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Localization Of Transforming Growth Factor-β1 In The Testis Of Freshwater Catfish, *Clarias Batrachus*, And Its Relation With Testicular Activity

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Abstract

TGF-β1, an isoform of transforming growth factor β, is involved in regulation of variety of physiological functions. In mammals, TGF-β1 is known to regulate reproductive activity but its role in reproduction of lower vertebrate, particularly fishes, is not yet clear. Therefore, study was planned to detect its presence in the testis of catfish, Clarias batrachus during different phases of its reproductive cycle. Expression of TGF-β1 was visualized through the immunohistochemistry along with the measurement of testicular testosterone. The immunoreactivity was present in Sertoli cell and germinal epithelium. As the testicular activity progressed the expression of TGF-β1 decreased gradually in testis. However, the level of testicular testosterone increased with the progression in spermatogenesis. This finding suggests that TGF-β1 is present in fish testis and its expression varies with changing reproductive status; it expresses more during the early reproductive phase. The correlation studies showed a negative correlation between TGF-β1 expression and testicular testosterone production.

Keywords: TGF-β1; Sertoli cells; Interstitial cells; Germ cell; Testosterone.

Introduction

Growth factors produced locally in the gonad exert very effective and impressive impacts on various reproductive events. These factors act on germ cells as well as somatic cells in autocrine and paracrine manner to complete various reproductive episodes. Out of several intra gonadal growth factors, members belonging to transforming growth factor-β (TGF-β) superfamily {TGF-β, bone morphogenetic protein (BMP), activin/Inhibin, anti-mullerian hormone (AMH)} have been reported to play important role in reproduction [1]. Among them, role of TGF-β1 has been study well in mammals but in fish studies are rare.

In mammals, three different isoforms of TGF-β are known, referred to as TGF-β1-3 [2], whereas TGF-β4 has been reported in birds [3] and TGF-β5 in *Xenopus laevis* [4]. Biologically active TGFβ1, TGFβ2 and TGFβ3 are 25 kDa homodimers linked by disulfide bonds sharing a high level of sequence conservation. In mammalian testis, TGF-β1 is expressed by somatic cells (Sertoli cells, peritubular myoid cells and macrophages), and germ cells, while TGFβ2 and TGFβ3 are expressed only by somatic cells[5-6].There are differential production of TGFβ receptors in testis during development, TGFβ-RI and II are highly expressed in the immature testis, which suggest that it plays a definite role in early testicular development [7]. Differential TGFβ expression also occurs during postnatal development of the testis and onset of spermatogenesis [8]. The actions of TGFβ in testicular target cells are influenced by endocrine hormones and sex steroids. Production of TGF-β1 by rat Sertoli cells can be up regulated by estradiol [9]. TGFβ inhibits Leydig cell steroidogenesis in primary culture [10-11]. TGFβ exerted both a stimulating and inhibitory effect on testosterone secretion at dose dependent manner [12].

In fish, TGF-β1 has been cloned and characterized in several fish species, including rainbow trout [13], goldfish [14], carp [15], and hybrid striped bass [16] and its transcript was reported in various tissues (thymus, head kidney, spleen, ovary, heart, liver, muscle etc.) of fish [17].

However, studies on existence of TGF-β1 polypeptide in fish gonads are rare. kohli et al. have repoted TGF-β1 in ovary of zebra fish and its role in inhibition of gonadotropin and 17α, 20β - dihydroxyprogesterone (DHP)-induced oocyte maturation action [18,19].Moreover, such type of information completely missing in the testis of fish. Therefore, the present study was undertaken to examine the presence of TGF-β1 in catfish testis and its relation with testicular activity.

2. Materials and Methods

2.1. Chemicals

The TGF-β1 antibody (cat. No. 55450) was purchased from the Anaspec. Goatanti-rabbit IgG-HRP secondary antibody (cat. No. 621140380011730) was procured from GeNei, Bangalore. The ELISA kit for testosterone (DKO002) was sourced from DiaMetra, Italy. Other laboratory chemicals of analytical reagent (AR) grade were procured from reputable suppliers, including SRL, Merck, Qualigens and HiMedia, India, through authorized vendors.

