Chapter 2

Cloning, expression and enzyme activity analysis of testicular 11β-hydroxysteroid dehydrogenase during seasonal cycle and after hCG-induction in air-breathing catfish *Clarias gariepinus*
Abstract

A full length cDNA encoding 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) was cloned from testis of air-breathing catfish, *C. gariepinus* which showed high sequence homology to zebrafish and eel. 11β-HSD2 ORF was then transfected to COS-7 cells. The transfected cells converted 11β-hydroxytestosterone (11-OHT) to 11-ketotestosterone (11-KT) at a considerable rate than mock transfected cells. Tissue distribution analysis by RT-PCR revealed prominent expression in testis, anterior kidney and liver. Expression of 11β-HSD2 in the testes was assayed by real-time PCR during four testicular phases (preparatory, prespawning, spawning and resting phases) and was found to peak during the prespawning phase and gradually decline during the spawning and resting phases. With NAD+, testicular microsomes oxidized 11-OHT with apparent $K_m$ of 56 ± 4 nM and $V_{max}$ of 55 ± 6 pmol/h/mg-protein, respectively. Seasonal 11β-HSD2 dehydrogenase activity in testicular tissues revealed highest production of 11-KT during the prespawning phase. Serum 11-KT levels corroborated well with the levels of transcripts and activity of 11β-HSD2. In vivo hCG administration enhanced 11β-HSD2 expression in the testis, especially during the prespawning phase, at 4, 8, 12 and 24h after induction. It also augmented 11-KT production by testicular microsomes at 8 and 24h. Ontogeny study indicated that this enzyme is expressed after the fate of gonad is determined. However, levels of 11β-HSD2 transcripts were significant during testicular differentiation. Thus the spatiotemporal expression results supported with dehydrogenase activity and circulating 11-KT. Based on these observations, present study clearly indicated a major role for 11β-HSD2 during testicular differentiation and seasonal testicular cycle in catfish.
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Introduction

It is widely accepted that sex steroids are involved during the process of sex differentiation, gametogenesis and sex reversal in fish (Devlin and Nagahama 2002). Role of estradiol-17β (the steroid hormone produced by cyp19a1) in ovarian differentiation, oogenesis and as a feminizing agent is well documented in many fishes (Baroiller et al., 1999; Andersen et al., 2003; Miura et al., 2007). The role of 11-oxygenated androgen, 11-ketotestosterone (11-KT) in teleost reproduction and testis formation is in its primitive stage with few studies suggesting its role, during testis formation and differentiation, (Liu et al., 2000; Rougeot et al., 2007) during sex change in sequential hermaphrodites (Miura et al., 2008) and during spermatogenesis and sperm maturation (Miura et al., 1991a; Miura et al., 2003). On the contrary, there are also report which states that 11β-hydroxylase, a penultimate steroidogenic enzyme involved in the synthesis of 11-KT, is not expressed at early stages or during male sex determination (Baron et al., 2005; Wang and Orban, 2007). Judging from the role of 11-KT, the expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and activity (the enzyme involved in 11-KT production) might be important for testicular differentiation (Miura et al., 2008). Thus, the involvement of 11β-HSD2 as a marker for testis determination in teleost is a contentious topic and needs further investigation. We have chosen an air-breathing, gonochoristic, male heterogametic annual breeding catfish, Clarias gariepinus having lobular testis with synchronous developing cyst as our experimental model because of the ease in breeding, rearing and maintaining them in laboratory/natural conditions. These features allow us to obtain catfish larvae from day one till they mature, to perform ontogeny study and to carry out seasonal expression and activity study on 11β-HSD2. Production of 11-KT can also be influenced by peripheral
conversion more specifically from liver and anterior kidney. However, the contribution from testis cannot be ruled out as studies from our laboratory showed the presence of 11-KT in testes which underwent changes during thyroid depletion (Chapter 4; Swapna et al., 2006) leading to impairment of testicular recrudescence. Further judging from the presence of 11β-HSD2 transcript and activity analyses, 11-KT production in testes might be essential for testicular function (Jiang et al., 2003, Kusakabe et al., 2003). This gene is primarily implicated in initiating and maintaining the spermatogenic cycle. Previous reports in catfish have emphasized the role of 11-KT in promoting and modulating puberty in juveniles, during spermatogenesis and in the development of secondary sexual characteristics (Cavaco et al., 1997a). Interestingly, testosterone (T) is also required for stimulating the hypothalamo-hypophyseal axis vis-à-vis the release of lutenizing hormone (Cavaco et al., 2001a) nevertheless excess T inhibited the production of 11-KT (Cavaco et al., 2001b). Identification of two different androgen receptors with different ligand binding specificity in teleosts (Ikeuchi et al., 1999) also provides evidence that T and 11-KT are required for the normal functioning of the testis and development of secondary sexual characters in males. There are vast number of reports that illustrates the serum profile of 11-KT corresponding to the varied testicular phases in different fishes (Scott et al., 1980; Cavaco et al., 1997a; 2001a; Koya et al., 2002). However, a comprehensive analysis of expression, enzyme activity backed with $K_m$ and $V_{max}$ value of 11β-HSD2 for 11-OHT and serum profile of 11-KT has not been carried out in a lower vertebrate so far to understand the role of 11β-HSD2 in testis. In addition no report exists, to substantiate the regulatory role of gonadotropins on 11β-HSD2 expression and activity after in vivo induction using hCG, a hormone that emulates the action of gonadotropins. Hence, the present study was
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aimed to clone 11β-HSD2, analyze its expression pattern, and measure 11-KT levels and enzyme activity to decipher specific role of 11β-HSD2 during gametogenesis including the seasonal testicular cycle and after hCG-induction.

