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Effects of 17α-methyltestosterone exposure on steroidogenesis and cyclin-B mRNA expression in previtellogenic oocytes of Atlantic cod (*Gadus morhua*)

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Abstract

Steroid hormone (estrogens and androgens) synthesis and regulation involve a large number of enzymes and potential biochemical pathways. In the context of these biochemical pathways, it is believed that the true rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein and the subsequent conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage (P450*scc*) enzyme. Oocyte development is a complex process that is triggered by the maturation-promoting factor (MPF) involving cyclin-B as a regulatory factor. In the present study, we evaluated the endocrine effects of 17α-methyltestosterone (MT) on steroidogenic pathways of Atlantic cod (*Gadus morhua*), using an *in vitro* previtellogenic oocyte culture technique that is based on an agarose floating method. Tissue was cultured in a humidified incubator at 10 °C for 1, 5, 10 and 20 days with different concentrations of the synthetic androgen MT (0 (control), 1, 10, 100 and 1000 μM) dissolved in ethanol (0.3%). Gene expressions for StAR, P450*scc*, aromatase-α (P450*aromA*) and cyclin-B were detected using validated real-time PCR with specific primer pairs. Cellular localization of the StAR protein and P450*scc* were performed using the immunohistochemical technique with antisera prepared against synthetic peptide for both proteins. Steroid hormones (estradiol-17β: E2 and testosterone: T) levels were estimated using enzyme immunoassay. Our data showed significant concentration-specific increase (at day 1 and 5) and decrease (at day 10 and 20) of the StAR mRNA expression after exposure to MT. P450*scc* expression showed a MT concentration-specific decrease during the exposure periods and cyclin-B mRNA expression was decreased in MT concentration-dependent manner at days 10 and 20 (reaching almost total inhibition after exposure to 1000 μM MT). MT exposure produced variable effects on the P450*aromA* mRNA expression that can be described as concentration-specific increase (day 1) and decrease (days 5 and 10). Cellular localization of the StAR protein and P450*scc* demonstrated their expression mainly in ovarian follicular cells. MT produced an apparent concentration-and time-dependent increase of E2 and T levels. Thus, the present study reveals some novel effects of pharmaceutical endocrine disruptor on the development of previtellogenic oocytes in cod. The impaired steroidogenesis and hormonal imbalance reported in the present study may have potential consequences for the vitellogenic process and overt fecundity in teleosts.
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1. Introduction

Pharmaceuticals are ubiquitous pollutants in the aquatic environment where their potential effects on non-target species like fish has only recently become subject of systematic investigations. Available scientific evidence indicate that the reproductive system, including its associated endocrine and neural controls, may be susceptible to alterations by occupational, pharmaceutical or environmental exposures to a variety of chemical and physical agents (Singleton and Khan, 2003); (DeRosa et al., 1998). The synthetic androgen, 17α-methyltestosterone (MT) is widely applied in aquaculture to control sex determination and induce sex-reversal of genetic females to phenotypic males (Hunter and Donaldson, 1983; Kitano et al., 2000; Papoulias et al., 2000). Nevertheless, the specific role or effect of MT remains to be resolved, and factors such as dose, timing and duration of MT treatment can influence the effects. It has been shown that high MT concentrations and/or prolonged exposure times, may induce phenotypic female characteristics (Rinchard et al., 1999; Orn et al., 2003). At the transcript level, a suppression of P450*aromA* gene expression after MT treatment

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has been reported in several studies (Kitano et al., 1999; Fenske and Segner, 2004). Furthermore, in several teleost species such as common carp (*Cyprinus carpio*; (Nagy et al., 1981), rainbow trout (*Oncorhynchus mykiss*; (Ostrowski and Garling, 1987) and tilapia (*Oreochromis mossambicus*; (Kuwaye et al., 1993), the anabolic effects of MT on growth have been demonstrated. Previous studies addressing the effects of MT treatment in female fish have mainly focused on monitoring sex reversal and proportions of intersex in fish (Kitano et al., 2000; Orn et al., 2003; Kanamori et al., 2006).

The synthesis and regulation of steroid hormones involve a large number of enzymes and potential biochemical pathways. In the context of these biochemical pathways, it is believed that the true rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, and the subsequent conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage enzyme (P450*scc*) (Stocco 1997; Stocco 2001a). The StAR protein and P450*scc* have been localized in most steroidogenic organs or tissues, including cod ovary (Kortner and Arukwe, 2007), and are rapidly synthesized in response to acute tropic hormone stimulation. Cytochrome P450 aromatase (P450*arom*) is another main enzyme involved in steroidogenesis, responsible for the conversion of a large range of androgens into estrogens (Simpson et al., 1994). It has been shown that teleost fish have two distinct mRNA isoforms of P450*arom*. P450*aromA* is predominantly expressed in the ovary and is involved in sex differentiation and oocyte growth, whereas P450*aromB* is highly expressed in brain (Callard et al., 2001; Kishida and Callard, 2001). Estrogens (17β-estradiol) and androgens (testosterone and 11-ketotestosterone) are known to be involved in a number of physiological functions such as sexual differentiation, ion and carbohydrate homeostasis, adaptation to stress, immune system functioning and reproduction (Dean and Sanders, 1996).

