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Metabolic signatures of bisphenol A and genistein in Atlantic salmon liver cells

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HIGHLIGHTS

- Atlantic salmon hepatocytes were exposed to bisphenol A and genistein for 48 h.
- Endpoints: cytotoxicity, DNA methylation, targeted transcriptomics and metabolomics.
- BPA and GEN both upregulated *esr1*, *vtg1* and *cyp1a* transcription.
- Metabolomics suggests main effects on glucose homeostasis and energy generation.
- BPA affected methionine degradation; GEN uridine and pyrimidine biosynthesis.

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ABSTRACT

Screening has revealed that aquafeeds with high inclusion of plant material may contain small amounts of endocrine disrupting agricultural pesticides. In this work, bisphenol A (BPA) and genistein (GEN) were selected as model endocrine disrupting toxicants with impact on DNA methylation in fish. Atlantic salmon hepatocytes were exposed *in vitro* to four concentrations of BPA and GEN (0.1, 1.0, 10 and 100 μM) for 48 h. Toxicity endpoints included cytotoxicity, global DNA methylation, targeted transcriptomics and metabolomic screening (100 μM). GEN was not cytotoxic in concentrations up to 100 μM, whereas one out of two cell viability assays indicated a cytotoxic response to 100 μM BPA. Compared to the control, significant global DNA hypomethylation was observed at 1.0 μM BPA. Both compounds upregulated *cyp1a1* transcription at 100 μM, while estrogenic markers *esr1* and *vtg1* responded strongest at 10 μM. *Dnmt3aa* transcription was downregulated by both compounds at 100 μM. Metabolomic screening showed that BPA and GEN resulted in significant changes in numerous biochemical pathways consistent with alterations in carbohydrate metabolism, indicating perturbation in glucose homeostasis and energy generation, and glutamate metabolism. Pathway analysis showed that while the superpathway of methionine degradation was among the most strongly affected pathways by BPA, GEN induced changes to uridine and pyrimidine biosynthesis. In conclusion, this mechanistic study proposes metabolites associated with glucose and glutamate metabolism, glucuronidation detoxification, as well as *cyp1a1*, *vtg1*, *esr1*, *ar*, *dnmt3aa*, *cdkn1b* and *insig1* as transcriptional markers for BPA and GEN exposure in fish liver cells.

1. Introduction

Atlantic salmon (*Salmo salar*) fillet, with its high content of fat, tend to accumulate lipid-soluble contaminants. Concerns have therefore been raised regarding human health risks of farmed salmon consumption (Hites et al., 2004; Nøstbakken et al., 2015). The diet is the main source of organic contaminants for farmed Atlantic salmon. Historically, these compounds were introduced mainly with marine ingredients such as fish oils (Berntssen et al., 2010). Today, with fishmeal and fish oil being increasingly replaced with plant protein and vegetable oils, contaminants might also stem from other sources. Potential sources include chemicals used in agriculture, as well as contaminants originating from food processing techniques, transport and storage. Farmed salmon might also be exposed to non-feed based contaminants during the production cycle. For example, modern Atlantic salmon aquaculture employ a relatively large amount of plastics in rearing tanks

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and piping, which has the potential to introduce plastic additives such as bisphenol A (BPA) into system water.

Recent screening has shown that novel Atlantic salmon aquaculture feeds, which consist of about 70% plant ingredients, may contain trace amounts of pesticides (Berntssen et al., 2010; Nacher-Mestre et al., 2014). In an effort to study the potential toxicological impact of contaminants associated with present-day salmon farming, we have used *in vitro* models to search for biomarkers of exposure in fish. This research has focused on compounds such as endosulfan, chlorpyrifos and pirimiphos-methyl (Krøvel et al., 2010; Søfteland et al., 2014, 2016; Olsvik et al., 2015a, 2017). Some of these chemicals, especially those that act as endocrine disruptors, may potentially impact mechanisms linked to DNA methylation. Feeds mainly based on plant ingredients may contain lower levels of B-vitamins and some indispensable amino acids compared to traditional feeds based on marine ingredient (Hansen et al., 2015; Hemre et al., 2016). Diets with suboptimal concentrations of nutrients necessary for folate-centered one carbon (1C) metabolism may thus render the farmed salmon more vulnerable to chemicals known to impact DNA methylation mechanisms (Dolinoy et al., 2007).

Two of the best-studied toxicants known to affect DNA methylation are BPA and genistein (GEN) (Dolinoy et al., 2006, 2007). Both chemicals are considered to be weak endocrine disruptors (Krishnan et al., 1993; Patisaul and Adewale, 2009). BPA is a ubiquitous environmental contaminant originating mainly from polycarbonate plastics and epoxy resins (Staples et al., 1998). As an endocrine disruptor, BPA has been shown to bind to estrogen receptors (ERs), resulting in feminizing effects in fish and other animals (Dolinoy et al., 2007). GEN is a plant-derived phytoestrogen with ability to bind to the ER beta receptor, activate PPARs and Nrf2 pathway, and inhibit tyrosine kinases (Morito et al., 2001; Dolinoy et al., 2006; Fan et al., 2006; Kim et al., 2009). Hence, both chemicals may interfere with vertebrate reproduction. In cells, these compounds can bind to nuclear receptors and initiate transcription of a number of estrogen-responsive genes (Kennedy et al., 2014). In male fish, increased transcription of ER genes and vitellogenin (VTG) are typical biomarkers of such chemicals (Sumpter and Jobling, 1995). Cross-talk between ER and the aryl hydrocarbon receptor (AhR) may also result in altered transcription of cytochrome P450 genes (Beischlag et al., 2008).

