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

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ABSTRACT

A full-length cDNA encoding 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) was cloned from testis of air-breathing catfish, *Clarias gariepinus* which showed high sequence homology to zebrafish and eel. The open reading frame of *11β-HSD2* was then transfected to COS-7 cells, which converted 11β-hydroxytestosterone (11-OHT) to 11-ketotestosterone (11-KT). Using NAD^+, *11β-HSD2* from testicular microsomes oxidized 11-OHT with apparent K_m 56 ± 4 nM and V_{max} 55 ± 6 pmol/h/mg protein values. Tissue distribution analysis revealed prominent expression in testis, anterior kidney, liver and gills. Expression of *11β-HSD2* in testis and serum levels of 11-KT were high in the prespawning phase. Administration of human chorionic gonadotropin (hCG) during prespawning and resting phases revealed initial rise in *11β-HSD2* transcript at 4 h followed by gradual increase at 8 h, 12 h and peaking at 24 h, only in testis of prespawning phase. Rate of conversion of 11-OHT to 11-KT by testicular microsomes during different testicular phases and after hCG administration corroborated well with the expression of *11β-HSD2*. Ontogeny study indicated that this enzyme is expressed during testicular development. Thus the spatio-temporal expression supported with putative dehydrogenase activity and circulating 11-KT levels clearly suggest a major role for *11β-HSD2* during testicular differentiation and seasonal testicular cycle in catfish.

1. Introduction

It is widely accepted that the sex steroids are involved during the process of sex differentiation, gametogenesis and sex reversal in fish [1]. The role of estradiol-17β (the steroid hormone produced by aromatase) in ovarian differentiation, oogenesis and as a feminizing agent is well documented in many fish species [2–4]. The role of 11-oxygenated androgen, 11-ketotestosterone (11-KT) in teleostean male reproduction is in its primitive stage with few studies suggesting its role during testis formation and differentiation [5,6], sex change in sequential hermaphrodites [7], spermatogenesis and sperm maturation [8,9]. On the contrary, there are also reports [10,11] which state that 11β-hydroxylase (*11β-H*), a penultimate steroidogenic enzyme involved in the biosynthesis of 11-KT, is not expressed at early stages of testis development or during male sex determination. Judging from the role of 11-KT, the expression and dehydrogenase activity of *11β-HSD2* (the enzyme involved in 11-KT production) might be important for testicular differentiation [7]. Thus, the involvement of *11β-HSD2* as a marker for testis determination in teleosts 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 (out-door tanks) conditions. These features allow us to obtain catfish larvae from day one until they mature to perform an ontogeny study and to determine seasonal expression and activity of *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 testis which underwent changes during thyroid hormone depletion [12,13] leading to the impairment of testicular recrudescence. Further judging from the presence of *11β-HSD2* transcript and activity analysis in the testis of few teleost species [14,15], 11-KT production in testis might be essential for testicular function. This gene is primarily implicated

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in initiating and maintaining the spermatogenic cycle [8,9]. Previous report [16] in catfish has emphasized the role of 11-KT in promoting and modulating puberty in juveniles, during spermatogenesis and in the development of secondary sexual characters. Interestingly, testosterone (T) is also required for stimulating the hypothalamo–hypophyseal axis vis-à-vis release of lutenizing hormone [17], nevertheless, excess T inhibited the production of 11-KT [18]. Identification of two different androgen receptors with differential transactivation potency among androgens using luciferase assay in eel [19] further provides evidence that T and 11-KT have discrete role in the normal functioning of the testis and development of secondary sexual characteristics in males. There are vast number of reports [16,17,20,21] that illustrate the serum profile of 11-KT corresponding to the varied testicular phases in different fish species. However, a comprehensive analysis of expression levels, enzyme activity with K_m and V_{max} values of *11β-HSD2* for 11β-hydroxytestosterone (11-OHT) and serum profile of 11-KT has not been carried out in lower vertebrates 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 human chorionic gonadotropin (hCG), a hormone that emulates the action of gonadotropins. Hence, the present study was aimed to clone *11β-HSD2*, analyze its expression pattern, and measure 11-KT levels and enzyme activity, to decipher the specific role of *11β-HSD2* during gametogenesis including the seasonal testicular cycle and after hCG induction.

2. Materials and methods

2.1. 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. [13]. Catfish were fed with live tube worms/minced goat liver/pelleted food *ad libitum* during acclimation and experimentation. They were also bred and reared in our aquaculture facility to obtain catfish larvae at different time periods for ontogeny study.

2.2. 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).

2.3. cDNA library construction and screening

A 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). 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 fragment as probe, obtained by RT-PCR, which shared 68% homology with other teleosts *11β-HSD2*. The probe was radiolabelled with ^{32}P-dCTP using random primer labeling kit (PerkinElmer) and the cDNA library was screened thrice to get positive clones. Single clone excision was performed for all the positive clones to obtain plasmid DNA for bidirectional nucleotide sequencing. Wherever necessary, we also performed 5′RACE as per the method described earlier [22].

