Gametogenic and steroidogenic action of kisspeptin-10 in the Asian catfish, *Clarias batrachus*: Putative underlying mechanistic cascade

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Highlights

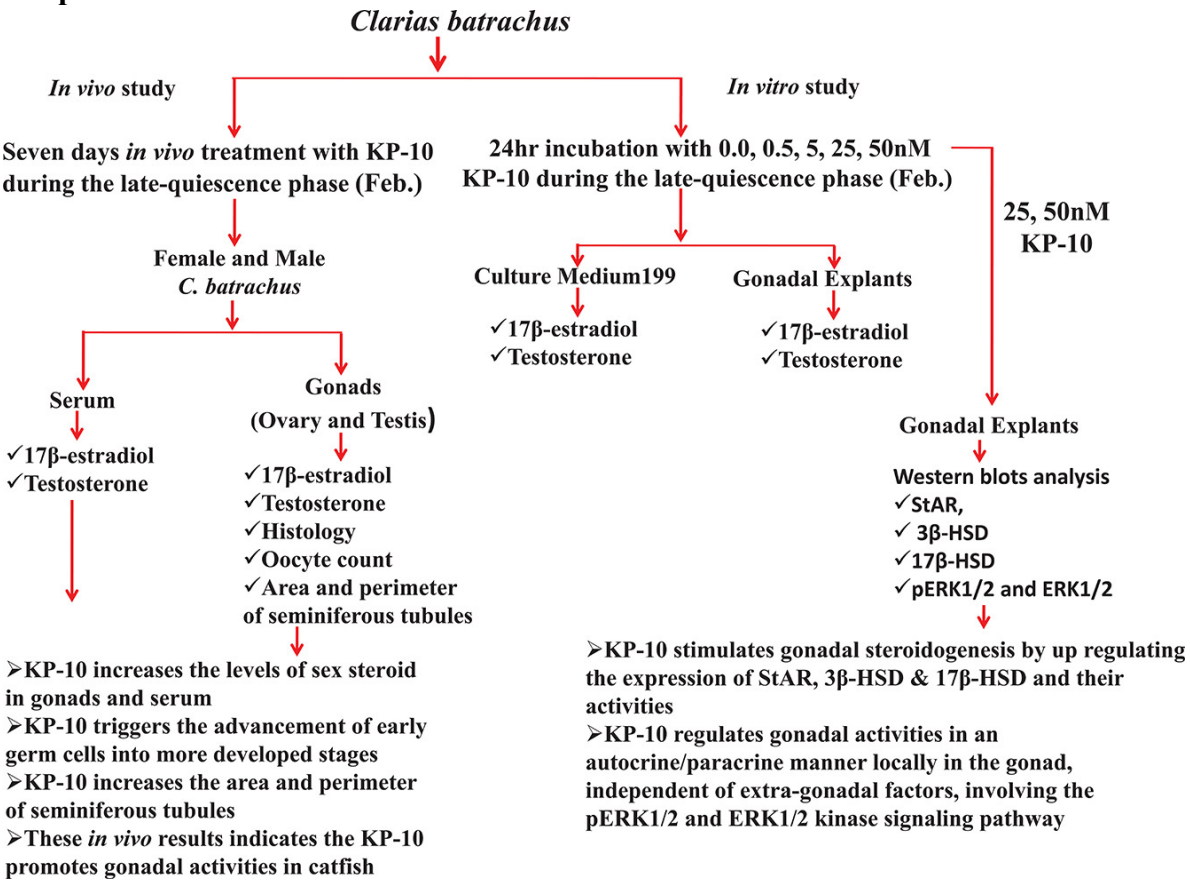
- Kisspeptin-10, KP-10 stimulates gametogenesis and steroidogenesis in catfish.
- KP-10 stimulates the expression of steroidogenic markers proteins, 3β-HSD, 17β-HSD & StAR in fish gonads.
- KP-10 also induces the phosphorylation of ERK1/2.
- KP-10 increases the activities of 3β-HSD, 17β-HSD in ovary.
- KP-10 acts in an autocrine/paracrine manner in fish gonads, independent of extra-gonadal factors.