2.2. Fish

The freshwater catfish, *Clarias batrachus* weighing 90–100gwere collected from ponds during different reproductive months. The *C. batrachus* is a seasonal breeder fish and its annual reproductive cycle is divided generally into four phases as per gonadal morphology, quiescence (January-February), recrudescence (March-June), spermiation (July) and the post-spawning (August-September). Acclimated catfish were anaesthetized in ice-chilled water, weighed and testes were removed quickly under aseptic condition and weighed to the nearest gram to calculate GSI. One of the testes was of the individual catfish was kept at -70º C till processed for the determination of testosterone while the other testis was fixed in Bouin's fluid and processed for histological examination and immunohistochemistry of TGF-β1.

2.3. Determination of testosterone level in testis

Testosterone level in testicular homogenate of fish collected during different phase of the reproductive cycle was measured by commercial available ELISA kits (DiaMetra, Italy) according to the manufacture's protocols. Steroids were extracted with diethyl ether before their estimation following the procedures of nee Pathak and Lal (2008) [20].

2.4. Immunohistochemistry of TGF-β1

The paraffin sections (6µm thin) of testis of different reproductive phase were deparaffinized in xylene, hydrated gradually through descending grades of ethanol. Then endogenous peroxidase activity was quenched by incubating sections with H_2O_2 in methanol (1:40) for 25min followed by washes thrice in 0.05M PBS (pH 7.4). Thereafter, sections were incubated with 5% normal goat serum for 2.5h at room temperature in moist chamber followed by addition of primary antibody of TGF-β1 at the dilution of 1: 300 for overnight at 4°C.

Next morning, sections were washed in PBS thrice for 10min each, followed by their incubation with HRP-tagged secondary antibody (dilution 1:100) for 1 h at room temperature, thereafter washed with PBS. Then sections were subjected to the chromogen substrate (0.06% DAB) with $H_2O_2$and kept in darkness for 10 min to develop color. Reaction was stopped by dipping the slide in distilled water followed by PBS, and then sections were dehydrated and mounted in DPX with glass cover. In corresponding negative control sections, primary antibodies were replaced by PBS. The images were captured at 40x by Leica DM2000 camera attached microscope. The intensity of immunoreactivity was analyzed by spot densitometry tool, Alpha Ease FC software (Alpha Innotech Corp., CA, USA). Integrated density values (IDV) in term of arbitrary unit were calculated in testicular sections for TGF-β1expression [21].To determine non-specific immunoreactions, control sections omitting the primary antibody were processed in parallel where total loss of immunoreactivity was observed in testicular sections (Fig.3 I-L).

2.5. Statistical analyses

Data were presented as mean ± SEM (n=5) and analyzed through one-way ANOVA followed by post-hoc, Duncan's multiple range test ($P< 0.05$) for comparisons amongst different groups. The correlation (r-value)

between the intensity of TGFβ-1 immunoreaction and levels of testosterone in testis were computed at significance level of 0.01. All statistical analyses were performed using SPSS 16 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. *Seasonal variation in the gonadosomatic index (GSI) and testicular histology*

GSI was low during the quiescence phase which increased significantly high in recrudescence phase then gradually declined to spermiation phase and further in post- spawning phase (Fig.1). During the quiescence phase, the seminiferous tubule consisted of germinal epithelium, Sertoli cells, and the lumen was full of primary spermatogonia. The interstitium was poorly developed (Fig.3A). In the recrudescence phase, diameters of seminiferous tubules were enlarged and active spermatogenesis was evident, as spermatogonial cysts with advanced stages of germ cells (spermatocyte/spermatid) were clearly seen in the lumen. Interstitium became prominent with large numbers of interstitial cells (Fig.3B). In the spermiation phase, seminiferous tubules were vacant and interstitial cells started undergoing hypotrophy (Fig.3C). The seminiferous tubules of the post-spawned catfish testis were started recruitment of spermatogonia. Interstitium was regressed and exhibited atrophied interstitial cells (Fig.3D).