2.4. Capacity of 11β-HSD2 to produce 11-KT from 11-OHT in COS-7 cells and determination of apparent K_m and V_{max} value for 11β-HSD2

Analysis of putative 11β-dehydrogenase activity of recombinant protein was performed as described in previous studies with few modifications [15]. Briefly, the deduced open reading frame (ORF) of *11β-HSD2* (1230 bp) 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 3×10^5 COS-7 cells were laid onto a 6 cm tissue culture plate containing 4 mL 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 (ORF) locked in reverse direction) and vector control (without any insert) were transiently transfected into COS-7 cells using Tfx-20 (Promega) according to the supplier's protocol. The COS-7 cells were incubated with 30 ng of 11β-OHT (Sigma) 24 h after transfection. The culture medium was then collected from each well after 24 h incubation with substrate, centrifuged at 1000 rpm, extracted twice with diethyl ether and evaporated in a vacuum centrifuge. Then the tubes containing steroids were reconstituted in 100 μL EIA buffer supplied in the 11-KT enzyme linked immunoassay (EIA) kit (Cayman). The 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 the 11-KT EIA kit according to the manufacturer's protocol. Cross-reactivity of the antibody against 11-KT to 11β-OHT was 1.7%, and the minimal detection threshold was 1.3 pg/mL for 11-KT. The entire assay characteristics including intra- and inter assay variations were described in detail by Swapna et al. [13]. 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 $P < 0.05$. Further, we studied the affinity and capacity of the enzyme *11β-HSD2* to oxidize 11-OHT with NAD^+ as cosubstrate. A kinetic study was performed following procedure described by Stewart et al. [23] with few alterations. After preliminary experiments on fractional conversion of 11-OHT versus time and protein concentration, testes microsomes (250 μg/mL of protein from pellet obtained at 105,000 × *g* after differential centrifugation) in 0.1 M potassium phosphate buffer, pH 7.4 (KPO_4) were incubated with various concentrations of 11-OHT (0.005–5 μmol/L) and 100 μmol/L NAD^+ for 15, 30, 45 and 60 min in a shaking water bath at 37 °C. 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 by 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 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 $1/V$ versus $1/[S]$, the apparent K_m, and the maximum velocity (V_{max}) was calculated. All incubates were

analyzed in triplicates. Data analysis and the Lineweaver–Burk plot was drawn using Graph Pad Prism 5 software (San Diego, CA, USA).

2.5. Real-time RT-PCR

The relative expression of the steroidogenic enzyme *11β-HSD2* in testicular samples was studied by real-time PCR using *β-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 *11β-HSD2* sequences in GenBank. With respect to *11β-HSD2*, the sense primer was located between exon 1 and exon 2, 5′-ATC ACA GGG TGC GAC 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 no template control was included as negative control. Analysis was done by using the RQ Manager to compare expression levels of *11β-HSD2* at different phases. 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 the calibrator, is given by $2^{-\Delta\Delta C_T}$. Data analysis was carried out using one-way ANOVA followed by Tukey–Kramer's multiple comparison test. Significance was accepted at $P < 0.05$.

2.6. Rate of production of 11–KT by testicular fragments at four testicular phases

The testicular tissues that were collected from five fishes each in different seasons to monitor expression level at four different testicular phases were simultaneously used to study the putative dehydrogenase activity of *11β-HSD2*. The conversion of 11-OHT to 11-KT was carried out as described previously by Stewart et al. [23] and Hu et al. [24] with few modifications. Testicular microsomes were prepared by homogenizing 500 mg of tissue in 3 mL of 0.1 M KPO_4 buffer, pH 7.4, clearing debris at 9000 × *g* for 20 min, and centrifuging at 105,000 × *g* for 1 h. The microsomal pellet was washed with 0.1 M KPO_4 buffer, pH 7.4 containing 0.1 mM EDTA, resuspended in 500 μL of 0.1 M KPO_4 buffer, 0.1 mM EDTA and 20% (v/v) glycerol. To 1 mL of assay medium, 300 μg of testicular microsome, 50 nM 11-OHT, 100 μM 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 100 μL of EIA buffer (Cayman). The amount of 11-KT formed was detected by using the 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 obtained from fish during different phases. Negative (heat-denatured microsome) and positive (recombinant *11β-HSD2*) controls were used to check the assay validity.

2.7. Measurement of 11-KT levels in catfish

Blood was collected by caudal puncture from five male catfishes each in different phases of testicular cycle before sacrificing. It was then allowed to coagulate and centrifuged at 1500 × *g* 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.

2.8. 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 g 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 24 h, fishes were sacrificed after immersing in ice-cold water, to collect testis. This procedure was repeated thrice with different batch of fish ($n = 5$). The testis samples were snap-frozen in liquid nitrogen and stored at −80 °C until assayed. Total RNA was then prepared using Tri-reagent (Sigma) as per the manufacturer's protocol, followed by first 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 tissues and activity measured as per the method described above.

2.9. 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 for 2 min, followed by 30 cycles at 94 °C for 45 s, 58 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 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 fragment. A no template control was included as negative control.

2.10. Ontogeny expression study of 11β-HSD2

Earlier finding [22] 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 5 biological samples ($n = 5$) for total RNA preparation in sterile condition, snap-frozen using liquid nitrogen and stored at −80 °C for later use. Total RNA was prepared using Tri-reagent (Sigma) as per the manufacturer's protocol. 2 μg RNA was reverse transcribed using random primer and Superscript III (Invitrogen). Subsequently real-time PCR was

performed as described for stage-dependent *11β-HSD2* expression study using 45 dph expression as calibrator.

