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Steroidogenic differential effects in neonatal porcine Leydig cells exposed to persistent organic pollutants derived from cod liver oil

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ABSTRACT

Seafood products, including fish and fish oils, are major sources of persistent organic pollutants (POPs) which may cause endocrine disruption related to reproductive dysfunction in males. Primary porcine neonatal Leydig cells were exposed to three extracts of POPs obtained from different stages in production of cod liver oil dietary supplement, in the absence and presence of luteinizing hormone (LH). No reduced viability was observed and all POP extracts showed increased testosterone and estradiol levels in unstimulated cells and decreased testosterone and estradiol secretion in LH-stimulated cells. A decrease in central steriodogenic genes including *STAR*, *CYP11A1*, *HSD3B* and *CYP17A1* was obtained in both culture conditions with all POP extracts. We implicate both small differences in composition and concentration of compounds as well as "old" POPs to be important for the observed steroidogenic effects.

1. Introduction

Persistent organic pollutants (POPs) pose a threat to human and wildlife health. Humans are exposed to these chemicals by ingestion, inhalation or dermal absorption [1]. POPs have been used in agriculture and other industries for decades and thus led to worldwide contamination including remote regions such as the Arctic where no chemicals have ever been made [2].

Despite 30 years of prohibited use, dichlorodiphenyltrichloroethane (DDTs) and polychlorinated biphenyl (PCBs) are still amongst most abundant anthropogenic chemicals found in nature due to their persistence (long half-life) and propensity to bioaccumulate in food chains. As a result, these POPs still pose major health threats to both wildlife and humans [3,4]. Newer emerging POPs, such as the brominated flame retardants (BFRs) and perfluorinated compounds (PFCs) are used and have been increasing [2,5]. Most POPs are highly lipophilic and readily bioaccumulate in fatty tissues of fish, and as such represent a major dietary source of these pollutants [6,7].

Despite fish consumption being a major contributor of POPs in humans, the Norwegian health authorities recommended an increase in lifetime fish consumption and daily intake of cod liver oil food supplement beginning at four weeks of age due to the high content of essential omega-3 fatty acids and vitamin A [8]. Omega-3 fatty acids are associated with positive effects on the nervous system, immune system function, serum triglyceride levels reduction, and have been associated with reduced risk of sudden death after a myocardial infarct [9]. Yet the range of effects of low but chronic exposure to such a mixture of contaminants is still unknown. It is, therefore, debated whether the health benefits of eating seafood due to the high omega-3 polyunsaturated fatty acids and other critical nutrients exceeds the risk of negative health effects caused by the high content of various POPs in these food products [9].

In this study we have revisited this topic by testing our hypothesis of endocrine disrupting effects of 3 different POP extracts taken from different steps in the manufacturing process of commercial grade cod liver oil. The first POP extract ("crude" oil) was obtained from crude cod liver oil before any clean-up treatment was performed, and all POPs including dioxin and dioxin-like PCBs were present. The second POP extract (industrial waste) was obtained from industrial waste fraction of the distillation process where dioxins and dioxin-like PCBs had been removed. The last POP

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extract ("clean" oil) was obtained from the final product, cod liver oil food supplement, which contains low levels of POPs.

Chemical risk assessment is traditionally performed on single compounds, but lately focus has shifted to address the reality of mixture effects [10]. There is unified agreement about additive effects of endocrine disrupters (EDs) with similar modes of action [10]. Endocrine disruption (ED) occurs if chemicals, including POPs, interfere with any aspect of hormone action where the developing stages of humans and animals are more sensitive to toxicants [11]. Thus, it is of concern that POPs can be carried from mothers to fetuses or newborns via the placenta or mothers milk [12]. Increased incidence of reproductive disorders such as sexual dysfunction, infertility, cryptorchidism, hypospadias and testicular cancer as well as declining sperm counts have been seen over the past 50 years and reports suggest that low-level environmental exposure to POPs may be related to these observations [13].

Most EDs impact multiple targets in the same cell type and also operate through different mechanisms by binding to receptors and enzymes, activating gene expression, post transcriptional modification of proteins, change of intracellular ion concentrations or cellular metabolism [11]. Thus Cytochrome P450 (CYP) enzymes responsible for synthesis of steriodogenic hormones must be considered targets for EDs. It has also recently been shown that several epigenetic mechanisms, including DNA methylation, histone modifications, and microRNA expression, can change genome function under exogenous influence of POPs [14].

Testosterone, produced mainly by Leydig cells, after stimulation by luteinizing hormone (LH) in the testicles, is critical during fetal development for male masculinization and reproductive tract development, as well as postnatally for initiation and maintenance of spermatogenesis and libido in males [15]. Consequently, perturbation of the hormonal balance due to POPs may produce lifelong consequences to individuals exposed at early life stages [11,15]. At the same time changes in LH levels that occur during the different life stages in men may be of importance as to how POPs exert their effects. We have in a previous study observed differential effects on steroidogenesis in Leydig cells with a metabolite of DDT, 3-MeSO_2-DDE dependent on the presence or absence of LH [16].

