EXAMINATION OF BDE-47 IN VITRO EXPOSURE ON STEROIDOGENESIS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) USING LC/MS/MS

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Introduction

Polybrominated diphenyl ether (PBDE) flame-retardants are lipophilic persistent organic compounds used in manufacturing of plastics, electronic equipment, polyurethane foam and textile materials. PBDEs have become an increasingly important environmental problem due to their ability to bioaccumulate and biomagnify. Since the late 1970's, substantial increases of these compounds have been measured in wildlife and human adipose tissue, with 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) as the predominant PBDE congener found. Recent studies have focused on the potential endocrine disrupting properties of PBDEs, which include effects on thyroid hormones and gonadal tissue steroidogenesis. Previous *in vitro* studies that have explored the influence of PBDEs on reproductive hormone production, cell proliferation and apoptosis have focused strictly on mammalian models. One such study by Gregoraszczuk et al. (*1*) showed that BDE-47 increased estradiol (E2) and testosterone (T) production in a porcine thecal cell *in vitro* assay; suggesting induction of aromatase (CYP17 and CYP19) activity. BDE-47 may have estrogenic activity through receptor mediated pathways (*2*,*3*), with the hydroxylated form exhibiting significantly higher potency (*4*). It has also been suggested that BDE-47 acts as an androgen receptor (AR) inhibitor by competing for binding sites (*4*,*5*). Canton et al. (*6*) found that hydroxylated metabolites significantly reduced the DHT-induced response in the yeast human androgen receptor (hAR) assay. They also found no evidence of androgenic activity as reported by others.

Recent work has shown that a BFR mixture-laden diet had no effect on adult zebrafish fecundity and gonadosomatic index, however there was an increase in the number of atretic oocytes, and the hatching success of offspring was affected (*7*). In a parallel study, Rattfelt et al. (*8*) demonstrated that BFRs can be transferred maternally to their offspring via ooycte yolk deposition. However, very little is known about their action on fish reproductive systems. To address this deficiency, the effect of environmentally relevant concentrations of BDE-47 on the *in vitro* steroidogenic capacity of rainbow trout (*Oncorhynchus mykiss*) oocytes and testicular tissue were examined.

Material and Methods

Chemicals. Native and C_{13}, d_4, d_2-mass labelled compounds were purchased from Cambridge Laboratories and Steraloids. 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) was purchased from Wellington Laboratories. OmniSolv methanol and water were from VWR and chemicals for the Cortland's incubation medium were from Sigma-Aldrich Canada.

Fish. Pre-spawning rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario, Canada. Six females (713.3 $\pm$ 35.7 g) and six males (1290.0 $\pm$ 80.6 g) were sacrificed, weighed and measured. Gonadal tissues were removed, weighed and placed in Cortland's incubation medium (pH 7.6) on ice.

In vitro incubations. *In vitro* incubations were performed as outlined by McMaster et al. (*9*). Briefly, 20 ovarian follicles or 20 mg of testicular tissue were placed in each well of the 24-well culture plates with 1 mL of Cortland's medium. BDE-47 was added to the wells in 5 µL of acetone carrier at the following concentrations: 0, 100, 500, 1000, 3000, and 5000 pg/µL. Each treatment was duplicated under basal and stimulated conditions using 100 IU Human Chorionic Gonadotropin (hCG), and acetone only solvent controls were added for each individual fish. The plates were incubated at 12.0 $\pm$ 0.5 ^{o}C for 24 hours. The incubation medium from each well was collected and stored separately at -80 ^{o}C until analysis.

LC/MS/MS analysis. Samples were first thawed, and vortexed to ensure homogeneity. The incubation medium was the centrifuged at 32 000 rpm for 12 minutes to remove any residual tissue fragments. Sub-samples of 100 µL were placed in autosampler vials and spiked with labelled internal standards. An external standard of

Cortland's medium spiked with 50 pg/µL each of monitored estrogens, and 5 pg/µL each of the selected androgens. Once prepared, the incubation medium was directly injected into the LC/MS/MS at volumes of 2 µL and 20 µL for the estrogens and androgens, respectively. Samples from the oocyte incubations were analyzed for the following estrogens and conjugates: Estradiol (E2), Estrone (E1), E2-3 Sulfate (E2-3S), E2-3 Glucuronide (E2-3G), E2-17 Glucuronide (E2-3G), E1-3 Sulfate (E1-3S), and E1-3 Glucuronide (E1-3G). Testicular tissue incubations were analyzed for Testosterone (T), T-sulfate (T-S), T-Glucuronide (T-G), and 11-Ketotestosterone (11-KT).

An Agilent 1100 HPLC was coupled to an API 2000 triple quadrupole mass spectrometer, which was operated in the ESI –ve ion mode for the estrogens, 11-KT, and T-S. Testosterone and T-G were analyzed in the +ve ion mode. Injections were made directly into a C_{18} column.