2. Materials and Methods

Animals

Air-breathing catfish, *C. gariepinus* were procured from local fish markets in and around Hyderabad at different phases of seasonal cycle. They were acclimated for 2-3 weeks by maintaining in aquarium tanks filled with filtered tap water under natural photoperiod and ambient water temperature conditions. Seasonal changes in catfish testis were described earlier by Swapna *et al.* (2006). Catfish were fed with live tube worms/minced goat liver/pelleted food in *ad libitum* during acclimation and experimentation. We also bred and reared the catfish in order to obtain fish at different time points for ontogeny studies.

RT-PCR amplification of partial cDNA homologous to 11β-HSD2

Degenerate primers were designed by aligning the existing sequences of vertebrate 11β-hydroxysteroid dehydrogenase type 2 obtained from DDBJ/EMBL/GenBank databases, to clone partial cDNA fragment of 11β-HSD2 from the testis of catfish. Using these degenerate primers, sense DF1, 5’ GCG GTS YTC ATC ACM GGY TGT GA 3’ and antisense DR1, 5’GCT GCY TTS GAG GYY CCA TA 3’, a cDNA fragment of 464 bp homologous to 11β-HSD2 was amplified by RT-PCR and cloned in pGEM-T-easy vector (Promega, Madison, WI, USA).

cDNA library construction and screening

cDNA library from testis of catfish was constructed using UNI-ZAP cDNA library synthesis kit (Stratagene). Total RNA from testis was prepared using TRI-reagent (Sigma).
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Using 2 mg of total RNA, mRNA was prepared by oligotex-mRNA kit (QIAGEN). Then 5 µg of mRNA was taken to synthesize cDNA using stratagene cDNA synthesis kit. The purified cDNA was ligated and packaged into UNI-ZAP-XR system using Gigapack II Gold packaging extract kit (Stratagene). Screening of the testis cDNA library for 11β-HSD2 was performed by using 464 bp cDNA fragments as probe, obtained by RT-PCR, which shared 68% homology with other teleost 11β-HSD type 2 genes. The probe was radiolabelled with $^{32}$P-dCTP using random primer labeling kit (Perkin Elmer). Single clone excision was performed for positive clones to obtain plasmid DNA for bidirectional nucleotide sequencing. Wherever necessary, we also performed 5’RACE as per the method described earlier (Raghuveer and Senthilkumaran, 2009).

Capacity of 11β-HSD2 to produce 11-KT from 11-OHT in COS-7 cells and determination of apparent $K_m$ and $V_{max}$ values of 11β-HSD2