3. Results

3.1. 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 7.5×10^5 recombinant phages from a testis library. After three rounds of screening, five positive clones were obtained and they were sequenced from both 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 a 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) super-family, which included the Rossmann fold and the catalytic domains, were clearly found in catfish *11β-HSD2*. 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 mammalian *11β-HSD2*, the teleost *11β-HSD2*, and non-vertebrate *HSD* clade. Catfish *11β-HSD2* grouped in the teleost clade sharing high homology with that of zebrafish followed by eel, rainbow trout and the Nile tilapia (68–63%), whereas *Ciona intestinalis-11β-HSD3* and *Caenorhabditis elegans* short-chain dehydrogenase used as an out group branched together in a separate clade (Fig. 3).

3.2. Transient transfection of 11β-HSD2 in COS-7 cells and apparent K_m and V_{max} values for 11β-HSD2

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 11-OHT (substrate) to 11-KT (product). *11β-HSD2* showed about 38% conversion of 11-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 11-OHT with apparent affinity value, K_m 56 ± 4 nmol/L and maximum velocity V_{max} 55 ± 6 pmol/h/mg microsomal protein (Fig. 4B).

3.3. Phase-dependent expression and activity of 11β-HSD2 in testis

Real-time RT-PCR analysis demonstrated seasonal fluctuation in the *11β-HSD2* transcripts with relatively high mRNA levels in

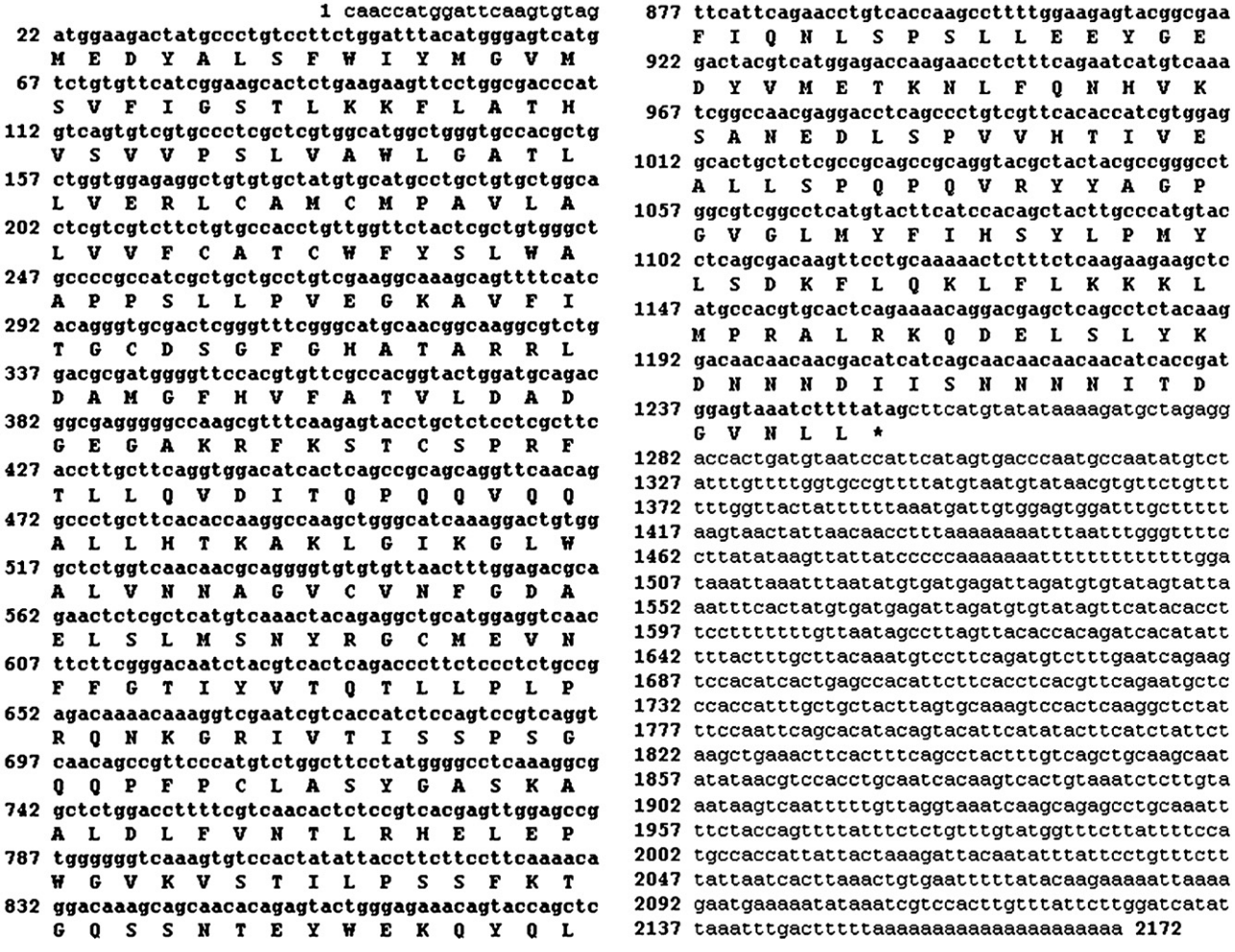

Fig. 1. Nucleotide and deduced amino acid sequence of catfish testis *11β-HSD2*. ORF was shown in bold letters; symbol '*' indicates stop codon.

