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Dynamic expression of 11β-hydroxylase during testicular development, recrudescence and after hCG induction, *in vivo* and *in vitro* in catfish, *Clarias batrachus*

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ABSTRACT

Cytochrome P450 11β-hydroxylase (11β-h), is involved in the production of 11-hydroxytestosterone, an immediate precursor for 11-ketotestosterone (11-KT), a potent androgen in teleosts. To understand the role of 11β-h in gonadal development, maturation, function and recrudescence in an annually reproducing teleost, the present study was conducted using *Clarias batrachus*. Four forms of *11β-h* cDNA, regular type (2.253 kb), variant 1 (1.290 kb), variant 2 (1.223 kb) and variant 3 (1.978 kb) were identified from the testis of catfish which expressed ubiquitously with high levels in testis. *11β-h* transcripts were detected as early as 0 days post hatch further, stage- and sex-dependent increase in the *11β-h* transcripts were seen during gonadal differentiation/development. In addition, high expression of *11β-h* (regular type) in pre-spawning phase was detected. Corroboratively, levels of 11-KT in serum and testicular tissue was high during pre-spawning and spawning phases, which might facilitate initiation and normal progression of spermatogenesis. The expression of *11β-h* was high after human chorionic gonadotropin induction *in vivo* (all forms), and *in vitro* (regular type). Immunohistochemical and immunofluorescence localization showed the presence of 11β-h in Sertoli and interstitial/Leydig cells of the testis. These results suggest that 11β-h is involved in late stages of testicular development, together with the regulation of seasonal reproductive cycle in catfish.

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1. Introduction

Sex steroid hormones play an important role in the sex differentiation and development of teleosts. The 11-ketotestosterone (11-KT) and testosterone (T) are the major sex steroids which regulate various processes including reproduction (Borg, 1994; Devlin and Nagahama, 2002; Mayer et al., 1990). 11-KT is essential for inducing male sexual phenotype, secondary sexual characteristics and spermatogenesis in many teleosts which can also cause female-to-male sex reversal (Cardwell and Liley, 1991; Kobayashi and Nakanishi, 1999; Mayer et al., 1990; Miura et al., 1991). In addition, 11-KT triggers Sertoli cells to synthesize activin βB which binds to its type I and II receptors on spermatogonia A leading to the initiation of mitosis, generating spermatogonia B, thereby inducing pre-meiotic spermatogonial proliferation (Dietrich and Krieger, 2009; Ge et al., 1997a,b; Nagahama et al., 1997). In most teleosts, 11-KT was higher than T during all stages of spermatogenesis and is essential for normal progression of spermatogenesis (Borg, 1994; Koldras et al., 1990; Weltzien et al., 2002). Hence, the regulation of enzymes involved in the production of 11-KT are critical for reproductive success of the teleosts.

The biosynthesis of 11-KT requires coordinated action of several steroidogenic enzymes and the final critical steps are catalyzed by the two enzymes, cytochrome P450 11β-hydroxylase (11β-h/P45011β/P450c11; encoded by *cyp11b1* gene) and 11β-hydroxysteroid dehydrogenase (11β-hsd). T is hydroxylated by 11β-h and, after oxidation by 11β-hsd, is converted into 11-KT, the whole process occur at testicular Leydig cells in addition to the peripheral conversion in adrenal and liver (Cavaco et al., 1997; Jiang et al., 1996, 1998; Kobayashi et al., 1998; Nagahama, 1994; Swart et al., 2013). It is also involved in the production of corticosterone and cortisol in the interrenal cells of kidney of several teleosts, while in mammals, the process occurs at adrenal cortex (Miller, 1988). Substantial changes in the mRNA levels of *11β-h* were seen during the reproductive cycle of *Oncorhynchus mykiss* (Kusakabe et al., 2002; Liu et al., 2000) and at spermiation in *Salmo salar*

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(Maugars and Schmitz, 2008). Treatment of hCG can induce *11β-h* mRNA in immature eel as well as increase in 11-KT production *vis-a-vis* spermatogenic process (Jiang et al., 1996; Miura et al., 1991). In the Nile tilapia, 11-oxygenated androgens are measurable only after the testis differentiation (Nakamura et al., 1998). In *Dicentrarchus labrax*, *Odontestes bonariensis* and *O. mykiss*, *11β-h* expression was detected well before the gonadal differentiation (Blasco et al., 2010; Blázquez et al., 2001; Liu et al., 2000). Evidences for the direct involvement of 11-hydroxytestosterone (11-OHT) and 11-KT in testis formation and differentiation along with the regulation of critical enzymes involved in their synthesis are very minimal (Socorro et al., 2007; Wang and Orban, 2007). In addition, the role of 11β-h and 11-oxygenated androgens in spermatogenesis and recrudescence were not studied in detail. Such an attempt to study the ontogeny and recrudescence cohesively in a seasonal breeding teleost like catfish might provide interesting insights. On this perspective, the present study was conducted by using the Asian catfish, *Clarias batrachus*, which undergo gonadal attenuation and maturation which might reveal coordinated regulation of steroidogenic enzyme gene expression. *C. batrachus* has five phases in its reproductive cycle, i.e. preparatory (February-April), pre-spawning (May–June), spawning (July–August) and post-spawning and/or resting (September–January) phases. The present study was conducted to understand the importance of 11β-h in testis development, and how its expression varies during the seasonal reproductive cycle together with the implications of gonadotropin (hCG) exposure, *in vivo* and *in vitro* in addition to sex steroid analogs treatment.