Oocyte maturation is a complex process that is triggered by the maturation-promoting factor (MPF) induced on the oocyte surface by the maturation-inducing hormone (MIH) that is secreted from follicle cells. During this process, cell division cycle 2 (cdc2) functions as a catalytic subunit while cyclin-B functions as a regulatory unit whose activity is controlled by inhibitory phosphorylation of cdc2 on threonine 14/tyrosine 15 (T14/Y15) (Morgan 1995; Kondo et al., 1997). Thus, the accumulation of cyclin-B in oocytes controls the timing of early embryonic cell cycle (Aegerter et al., 2004). It has been shown that natural occurring steroids as well as endocrine-disrupting chemicals (EDCs) are able to affect cyclin-B synthesis (Kudo et al., 2004; Tokumoto et al., 2004; Tokumoto et al., 2005; Kortner and Arukwe, 2007). Thus, gene expression patterns of cyclin-B may be used as a marker for oocyte developmental competence.

The present study was designed to evaluate the effects of MT on gene expression patterns, whose functional products may regulate steroidogenesis and modulate development of previtellogenic oocytes. In addition, we investigated the effects of MT on steroid hormone levels in previtellogenic oocytes. These were performed using an *in vitro* organ culture technique that was based on an agarose floating method and Atlantic cod (*Gadus morhua*) as a model species. The Atlantic cod is a popular species with large economic value and the farming potential on a global basis is therefore regarded as extremely high. Despite its high economic significance, the Atlantic cod is not a well-studied species neither from an endocrinological or toxicological standpoint. We hypothesize that exposure of previtellogenic oocytes to MT will produce differential gene expression and hormone patterns, whose functional products may modulate steroidogenesis with significant effect on early oocyte growth and development. After thorough validation, these responses may be prognostic, diagnostic and indicative of the effects of pharmaceuticals and chemical endocrine disruptors on the growth and development of previtellogenic oocyte in teleosts with potential implication for overt fecundity.

2. Materials and methods

2.1. Chemicals and reagents

17α-Methyltestosterone was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). Trizol reagent for RNA purification, TA cloning kit and Leibovitz L-15 medium were purchased from Gibco-Invitrogen life technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) and *N*-[2-hydroxyethyl]piperazine-*N*′*e*-[2-ethanesulfonic acid] (Hepes) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Iscript cDNA synthesis kit and iTaq™Sybr® Green supermix with ROX were purchased from Bio-Rad laboratories (Hercules, CA, USA). Generuler™ 100 bp DNA ladder and dNTPs were purchased from Fermentas GmbH (Germany). Superpicture™ polymer detection kit (Cat. No. 87-9263) was purchased from Zymed (San Francisco, CA, USA), and Tissue-clear® and Tissue-mount was purchased from Sakura Finetek Europe (Zoeterwoude, The Netherlands). Testosterone and estradiol-17β enzyme immuno-assay (EIA) kits (Cat. No. 582701 and 582251) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of the highest commercially available grade.

2.2. Animals and floating agarose in vitro culture technique

Juvenile female Atlantic cod, 150–350 g body weight were purchased from Akvaforsk genetic center (Sunndalsøra, Norway) and kept in circulating water at 10 °C and a 12 h light:12 h dark photoperiod. The *in vitro* organ culture techniques employed were based on the agarose floating method (Nader et al., 1999; Kortner and Arukwe, 2007). Briefly, juvenile female cod were anesthetized, sacrificed and washed in 70% ethanol. Ovaries were removed, cut into small pieces (1 × 1 × 1 mm) and grown in 6-well dishes on a floating agarose substrate covered with a nitrocellulose membrane in basal culture media. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 0.1 mM L-glutamic acid, 0.1 mM L-aspartic acid, 1.7 mM L-proline, 0.5% BSA, and 10 mM HEPES (pH 7.4). The gonadal tissue was cultured randomly in triplicates ($n=3$) for 1, 5, 10 and 20 days with different concentrations of methyltestosterone (0 (control), 1, 10, 100 and 1000 μM) in a humidified incubator at 10 °C. The control

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