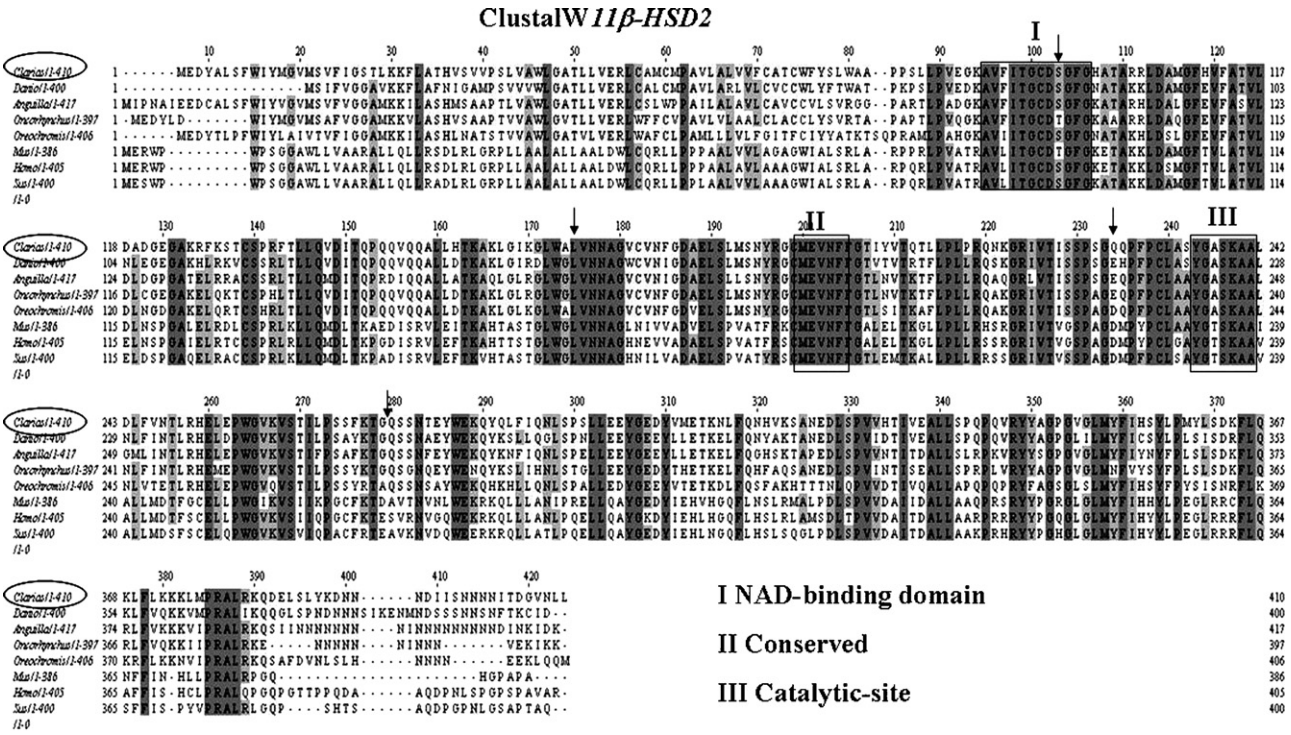

Fig. 2. Alignment of deduced amino acid sequences of catfish *11β-HSD2* with that of other vertebrate *11β-HSD2* using ClustalW multiple alignment tool. Conserved domains are shown in rectangles. I: NAD-binding domain, II: *11β-HSD* conserved sequence, III: catalytic site. Highly conserved regions are shaded. Mus: *Mus musculus*, Homo: *Homo sapiens*, Sus: *Sus sacrofa*, Clarias: *Clarias gariepinus*, Danio: *Danio rerio*, Anguilla: *Anguilla japonica*, Oncorhynchus: *Oncorhynchus mykiss*, Oreochromis: *Oreochromis niloticus*. The GenBank accession numbers for teleostean and mammalian *11β-HSD2* are provided in Fig. 3. The four intron positions are marked by arrows.

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.