Analysis of putative 11β-dehydrogenase activity of recombinant protein was performed as described in previous studies with few modifications (Kusakabe et al., 2003). Briefly, the deduced complete open reading frame (ORF) of 11β-HSD2 was inserted into the pCDNA3.1+ TOPOV5-His mammalian expression vector (Invitrogen). The sequence integrity of the insert was verified by nucleotide sequence analysis. Approximately $3\times10^5$ COS-7 cells were laid onto a 6-cm tissue culture plate containing 4ml of DMEM with or without (during transfection) 10% (v/v) fetal calf serum. The cells were cultured at 37°C in 5% CO$_2$ until confluent. Then 1–2 µg of recombinant plasmids, mock (insert locked in reverse direction) and control vector (without insert) were transiently transfected into COS-7 cells using Tfx-20 (Promega) according to the supplier’s protocol. 24h after transfection, the COS-7 cells were incubated with 30 ng of 11β-OHT (Sigma). 24h after
incubation with substrate the culture medium was collected from each well by centrifugation at 1000 rpm, extracted twice with diethyl ether followed by evaporation in a vacuum centrifuge. The steroids were reconstituted in 100µl EIA buffer supplied in the 11-ketotestosterone enzyme linked immunoassay (EIA) kit (Cayman). Entire protocol was repeated thrice with three replicates each time to get concomitant values. The 11-KT produced in the culture medium was measured using 11-KT- EIA kit according to the manufacturer’s protocol. Cross-reactivity of the antibody against 11-KT to 11β-OHT was 1.7%, the minimal detection thresholds was 1.3 pg/ml for 11-KT. After measurements, the conversion rates were calculated and the values of cross-reactivity were subtracted. Results were expressed as mean ± SEM of three replicates. Data analysis was carried out using one-way ANOVA followed by Dunnett’s test. Significance was accepted at $P < 0.05$. Next we studied the affinity and capacity of the enzyme 11β-HSD2 to oxidize 11-OHT with NAD$^+$ as cosubstrate. Kinetic study was performed following procedure described by Stewart et al. (1994) with few alterations. After preliminary experiments on fractional conversion of 11-OHT versus time and protein concentration, testes microsomes (250µg/ml of protein from pellet obtained at 1,05000g after differential centrifugation) in 0.1M potassium phosphate buffer, pH 7.4 (KPO$_4$) were incubated with various concentrations of 11-OHT (0.005-5µmol/L) and 100 pmol/L NAD for 15, 30, 45 and 60 min in a shaking water bath at 37ºC. This was performed on microsomes obtained from five separate testis of prespawning phase male. The reaction volume was 500 µL and the experiment was terminated placing the tube on ice. Steroids were extracted with diethyl ether (thrice the incubation volume), dried, dissolved in EIA buffer, and 11-KT levels estimated using 11-KT- EIA kit. The percentage conversion of 11-OHT to 11-KT was
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calculated. For the kinetic studies, the reaction rate (V), expressed as picomoles of 11-KT formed per h/mg-protein, was linear, for each substrate concentration (S). From a Lineweaver-Burk plot of \(\frac{l}{V}\) vs. \(\frac{l}{[S]}\), the apparent \(K_m\), and maximum velocity (\(V_{max}\)) was calculated. All incubates were analyzed in triplicate. Data analysis and Lineweaver-Burk plot was drawn using Graph Pad Prism 5 software (San Diego, California, USA).

**Real-time RT-PCR**

The relative expression of the steroidogenic enzyme gene 11\(\beta\)-HSD2 in testicular samples was studied by real-time PCR using \(\beta\)-actin (sense: 5’-ACC GAA TGC CAT CAC AAT ACC AGT-3’; antisense: 5’-GAG CTG CGT GTT GCC CCT GAG-3’) as endogenous control at four phases of the reproductive cycle i.e. preparatory, prespawning, spawning and resting. Gene specific primers were designed at the intron–exon boundaries by comparing the ORF with already available 11\(\beta\)-HSD2 sequences in GenBank. With respect to 11\(\beta\)-HSD2 the sense primer was located between exon 1 and exon 2, 5’ATC ACA GGG TGCGAC TCG GGT TTC GGG 3’ whereas the antisense primer was located in exon 2, 5’CGG CTG AGT GAT GTC CAC CTG A 3’, which amplified 168 bp fragments. Real-time PCR was carried out in a 7500 Fast thermocycler (Applied Biosystems) at 95°C denaturing temperature and 60°C annealing temperature for 40 cycles according to the manufacturer's recommendations. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the sequence detector software (Applied Biosystems). Comparative C\(_T\) method was used to quantify the target gene abundance. Each sample (n=5) was run in triplicates with a final volume of 25 µl containing 0.3 µl of cDNA representing the four different phases of the testis, 10 pmol of each primer, and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems). A non-template control was
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included as negative control. Analysis was done by using the RQ Manager to compare expression levels among genes. The RQ (relative quantification) was carried out using preparatory phase expression as calibrator. The amount of target normalized to an endogenous control and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$. Data analysis was carried out using one-way ANOVA followed by Tukey-Kramer’s multiple comparison test. Significance was accepted at $P < 0.05$.