The aim of this study was to study the mechanistic effects of two endocrine disrupting toxicants affecting DNA methylation in Atlantic salmon liver cells. Based on their known mode of action, BPA and GEN were selected as model toxicants. Identified biomarkers will be applied as potential markers in follow-up *in vivo* examinations of the impact of compounds associated with present-day salmon farming. Atlantic salmon hepatocytes were exposed to four concentrations of BPA and GEN (control, 0.1, 1.0, 10.0, 100 μM) for 48 h. Cytotoxicity was examined with the MTT assay and the xCELLigence system. Global DNA methylation was determined with a HPLC-based method. Molecular endpoints included targeted transcription and cellular metabolites. Eighteen potential markers for cellular stress and DNA methylation were selected for transcriptional analysis using RT-qPCR. Cells exposed to the highest BPA and GEN concentration (100 μM), were selected for metabolomic profiling with GC/MS and LC/MS/MS platforms (polar and non-polar metabolites). Pathway analysis was used to search for causal relationships and biomarkers.

2. Materials and methods

2.1. Cell harvesting

Atlantic salmon was maintained at the Industrilaboratoriet (ILAB) animal holding facility, Bergen, Norway. The fish were kept in flowing sea water at 7–9.5 °C, 34.4‰ and a 12/12 light/dark cycle. Wastewater O_2 levels were always above 7 mg/l and the pH was 8.1–8.2. The fish were fed once a day with a special-made feed produced without synthetic antioxidants and with low levels of contaminants, delivered by EWOS, Norway (Spirit 400-50A HH, 6.0 mm). Hepatocytes were isolated from male juvenile Atlantic salmon (n = 5, mean ± SEM: 214 ± 7 g) with a two-step perfusion method described by Søfteland et al. (2009). The fish were sacrificed by terminal anaesthetization with tricaine methanesulfonate (MS-222) (200 mg/l). Fish sacrifice and harvesting of cells were conducted by the authors and approved by the Norwegian Animal Research Authority (NARA) via NIFES' Animal Care and Use Committee.

Prior to exposure, the Trypan Blue exclusion method was used to determine cells viability (Lonzo, Medprobe, Oslo, Norway). Cell suspensions were put on 5 μg/cm^2 laminin (Sigma-Aldrich, Oslo, Norway) coated culture plates (TPP, Trasadingen, Switzerland) and the hepatocytes were kept at 10 °C in a sterile incubator without additional O_2/CO_2 supply (Sanyo, CFC FREE, Etten Leur, Netherland). The following cell densities were used: A) for cytotoxicity evaluation in xCELLigence 96-well plates and regular 96-well plates for the MTT assay: 2 × 10^5 cells per well, and B) for RT-qPCR and metabolite profiling: 7.2 × 10^6 cells per well in 6-well plates (in 3 mL complete L-15 medium).

2.2. BPA and GEN exposure

Hepatocyte cells were cultured for 36–40 h prior to chemical exposure with exchange of medium after 18–20 h. Cells were kept as controls or treated with BPA and GEN (0.1, 1.0, 10 and 100 μM) and harvested after 48 h exposure. Cells were also exposed to the demethylating agent 5-aza-2′-deoxycytidine (0, 0.001, 0.01, 0.1, 1 and 10 μM 5-AZA), which was included in the study as a positive control. BPA, GEN and 5-AZA were dissolved in DMSO, with an equal amount of DMSO (0.1%) used in the control group. BPA, GEN and 5-AZA were obtained from Sigma (Sigma-Aldrich, Oslo, Norway). Hepatocyte cells were exposed in triplicate wells for RT-qPCR and metabolite profiling, and in 96-wells culture plates for the xCELLigence and MTT cytotoxicity screening (single wells). The exposure medium was exchanged after 18–20 h. The number of biological replicates was 5 for all analytical methods. The final cell pellet was resuspended in L-15 medium (Sigma Aldrich, Oslo, Norway) containing 10% FBS (Sigma Aldrich, Oslo, Norway), 1% glutamax (Invitrogen, Norway) and 1% penicillin–streptomycin–amphotericin (10,000 units/ml potassium penicillin, 10,000 μg/ml streptomycin in sulfate and 25 μg/ml amphotericin B) (Lonzo, Medprobe, Oslo, Norway).

2.3. Cytotoxicity screening

Two methods were applied to determine the cytotoxicity of BPA and GEN (5-AZA was not evaluated for cytotoxicity). Cell viability was determined with the MTT method using the *In Vitro* Toxicology assay kit according to the manufacturer's protocol (Sigma Aldrich) and by the xCELLigence system (Real-Time Cell Analyzer RTCA-SP, ACEA Biosciences, San Diego, USA) (Abassi et al., 2009). For the latter method, recording of cell index (CI) values and normalization was performed using the RTCA Software version 1.2.1. The real-time cell monitoring was conducted at 10 °C in an incubator without additional O_2/CO_2 supply (Sanyo, CFC FREE, Etten Leur, Netherland). Data was collected with 2 min intervals after BPA and GEN exposure for 12 h and then every 15 min for 60 h. For calculation of cell viability after 48 h of exposure, the impedance signal was analyzed by normalizing data of each singe well to a reference time point set