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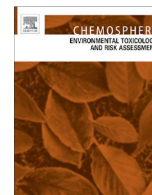

Effects of polar oil related hydrocarbons on steroidogenesis *in vitro* in H295R cells

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HIGHLIGHTS

- H295r cells were exposed to polar hydrocarbons.
- Alkylphenols, naphthenic acids and produced water induced E_2 and P_4 production.
- Exposure to naphthenic acids caused a decrease in testosterone.
- All compounds cause an up-regulation in CYP1A.

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ABSTRACT

Oil pollution from various sources, including exploration, production and transportation, is a growing global concern. Of particular concern is the environmental impact of produced water (PW), the main waste discharge from oil and gas platforms. In this study, we have investigated the potential of polar hydrocarbon pollutants to disrupt or modulate steroidogenesis *in vitro*, using a human adrenocortical carcinoma cell line, the H295R assay. Effects of two of the major groups of compounds found in the polar fraction of crude oil and PW; alkylphenols (C_2- and C_3-AP) and naphthenic acids (NAs), as well as the polar fraction of PW as a whole has been assessed. Endpoints include hormone (cortisol, estradiol, progesterone, testosterone) production at the functional level and key genes for steroidogenesis (17β-HSD1, 17β-HSD4, 3β-HSD2, ACTHR, CYP11A1, CYP11B1, CYP11B2, CYP17, CYP19, CYP21, DAX1, EPHX, HMGR, SF1, STAR) and metabolism (CYP1A) at the molecular level. All compounds induced the production of both estradiol and progesterone in exposed H295R cells, while the C_3-AP and NAs decreased the production of testosterone. Exposure to C_2-AP caused an up-regulation of DAX1 and EPHX, while exposure to NAs caused an up-regulation of ACTHR. All compounds caused an up-regulation of CYP1A1. The results indicated that these hydrocarbon pollutants, including PW, have the potential to disrupt the vitally important process of steroidogenesis.

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1. Introduction

There is increasing public concern over the environmental impact of hydrocarbon pollutants resulting from oil exploration, production and transport (Ivanov, 2011). Of particular concern is the potential impact of both produced water (PW), the main waste discharge from offshore oil production facilities, and crude oil resulting from oil-leakages and spills, on aquatic wildlife. Crude oil and PW both contain a complex mixture of hydrocarbons, several of which are known to cause adverse biological effects. Among these compounds are PAHs (polyaromatic hydrocarbons), as well as several polar compounds, for example alkylphenols (APs) and naphthenic acids (NAs) (Røe Utvik, 1999).

In the literature APs are widely reported as estrogenic compounds, with their potency depending on the molecular structure (Routledge and Sumpter, 1997). The majority of APs present in PW are the more water soluble short-chained APs (C_1–C_3), reported to have ER agonistic properties *in vitro* (Thomas et al., 2004; Tollefsen and Nilsen, 2008).

NAs are a highly complex mixture of polar organic carboxylic acids, composed of a carbon backbone of between 9 and 20 carbon atoms, and with a molecular weight between 120 and 700+. NAs are a natural component of all fossil fuels (Whitby, 2010), and may account for as much as 4% of the weight of crude petroleum (Clemente and Fedorak, 2005; Whitby, 2010). As such, NAs are also a major component of oil spills and oil production discharges (Rowland et al., 2011a). Further, NAs are also present in bitumen and most of the acute toxicity associated with oil sand process water (OSPW) is believed to be explained by the presence of high

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levels of NAs (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986; Madill et al., 2001; Frank et al., 2009; He et al., 2010). Although the mode of action of its reported toxicity is still uncertain, (He et al., 2010), with a suggested primary mode of action being cell narcosis (Frank et al., 2009), adverse biological effects of NAs have been reported in several wildlife species (reviewed in Clemente and Fedorak (2005) and Whitby (2010)). Several studies have reported altered sex steroid levels in aquatic wildlife following exposure to OSPW. For example, yellow perch (*Perca flavescens*) exposed to OSPW displayed decreased plasma levels of sex steroids (van den Heuvel et al., 1999a,b, 2012). Similarly, plasma levels of both testosterone (T) and estradiol (E_2), as well as cortisol, were significantly reduced in goldfish (*Carassius auratus*) exposed to OSPW (Lister et al., 2008). In the same study, OSPW also inhibited gonadal T production *in vitro*. Recently, studies have reported reduced plasma levels of T and 11-ketotestosterone (11kT), and reduced levels of E_2 in male and female fathead minnow (*Pimephales promelas*) respectively when exposed to either OSPW or NAs extracts (Kavanagh et al., 2011, 2012). Using the H295R steroidogenic assay, He et al. (2010) recently demonstrated that OSPW was capable of disrupting normal steroidogenesis *in vitro*, with a significant decrease in T and increase in E_2 production. This supports earlier findings that PW extracts, containing NAs, displayed both estrogenic and anti-androgenic properties *in vitro* (Thomas et al., 2009).

