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Precision-cut liver slices of Atlantic cod (*Gadus morhua*): An *in vitro* system for studying the effects of environmental contaminants

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ABSTRACT

The Atlantic cod (*Gadus morhua*) is an economically important species commonly consumed by humans. The widespread distribution of cod in the North Atlantic Ocean makes it vulnerable to effluents from human activities, such as coastal industries and offshore petroleum exploration. It has been demonstrated that many effluents have adverse effects on cod reproduction and health, *e.g.* by disrupting endocrine signaling pathways. The liver, expressing important components of the biotransformation and the endocrine system, is one of the main target organs. Thus, reliable and reproducible *in vitro* systems of the liver are important for studying effects of environmental contaminants.

The aim of this study was to investigate precision-cut liver slices (PCLS) as an alternative *in vitro* system for toxicological studies of the Atlantic cod liver. Slices of 8 mm in diameter and 250 μm thickness were prepared and cultivated from immature cod. Several analyses to measure the liver slice viability were performed: enzyme assays, histology, and morphometric analysis, all confirming cell viability for up to 72 h in culture. The liver slices were also exposed to two well-known model environmental contaminants, β-naphthoflavone (BNF) and 17α-ethynylestradiol (EE2), representing established agonists for the aryl hydrocarbon receptor (AHR) and the estrogen receptor (ER), respectively. The results showed increased transcription of the target genes cytochrome P450 1A (*CYP1A*) and vitellogenin (*VTG*), both well-established biomarkers for exposure of fish to the selected compounds.

In conclusion, PCLS is a promising *in vitro* system for toxicological studies of cod liver cells. The liver slices are viable in culture for several days and respond to environmental contaminants in a dose- and time-specific manner.

1. Introduction

The Atlantic cod (*Gadus morhua*) is an important commercial fish species and a common human food source (Kurlansky, 1997). This teleost species (*Gadiformes*; *Paracanthopterygii*) is widely distributed in the North Atlantic Ocean. Human activities, such as onshore and offshore petroleum industries, continue to invade these areas, releasing effluents that may pose a threat to oceanic wildlife. Several of these chemicals have previously been shown to affect the growth, reproduction and health of the Atlantic cod (Aas et al., 2000; Meier et al., 2010; Sundt and Bjorkblom, 2011; Yadetie et al., 2013). To further reveal the possible impacts of environmental contaminants on cod, reproducible, reliable, and effective *in vitro* systems are necessary.

The Atlantic cod genome was recently sequenced and annotated (Star et al., 2011). The availability of a sequenced genome facilitates comprehensive toxicogenomic analyses, making Atlantic cod an attractive model for analysing the effects of environmental contaminants in the North Atlantic Ocean (Karlsen et al., 2011; Yadetie et al., 2013). The liver is the main site of detoxification in cod, and expresses a wide range of components of the biotransformation system, such as nuclear receptors (xenobiotic sensors) and cytochrome P450 (CYP) enzymes. Knowledge of the biotransformation enzymes and responses in cod is well established (Goksøyr, 1985; Goksøyr et al., 1986, 1987, 1988; Goksøyr and Solberg, 1987; Karlsen et al., 2012). The vertebrate liver is also the site where many environmental compounds tend to accumulate. The composition of vertebrate liver architecture, as well as toxicant-induced liver damages, has been presented in several recent papers (Hardman et al., 2007; Howarth et al., 2010; Van Wettere et al., 2013). However, the cod hepatocytes differ from many other species in that they have increased cellular volume and contain several lipid droplets (Fujita

Abbreviations: PCLS, precision-cut liver slices; AHR, aryl hydrocarbon receptor; ER, estrogen receptor; BNF, β-naphthoflavone; EE2, 17α-ethynylestradiol; CYP, cytochrome P450.

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et al., 1986), suggesting even higher susceptibility for accumulation of lipophilic environmental contaminants in the liver. In several other animals, primary hepatocytes or immortalized cell lines are often used as *in vitro* systems for studying physiological responses the liver (Segner, 1998). Isolation of primary hepatocytes from cod were previously described by Søfteland et al. (2010) and Ellesat et al. (2011), but these isolation techniques are less efficient than with other species due to the unique hepatocyte morphology of Atlantic cod. Furthermore, isolated cell systems have limited ability to predict *in vivo* toxicity due to cell differentiation and mutagenic events during their cultivation (Segner, 1998).

An alternative *in vitro* system for toxicological studies is presented here; cultivation of precision-cut liver slices (PCLS) (Lerche-Langrand and Toutain, 2000). PCLS are thin (100–300 μm thick) slices of liver that are viable for several days in culture and that can be exposed to chemicals as normal cell systems. In PCLS, all cell types are retained in their normal distribution. Hence, though the hepatocytes provide the highest response to contaminant exposure, also vascular and biliary endothelial cells, perisinusoidal fat-storing cells, and erythrocytes are involved directly or indirectly in the liver slices, as in the liver *in vivo*. The liver slices also have the advantage that the cells are not exposed to enzymatic stressors during preparation and retain their normal tissue integrity. Several studies using microarrays confirm a closer prediction of *in vivo* toxicity by PCLS than by primary cell cultures and immortalized cell lines (Boess et al., 2003; Elferink et al., 2008). Whereas PCLS primarily have been applied in mammalian studies, the system has previously been published using piscine species as well, including Atlantic species such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Cravedi et al., 1998; Lemaire et al., 2011). In fact, Singh et al. (1996) concluded that PCLS are an excellent *in vitro* model for metabolism and toxicity studies in aquatic species. However, PCLS has to our knowledge never been applied on liver of Atlantic cod.