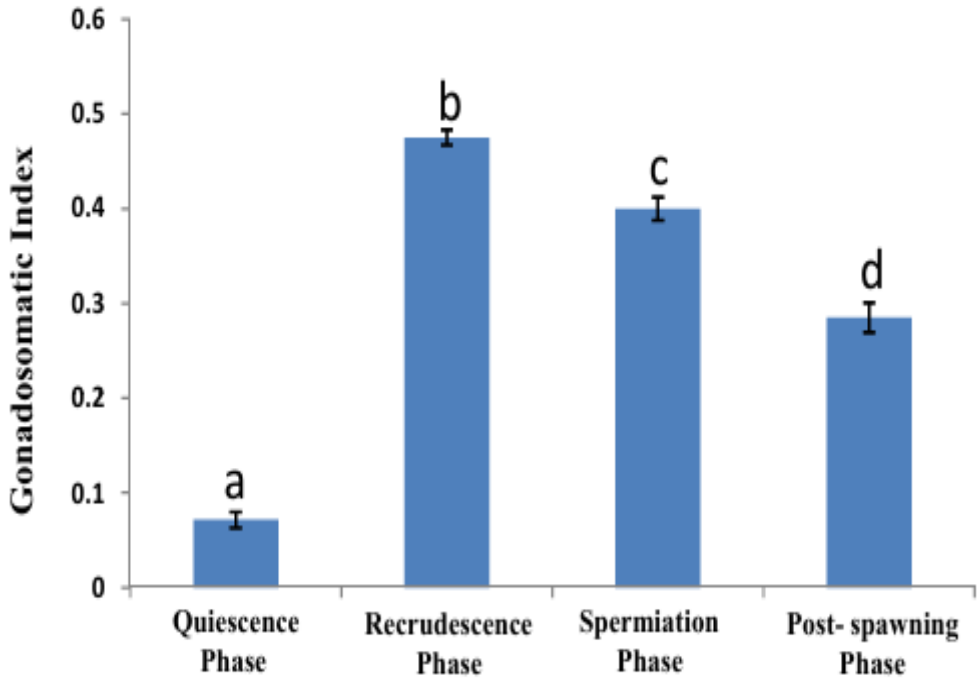

Fig.1. Seasonal variations in gonadosomatic index of *Clarias batrachus* during different phases of the reproductive cycle. Each bar represents Mean ± SEM (n = 5). Means bearing same superscript do not differ from each other, while means bearing different superscripts are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts a, b, c & d are used to compare GSI.

3.2. Seasonal variations in testosterone level in testis

Testosterone content in testis was low during quiescence phase. Their level increased and reached maximum in recrudescence phase. Then, their concentration gradually decreased in spermiation and further declined in post-spawning phase (Fig.2).

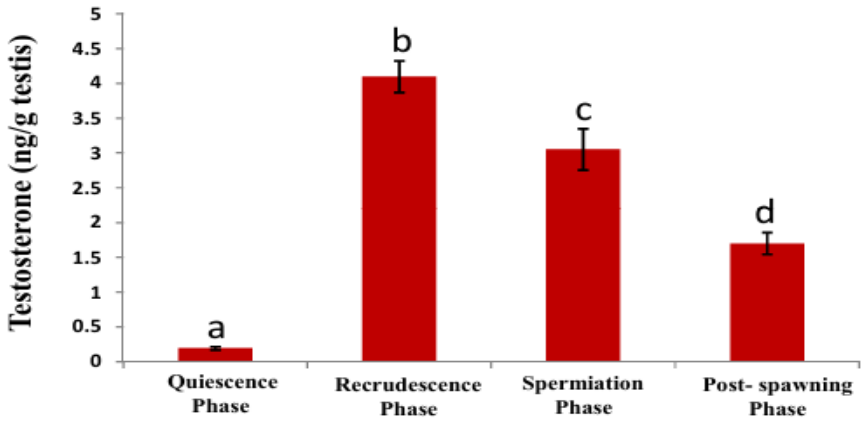

Fig.2. Seasonal variations in the levels of testosterone in the testis of *Clarias batrachus* during different phases of the reproductive cycle. Each bar represents Mean ± SEM (n = 5). Means bearing different superscripts are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts a, b, c & d are used to compare testosterone level in testis.

3.3. Immunohistochemical localizationTGF-β1 in testis

In quiescence phase, expression of TGF-β1 was intensely observed in Sertoli cell and germinal epithelium of testis (Fig.3E).In recrudescence phase, immunoprecipitation of TGF-β1 distinct in spermatocyte within the seminiferous tubules and interstitial cells also showed TGF-β1 immunoprecipitation but immunoreactivity of TGF-β1 declined in Sertoli cell and germinal epithelium (Fig.3F). In spermiation phase expression of TGF-β1 decreased in both the compartment of testis and further declined in post spawning phase (Fig.3G-H).The overall expression of TGF-β1 declined from quiescence to post-spawning phase in catfish testis (Fig.4).