A wide variety of organic pollutants, including PAHs and APs have now been shown to interfere with the cytochrome P450 enzymes, notably aromatase (CYP19), the key enzyme controlling the conversion of T to E_2 (Kazeto et al., 2004; Hinfray et al., 2006; Meucci and Arukwe, 2006; Bonefeld-Jørgensen et al., 2007). Aromatase might be impaired directly through competitive ligand binding or indirectly through feedback loops increasing aromatase to compensate for change in T or E_2 (Ung and Nagar, 2009). Similarly, APs are known to inhibit the enzymes CYP11A, CYP17 and CYP21B, causing a decrease in dibutyryl-cAMP induced cortisol secretion by exposed H295R cells (Nakajin et al., 2001). Several *in vivo* studies have also found that exposure to long- (nonylphenol) and middle-chained (4-tert-pentylphenol) APs down-regulates the gene expression of CYP11A in testis of medaka (*Oryzias latipes*) (Yokota et al., 2005) and the brain of Atlantic salmon, *Salmo salar* (Arukwe, 2005).

There are still major knowledge gaps in our understanding of the mode of action of hydrocarbon pollutants, notably the NAs and short-chain APs found in high concentration in the polar fraction of crude oil and PW. As such, the primary aim of this study was to evaluate further the effects of these compounds, as well as the polar fraction of PW, on steroidogenesis, both at the functional (hormone production) and the molecular level (key steroidogenesis and metabolism gene expression). To that end, H295R cells were exposed to test compounds/mixtures via the cell medium, and the effect of exposure on steroid hormone secretion evaluated. Further, in addition to CYP1A1, the expression of 15 key genes regulating the steroidogenic pathway were analysed by quantitative RT–PCR. Together, these results would enable a more comprehensive biological risk assessment of exposure to hydrocarbon pollutants to aquatic wildlife, specifically indicating their potential to impact reproductive function via deleterious effects on the steroidogenic pathway.

2. Materials and methods

2.1. Experimental design

The impact of four groups of compounds (NAs, C_2-AP, C_3-AP and the polar phase of PW) on steroidogenesis was evaluated *in vitro* using the H295R cell bioassay; a human adrenocortical cell-line capable of full steroidogenesis (Gazdar et al., 1990). The major end-points of this steroidogenesis assay were the measurement of the mRNA of key genes involved in the steroidogenic pathway (analysed with quantitative RT–PCR) and the quantitative measurement of the key steroid hormones cortisol, E_2 and T (analysed by radioimmunoassay). The hypothesis that was tested was: polar organic hydrocarbons have the potential to alter hormone levels and mRNA levels of genes involved in the steroidogenic pathway.

2.2. Chemicals and oil compounds

Ethanol, dimethylsulfoxide (DMSO), forskolin (CASNR 66575-29-9) 98%, dichloromethane (DCM) and acetonitrile were all purchased from Sigma–Aldrich (Oslo, Norway). PW was obtained from Statoil's platform Oseberg C, located in the Norwegian sector of the North Sea (60°36′28″ N, 2°46′28″ E). The PW was transported (within 24 h after collection) from the platform to the Department of Biology, University of Bergen, where it was immediately aliquoted into 10 L containers and frozen at −20 °C. The organic fraction of the PW was extracted with DCM and the polar phase was separated from the nonpolar hydrocarbons by partitioning between hexane and acetonitrile as described in Boitsov et al. (2007). In brief, 2 L of PW were extracted three times with DCM (150 mL, 50 mL, 50 mL) and the combined extracts were dried with $MgSO_4$ and filtered through a glass filter funnel. The extracts were reduced to dryness at 20 °C by rotary evaporation. The residues was dissolved in 4 mL of hexane and shaken twice with 6 mL of acetonitrile saturated with hexane. The combined acetonitrile layer was evaporated to dryness by rotary vaporation. The residues were re-dissolved in DCM and transferred to a weighed glass tube and evaporated to dryness under nitrogen gas flow, and the residues were dissolved in ethanol to concentration of 3.7 mg $m^{-1}L^{-1}$. Commercial petroleum derived NAs were purchased from Sigma–Aldrich (Oslo, Norway) and short chained APs were purchased from Chiron (Trondheim, Norway). Environmentally relevant artificial mixtures were formulated in the laboratory to closely mimic the composition C_2- and C_3-APs found in waste discharges from North Sea offshore oil platforms (Harman et al., 2009) (see Table 1 for chemical composition of stock solutions).

2.3. Chemical characterization

The stock solutions were characterized at the Laboratory for Environmental Chemistry at the Institute of Marine Research, Bergen, Norway. The AP and the PAH concentration in the PW was determined by chromatography–mass spectrometry GC–MS according to previously described methods (Boitsov et al., 2007, 2011). The composition of the different APs and the PAH in the PW fraction is shown in Fig. 1, and the isomers composition of the C_2-AP and C_3-AP solutions are given in Table 1.

The distribution of the different molecular size and ring structures of the commercial petroleum derived NAs were tentatively analysed after derivatization with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) using previously described GC–MS method (St. John et al., 1998; Young et al., 2010). The samples were analysed by full scan from 50 to 500 m/z and the integrated areas were obtained for clusters of all the theoretical possible masses for the $[M + 57]^+$ ions for $C_nH_{2n} + ZO_2$ (where n is the number of carbon atoms and Z the hydrogen deficiency resulting from ring formation. There were scanned for $n = 5–29$ and $Z = 0–12$, in total 128 masses. Extracted ion mass and distribution of NAs shown in Tables 2A and 2B.

2.4. Cell culture and exposure

Human adrenocortical carcinoma H295R-cells obtained from ATCC (CRL-2128; ATCC, Manassas, VA, USA) were cultured and

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