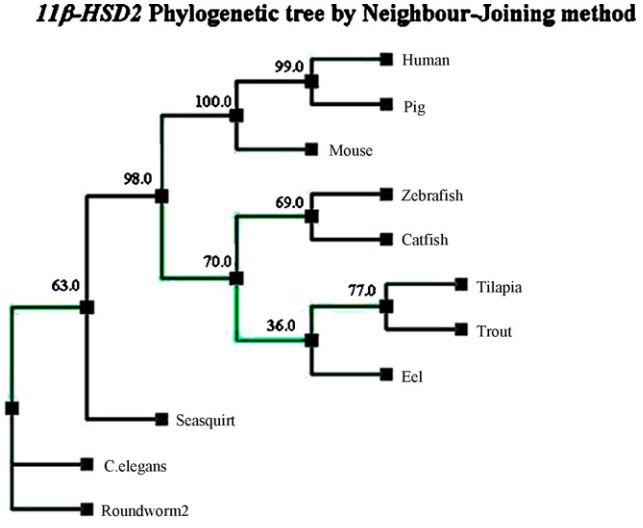

Fig. 3. Phylogenetic analysis of vertebrate *11β-HSD2* showing evolutionary relationship. POWER tool (www.power.nhri.org) with 100 bootstrap trials was used to construct the phylogenetic tree using *C. elegans* short-chain dehydrogenase protein belonging to SDR family as outgroup. Branch length is proportional to the distance between each protein. Bootstrap values are the number of trials that this cluster was found in 100 trials. Accession Numbers: Human BC036780; Mouse BC066209; Pig NM213913; Tilapia DQ991146; Trout AB104415; Eel AB252646; Zebrafish NM212720; Catfish GU220074; Seasquirt: *Ciona intestinalis* AK116129; C.elegans: *Caenorhabditis elegans* AF022968; Roundwom2: *Caenorhabditis elegans* AF00310.

3.4. *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 the saline. The sustained rise in *11β-HSD2* transcript and enzyme activity at different time points was evident from 4 h after induction with a maximum at 24 h (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 8 h and was further maintained in line with the control group (Fig. 7A–C).

3.5. *Tissue distribution of 11β-HSD2*

Semi-quantitative RT-PCR analysis detected *11β-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).

3.6. *Ontogeny of 11β-HSD2*

Temporal expression of *11β-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 testis of 45 dph group, no amplification of *11β-HSD2* transcript was observed. The transcript was first detected at 55 dph with subsequent rise in expression measured up till 260 dph (Fig. 9).

4. Discussion

The role of *11β-HSD2* during testicular differentiation and in the maintenance of reproductive cycle in gonochoristic male catfish was demonstrated in the present study by a comprehensive

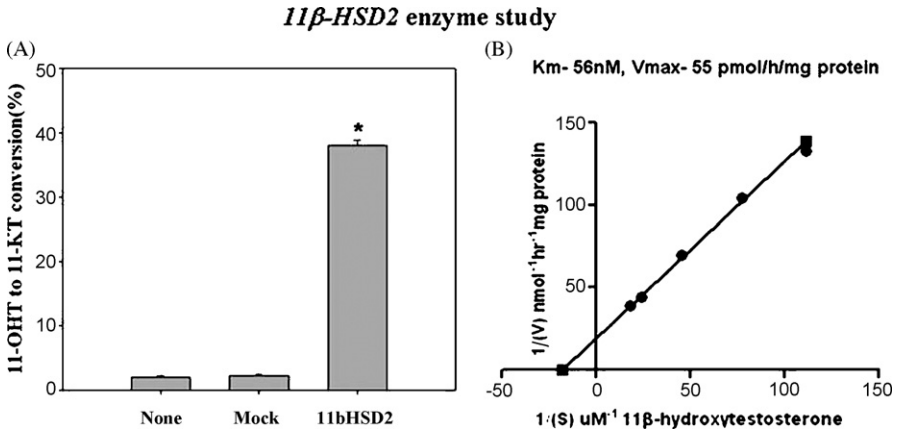

Fig. 4. (A) Histogram showing the percentage (%) conversion of 11β-hydroxytestosterone to 11-ketotestosterone by recombinant 11β-HSD2 protein transiently expressed in COS-7 cells (3 different transfection with triplicate assays at each time); symbol '*' indicates the significance, (B) *11β-HSD2* activity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

analysis of *11β-HSD2* expression pattern, its putative steroidogenic capacity to produce 11-KT and subsequently correlating with circulating 11-KT levels during various phases of testicular development and recrudescence. We 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 teleosts the capacity and affinity of the enzyme *11β-HSD2* for 11-OHT. The apparent K_m value of *11β-HSD2* for 11-OHT was in range with K_m value obtained for glucocorticoids in higher vertebrates [25], however, the capacity of this enzyme was low when compared to the data from avian and mammalian *11β-HSD2* kinetic study. Likewise our transfection and