Leydig cells have a high lipid content and may be vulnerable to endocrine disruption by POPs [17]. Porcine Leydig cells are highly similar to human neonatal Leydig cells [18] and, therefore, constitute a very useful model to study potential endocrine disruption by persistent POPs. The objective of the present study was to enlighten the steroidogenic effects of "natural" POP mixtures on steroidogenesis in primary porcine neonatal Leydig cells in the presence or absence of LH.

2. Materials and methods

2.1. Mixtures of persistent organic pollutants

Three different POP extracts were obtained from (a) 700 ml crude cod liver oil from livers of wild caught Atlantic cod (*Gadhus morhua*), after multiple freezing and thawing steps, (b) 20 g industrial waste from the distillation process in manufacturing of dietary supplement cod liver oil (containing nondioxin-like (ndl) compounds) and (c) 700 ml dietary supplement cod liver oil (Møller's tran®, Møller's, Oslo, Norway) as described by Montaño et al. [19]. The fish oil was cleaned by a stepwise procedure adding the oil to *cyclohexane* and 96% *sulphuric acid* (H_2SO_4) (Chem Scan, Elverum, Norway) for clean-up. To avoid uncontrolled heat reactions, no more than 100 ml oil were added to a batch, and the oil was added in small steps to maintain a controlled temperature. The cleaning was performed by shaking the acid with solvents, and repeatedly transferring the solvent to new acid while the volume was reduced by evaporation as described in Zimmer et al. [20]. The extracts were finally transferred to *DMSO* (D2650, Sigma Aldrich Co, St. Louis, MO) and the *cyclohexane* gently evaporated under N_2 stream. The POP extracts were stored in glass tubes with screw cap in the refrigerator. Aliquots of the extracts were diluted in cyclohexane for chemical analyzes.

Dioxin and dioxin like compounds were only present in the "crude" oil, but extracted out from the industrial waste and "clean" oil extracts. These compounds were not measured in this study, but an aryl hydrocarbon receptor (AhR) assay was performed on similar extracts going through the same extraction process from the same source. The findings of this assay indicated dioxin and dioxin-like compounds were present in "crude" oil and only 10% of this activity was found in "clean" oil [19].

Chemical characterization and quantification of each of the three diluted extracts were performed by the Laboratory of Environmental Toxicology at the Norwegian University of Live Sciences, School of Veterinary Science, Oslo, Norway. The laboratory is accredited by the Norwegian Accreditation for testing biological material of animal origin according to the requirements of NS-EB ISO/UEC 17025:2000, TEST (137). Internal standards for *polybrominated diphenylethers (PBDEs)* and *hexabromocyclododecane (HBCD) (BDE- 77, 119* and *181*) and for *PCBs* and *pesticides (PCB- 29, 112 and 207)* were added to the samples used for analysis after dilution. Extractions and quantification of POPs were performed as described in Montaño et al. [19]. *HBCD* and *PBDEs* were determined by gas chromatography-mass spectrometry (GC-MS), whereas *PCBs*, *HCHs*, *hexachlorobenzene (HCB)*, *chlordanes* and *DDTs* were determined by gas chromatography with electron-capture detection (GC-ECD). *Toxaphenes* [*chlorinated bornanes (CHBs)*] were analyzed by GC-MS. The analytical quality of the laboratory has been approved in several inter-calibration tests, and certified international reference materials (CRM 349 and 350, ICES cod liver oil and mackerel oil) are analyzed regularly with results within the given ranges.

2.2. Collection of porcine testicular tissue

Testicles were collected from 9 to 12 day old Norwegian Landrace pig litters as previously described [21]. The number of testicles obtained during each collection ranged from 50 to 90. Local anaesthesia, 1% lidocaine without adrenalin (Haukeland Hospital Pharmacy, Bergen, Norway) was given subcutaneously after the skin of the testicles was disinfected. The piglets were given 6 mg/kg ketoprofen i.m. (Romefen Vet®; Merial GmbH, Hallbergmoos, Germany) as pain reliever after castration. Extracted testes were left encapsulated and stored in medium on ice consisting of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM Hepes, pH 7.4 (Gibco Invitrogen, Carlsbad, CA, USA) in presence of penicillin/streptomycin/neomycin (PSN) (10 ml per 500 ml medium; Invitrogen). Leydig cell isolation was performed in the morning after castration of the piglets. The maximum time for the testicles to arrive at the laboratory after harvesting was 2 h.

2.3. Porcine Leydig cell isolation

Isolation, purification and culture of porcine Leydig cells were adapted from Lervik et al. [21]. In short, testes were de-capsulated, tissue chopped and washed in DMEM medium several times, and then digested with 1.1 mg/ml collagenase/dispase (*Vibrio alginolyticus/Bacillus polyxema*, Roche Neuss, Düsseldorf, Germany) and 5.5% Foetal Calf Serum (Fisher Scientific, Pittsburgh, PA, USA) in DMEM/F12 medium at 34 °C under agitation. Digested tissue was collected after 45, 90, and 120 min and approximately 50 ml cell suspension from each time point was filtered through a metal