Rate of production of 11-KT by testicular fragments at four testicular phases

The testicular tissues that were collected from five catfishes each in different seasons to monitor expression level at four different testicular phases were simultaneously used to study the enzyme activity of $11\beta$-HSD2. The conversion of 11-OHT to 11-KT was carried out as described by Stewart et al. (1994) and Hu et al. (2008) using cold steroids with few modifications. Testicular microsomes were prepared by homogenizing 500 mg of tissue in 3ml of 0.1M KPO$_4$ buffer pH 7.4, clearing debris at 9,000xg for 20 min, and centrifuging at 1,05,000xg for 1h. The microsomal pellet was washed with 0.1M KPO$_4$ pH 7.4 buffer containing 0.1mM EDTA, resuspended in 500 µl of 0.1M KPO$_4$ buffer, 0.1mM EDTA and 20% v/v glycerol. To 1ml of assay medium, 300 µg of testicular microsome, 50nm 11β-OHT, 100µM NAD was added and incubated in a water-bath with shaker at 37°C for 60 min. The reaction was stopped by adding ice-cold diethyl ether. The steroids were extracted with diethyl ether and the organic layer was dried under N$_2$ gas and dissolved in 100µl of EIA buffer (Cayman). The amount of 11-KT formed was detected by using Cayman 11-KT-EIA kit as per the method described above. Data analysis was carried out using one-way ANOVA followed by Kruskal-Wallis’ test. Significance was accepted at $P < 0.05$ for the testicular fragments of four different phases. Negative (heat-denatured
Measurement of 11-KT levels in catfish

Blood was collected by caudal puncture from five male catfishes each at different phases of testicular cycle before sacrificing the fish. It was then allowed to coagulate, centrifuged at 1,500xg for 10 min to collect the serum. The 11-KT levels in the serum were measured using the 11-KT-EIA kit as described previously. Results were expressed as mean ± SEM of five samples that were done in three replicates each.

Effect of in vivo hCG treatment on 11β-HSD2 expression and 11-KT production

To study the seasonal effect of gonadotropins on the expression of 11β-HSD2 transcript and 11-KT production, especially during late testicular recrudescence (May) and quiescent (December) phases, five catfishes weighing about 400-500 gm were injected intraperitoneally with single dose of hCG (1000 IU/Kg body weight) after standardizing the dosage in our laboratory. Control fish were injected with fish physiological saline. Further, at an interval of every four hours up to 24h, fishes were sacrificed after immersing in ice cold water, to collect testes. This procedure was repeated thrice with different batch of fish (n=5). The testicular samples were snap-frozen in liquid nitrogen and stored in -80°C until assay. Total RNA was then prepared using Tri-reagent (Sigma) as per the manufacturer’s protocol, followed by 1st strand cDNA synthesis using random primer-Superscript III (Invitrogen). To study the changes in the expression level of the 11β-HSD2 transcript, semi-quantitative RT-PCR was performed using specific primers and the intensity of the gel bands was analyzed by densitometric method using Bio-Rad Gel Documentation 1000 system and multi-analyst software program (Bio-Rad, CA, USA). To
measure the rate of production of 11-KT by putative dehydrogenase activity of 11β-HSD2 at different time points, microsomes were prepared from the testicular tissue and activity measured as per the method described above.

**Tissue distribution of 11β-HSD2 in catfish by RT-PCR**

Total RNA was prepared from various tissues of adult male catfish (prespawning phase) using Tri-reagent (Sigma) as per the manufacturer’s protocol. First strand cDNA was then synthesized using oligodT18-Superscript III (Invitrogen) and semi-quantitative RT-PCR was performed to study the spatial expression of 11β-HSD2 in various tissues. The PCR cycle employed for analyzing expression was 94°C-2 min followed by 30 cycles at 94°C-45 (sec), 58°C-30 (sec), 72°C-1 (min) followed by final extension at 72°C-10 (min), specific primers were designed for this purpose (sense 5’-TAC CTG CTC TCC TCG CTT CAC CTT 3’ and antisense primer 5’-GCT GTT CAC CTG ACG GAC TGG AGA 3’) which amplified 296 bp fragments. A no template control was included as negative control.

**Ontogeny expression study of 11β-HSD2**

Earlier finding (Raghuveer and Senthilkumaran 2009) from our laboratory reported that the morphological signs of sex differentiation in catfish were evident around the period of 40-50 days post hatch (dph). To study the temporal expression of 11β-HSD2, catfish larvae were collected at 45, 55, 75, 90, 150 and 260 dph. 15-20 larvae were dissected for each time period under dissection microscope (Carl-Zeiss, Germany) and the gonads were pooled to have five biological samples (n=5) for total RNA preparation in sterile condition, snap-frozen using liquid nitrogen and stored in -80°C for later use. Total RNA was prepared using Tri-reagent (Sigma) as per the manufacturer’s protocol. 2µg RNA was reverse transcribed using random primer and Superscript III (Invitrogen). Subsequently
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real-time PCR was performed as described for stage-dependent 11β-HSD2 expression study using 45 dph expression as calibrator.