2. Materials and methods

2.1. Animals, sampling and sex steroid analogs exposure

C. batrachus hatchlings at different age groups were obtained by *in vitro* fertilization during breeding season (July–August) and reared in fresh water tanks under ambient photothermal conditions in the aquaculture facility at University of Hyderabad as described previously (Rajakumar et al., 2012). Catfish at different age groups (0, 10, 20, 30, 40, 50, 100, 150, 200, 250 dph and adult) were collected for ontogenic studies. Morphological gonadal distinction occurs at 50 dph in *C. batrachus*, and hence, gonads were dissected from 50, 100, 150, 200, 250 dph and adult catfishes, while mesonephric gonadal complex (MGC) were isolated from 10, 20, 30 and 40 dph larvae and used for total RNA preparation. Isolation of MGC was difficult in 0 dph hatchlings and hence the whole trunk was used for total RNA preparation. During gonadal development, the sex of the gonads was determined by histological analysis. Testes appear like a slender thread while ovaries appear as a transparent pouch devoid of sperm and oocytes, respectively. Histological examination of gonads showed that the gonadal differentiation into testis and ovary occurs around 35–50 dph in this and another co-existing species *Clarias gariepinus* (Raghuveer and Senthilkumaran, 2009). Hence from 50 dph onwards to adult stages, testes and ovaries were dissected for total RNA preparation. Catfish takes around a year to mature and undergo reproduction. Reproductive phases begin only after initial gonadal development and maturity. Both males and females undergo a seasonal reproductive cycle. The seasonal breeding cycle of catfish has been well characterized from our laboratory. The preparatory phase testis contains predominantly spermatogonia and spermatocytes and spermatids. Pre-spawning phase testis contains more number of spermatids and lesser number of sperms, while the number of spermatogonia and spermatocytes were decreased than the testis of preparatory phase. Testis of spawning phase contains spermatids and sperms in high number, while spermatogonia and spermatocytes were negligible. Post-spawning/resting phase testis shows lots of empty lumens with relatively less number of spermatogonia and almost no sperm. Adult *C. batrachus* were reared further in the aquaculture facility for further more a year, before it was sacrificed for experiments on the seasonal cycle and hCG (Pubergen, Uni-Sankyo Pvt., Ltd., India) induction *in vivo* and *in vitro*. Prior to sacrifice, blood was collected from different stages of catfishes by caudal puncture and the collected blood was centrifuged at 2500g in 20 °C for 20 min for serum separation. For tissue expression, different tissues of adult male catfish (testis, brain, liver, kidney, gills, intestine, muscle, heart and spleen) and female catfish (ovary) during pre-spawning phase of reproductive cycle was collected and used for total RNA preparation and expression analysis. Further, for sex-steroid analog studies, juvenile catfish of 50 dph were divided into three groups of 50 larva each and maintained in well-aerated aquarium tanks containing filtered water with or without treatment compounds of 1 μg/L of 17α-methyltestosterone (MT; 97% purity; Wako, Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan) or 1 μg/L of 17α-ethinylestradiol (EE_2; 98% purity; Sigma, St. Louis, MO, USA). Filtered water containing treatment compounds were replenished daily. After 21 days of exposure, catfish were sacrificed by anesthetizing with MS 222 (Sigma) in mild ice-cold water. All experiments and sacrifice procedures were done by following the general animal ethical guidelines of Institutional Animal Ethical Committee. Both EE_2 and MT used in the study were first dissolved in absolute ethanol, air-dried and reconstituted in milliQ water. The dosages were chosen based on pilot studies which were comparable to other related studies (Orn et al., 2003; Pawlowski et al., 2004; Sridevi et al., 2013).

2.2. Molecular cloning of full length 11β-h cDNA from catfish testis

Total RNA was prepared from catfish testis using TRI-reagent (Sigma) and the quality and quantity were determined using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies and Wilmington, Delaware, USA). Total RNA (5 μg) was reverse transcribed to cDNA using SuperScript® III according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). A set of degenerate primers were designed based on the available *11β-h* nucleotide sequences of different teleosts from the NCBI GenBank database. PCR amplification was performed at 94 °C (1 min) and 35 cycles of 94 °C (30 s), 53 °C (30 s) and 72 °C (1 min) using degenerate primers (DF and DR; Table 1). The partial cDNA fragment of 402 bp obtained was cloned in pGEM®-T easy vector (Promega, Madison, WI, USA), sequenced, analyzed and was confirmed as catfish *11β-h* partial cDNA. RACE strategy was performed to obtain the full length 5′ and 3′ cDNA of *11β-h* using the gene-specific primers (GSP) designed based on the sequence information of *11β-h* partial cDNA. The cDNA templates for 5′ and 3′ RACE were prepared using SMARTer™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Later, to obtain the 5′ and 3′ ends of *11β-h* touchdown PCR reactions were performed using 5P, 5N, 3P and 3N primers (Table 1) as well as anchor primers, universal primer A mix, nested universal primer and the advantage® 2 PCR kit (Clontech) by following manufacturer's universal thermal cycling conditions (Clontech). All the nested PCR amplified fragments were gel purified, cloned in pGEM®-T easy vector (Promega), sequenced, and analyzed using Lasergene software 7.1.0 (DNASTAR, Madison, WI, USA), which showed high similarity with the already known (in other teleosts) *11β-h* cDNA but shorter in length. Hence, a degenerate primer (DR1/Table 1) was designed which along with a specific primer (SpF) were used for RT-PCR, upon cloning, sequence analysis and confirmation, a new set of RACE primers was designed and 3′ RACE was repeated, A specific single band was amplified, upon cloning,

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