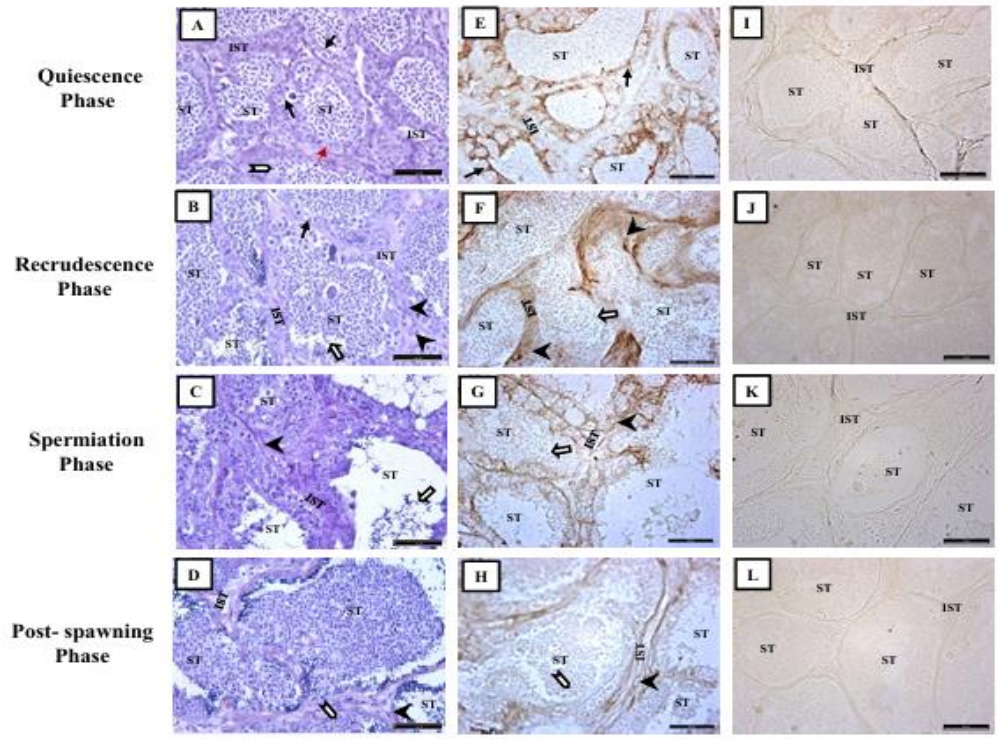

Fig.3. Representative images of transverse sections of the testis of Clarias batrachus during different phases of the reproductive cycle. Fig.3 A-D represent hematoxylin/eosin stained sections, E-H represent immunoreactivity for TGF-β1 and I-L represent negative control section (by omitting the primary antisera).

Note- Interstitium (IST), seminiferous tubule (ST), spermatogonial stem cells (⇢), spermatogonia(⇨) Sertoli cells (→), interstitial cells(➤), advance germ cell (⇨).

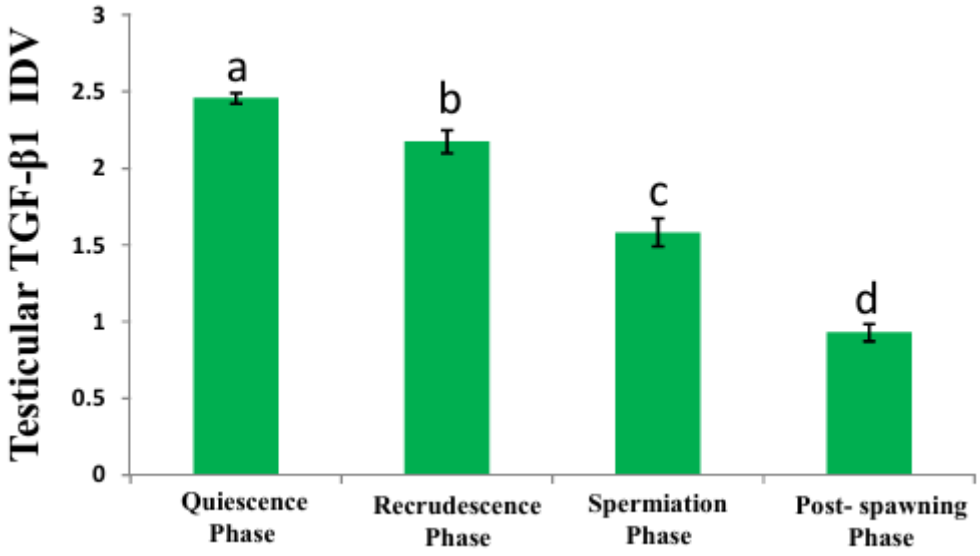

Fig.4. Seasonal variations in expression of TGF-β1in the testis of *Clarias batrachus* during different phases of the reproductive cycle. Expression was quantified in term integrate density value per unit area (IDV). Each bar represents Mean ± SEM (n = 5). Means bearing same superscript do not differ from each other, while means bearing different superscripts are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts a, b, c & d are used to compare variance of TGF-β1 intensity.

