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EXPRESSION KINETICS OF VITELLOGENIN GENE IN ESTRADIOL-17β INDUCED *CATLA CATLA*

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Vitellogenin (Vtg) is the yolk precursor protein in fish and is synthesized in the liver under estradiol-17β (E2) stimulation. In all oviparous animals, Vtg is synthesized as a response to endogenous estrogen in females in the breeding season. It also could be induced exogenously by E2 in males and juveniles. In the current study the inducibility of Vtg gene in Indian major carp, *Catla catla* was tested. A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed for kinetic study of Vtg mRNA level in *C. catla* after primary and secondary inductions by E2. It was found that the level of Vtg-specific mRNA was highest following 12 h post treatment in primary induction, and after 1 d post treatment in secondary induction. There was a sharp decrease in Vtg mRNA level on day one and day two in primary and secondary induction, respectively. Thus the 'memory effect' of vitellogenin induction by E2 in catla could not be confirmed.

INTRODUCTION

Vitellogenins (Vtgs) are the precursors of the major egg yolk proteins in oviparous species including teleost fish. In maturing females, the synthesized Vtg is transported from liver to the ovary through the bloodstream and incorporated into the growing oocytes by receptor-mediated endocytosis during prespawning period (Wallace, 1985; Specker and Sullivan, 1994). Therefore, vitellogenesis is crucial for gonad maturation and hence, for spawning success. The synthesis of Vtg is induced by estrogens in females and is inducible in immature fishes by a synthetic estrogen, estradiol-17β (E2). Normally Vtgs are produced in females due to endogenous estrogen; however, males and juveniles also begin synthesizing Vtgs when they are exposed to exogenous estrogens (Mommsen and Walsh, 1988; Rankouhi *et al.*, 2002). The study of vitellogenin expression has many implications. It is well established that steroid hormones, such as E2, play a strong regulatory role in physiological processes such as reproduction (Sabo-Attwood *et al.*, 2004). Kinetic study of Vtg induction has been conducted in a number of species during the primary response (Flouriot *et al.*, 1996; Lim *et al.*, 1991; Searle and Tata 1981;

Baker and Shapiro 1978; Brock and Shapiro 1983). For the evaluation of environmental estrogens, quantification of Vtg mRNA expression in the male liver has been utilized (Lech *et al*., 1996; Islinger *et al*., 1999; Korte *et al*., 2000; Costa *et al*., 2010). The Vtg mRNA detection through highly sensitive tool such as real time PCR has advantage over Vtg protein detection in ELISA; although the half-life of the Vtg mRNA is short as it is quickly degraded in the absence of estrogen. Furthermore, the Vtg mRNA detection is faster than Vtg protein detection (Korte *et al*., 2000).

The Vtgs from several fish species have been studied over the years (Review: Hiramatsu *et al*., 2006; Nath *et al*., 2007). Catla (*Catla catla*) is one of the fastest growing Indian major carps (IMCs) and widely cultured species in India. Intensive and profitable culture of any species requires a continuous supply of seed throughout the year. Although, multiple spawning within the breeding season (i.e. during monsoon) has been standardized in this species (Gupta *et al*., 1995), out of season maturation and breeding requires detail knowledge of the inducibility of Vtg gene. Vitellogenin protein in catla has already been characterized (Jena *et al*., 2012). Further, two Vtg mRNAs have been isolated from catla by these authors (unpublished data). In this experiment, the expression pattern of Vtg mRNA has been studied in E2-treated immature fishes after primary and secondary inductions. The expression levels of Vtg were determined by semi-quantitative RT-PCR.

MATERIALS AND METHODS

Fish

Catla of 200 to 400 g body weights, of unknown sex were used for induction of vitellogenesis by hormone administration. About 100 numbers of fish were collected from CIFA farm and were acclimatized in 1000 l capacity cement tanks in the wet laboratory of Central Institute of Freshwater Aquaculture (CIFA) for a week prior to hormone injection. During the experiment, the fish were fed with a commercial floating fish feed available in the local market (Godrej India Pvt. Ltd). Hormone E2 (Sigma, USA) was first dissolved in acetone followed by addition of coconut oil as carrier. The mixture was stirred overnight to evaporate acetone. The preparation was injected intraperitoneally to individual fish at a dose of 0.2 ml/ fish containing 100 µg of E2.