Abstract:

Unlike mammals, two kisspeptins genes encoding, *kiss1* and *kiss2* are detected in fishes with highly varied and contradictory difference in their reproductive activities. The present study was undertaken to examine the direct action of kisspeptin-10 and its role in gonadal activities in the gonadally quiescent Asian catfish using native mammalian kisspeptin decapeptide (KP-10) involving *in vivo* and *in vitro* approaches. The *in vivo* KP-10 treatment caused precocious onset of gametogenesis and its rapid progression, as was evident from the appearance of advanced stages of ovarian follicles in ovary, and advanced germ cells (spermatocytes/ spermatids) in the testis of the treated *Clarias batrachus* in comparison to the control gonads. It also elevated the steroid levels in gonads of the catfish *in vivo* and *in vitro* conditions. Simultaneously, it increased the expressions of key steroidogenic enzymes like 3β-HSD, 17β-HSD, and StAR protein, responsible for transfer of cholesterol from outer to inner membrane of the mitochondria of steroidogenic cells. Concurrently, it augmented the activities of 3β-HSD and 17β-HSD in the ovarian explants. The expressions of MAPK component (pERK1/2 and ERK1/2) were also up-regulated by KP-10 in gonadal explants. Thus, the data suggest that kisspeptin-10 stimulates gametogenesis by enhancing gonadal steroid production. The study also describes the putative mechanistic cascade of steroidogenic actions of kisspeptin-10 in the catfish so much so in teleost fish. The study also suggests that, kisspeptin may act locally to regulate gonadal activities in an autocrine/paracine manner, independent of known extra-gonadal factors in the catfish.

Keywords: Kisspeptin-10, 3β-HSD, 17β-HSD, Steroids, pERK1/2 and ERK1/2, gametogenesis

Graphical abstract



1. Introduction

Although a variety of reproductive strategies are adopted by vertebrates, the regulatory network of reproduction originates in the hypothalamic-pituitary gonadal (HPG) axis, culminating into well-coordinated release of gonadotropin-releasing hormone (GnRH), gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and sex-steroids. The discovery of kisspeptins and their G-protein coupled receptors (GPR54/KISS1R) has added a new dimension in researches on the hormonal regulation of vertebrate reproduction, in recent decades, as the kisspeptins act upstream of GnRH (Clarke et al., 2015; De Roux et al., 2003; Seminara et al., 2003; Tena-sempere, 2006). In mammals, kisspeptins belong to RFamide family of neuropeptides and are derived from preprohormone, composed of 145 amino acids. It

is proteolytically cleaved into KP-54, KP-14, KP-13 and KP-10 fragments (Kotani et al., 2001). These entire kisspeptins share a common C-terminal region decapeptide sequence, highly conserved across vertebrates (Felip et al., 2009; Osugi et al., 2013; Pasquier et al., 2014; Ohga et al., 2018).

Kisspeptin has emerged as important regulators of mammalian reproduction, as the interruption of kisspeptin signaling (Kiss1/GPR54) results in reproductive failure (De Roux et al., 2003; Mei et al., 2013; Seminara et al., 2003; Topaloglu et al., 2012). The impacts of the shortest kisspeptin, KP-10, on the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) have been examined following its exogenous administration in several rodents (De Tassigny et al., 2017; Gottsch et al., 2004; Thomson et al., 2004) and humans (Calley and Dhillo, 2014; George et al., 2012, 2011; Jayasena et al., 2011; Nabi et al., 2018). The kisspeptin has been shown to control reproductive activities of the mammalian species by stimulating gonadotropin secretion (Kauffman et al., 2007; Popa et al., 2008).

However, in fishes two variants of the Kiss peptides (Kiss1 and Kiss2) with their receptors as Kissr1 and Kissr2 are reported in brain and gonads, besides other peripheral organs (Kitahashi et al., 2009, van Aerle et al., 2008; Felip et al., 2009; Song et al., 2015; Fairgrieve et al., 2016; Rather et al., 2016; Shahi et al., 2017; Bakshi and Rai, 2019; Chaube et al., 2020; Somoza et al., 2020). The *kiss1* and *kiss2* mRNAs are reported in the gonads of sea bass, medaka and zebrafish. Recently, the authors have shown the expression of Kiss1 like peptide immunoreactive to zebrafish kiss1 in the follicular cells in ovarian follicles and interstitial cells in testis of the catfish, *C. batrachus,* which varied temporally and spatially with changing reproductive status (manuscript under revision).

Moreover, KP-10 has been shown to elevate levels of GnRH, LH and sex-steroids in goldfish, *Carassius auratus* (Li et al., 2009; Yang et al., 2010). The KP-10 treatment up-regulates the expression of *fshβ* and *lhβ* mRNAs in yellowtail kingfish, *Seriola lalandi* (Nocillado et al., 2013). In other studies, KP-10 has been demonstrated to promote gonadal development in Marone and Chub mackerel fish by stimulating GnRH/gonadotropin/sex-steroids (Beck et al., 2012; Ohga et al., 2018). In recent past, researchers have made substantial efforts to investigate the effects of kisspeptins and the underlying mode and mechanism of its action at the brain-pituitary level. But effect of KP-10 at gonadal level is rarely studied in fish despite that kisspeptins transcript have been detected in the gonads of some fishes. The precise mode and mechanism of Kiss/Kissr signaling at gonadal level is completely missing in fishes and yet to be thoroughly addressed.