3.4. Correlation between TGF-β1 *expression and testosterone content in testis*

A negative correlations ($p<0.01$) were observed between testicular TGF-β1 expression intensity and testosterone levels in testes of *C. batrachus*.

4. Discussion

This study reports two important aspects of catfish testis: 1) TGF-β1 expressed in catfish testis in different cytotypes (Sertoli, germ and interstitial cells); and 2) their expressions vary with changing reproductive status of fish. The presence of TGF-β1in both the compartment of fish testis: interstitium and seminiferous tubules suggesting its possible role in spermatogenesis and steroidogenesis.

The marked seasonal changes in the testicular morphology with GSI in the catfish, *C. batrachus* are in agreement with the previous reports on this catfish [22]. The dividing spermatogonial stem cells in the germinal lining of the seminiferous tubules during the quiescence phases suggest the beginning of spermatogenesis through renewal of spermatogonial stem cells. Spermatogenic activity increased in recrudescence phases as they exhibited advanced germ cells (secondary spermatocytes and spermatids). In spermiation phase the release of sperms from the tubules (evacuation) was observed and the lumens of most of the seminiferous tubule were vacant. During the post-spawning phase (August-September), seminiferous tubules were devoid of spermatids/spermatozoa, and were dominated by the spermatogonial cells.

The presence of TGF-β1 in the quiescent testis is indicating about its possible role in the beginning of spermatogenesis through renewal of spermatogonial stem cells. Comparatively reduced expression in the recrudescence phases is suggesting the opposite effect of TGF-β1 on the advancement of germ cells (secondary spermatocytes and spermatids). Further on, continuous decease in the immunoreactivity of TGF-β1 in the testis indicates about the very small role of TGF-β1 in the terminal events of testicular functions i.e. sperm maturation and spermiation phase.

During recrudescence phase, increasing number of interstitial cells (most probably Leydig cell) in the interstitium with rise in levels of testicular testosterone suggests that Leydig cells also proliferate and revolve into hypertrophied in parallel with the progression of spermatogenesis. During post-spawning phase Leydig cells undergo hypotrophy. The increased in testicular testosterone concentration from the quiescence phase to the maximum during recrudescence phase in parallel with advancing spermatogenesis indicates the involvement of the testosterone in regulation of fish spermatogenesis. Role of testosterone has been well-known in spermatogenesis of fishes [23-24].

Moreover, the present study reports expressions of TGF-β1 in Sertoli cells, germ cells as well as interstitial cells. An intense immunoreaction for TGF-β1 was observed in Sertoli cells and germinal epithelium in the

quiescence testis which decreased in recrudescence phase. Though the presence of TGF-β1 in Sertoli cells, peritubular myoid cell, macrophages and germ cells has previously been shown in mammalian testis [5,6] and high level of expression of TGFβ receptors in immature testis, suggest that TGFβ acting a role in early testicular development [7]. TGFβ also plays a crucial role in the postnatal development of the testis and the onset of spermatogenesis, with differential expression occurring during these processes [8]. In organ culture, TGFβ is capable of regulating the number of germ cells in developing testes by inducing apoptosis [25].A moderate immunoprecipitation of TGF-β1 was observed in interstitial cells in recrudescence testis of *C. batrachus* which gradually decreases with advancement of testicular activity. In mammal, also the actions of TGFβ in testicular cells are influenced by endocrine hormones and sex steroids, with estradiol upregulating the production of TGF-β1 by rat Sertoli cells [9]. TGFβ also inhibits Leydig cell steroidogenesis in primary culture [10,11], with a dose-dependent effect on testosterone secretion. Low doses of TGFβ stimulate testosterone secretion by increasing 3-β hydroxy steroid, while high doses inhibit pregnenolone formation, leading to decreased testosterone secretion. However, such type of repots in fishes is practically unavailable. Unfortunately due to non-availability of such studies in fishes, it is difficult to discuss these findings. However, the changing expression of TGF-β1 in catfish testis and more expression in quiescence phase suggest its role in early testicular development.

Thus, the present study provides morphological evidence of expression of TGF-β1 in different cell types in fish testis and its expression gradually declined with advancement of testicular activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this scientific work.

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