Hundred experimental fishes were randomly divided into two equal groups, one for primary and another for secondary induction. Fishes of both the groups were administered with 1st dose while 2nd dose was administered after one month of the 1st injection, and only to those fishes kept for secondary induction.

Tissue sample collection

Liver tissues were collected from experimental fishes by sacrificing the animals after euthanizing by MS-222. For primary induction experiment, tissues were collected at

0, 2, 4, 8, 12 h, 1st, 2nd, 3rd, 7th day and one month post treatment of E2. For secondary induction experiment, tissues were collected at 0, 4, 12 h, 1st, 2nd day and one month post treatment (2nd) of E2. The tissues collected at 0 h were considered as control. Tissue samples were collected in triplicates for each time point, frozen immediately in liquid nitrogen and stored at -80 ˚C till processed for RNA extraction.

Semi-quantitative RT-PCR analysis of mRNA expression of catla Vtg

Total RNA from the collected tissue samples of catla at different time intervals were isolated using TRI Reagent (Sigma, USA) following manufacturer's instructions. RNA was treated with DNase I (Invitrogen, USA) to remove genomic DNA contamination. The purity and integrity of RNA was checked spectrophotometrically (A_{260}/A_{280}) and by agarose gel electrophoresis. One microgram of total RNA was converted to cDNA using oligo $d(T)_{18}$ as primers and M-MLV reverse transcriptase (NEB, UK) at 37 ^{0}C for one hour according to the manufacturer's instructions. Primer pairs (VTG-1F-360 and VTG-1R-360; BACTINF and BACTINR) were used for PCR amplification (Table 1). The primers used were from the already available sequence in the GenBank for catla vitellogenin (Accession # EF190987) submitted by the same authors. Beta actin (517 bp) was amplified with PCR conditions of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C, with final extension of 7 min at 72 °C. Primers VTG-1F-360 and VTG-1R-360 were used for amplification of Vtg fragment (360 bp) using similar parameters to that of beta actin.

Table 1. Primers used for RT PCR

Primer name	Sequence (5′→3′)	Target gene	Optimum cycle no.	Amplicon size (bp)	Reference accession no.
BACTINF	ACTACCTCATGAAGATCCTG	Beta actin	25	517	EU184877
BACTINR	TTGCTGATCCACATCTGCTG				
VTG-1F-360	AGTTCTGGACAATGCTGGTCA	Vtg	35	360	EF190987
VTG-1R-360	CTGCCATATCAGGAGCAGTGAT				

For Vtg kinetic study, relative expression through RT-PCR was carried out in both primary and secondary E2-induced fish. The semi-quantitative RT-PCR experiment for relative expression study was established as described previously (Zhang *et al*., 2007). Briefly, beta actin was considered as a reference gene. The ratio of Vtg of each hour and its respective beta actin level as revealed densitometrically was compared with each other to have a relative quantification analysis for each group. The intensity of each band was analyzed with Gel Doc 2000 system and Molecular Analyst Software (UVTech, USA). The optimal numbers of PCR cycles, within the linear range of the reaction, were determined for each gene separately.

The significance of the differences in level of mRNA between groups of means was determined by one-way analysis of variance (ANOVA) at $p \leq 0.05$ and was then subjected to Duncan's multiple range post hoc tests (SAS Inc., USA).

RESULTS

A single band at 360 bp size was obtained for catla putative Vtg cDNA (Fig. 1). Sequencing of the purified PCR products were carried out to confirm the sequence as catla Vtg. Several rounds of polymerase chain reaction with different cycle numbers *viz,* 11, 13, 15, 19, 25, 35, 40 for beta actin gene and Vtg gene in primary E2 induction experiment were performed to determine the cycles in log phase. Cycle numbers 20, 35, 40 and 45 were similarly done for secondary E2 induction of Vtg gene. PCR was carried out for the beta actin primers to obtain PCR band at 517 bp. For each PCR performed a single band of the target gene was observed in agarose gel electrophoresis. The optimal numbers of PCR cycles, all within the linear range of the reaction, were 25 cycles for beta actin and 35 cycles for Vtg for both primary and secondary induction.