The present study, thus, was undertaken to investigate the effects of mammalian kisspeptin-10 on gametogenesis and steroidogenesis in the Asian freshwater catfish, *Clarias batrachus* during the late-quiescence phase, when hypothalamo-hypophseal-gonadal axis remain inactive. The *in vitro* effect of KP-10 was also evaluated to decipher the direct action of kisspeptin in gonad, independent of extra-gonadal factors, and also to explore the putative underlying mechanistic cascade. The present catfish is a seasonal breeder and cultured commercially in India due to its high nutritional and medicinal values.

2. Materials and Methods:

2.1. Chemicals

Native mammalian kisspeptin decapeptide, KP-10, was provided by Prof. R.P Millar, Department of Immunology, University of Pretoria, South Africa. Detail of KP-10 is described elsewhere (Millar and Newton, 2013). The ELISA kits for 17β-estradiol (DKO003) and testosterone (DKO002) were purchased from DiaMetra, Italy. Medium 199 (AL094A) and streptomycin sulphate (RM220) was obtained from Himedia Laboratories Pvt. Ltd., India. Other routine laboratory chemicals (AR grade) were procured from Qualigens, Merck, SRL and HiMedia through local authorized vendors. Specificities of antibodies against steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hysdroxysteroid dehydrogenase (17β-HSD) have already been validated in the present catfish and published elsewhere (Yadav and Lal, 2017).

The specificities of antibodies against extracellular signal regulated kinase (pERK1/2 and ERK1/2) were validated for *C. batrachus*, using mouse ovary and testis as positive controls. Their antibodies (Cat #9101 and Cat #9102) were purchased from the Cell Signaling Technology (USA) (see table). The immunoblot analyses of pERK1/2 and ERK1/2 revealed two bands at ~44 kDa and ~42kDa in ovary and testis of the catfish like mouse (Fig. 9). The two bands at ~44 kDa and ~42kDa against pERK1/2 and ERK1/2 have been identified and reported earlier (Hanchate et al., 2012; Szereszewski et al., 2010; Wang et al., 2019; Zhang et al., 2018).

Table 1: Details of the antisera used in immunoblot analyses

Antibody	Host	Dilution	Source	References
Primary				
StAR	Rabbit polyclonal	1:1000	Santa Cruz Biotechnologies Inc., CA, USA	(Yadav and Lal, 2017, 2019)
3β-HSD	Rabbit polyclonal	1:1000	Santa Cruz Biotechnologies Inc., CA, USA	
17β-HSD	Rabbit polyclonal	1:1000	Santa Cruz Biotechnologies Inc., CA,USA	
Phospho-p44/42 MAPK(ERK1/2)# 9101	Rabbit polyclonal	1:500	Cell Signaling Technology, USA	Fig.9 (A,B,C,D)
p44/42 MAPK(ERK1/2)# 9102	Rabbit polyclonal	1:500	Cell Signaling Technology, USA	
β-actin	Mouse monoclonal	1:100000	Sigma-Aldrich, USA (A3854)	(Yadav and Lal, 2017, 2019)
Secondary				
Rabbit IgG	Goat	1:2000 (StAR, 3β-HSD, 17β-HSD) 1:3000 (pERK1/2,ERK 1/2)	GeNei, Bangalore, India	(Yadav and Lal, 2017, 2019)

2.2. Procurement of catfish, their acclimation and in vivo treatment

The freshwater catfish, *C.batrachus* were collected in the first week of February (late-quiescence phase). Fish were acclimated for two weeks to the laboratory conditions in cemented tanks (1000x80x70cm) carrying 200 liters of water and fed with minced goat liver *ad libitum*. After acclimatization to the ambient photoperiod and temperature, fish were sorted in very close

weight range (90-95g) and divided into three groups, 15 catfish in each. Group-I served as control and received fish saline, while fish in group-II and III were injected with 0.5µgand 5µg of KP-10/100g body weight, respectively. Injections (100µl/injection) were given intra-muscularly using insulin needle 30Gx5/16'' (0.30x8mm) daily for seven days between 09.00AM -10.00A.M. Twenty four hours after the last injection, fish were cold anaesthetized by immersing them in ice-chilled water (6-7^{0}C) for 6-8min following the method of Mittal and Whitear (1978) and then sacrificed. Blood was collected in sterile glass tubes through caudal puncture and centrifuged at 4^{0}Cat 2250g for 10min to obtain serum. Both the lobes of ovary and testis were excised rapidly after opening the abdomen of the five catfish of each sex and rinsed in fish saline, blotted on the Whatman paper no. 1, and one lobe of the ovary and testis were cut into pieces and fixed in aqueous Bouin's solution for 22hrs for their histological examination. The remaining lobe of gonad was stored at -80°C for the estimation of sex-steroids. The entire experiment were conducted in accordance to the guidelines of Institutional Animal Ethics and Care of Banaras Hindu University, India for catfish and mice respectively (approval letter No. F.Sc./IAEC/2016-17/1136, BHU/DoZ/IAEC/2019-20/T003).