Results

Molecular cloning of 11β-HSD2 from testis of catfish

A 464 bp partial cDNA fragment homologous to 11β-HSD2 was obtained from catfish testis by RT-PCR. This was used as a probe to screen approximately 7.5 x 10^5 recombinant phages from testis library. After three rounds of screening, five positive clones were obtained and they were sequenced from both the ends. Four of them were 5’ truncated while one clone had full length sequence. The full length sequence was also confirmed by performing 5’RACE with the sequence data of 5’ truncated clones. The testicular 11β-HSD2 was 2172 bp long with 21 bp 5’ untranslated region (UTR) and 918 bp 3’ (UTR). The ORF encoded a protein of 410 amino acids with four ATTAAA as poly-adenylation signals which are 636, 598, 60 and 11 bp apart from the 21 bp poly (A) tail (Fig.1). The sequence data of 11β-HSD2 has been submitted to GenBank (Accession Number: GU220074). The clone exhibited conserved NAD binding domain typical of type-2 11β-HSD, and the presence of characteristic five amino acid residues (MEVNF) common for both type 1 and 2 11β-HSD. The signature domains typical of short-chain dehydrogenase reductase (SDR) superfamily, which included the Rossmann fold and the catalytic domains, were clearly found in the catfish testicular 11β-HSD2 gene. ClustalW multiple alignment analysis demonstrated that these regions were highly conserved among vertebrates (Fig. 2). Phylogenetic analysis constructed using POWER program showed three distinct clades, the mammalian11β-HSD2, the teleost11β-HSD2, and non-vertebrate 11β-HSD clade. Catfish 11β-HSD2 grouped in the teleost clade shared high homology with
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that of zebrafish followed by eel, rainbow trout and the Nile tilapia (68\%-63\%), whereas

*Ciona intestinalis*-\(11\beta\)-HSD3 and *Caenorhabditis elegans*-short chain dehydrogenase used

as an out group branched together in a separate clade (Fig. 3).
Fig. 1. Nucleotide and deduced amino acid sequence of catfish testis 11β-HSD2. UTRs, (5’ 1-21 bp, 3’ 1254-2142 bp) are boldfaced and the polyadenylation signals are underlined.
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Fig. 2. Alignment of deduced amino acid sequences of catfish 11β-HSD2 (GU220074) with that of other teleosts 11β-HSD2 using ClustalW multiple alignment tool. Conserved domains are shown in rectangles. I: NAD-binding domain, II: 11β-HSD2 conserved sequence, III: catalytic site. Highly conserved regions are shaded. The four intron positions are marked by arrows. Clarias: Clarias gariepinus, Danio: Danio rerio, Anguilla: Anguilla japonica, Oncorhynchus: Oncorhynchus mykiss, Oreochromis: Oreochromis niloticus, Mus: Mus musculus, Homo: Homo sapiens, Sus: Sus scrofa. The GenBank accession numbers for the teleostean and mammalian 11β-HSD2 are given in fig. 3.

Fig. 3. Phylogenetic analysis of vertebrate 11β-HSD2 showing evolutionary relationship. POWER tool (www.power.nhri.org) with 100 bootstrap trial was used to construct the Phylogenetic tree. C. elegans short chain dehydrogenase protein, belonging to SDR family, was used as outgroup. Branch length is proportional to the distance between each protein. Bootstrap values are the number of trials that this cluster was found in 100 trials. Accession No.: Human BC036780; Mouse BC066209; Pig NM213913; Tilapia DQ991146; Trout AB104415; Eel AB252646; Zebrafish NM212720; seasquirt: Ciona intestinalis AK116129; C. elegans: Caenorhabditis elegans AF022968; Roundworm2: Caenorhabditis elegans AF00310 and catfish: Clarias gariepinus GU220074

Transient transfection of 11β-HSD2 in COS-7 cells and apparent $K_m$ and $V_{max}$ values of 11β-HSD2
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The result of transient expression study in non-steroidogenic COS-7 cells transfected with pCDNA3.1+ vector harboring putative ORF of 11β-HSD2, was expressed as, percentage conversion of 11β-OHT (substrate) to 11-KT (product). 11β-HSD2 showed about 38% conversion of 11β-OHT to 11-KT (P<0.05), compared to blank (only vector) and mock transfection (the ORF locked in reverse direction) Fig. 4A. Kinetic analysis of testicular microsomes incubated with increasing concentrations of 11-OHT, revealed a high affinity for 11β-hydroxy testosterone with apparent affinity value, K_m 56 ± 4 nmol/L and maximum velocity V_max 55 ± 6 pmol/h/mg microsomal protein (Fig. 4B).

![Figure 4A](image1.png)

**Fig. 4.** (A) Representative histogram showing the percentage (%) conversion of 11β-hydroxytestosterone to 11-ketotestosterone by recombinant 11β-HSD2 protein transiently expressed in COS-7 cells (n=3 different transfection with triplicate assays at each time), * indicates the significance (B) 11β-HSD2 activity in catfish testis microsomes depicting apparent Km and V_max for 11-OHT. Each point represents the mean of three separate experiments [K_m 56 ± 4 (±SE) nM and V_max 55 ± 6 pmol/h/mg protein].