The objective of this study was to establish a method for PCLS of Atlantic cod as an *in vitro* system to study effects of environmental contaminants. Several analyses to measure the liver slice viability were performed, including enzymatic assays, histology, and morphometric analysis, which all confirmed cell viability for several days in culture. The liver slices were also exposed to two well-known model compounds, β-naphthoflavone (BNF) and 17α-ethynylestradiol (EE2), representing established agonists for the aryl hydrocarbon receptor (AHR) and the estrogen receptor (ER), respectively. Effects on the transcriptional level revealed clear dose- and time-specific responses of the environmental contaminants.

2. Materials and methods

2.1. Fish

Atlantic cod (*G. morhua*) was provided from Fjord Gadus AS (Fiskå, Norway), and maintained at The Industrial and Aquatic Research Laboratory (ILAB, Bergen, Norway). The fish were kept in 500 L tanks in natural seawater at 9 °C with a 12:12 h light cycle regime. The fish were fed with a commercial diet *ad libitum* (EWOS, Bergen, Norway). Seven individual cod weighing approximately 1 kilogram were used for the experiments. None of the individuals had reached sexual maturity.

2.2. PCLS procedure

The fish were killed by a blow to the head, and the liver transferred to a tray containing cold buffer (4 °C) consisting of NaCl (122 mM), KCl (4.8 mM), $MgSO_4$ (1.2 mM), Na_2HPO_4 (11 mM) and $NaHCO_3$ (3.7 mM), pH 8.4 (as used for cod liver perfusion by Ellesat et al. (2011)). A cylinder-shaped coring tool with diameter of 8 mm was used to core out pieces of the central part of the cod liver. Subsequently, the pieces were glued to the tray of a DSK Microslicer DTK-1000 (Ted Pella Inc., Redding, CA, USA), and again submerged in cold buffer. Slices of 250 μm were cut using low forward speed and high cutting frequency, as recommended by the provider. Prepared slices (17.2 ± 6.4 mg wet weight, $n = 24$) were kept in cold buffer (4 °C) during the procedure.

2.3. PCLS culture and exposure assays

Cod liver slices were cultivated in 24-well plates (Thermo Scientific Nunc, Langenselborg, Germany) with 500 μL Leibowitz-15 cell media (Life Technologies™ Gibco®, Paisley, UK) supplemented with 10% charcoal-stripped and heat-inactivated fetal bovine serum (Gibco®) and 1% penicillin–streptomycin–amphotericin (10,000 U/mL potassium penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B; Sigma-Aldrich), as described by Søfteland et al. (2010). The slices were kept in a sterile cell incubator at 10 °C in an ambient environment.

After a 2 h-long acclimatization period for the PCLS, half of the growth medium was replaced by medium containing either the aryl hydrocarbon receptor (AHR)-ligand β-naphthoflavone (BNF) or the estrogen receptor (ER)-ligand 17α-ethynylestradiol (EE2) (purchased from Sigma-Aldrich, Oslo, Norway). Whereas the BNF exposure was performed on PCLS from both male and female fish, only male fish were exposed to EE2 to avoid any influences of endogenous estrogens present in female livers. Both BNF and EE2 were dissolved in Hybri-Max™ Dimethyl sulfoxide (DMSO, Sigma Aldrich) to final concentrations of 0.1 μM and 10 μM, and 1 nM and 100 nM, respectively. 0.01% DMSO was used as a solvent control. The slices were exposed for 24 and 48 h to the compounds. Cytotoxicity of the chemicals was tested by measuring the release of lactate dehydrogenase (LDH; EC 1.1.1.27) into the culture medium in triplicates, using the Cytotoxicity Detection Kit^{PLUS} (Roche Applied Sciences, Basel, Switzerland) according to the manufacturer's protocol.

2.4. PCLS viability assays

2.4.1. ATP content of the slices

Slices stored at −80 °C were thawed, weighed, and sonicated by Bioruptor® Sonication Device UCD-300 (Diagenode, Liège, Belgium) in ice water with intervals of 1 min on, 30 s off for 2–5 repetitions. Adenosine triphosphate (ATP) content of the slices was measured using the ATPlite 1step kit (Perkin Elmer) following the provider's instructions and an EnSpire plate reader (Perkin Elmer). Statistical significant increase in ATP content was determined using Student's *t*-test.

2.4.2. Histological analysis

Following removal of the growth medium and washing with PBS, the liver slices were fixed in a fixative containing 0.04 M cacodylate buffer, 2.5% glutaraldehyde, and 2% formaldehyde in phosphate buffered saline (pH 7.2), and mounted in resin (Technovit 7100, Heraeus Kulzer GmbH & Co, Germany). Each liver slice was then divided into two halves, before this cross section surface was cut to 2 μm sections by a Leica RM 2155 microtome and stained in 1% toluidine blue. By this method, both surface and deeper layers were visualized in all the sections. Pictures were obtained in an Olympia AHB3T, operated at 200× or 400× magnification.

2.4.3. Morphometric analysis

Estimations of the ratios of intact cells relative to dead cells were made by morphometric analysis in the light microscope