2.3. In vitro treatments with KP-10

In order to evaluate the direct effect of KP-10 (independent of extra-gonadal factors) on gonadal steroidogenesis, an *in vitro* study was performed using ovarian and testicular explants. *In vitro* experiments were conducted during the late-quiescence phase of reproductive cycle of the catfish. Fish were sacrificed; their ovaries and testes were dissected out and cleaned of any adhered fat tissues. Then gonadal tissues were cut into small fragments (approximately 10mg/fragment) in culture medium199 supplemented with 0.2% $NaHCO_3$, penicillin at the dose of 100IU/ml, streptomycin 100µg/ml and 40µg/ml gentamycin, and pre-incubated for 3h at

25°C. Subsequently, the culture medium was replaced with fresh medium containing 0.5, 5, 25 and 50nM/ml KP-10, separately, under humidified atmosphere with 95% air and 5% CO_2 at pH 7.4 for 24hr at 25°C. Control gonadal tissues were also incubated with fresh medium199 without KP-10. After 24hr, the KP-10-treated ovarian and testicular fragments were washed with PBS and stored at -80°C for further analysis of steroid levels, Western blotting of steroidogenic markers and signaling molecule as well as the activities of 3β-HSD &17β-HSD enzymes. Culture medium was also stored, separately, at -80°C for steroid analyses. For the Western blotting analyses of StAR, 3β-HSD, 17β-HSD, pERK1/2 and ERK1/2, the gonadal explants incubated with 25 and 50nM/ml KP-10 were used because the maximum steroidogenic response was noted at these doses of KP-10. The treatments were repeated thrice, each time with five fish ovary or testis, separately. The *in vitro* protocol to study of the effects of drug/ hormone on steroidogenesis has been well established in the author's laboratory (Singh and Lal, 2017; Yadav and Lal, 2017; Priyadarshini and Lal, 2018; Singh nee Priyadarshini and Lal, 2018).

2.4. Histology of gonads

Testicular and ovarian tissues were processed as described elsewhere (Singh nee Priyadarshini and Lal, 2018; Singh and Lal, 2016). Briefly, the gonadal tissues were paraffin embedded and 6μm thin sections were cut and mounted on clean glass slides. The slides were dried properly at 40^0C. The sections were then deparaffinized in xylene and processed for hydration using descending ethanol series. The sections were then put in the Mayer's Ehrlich's hematoxylin solution for 45min to stain nuclei, followed by differentiation with 1% acid water for 3-5sec. The sections were then stained with 1% eosin solution for 5min. Slides were then dehydrated through ascending ethanol series, cleared in xylene and mounted with DPX. Thereafter, the sections were examined under Leica microscope (LEICA DM 2000) for

histological organization. Images of ovarian sections were captured at 10 and 40x, while at 40 and 100x for testicular sections. The morphometric analyses of ovarian and testicular sections were done using Motic Image Plus V2.0 software as per method described elsewhere (Singh and Lal, 2016, 2017). Following calibration of images, perinucleolar oocytes, oocytes-I, II and III were counted manually from randomly selected areas (10^2mm/area) in ovarian sections. Three such areas from a single ovarian section and three sections of single ovary were taken into account. Similar counting was done for three catfish ovaries and the final counting was expressed in percent as Mean±SEM. The identification and classification of different types of oocytes was based on specific features of each oocyte described elsewhere (Sundararaj and Sehgal, 1970; Singh and Lal, 2017). In brief, the perinucleolar oocytes are very small with large nucleus showing nucleoli at its periphery, and are surrounded by very little cytoplasm. The oocytes-I are non-yolky oocytes and are differentiated by its larger size with big nucleus & substantial cytoplasm. Oocytes-I are also surrounded by some somatic cells. The oocytes-II is yolky oocytes and is distinguished from oocytes-I by its further larger size with distinct cortical alveoli. It is also surrounded by single layer of granulosa cells and thecal layers. The oocytes-III are fully grown oocytes and are full of yolk granules, and also has fully developed single layer of granulosa enveloped by multi layers of thecal cells and connective tissue. The area and perimeter of seminiferous tubules were also calculated in a similar fashion.