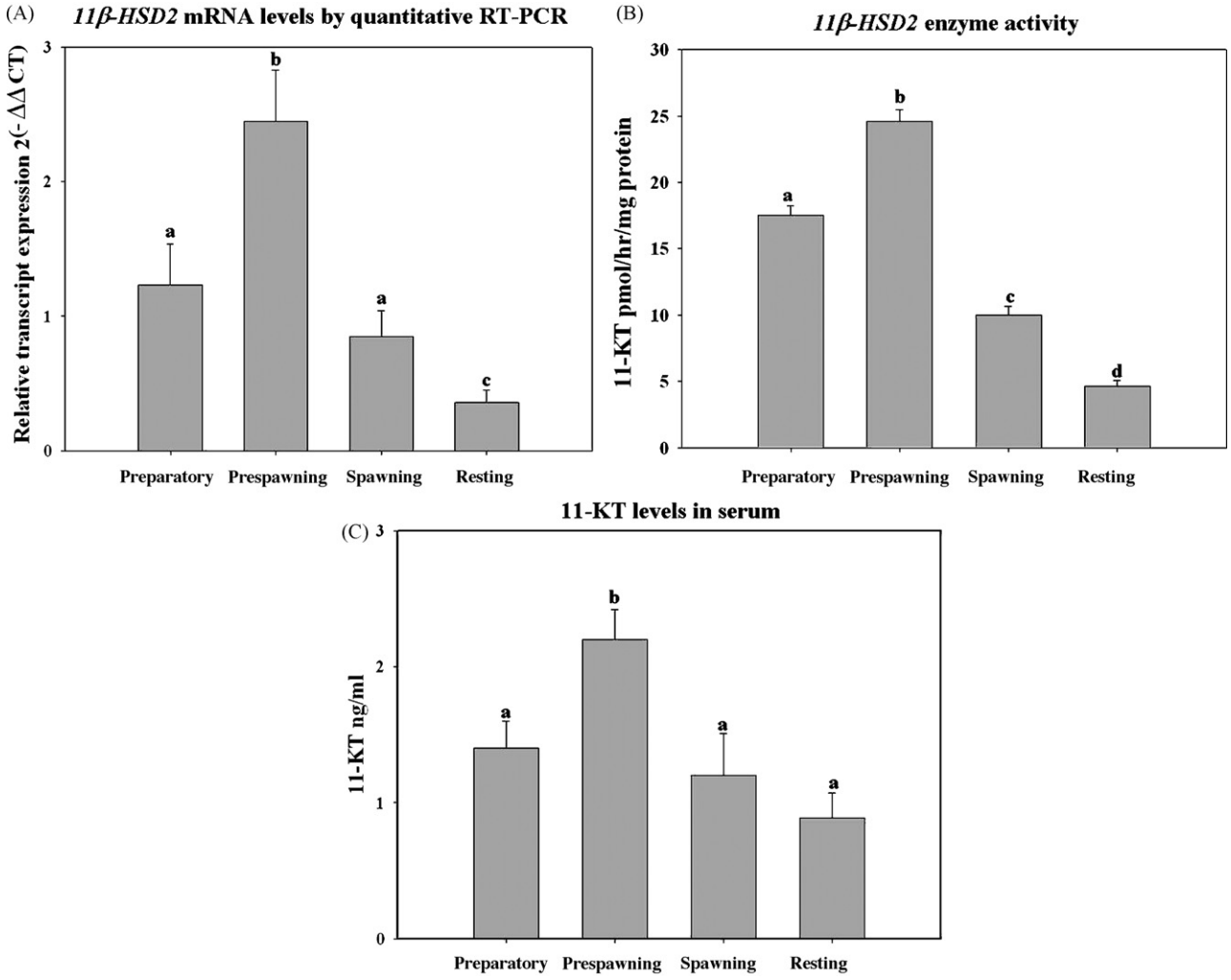

Fig. 5. (A) Real-time RT-PCR analysis of *11β-HSD2* expression, (B) change in the rate of production of 11-KT and (C) 11-ketotestosterone levels in the serum during catfish testicular cycle. Means with different alphabets differ significantly ($P < 0.05$, ANOVA).