Phase-dependent expression and activity of 11β-HSD2 in testis
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Real-time RT-PCR analysis demonstrated seasonal fluctuation in the 11β-HSD2 transcripts with relatively high mRNA levels in preparatory phase, which peaked in the prespawning phase followed by a drop in spawning and regressed phases (Fig. 5A) The putative dehydrogenase activity of 11β-HSD2 (Fig. 5B) and serum 11-KT levels (Fig. 5C) measured in four different phases showed positive correlation with the transcript levels, displaying maximum 11-KT levels in the prespawning phase.

Fig. 5. (A) Real-time RT-PCR analysis of 11β-HSD2 expression, (B) change in the rate of production of 11-KT and (C) 11-ketotestosterone levels in the serum during catfish testicular cycle. Means with different letters differ significantly, (P<0.05, ANOVA).
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11β-HSD2 expression and rate of 11-KT production after in vivo hCG induction

The hCG injection in the prespawning phase significantly enhanced 11β-HSD2 expression and activity when compared to saline treated group. The sustained rise in 11β-HSD2 transcript and enzyme activity at different time points was evident from 4h after induction with a maximum at 24h (Fig. 6A-C). On the other hand in the resting phase, fishes responded with an initial spurt in 11β-HSD2 mRNA levels and protein dehydrogenase activity which later dwindled at 8h and was further maintained in line with the control group (Fig. 7A-C).

![Image](image.png)

**Fig. 6.** Semi-quantitative RT-PCR analysis of 11β-HSD2 (A) expression (B) densitometric analysis of expression, small letter over bars a, b and c represent significant change compared to control and (C) rate of production of 11-KT in testis,
after hCG induction in the prespawning phase. X-axis represents hours after treatment, \((P<0.05, ANOVA)\).

Fig. 7. Semi-quantitative RT-PCR analysis of \(11\beta\)-HSD2 (A) expression (B) densitometric analysis of changes in the expression and (C) rate of production of 11-KT in testis, after hCG induction in the resting phase. X-axis represents hours after treatment, \((P<0.05, ANOVA)\).

**Tissue distribution of \(11\beta\)-HSD2**

Semi-quantitative RT-PCR analysis detected \(11\beta\)-HSD2 expression in several tissues other than testis including brain, gills, heart, muscle, spleen, liver, kidney and ovary. However, the expression was prominent in testis, liver, kidney and gills (Fig. 8).
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Fig. 8. Semi-quantitative RT-PCR analysis of spatial expression pattern of catfish $11\beta$-HSD2 in different tissues. Negative control contains no cDNA template.

**Ontogeny of $11\beta$-HSD2**

Temporal expression of $11\beta$-HSD2 by real-time PCR was performed from 45 dph up to 260 dph to study its role during testicular growth and differentiation. In the testes of 45 dph group, no amplification of $11\beta$-HSD2 transcript was observed. The transcript was first detected at 55 dph with subsequent rise in expression measured up till 260 dph (Fig. 9).
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Fig.9. Real-time RT-PCR analysis of temporal expression pattern of catfish 11β-HSD2 in developing larvae at 45, 55, 75, 90, 150 and 260 dph. Means with different alphabets differ significantly (P<0.05, ANOVA). No detection was seen in 45 dph