2.5. Measurement of 17β-estradiol and testosterone

Analyses of 17β-estradiol and testosterone in gonadal tissue, serum and culture medium were achieved by using commercially available ELISA kits (DiaMetra, Italy) following the manufacturer's instructions. Prior to estimations, 17β-estradiol and testosterone were extracted from the gonadal tissues, serum and culture medium, separately, with diethyl ether twice. In

brief, 1ml of gonadal homogenates (10% in 0.01M PB, pH 7.3), serum or culture medium was thoroughly mixed with 5ml of diethyl ether on vortex for 5min. The mixture was allowed to settle for a min and then the aqueous phase was frozen at -80^{0}C followed by the collection of ether phase in a test tube. The aqueous phase was melted and again thoroughly mixed with 3ml of diethyl ether, vortexed and aqueous phase was frozen at -80^{0}C. The ether phase was again collected in the same test-tube. This process of extraction was performed for each sample, separately. After evaporation of diethyl ether, the residue in the test tube was reconstituted in 1ml of PBS and processed for ELISA. Briefly, 25µlof different concentrations of standard 17β-estradiol and testosterone, reconstituted extract of serum, culture medium 199 and gonadal homogenate were added in the wells of ELISA plates, separately. Thereafter, either 200µl of estradiol conjugate or 100µl of testosterone conjugate was added, in respective wells, mixed and incubated at 37°C for 2hr for estradiol and 1h in case of testosterone. Following appropriate duration of incubation, content of the each well were carefully flicked and washed thrice with 300µl of wash buffers provided with kits. Thereafter, 100µl of substrate (tetramethylbenzidine (TMB)) was added to each well and incubated in dark at room temperature for 30min & 15min for 17β-estradiol & testosterone, respectively. Stop solution (100µl) was then added in each well and plate was analyzed at 450nm in Multiskan EX Elisa Reader (Thermo Labsystem). The sensitivities of the kits (0.0086 ng/ml for 17β-estradiol & 0.10 ng/ml for testosterone), as per the manufacturer's claims, are very well within the detectable and acceptable limits of estradiol and testosterone levels in fishes. The variations in the intra- and inter- assays precisions were 5.4% & 6.8% for 17β-estradiol and 6.4%& 7.2% for testosterone, respectively. The cross-reactivities of 17β-estradiol kit with other steroids, as claimed by the manufacturer, are 100% with 17β-estradiol, while 2%, 0.39%, 0.09%, 0.02%, $<7x10^{-3}$%, $<3x10^{-3}$% and $1x10^{-3}$% with estrone,

estriol, fulvestrant, testosterone, cortisol, progesterone and DHEA-S, respectively. Whereas the cross-reactivities of testosterone kit with testosterone was 100%, while 2.03%, 0.01%, 0.05%, 0.0%, 0.01%, 0.0%, 0.16%, 0.0%, 0.01% with dihydrotestosterone, androstenedione, DHEA-S, cortisol, cortisone, 17β-estradiol, predisone and estrone, respectively.

2.6. Western blotting

Homogenates of testicular and ovarian explants (10%) were prepared by sonicating gonadal tissues for 30sec on ice, in lysis buffer (1% NP40 (W⁄V), 0.1% SDS, 0.1% aprotinin, 20µl of 50mM sodium orthovanadate, 1% PMSF in PBS). The whole tissue protein lysate was then centrifuged at 12,000 rpm for 20min at 4^0C to remove cellular debris. The supernatant was used as the soluble protein extract. The protein concentrations of the homogenates were determined by Bradford method. The homogenate were further processed to prepare gel samples by mixing with gel loading buffer (4% SDS, 0.05% bromophenol blue (w/v), 20% glycerol, 1% mercaptoethanol (v/v) in 0.1M Tris buffer, pH 6.8). Samples then incubated at 100ºC for 10min. Equal amount of protein (50µg) were subsequently loaded on 12% SDS-polyacrylamide gel. After separation of samples on SDS-PAGE, proteins were transferred to a nitrocellulose membrane (0.45µm Millipore) overnight at 4^0C. The membrane blocking was done with Tris-buffered saline [TBS; 10mM Tris, 150mM NaCl, 0.05% Tween, v/v] containing 5% (w/v) non-fat milk for 2h. The membranes were then incubated with primary antibodies against StAR, 3β-HSD, 17β-HSD, pERK1/2 and ERK1/2 separately, at 4^0C for overnight. Details of all the antibodies and its respective dilutions used in this experiment are given in the table. The next day morning, membranes were washed thrice with TBST. There after membranes were incubated with goat anti-rabbit-HRP conjugated secondary antibody (1:2000 diluted for StAR, 3β-HSD, 17β-HSD and 1:3000 for pERK1/2 & ERK1/2) in TBST containing 2% (w/v) non-fat milk. The membranes were then washed for four times,10min each, in TBST, followed by signal development using an enhanced chemiluminescence (ECL) reagents kit (Thermo Scientific, Rockford, IL, USA) as per manufacturer's instructions on X-Ray film. Resulting immunoreactive bands were quantified by Image J software (NIH, Bethesda, USA). Equal loading was normalized by β-actin and total ERK. The similar procedure of western blotting was followed for the validation of pERK1/2 & ERK1/2. Briefly, female and male mice (n=3) were decapitated

under sodium pentobarbital (40mg/kgbw, i.p.) (Patel and Singh, 2020). Abdomen was opened by making incision in the skin and body wall of the mice. The ovary and testis from each animal were quickly dissected out aseptically and their homogenate (10%) was prepared in lysis buffer.