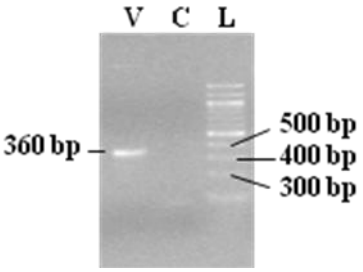

Fig. 1. Amplified fragment of Vtg specific primers. Lanes V: 360 bp (Vtg), C: Control with RNA as template and L: 100 bp DNA Ladder.

The analysis of semi-quantitative RT-PCR result confirmed variable expression of vitellogenin in primary and secondary induction test samples (Figs. 2 and 4). In primary induction experiment, the bar diagram showing beta actin normalized values of band intensities were of the order 12 h > 8 h > 4 h > 1 day > 2 day > 7 day > 3 d > 2 h (Fig. 3). While there was no significant difference in the relative expression levels among 0 h, 2 h and one month post treatment; 12 h expression differed significantly from the rest of the treated samples. Although statistically no difference could be observed between Vtg expressions at 0 h and 2 h, an increase in Vtg expression level could be appreciated from 2 h onwards reaching its peak at 12 h. This was followed by a decrease from day one onwards reaching to control level by one month.

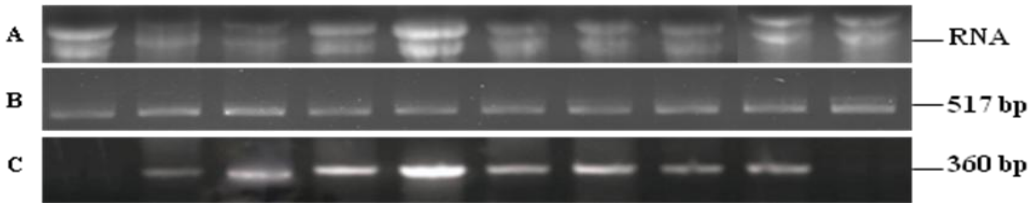

Fig. 2. Formaldehyde denaturing agarose gel electrophoresis of primary induction of Vtg in catla by E2. Liver tissues collected at different time intervals are given. Panel A shows total RNA; B and C represent RT-PCR products for beta actin and Vtg in catla, respectively.

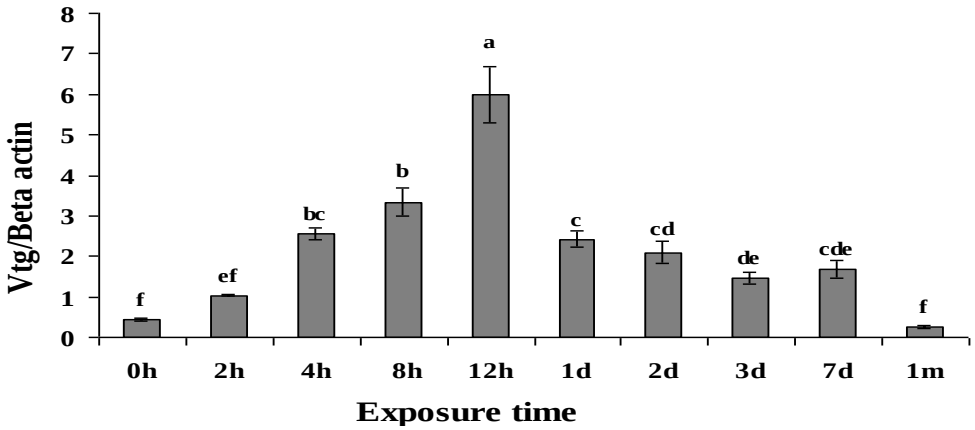

Fig. 3. Primary induction of Vtg by E2 in catla after normalization with beta actin gene. All the bars are given with standard error of means (SEM) of three samples at each time interval. Bars having no significant difference were marked with same letters

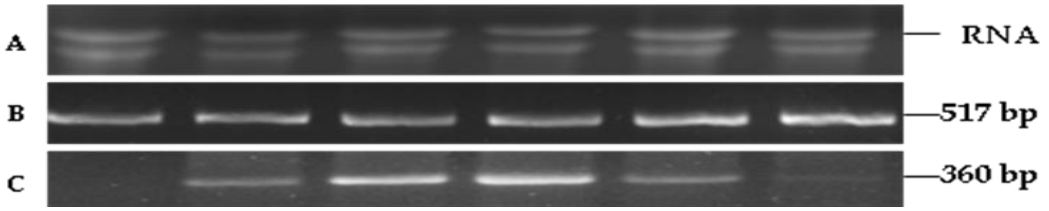

Fig. 4. Formaldehyde denaturing agarose gel electrophoresis of secondary induction of Vtg in catla by E2. Liver tissues collected at different time intervals are given. Panel A shows total RNA; B and C represent RT-PCR products for beta actin and Vtg in catla, respectively