Results and Discussion

Oocyte Incubations. Analysis of incubation medium using the LC/MS/MS detected E2, E1, E2-3S and E2-17G, however only E2 and E2-3S were consistently measured in all samples. Analysis of variance (ANOVA) showed there was no significant difference ($p>0.05$) in the production of free E2 with increasing BDE-47 concentrations under either basal or stimulated conditions (Fig. 1). These results are contradictory to those observed by Gregoraszczuk et al. (*1*) in porcine thecal cells. However, the highest dose of BDE-47 used in this experiment was 200x lower than that used in the mammalian study and are more representative of realistic concentrations found in fish tissues. One *in vivo* study using male juvenile turbot (*Schophtalamus maximus*) reported no effects on circulating T and E2 concentrations with waterborne exposures to BDE-47 (*10*).

E2-3S conjugate accounted for a significant proportion of the total estrogens produced by the fish oocytes; over 50% under hCG stimulated conditions. These results are different from those of Pankhurst (*11*) who found that the glucuronide conjugate form of E2 was a predominant product of rainbow trout ovarian follicle *in vitro* steroidogenesis. There were also no significant differences in follicle E2-3S production between treatments under both basal and stimulated conditions (ANOVA $p>0.05$, Fig. 2). Previous studies have reported that BDE-47 inhibits the activity of E2-sulfotransferase (*3, 4*), but we found no evidence for this in the current study.

Testicular Tissue Incubations. Preliminary analyses of the Cortland's incubation medium with the rainbow trout testicular tissue showed that only testosterone and 11-KT under hCG stimulated conditions were within our current LC/MS/MS detection limits.

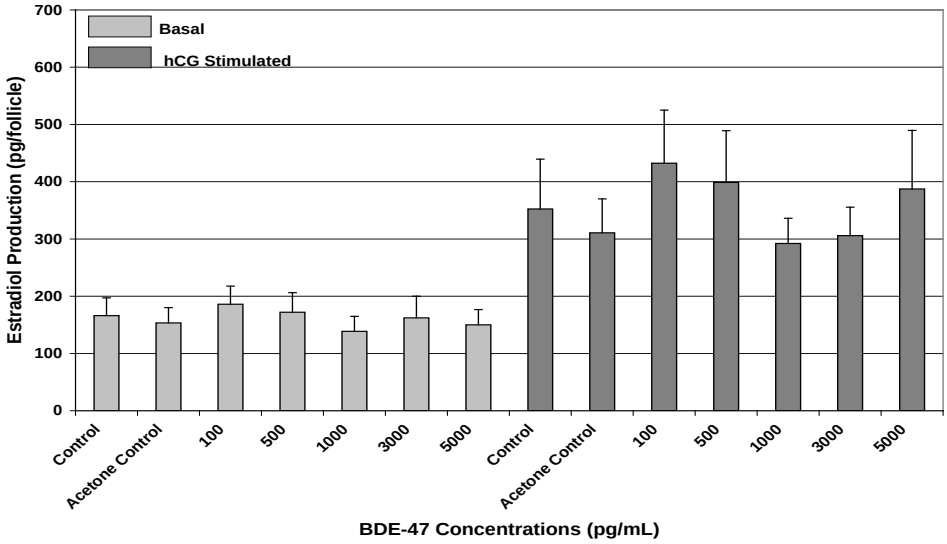

Figure 1. Mean estradiol (E2) concentrations (± SEM) produced by ovarian follicles *in vitro* treated with BDE-47 (pg/mL) under basal and hCG stimulated conditions.

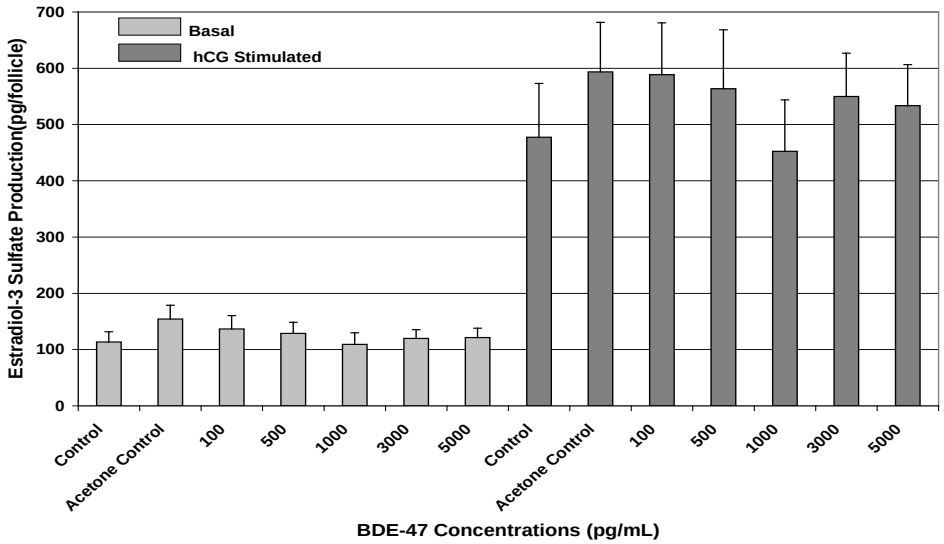

Figure 2. Estradiol-3 Sulfate (E2-3S) produced by rainbow trout ovarian follicles *in vitro* treated with various concentrations of BDE-47 (pg/mL) under basal and hCG stimulated conditions. E2-3S concentrations are expressed as mean ± SEM.

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