2.7. Analyses of activities of ovarian 3β-HSD and 17β-HSD enzymes

The activity of steroidogenic enzymes like 3β-HSD and 17β-HSD were measured only in the ovarian explants in the quiescent phase. However, their activities in testicular fragments could not be measured due to lack of sufficient testicular tissue. As during the quiescent phase the testicular size and weight is very small as compared to the ovary, hence, required amount of tissue could not be obtained. The 3β-HSD and 17β-HSD activities were determined as per the original methods of Wiebe (1978) and Jarabak (1969), respectively, with slight modification described elsewhere (Priyadarshini and Lal, 2018; Singh nee Priyadarshini and Lal, 2018; Singh and Lal, 2016). In brief, 10% ovarian homogenate was prepared in sucrose solution buffered with potassium phosphate (5mM) and EDTA(1mM) and spanned at 12,000g at 4^0Cfor 30min. The supernatants were collected and used for enzyme assay. For 3β-HSD activity, 250ml of the supernatant was added in cuvette containing sodium phosphate buffer (0.1M, pH7.5) & 0.3mM dehydroepiandrosterone, as substrate and incubated for 5min at 30^0C. Then after, 2.8 mM β-NAD was added and readings were taken every 15sec in a spectrophotometer at 340 nm against blank without β-NAD. The enzyme activity was estimated as change in absorbance with the time after addition of β-NAD. The enzyme activity was expressed as unit/hour/g ovary. The activity of 17β-HSD was measured following similar sequence of steps, except that sodium phosphate buffer (0.44mM, pH 10.2) was used as buffer and androstenedione (0.3mM) as a substrate.

2.8. Statistical analyses

Data related to 17β-estradiol, testosterone, area and perimeter of seminiferous tubules of testes are presented as Mean±SEM (n=5). Data were analyzed by one way ANOVA followed by Duncan's multiple range tests at 95% confidence limit ($P< 0.05$) for the comparison amongst different groups. The counts of the different oocytes (in per cent), Western blot analyses in term of IRDV and activities of 3β-HSD & 17β-HSD are presented as Mean±SEM (n=3). The IRDV stands for "Integrated Relative Density Value" and expressed as an arbitrary unit. The computer assistance analysis of bands of immunoblot using advanced digital image processing systems is frequently used by researcher these days. The Western Blots in the present study were quantified through densitometry analyses using Image J software, NIH, in term of IRDV. Briefly, integrated density sums all of the pixels within a region and gives a total value. Integrated density captures very bright and very dim pixels in the same object more accurately in accordance with their contribution to the biological phenomenon versus an average. The procedure of densitometric quantification of bands in term of IRDV using Image J software is well established and has been extensively used by several workers (Andric et al., 2007; Kostic et al., 2010; Pires et al., 2009; Singh et al., 2015; Yadav and Lal, 2019, 2017).

For *in vitro* studies, the mean of five separate gonadal explants, based on incubations in triplicate, for each treatment of the three separate experiments were analyzed by ANOVA supplemented with above post-hoc test (Duncan's multiple range test at $P<0.05$).The levels of 17β-estradiol or testosterone are expressed as ng/g ovary or testis and ng/ml serum or culture medium. The enzyme activities are presented as unit/hour/g ovary. All the statistical analyses were performed in SPSS16 software (SPSS Inc., Chicago, IL, USA).