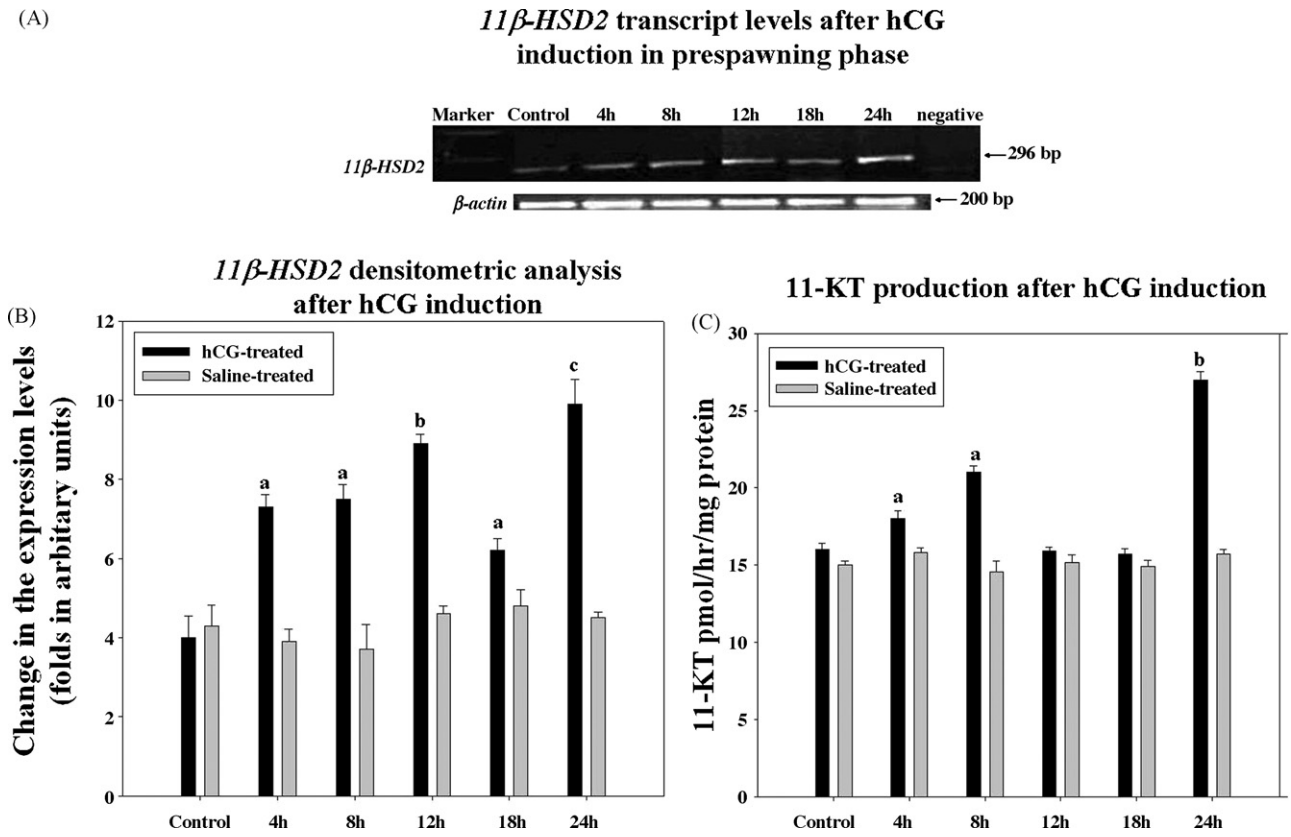

Fig. 6. Semi-quantitative RT-PCR analysis of *11β-HSD2* (A) expression (B) densitometric analysis of expression and (C) rate of production of 11-KT in testis, after hCG induction in the prespawning phase. *X*-axis represents hours after treatment. Alphabets (a, b and c) over bars represent significant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

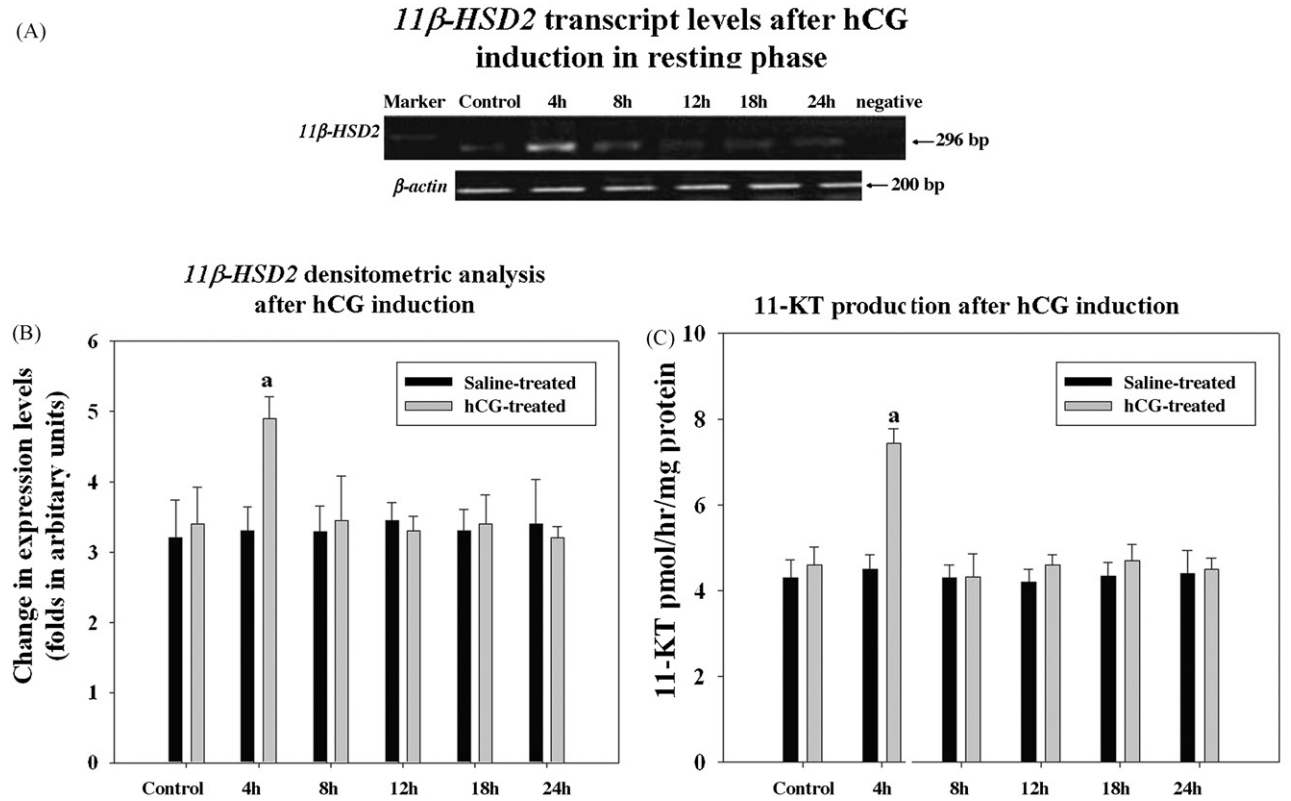

Fig. 7. Semi-quantitative RT-PCR analysis of *11β-HSD2* (A) expression (B) densitometric analysis of changes in the expression and (C) rate of production of 11-KT in testis, after hCG induction in the resting phase. *X*-axis represents hours after treatment. Alphabets (a, b and c) over bars represent significant change compared to control ($P < 0.05$, ANOVA).