In secondary induction experiment, the normalized value of target Vtg transcript level was highest at day one followed by 12 h post treatment of E2; differing significantly between them. In all other samples the Vtg transcript levels were significantly lower than the above two groups. Among them, 4 h and 2nd day levels showed no statistical difference and, the level dropped significantly after one month (Fig. 5). Thus in secondary induction, the Vtg transcript level increased gradually to peak at day one followed by a significant decrease by day two.

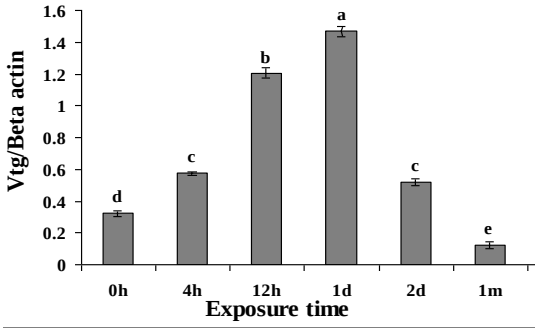

Fig. 5. Secondary induction of Vtg by E2 in catla after normalization with beta actin gene. All the bars are given with standard error of means (SEM) of three samples at each time interval. Bars having no significant difference were marked with same letters.

DISCUSSION

Vitellogenin transcript in catla peaked at 12 h in primary induction and 1 d in secondary induction as revealed from semi-quantitative RT-PCR study. Similar experiments of Vtg induction were carried out in fishes by several authors. Vtg transcript was induced by estradiol-17β-propionate in male tilapia, *Oreochromis aureus* as revealed by Northern blot result. The vitellogenin mRNA was reported to peak at 72 h and 48 h for primary and secondary stimulations, respectively (Lim *et al*., 1991). In the present study, Vtg was detected in gel after 2 h of primary induction. Similarly, Vtg mRNA transcripts have been detected as early as 1 h following primary and secondary estradiol stimulations in trout (Flouriot *et al*., 1996). This finding was similar except that the Vtg trascripts could be detected in immature fishes at 0 h in our investigation. In tilapia, Lim *et al*. (1991) also reported a basal level of Vtg mRNA in untreated male. As reported earlier in trout and tilapia, there exists a 'memory effect' of E2 induction that prolongs the Vtg degradation in case of secondary stimulation as well as the peak reaches much earlier than the primary induction. Estrogen is known to selectively stabilize *Xenopus* liver vitellogenin mRNA against cytoplasmic degradation. The half-life of vitellogenin mRNA is approximately 3 weeks in the presence of estrogen and 16 h after estrogen is withdrawn from the culture medium. The rapid cytoplasmic degradation of vitellogenin mRNA in the absence of estrogen is fully reversible upon restimulation with estrogen (Brock and Shapiro, 1983). Baker and Shapiro (1978) demonstrated that prior administration of estrogen alters the pattern of gene expression observed upon secondary estrogen stimulation. Administration of estrogen to male *Xenopus laevis* evokes stable long lived changes in the pattern of vitellogenin gene expression and constitutes a type of cellular "memory effect". Searle and Tata (1981) carried out a short-term time course analysis, which showed that vitellogenin mRNA was detectable within 3 h of exposure to estrogen during primary stimulation, and that the maximum rate of accumulation was reached at 5-6 h in primary cultures of male *Xenopus* liver parenchymal cells.

In the present study, there was a sharp decrease in Vtg level on day 2 in secondary induction and the Vtg peak appeared later as compared to primary induction. Thus no trend of 'memory effect' of E2 induction could be confirmed in catla and, that requires further studies.

CONCLUSION

This study compared the induction of Vtg mRNA in catla after single or double injections of E2. It was observed that the level of mRNA expression was highest after 12 h and 1st day post treatment for primary and secondary induction experiments, respectively. Unlike other species, a 'memory effect' of Vtg induction by E2 in catla could not be confirmed.

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