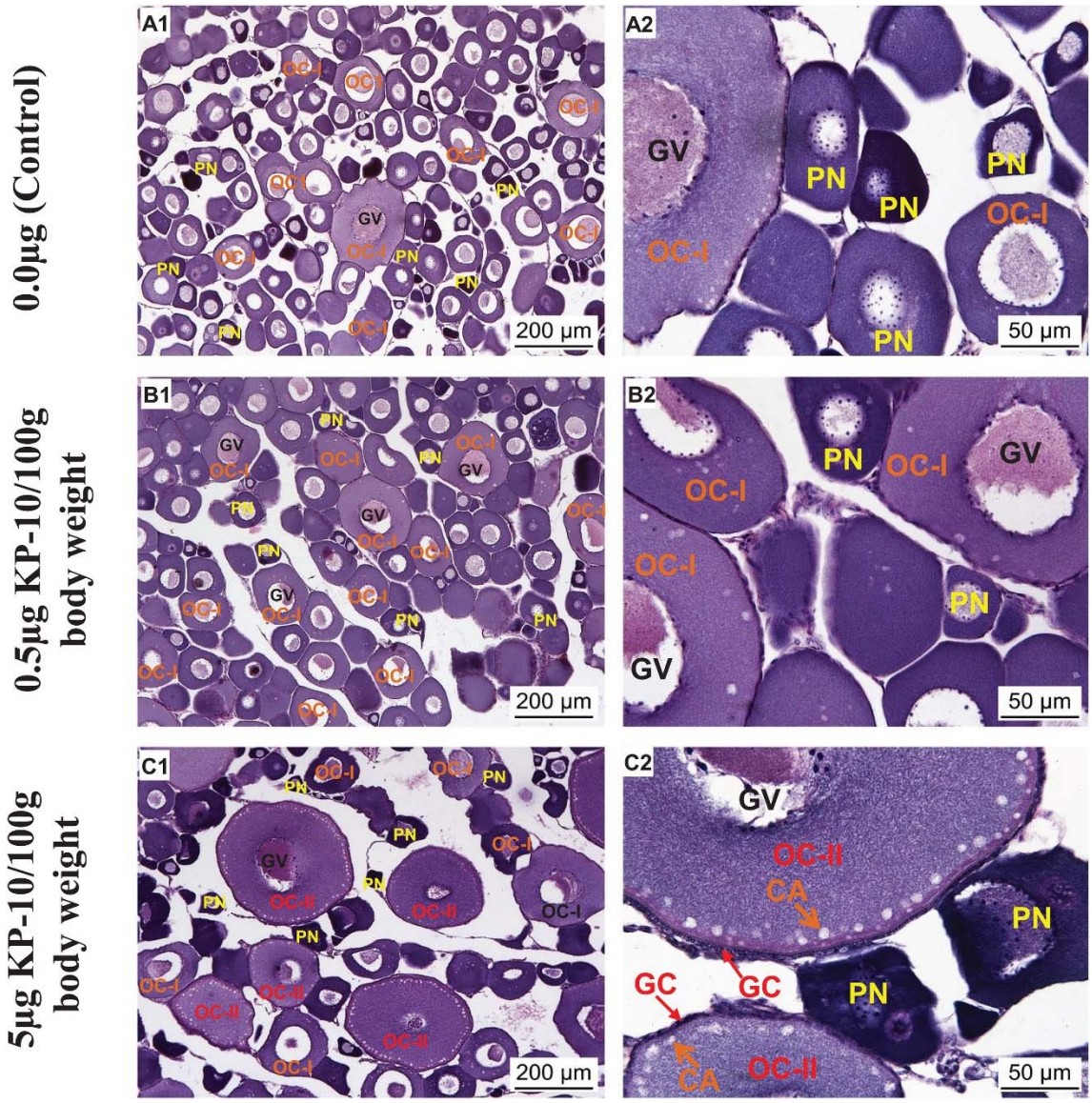


Fig.1. Representative images of hematoxylin/eosin stained transverse sections of the ovary of *Clarias batrachus,* during the late-quiescence (February) after seven days *in vivo* treatment with KP-10 (A1 to C1 at 10x and A2 to C2 at 40x magnification). Note- Perinucleolar oocytes (PN), Oocytes-I (OC-I), Oocytes-II (OC-II), Germinal vesicle (GV), Cortical Alveoli (CA), Granulosa Cell (GC)

