cod liver oil/fish oil supplements during the first 4–5 months of pregnancy, while similar studies reported 23% and 4.3% consumption in Ireland and Denmark, respectively (Haugen et al., 2008). However, several studies also indicated fish and other sea food as sources of POPs (Domingo and Bocio, 2007).

The aim of this paper is to characterize and quantify the steroidogenic effects of POP mixtures, extracted from crude Atlantic cod liver oil (Cod), its derived pharmaceutical product (Pharm) and its concentrated industrial residue product (Ind Res) on the H295R human adrenocortical carcinoma cell line. This cell line expresses all steroidogenic key pathways (Hecker and Giesy, 2008). In addition, methods for quantitating gene expression, enzyme activity and hormone production have been reported previously (Gracia et al., 2006; Hecker et al., 2006; Hilscherova et al., 2004). These cells were used to better understand the effects of a diversity of single substances and complex mixtures on steroidogenesis (Xu et al., 2006; Cantón et al., 2006; Gracia et al., 2007; Sanderson et al., 2001; Gustavsen et al., 2009). In the current study we: (a) compare the effects of mixtures where groups of compounds have been serially removed, and (b) analyze effects of contaminants on steroidogenesis through hormone production and gene regulation.

## 2. Materials and methods

### 2.1. Test chemicals and isolated POP mixtures

Dimethylsulfoxide (DMSO, CASNR 67-68-5), and forskolin (CASNR 66575-29-9) 98% were purchased from Sigma Aldrich Co. (St. Louis, MO). POP mixtures were originated from 60 g of crude Atlantic cod (*Gadus morhua*) liver oil, 50 g of commercially available pharmaceutical cod liver oil, and 5 g from the concentrated industrial residue. Samples were extracted with cyclohexane (Rathburn Chemicals, Walkerburn, Scotland) and cleaned with 96% sulfuric acid ($H_2SO_4$) (Chem Scan, Elverum, Norway) as previously published (Zimmer et al., 2011). The extracts were then transferred to DMSO and the cyclohexane was evaporated gently under $N_2$ stream. Approximately 500 μL DMSO extract was obtained after leaving some insoluble solids behind.

### 2.2. Chemical characterization

From the DMSO stock solutions of each extract, aliquots were diluted with cyclohexane for chemical identification and quantification at the Laboratory of Environmental Toxicology at the Norwegian School of Veterinary Science, Oslo, Norway. The laboratory is accredited for analyzing the components reported here, according to the requirements of NS-EN ISO/IEC 17025:2000 (Test 137). PBDEs and hexabromocyclododecane (HBCD) were determined by gas chromatography–mass spectrometry (GC–MS), while PCBs, hexachlorocyclohexanes (α, β, and γ HCHs), hexachlorobenzene (HCB), chlordanes and DDTs were determined by gas chromatography with electron-capture detection (GC–ECD) according to published methods (Murvoll et al., 2006). Toxaphenes were analyzed on GC–MS according to published method (Føreid et al., 2000). Internal standards for PCBs and pesticides (PCB- 29, 112 and 207) and for BDEs and HBCD (BDE- 77, 119 and 181) were added after dilution of the aliquots.

### 2.3. Cell culture and exposure

H295R culture was conducted as previously described (Gazdar et al., 1990; Gustavsen et al., 2009). Briefly, H295R cells were obtained from the American type culture collection (ATCC CRL-2128; ATCC, Manassas, VA,USA) and cultured in 75 $cm^2$ flasks at 37 °C and 5% $CO_2$ in a humidified atmosphere with in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) containing, phenol red, HEPES buffer, L-glutamine, pyridoxine HCl (Gibco Invitrogene, Paisley, UK), plus 1% insulin–transferrin–selenium (ITS) premix (BD Biosciences, Bedford, MA) and 2.5% Nu-Serum (BD Biosciences, Bedford, MA). The medium was changed three times a week and cells were sub-cultured once a week at approximately 80% confluence using trypsin 0.25% /EDTA 0, 53 nM (Gibco Invitrogen, Paisley, UK). Cells were used between passages 5–13. A quality control plate including medium, solvent control and the steroidogenesis inducer forskolin (CASNR 66575-29-9, 98%, Sigma Aldrich Co., St. Louis, MO) in triplicate wells were included for each cell batch to control cell growth and basal hormone production.

H295R cells were seeded at a density of $3 \times 10^5$ cell/mL in 24-well transparent flat bottom plates (Falcon, Franklin Lakes, NJ) in 1 ml of supplemented DMEM/F12 medium. Mixtures were dissolved in DMSO to reach desired dilutions of the original extract. Chemicals and mixtures were then mixed with culture medium at a fixed solvent concentration of 0.25%. Cells were seeded and after 24 h, the medium was refreshed and cells were exposed to the mixture extracts. Test plates included triplicates of 0.25% DMSO as solvent control as well as 5 μM forskolin in DMSO as positive control. After 48 h of exposure, the medium was transferred to an Eppendorf tube and stored at −20 °C for to hormone analysis; otherwise, it was discarded and the plates snap-frozen on dry ice and stored at −80 °C prior to gene expression analysis.

### 2.4. Cell viability assay

After removal of exposure medium for hormone analysis, the cell viability was determined on the remaining cells by adding 100 μL of AlamarBlue™ (Invitrogen, Carlsbad, CA) to 1 mL of newly added medium per well. Cells were incubated at 37 °C and 5% $CO_2$ in a humidified atmosphere for 3 h. A 100 μL aliquot was collected from each well and transferred into a 96-well transparent well plate (Falcon, Franklin Lakes, NJ). Absorbance was measured at 570 and 600 nm in a VICTOR$^3$™ spectrophotometer (Perkin Elmer, Shelton, CT).

### 2.5. Hormone analysis

Hormones levels in culture medium were measured by solid phase radioimmunoassay (RIA) kits. The kits protocols were modified by replacing the standard curve in serum with standards prepared in cell culture medium. Prior to hormone analysis

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