Discussion

The role of 11β-HSD2 during testicular differentiation and in the maintenance of reproductive cycle in the gonochoristic male catfish was demonstrated in the present study by a comprehensive analysis of 11β-HSD2 expression pattern, its putative steroidogenic capacity to produce 11-KT and subsequently correlating with the circulating 11-KT levels during various phases of testicular development and recrudescence. Present work also demonstrated modulation in steroidogenic capacity and 11β-HSD2 transcript expression in testis of prespawning and resting phase by hCG administration. Further we reported for the first time in teleost the capacity and affinity of the enzyme 11β-HSD2 for 11-OHT. The apparent Km value of 11β-HSD2 for 11-OHT was in range with Km value obtained for glucocorticoids in higher vertebrates (Katz et al., 2008), however, the capacity of this enzyme was low when compared to data from avian and mammalian 11β-HSD2 kinetic study. Likewise transfection study also showed considerable synthesis of 11-KT by recombinant 11β-HSD2 and testicular fragments using 11-OHT and NAD+ as substrate similar to the finding by Kusakabe et al. (2003) and Ozaki et al. (2006). Homology study of catfish 11β-HSD2 with Japanese eel showed 65% sequence identity suggesting common function of the gene. Further eel 11β-HSD2 converted cortisol to cortisone but cortisone to cortisol conversion was negligible (Ozaki et al., 2006). However, this was not probed in the present study as we focused on 11β-HSD2 and 11-KT. It would also be inadequate to overlook the role of 11β-HSD3 (an isoform of 11β-HSD2) in contributing to the
production of 11-KT from 11-OHT (if any) which is also a unidirectional oxidative enzyme with proven dehydrogenase activity in pig, chicken and humans using NADP⁺ as cosubstrate (Katz et al., 2008, Robinzon and Prough et al., 2005; 2009). Furthermore Baker (2004) reported the existence of \(11\beta\)-HSD3 isoform in medaka, zebrafish and fugu. He also confirmed the absence of \(11\beta\)-HSD1 in the genome of these fishes proposing that \(11\beta\)-HSD3 may be the ancestral form of \(11\beta\)-HSD1 that arose in terrestrial forms after the divergence of ray-finned and lobed-finned fishes. At the same time no report exists from teleost that could accounts for either the involvement or up regulation of \(11\beta\)-HSD3 isoform during spermatogenesis. BLAST search of catfish \(11\beta\)-HSD2 showed high identity with \(C_{11}\) and \(C_{17}\) hydroxysteroid dehydrogenase type 2 genes. Part of the sequence also matched with 3-hydroxyl butyrate dehydrogenase type 1 and retinol dehydrogenase gene which is in concurrent with Baker’s (1998) findings on hydroxysteroid dehydrogenase evolution in animal kingdom. Although data on 11-KT are available in catfish to support its role in spermatogenesis by hormone implantation studies in juveniles and by measurement of plasma and tissue levels of steroids along with \textit{in vitro} and \textit{in vivo} bioconversion of precursor steroids by testicular fragment at the time of puberty (Cavaco et al., 1997a, Schulz et al., 2008). Nonetheless there exist neither any reports on early expression of \(11\beta\)-HSD2 during gonad development nor any reports on stage-wise expression pattern and activity during testicular cycle, to implicate a specific role to \(11\beta\)-HSD2 during testis formation and development in catfish. To start with, we cloned \(11\beta\)-HSD2 cDNA encoding 410 amino acid residues that displayed conserved catalytic and characteristic GlyXXXGlyXGly regions, which are hallmarks of the SDR super family. Studies pertaining to structure function relationship utilizing site-directed mutagenesis and
X-ray crystallography demonstrated that the co-factor binding domain (NAD-binding), Rossmann fold and active site motif are crucial (Daux and Ghosh, 1997). The SDR superfamily is one of the biggest families with more than 2000 known primary structures (Kallberg et al., 2002). In spite of highly divergent primary structures in this family, they all have super-imposable tertiary structures, highly conserved signature domains and these motifs are well conserved in catfish 11β-HSD2.

Real-time PCR analysis demonstrated a steady elevation in the 11β-HSD2 transcripts during the proliferation of spermatogonial cells followed by a gradual decline during maturation and spermiation in catfish. These results corroborate well with the seasonal pattern of plasma 11-KT levels measured in the present study and by Cavaco et al. (1997a) in the same species during puberty. The pattern of seasonal change of 11β-HSD2 expression and 11-KT production by testis clearly reflected the testicular phases. In concurrent with our findings, expression data from rainbow trout, Pacific herring, and sea bass also reported similar seasonal fluctuation of genes involved in 11-KT production (Liu et al., 2000; Kusakabe et al., 2003; Koya et al., 2002; Vinas and Piferrer, 2008). However in salmonids, plasma 11-KT levels and expression of steroidogenic genes implicated in 11-KT production were low during early spermatogenesis yet peaked at spermiation (Borg 1994; Maugars and Schimtz 2008). The presence of high expression of 11β-HSD2 in preparatory/prespawning testes is also in agreement with previous findings in eel, which proved an induction of 11β-HSD2 transcript by hCG treatment in immature testes (Jiang et al., 2003; Miura et al., 1991b) leading to initiation of the spermatogenic cycle and production of spermatoocytes, spermatozoa and spermatids. The events that ensued after induction of 11β-HSD2 transcript by gonadotropins were activation of Sertoli cells, which
in turn produced activin B and proteins involved in initiation of mitotic cycle (Nagahama, 1994). The waning of 11β-HSD2 expression in spawning and resting phase is also in accordance with the existing data on eel’s steroid profile which testifies a shift in steroid from 11-KT to 17α-20β-dihydroxy-4-pregnen-3-one (17α-20β-DP) with the onset of spawning season (Sakai et al., 1989). On the other hand, (Borg, 1994; Ozaki et al., 2006; Maugar and Schmitz, 2008) reported the requirement of both 11-KT and 17α-20β-DP at the time of sperm maturation and spermiation. Analysis of putative 11β-HSD2 oxidation activity in the present study using testicular explants from different reproductive phases showed similar results with the dehydrogenase activity peaking in the prespawning phase, might be due to abundant number of spermatogonial cells present after its proliferation, which along with interstitial cells, expresses steroidogenic genes (Vinas and Piferrer, 2008) required for the synthesis of 11-oxygenated androgens. Further, the expression of 11β-HSD2 and activity pattern of 11-oxo-androgen production (11-KT) studied at different time points after administration of hCG in the prespawning phase revealed steady increase in 11β-HSD2 transcript levels and 11-KT production up to 24h accompanied by induction of spermatogenesis. However, results obtained in the resting phase, where hCG administration could not induce sustained elevation in the expression of 11β-HSD2, indicates that gonadotropin input alone cannot trigger 11-KT production vis-à-vis spermatogenic cycle during testicular quiescence. These results are in agreement with the previous hCG induction studies performed on eel testes belonging to various developmental stages where hCG promoted spermatogenesis and increased the milt volume in developed testis but could not sustain completion of spermatogenic cycle in the quiescent testis but for initiating few mitotic division in spermatogonia (Miura et al.,
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1997). These findings together with the present study suggests that various factors, for example sex steroids, androgen receptors, environmental cues and signals from the hypothalamo-hypophyseal axis may act collectively in a complex coordinated manner to initiate the spermatogenic cycle after testicular quiescence. Nevertheless, judging from changes in 11β-HSD2 during seasonal cycle and after hCG-induction in prespawning phase in the present study, it is plausible to infer that gonadotropins targets up regulation of 11β-HSD2 at the level of testis to promote testicular recrudescence. This may be one of the mechanisms to entrain testicular cycle.