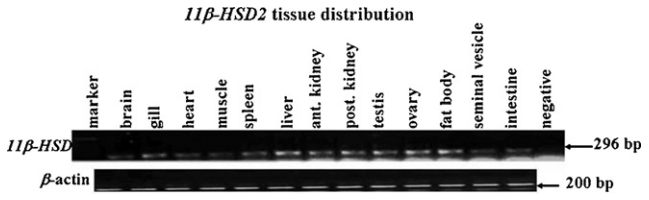

Fig. 8. Semi-quantitative RT-PCR analysis of spatial expression pattern of catfish *11β-HSD2* in different tissues. Negative control contains no cDNA template.

in vitro study also demonstrated 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. [15] and Ozaki et al. [26]. 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 [26]. However, this was not probed in the present study as we focused on *11β-HSD2* and 11-KT. Another enzyme that should be considered is *11β-HSD3*, which was reported to have dehydrogenase activity in pig, chicken and humans using $NADP^+$ as cosubstrate [25,27,28]. Furthermore, Baker [29] reported the existence of *11β-HSD3* isoform in medaka, zebrafish and fugu. This report also confirmed the absence of *11β-HSD1* in the genome of these fishes proposing that *11β-HSD3* may be the ancestral form of *11β-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 account for either the involvement or up regulation of *11β-HSD3* isoform during spermatogenesis. A BLAST search of catfish *11β-HSD2* showed high identity with C11 and C17 hydroxysteroid dehydrogenase type 2 genes. Part of the sequence also matched with 3-hydroxyl butyrate dehydrogenase type 1 and retinol dehydrogenase gene in concurrent with Baker's [30] finding on hydroxysteroid dehydrogenase evolution in animal kingdom. Earlier reports [16,31] using catfish demonstrated a role for 11-KT in spermatogenesis by hormone implantation studies in juveniles and also by the measurement of plasma and tissue levels of steroids along with *in vitro* and *in vivo* bioconversion of precursor steroids by testicular fragments at the time of puberty. Nonetheless, there exists neither any report on early expression of *11β-HSD2* during gonadal development nor any report on phase-wise expression pattern and activity during testicular cycle, to implicate a specific role to *11β-HSD2* during testis formation and development in catfish. To start with, we cloned *11β-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 [32]. The SDR super-family is one of the biggest families with more than 2000 known primary structures [33]. 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 all well conserved in catfish *11β-HSD2*.

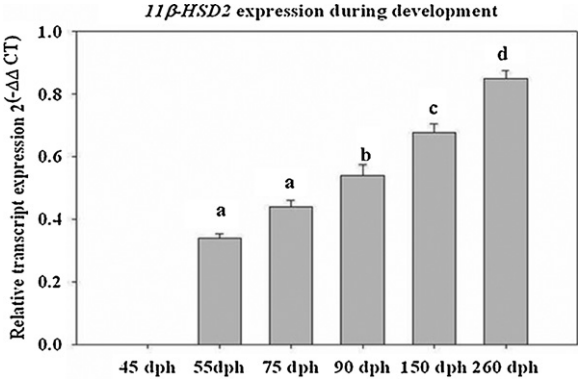

Fig. 9. Real-time RT-PCR analysis of temporal expression pattern of catfish *11β-HSD2* in developing larvae at 45, 55, 75, 90, 150 and 260 days post hatch (dph). Note that the expression of *11β-HSD2* was not in detectable limits at 45 dph. Means with different alphabets differ significantly ($P < 0.05$, ANOVA).

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 also by Cavaco et al. [16] 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 cycle. In concurrent with our findings, expression data from rainbow trout, the Pacific herring, and sea bass also showed similar seasonal fluctuation of genes involved in the 11-KT production [5,15,21,34]. However in salmonids, the plasma 11-KT levels and expression of steroidogenic enzyme genes implicated in 11-KT production were low during early spermatogenesis, and peaked at spermiation [35,36]. The elevated transcript levels of *11β-HSD2* in preparatory/prespawning testes is also in agreement with previous findings from eel, which displayed an induction of *11β-HSD2* mRNA by hCG treatment in immature testis [14,37] leading to the initiation of spermatogenesis and production of spermatocytes, spermatids and spermatozoa. The events that ensued after induction of *11β-HSD2* transcript by gonadotropins were activation of Sertoli cells, which in turn produced activin B and other proteins involved in initiation of mitotic cycle [38]. The waning of *11β-HSD2* expression in spawning and resting phases is also in accordance with the existing data on the steroid profile of eel, which testifies a shift in steroidogenesis from 11-KT to 17α-20β-dihydroxy-4-pregnen-3-one (17α-20β-DP) with the onset of spawning season [39]. On the other hand, few studies [26,35,36] 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 enzyme genes [34] 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 24 h accompanied by the 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 testis 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 divisions in spermatogonial cells [40]. 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 target 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 [14,15]. 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-HSD2*, 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 [41,42]. Earlier reports in teleosts ascertained the existence of genes coding for enzymes involved in corticosteroid biosynthetic pathway and mineralocorticoid receptor in kidney, reconfirming the protective role of *11β-HSD2* from cortisol, the main corticosteroid in teleosts [43]. 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 [44]. A 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 [45]. 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 [15,46].

An 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 of *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. The present study provided substantial evidence on phase-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 implicate an important role for *11β-HSD2* during testicular development and recrudescence in catfish.

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