Spatial expression pattern of catfish 11β-HSD2 by semi-quantitative RT-PCR demonstrated ubiquitous expression with predominant expression in testis, gill, anterior kidney and liver. The occurrence of extra testicular expression is in agreement with reports in the Nile tilapia, eel and rainbow trout, (Jiang et al., 2003; Kusakabe et al., 2003). The presence of 11β-HSD2 in gill suggests a role in osmoregulation. In kidney, 11β-HSD2 might play a protective role as that of mammalian 11β-HSD type-2 gene, where it is involved in the protection of mineralocorticoid receptor from over stimulation by excess corticosteroid and also in the prevention of inhibitory action of cortisol on androgen synthesis (Monder et al., 1994; Bambino and Hsueh, 1981). Earlier reports in teleosts ascertained the existence of genes coding for enzymes involved in corticosteroid synthesis pathway and mineralocorticoid receptor in kidney, reconfirming the protective role of 11β-HSD2 from cortisol, the main corticosteroid in teleosts (Colombe et al., 2000). The presence of abundant expression in liver is in agreement with the previous report in this species where they indicated, extra testicular conversion of T to 11-KT by liver that contributed to the 11-KT level measured in plasma (Cavaco et al., 1997b). Biological role
of 11β-HSD2 has been implicated in teleost reproduction but the presence of 11β-HSD2 transcripts in non-steroidogenic tissues such as heart and muscle is unclear. Expression of 11β-HSD2 in brain is not unusual since steroidogenic enzyme genes are often detected in brain (Tomy et al., 2007). In rainbow trout, in situ hybridization with 11β-HSD2 mRNA yielded positive signals in the thecal layer of the ovarian follicle, which supports the occurrence of 11β-HSD2 expression in catfish ovary, assigning it a protective role in ovary from the excessive circulatory cortisol, as suggested in rainbow trout and carp (Kusakabe et al., 2003; Nematollahi et al., 2009).

Ontogeny study was undertaken to confirm 11β-HSD2 role during testicular differentiation in catfish which displayed 11β-HSD2 transcripts in testis from 55 dph onwards followed by stability in transcript levels at 75, 90, 150 and 260 dph catfish larvae strongly emphasizing its role at least in testicular development.

In summary, a full-length cDNA for 11β-HSD2 was cloned from testis of catfish. Catfish 11β-HSD2 cDNA showed high homology to that of zebrafish followed by eel. Dehydrogenase capacity of the recombinant 11β-HSD2 protein was demonstrated in COS-7 cells. We also studied the affinity and capacity of testicular 11β-HSD2 enzyme towards 11-OHT. Present study provided substantial evidence on stage-dependent expression of 11β-HSD2 and 11-KT production in maintaining the testicular cycle. Further, we demonstrate the responsiveness of testis to hCG induction, in vivo at recrudescence but not in quiescent phase to validate our hypothesis that gonadotropins might regulate 11β-HSD2 vis-à-vis 11-KT to entrain testicular cycle. It is apparent from the ontogeny expression study in catfish that 11β-HSD2 might be required only during late stages of testicular differentiation or development. Based on our comprehensive study, it is possible to
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implicate an important role for $11\beta$-HSD2 during testicular development and recrudescence in catfish.

References


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