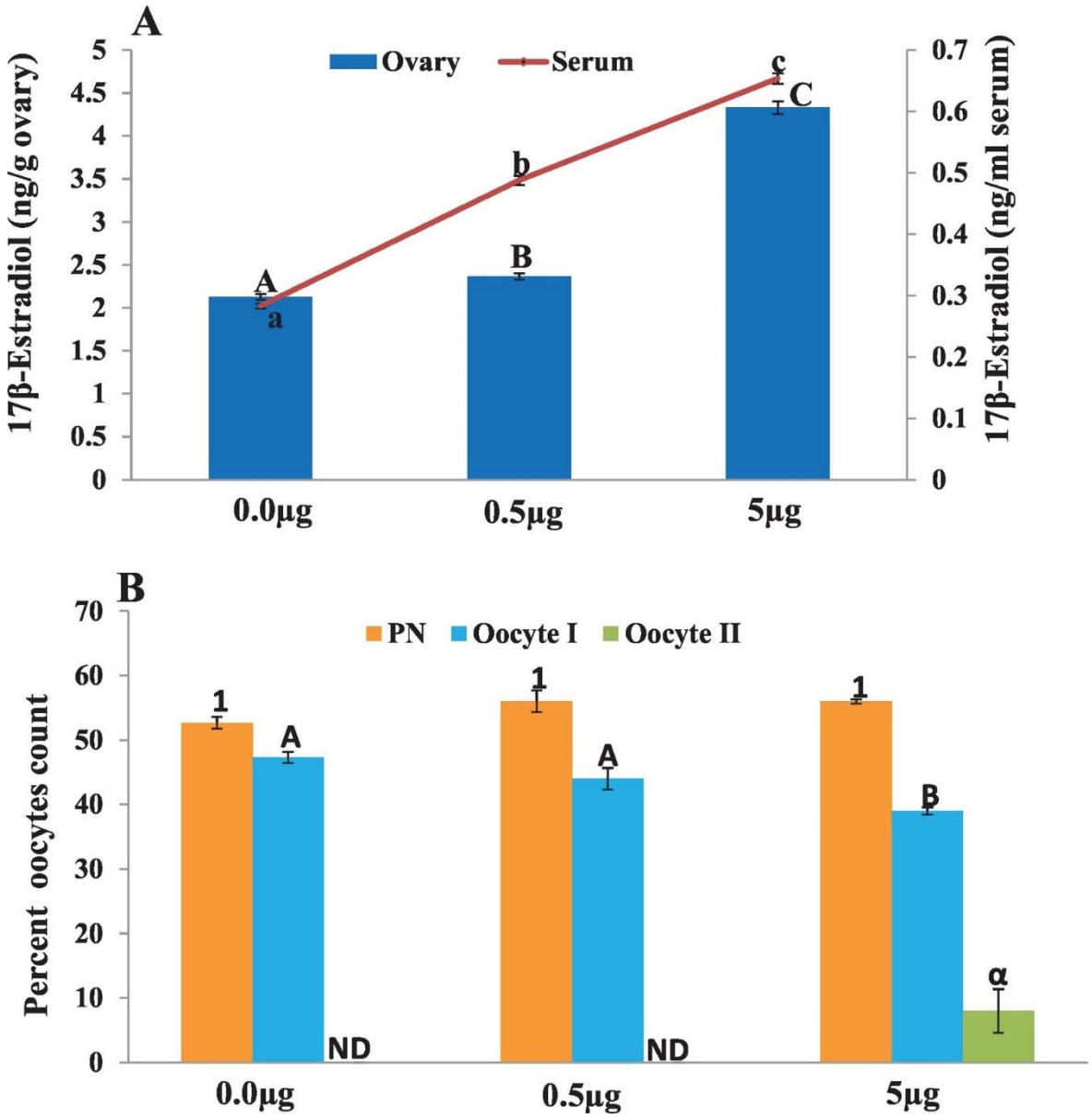


Fig.2. Levels of 17β-estradiol in ovary and serum (A) and percent of oocytes count (B) of *Clarias batrachus* during the late-quiescence phase (February) after seven days *in vivo* treatment with KP-10. Each bar represents Mean±SEM (n=5). Means with same superscript do not differ from each other, while means with different superscripts are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts a, b& c are used for 17β-estradiol level in serum and A, B & C for ovary. Superscripts A, B, 1 & α are used for percentage count of different oocytes. Note: ND represents not detected.

3. Results

3.1. Effects of KP-10 peptide on ovarian morphology and 17β-estradiol

The quiescent ovary of *C.batrachus* largely contained perinucleolar oocytes and non-yolky oocytes-I in the ovigerous folds (Figs.1 A1&A2 and 2B). The histo-morphology of the ovary of catfish treated with low-dose of KP-10 was almost similar to the control ovary (Figs.1 B1 & B2 and 2B). However, the ovary of catfish treated with high dose of KP-10 showed higher number of developing oocyte-II and lesser number of oocytes-I, when compared with ovary of the control catfish (Figs.1C1 & C2 and 2B).

17β-estradiol levels in ovary and serum were at the basal level in the control female catfish during the late-quiescence phase which were significantly raised by the mammalian kisspeptin, KP-10, in a dose-dependent fashion (Fig. 2A).

3.2. Effects of KP-10 on testicular morphology and testosterone

During the late-quiescence phase, the control testis exhibited poorly developed interstitium and smaller seminiferous tubules. The germinal epithelium of seminiferous tubules were fairly organized and filled with cysts containing spermatogonia. Sertoli cells were visible surrounding the spermatogonial stem cells (Figs. 3 A1 & A2). The KP-10 treatment enlarged the area and perimeter of seminiferous tubules in a dose-dependent manner (Fig.4B). The interstitium were also developed with distinct interstitial cells (Figs. 3 B1 and B2). The high-dose treated testis displayed germ cells in different developmental stages in cysts of the seminiferous tubules with fair amount of advanced germ cells (spermatocytes/spermatids) in some of the cysts (Fig. 3C2). The interstitium with distinct interstitial cells was also recorded (Figs. 3C1 and C2).

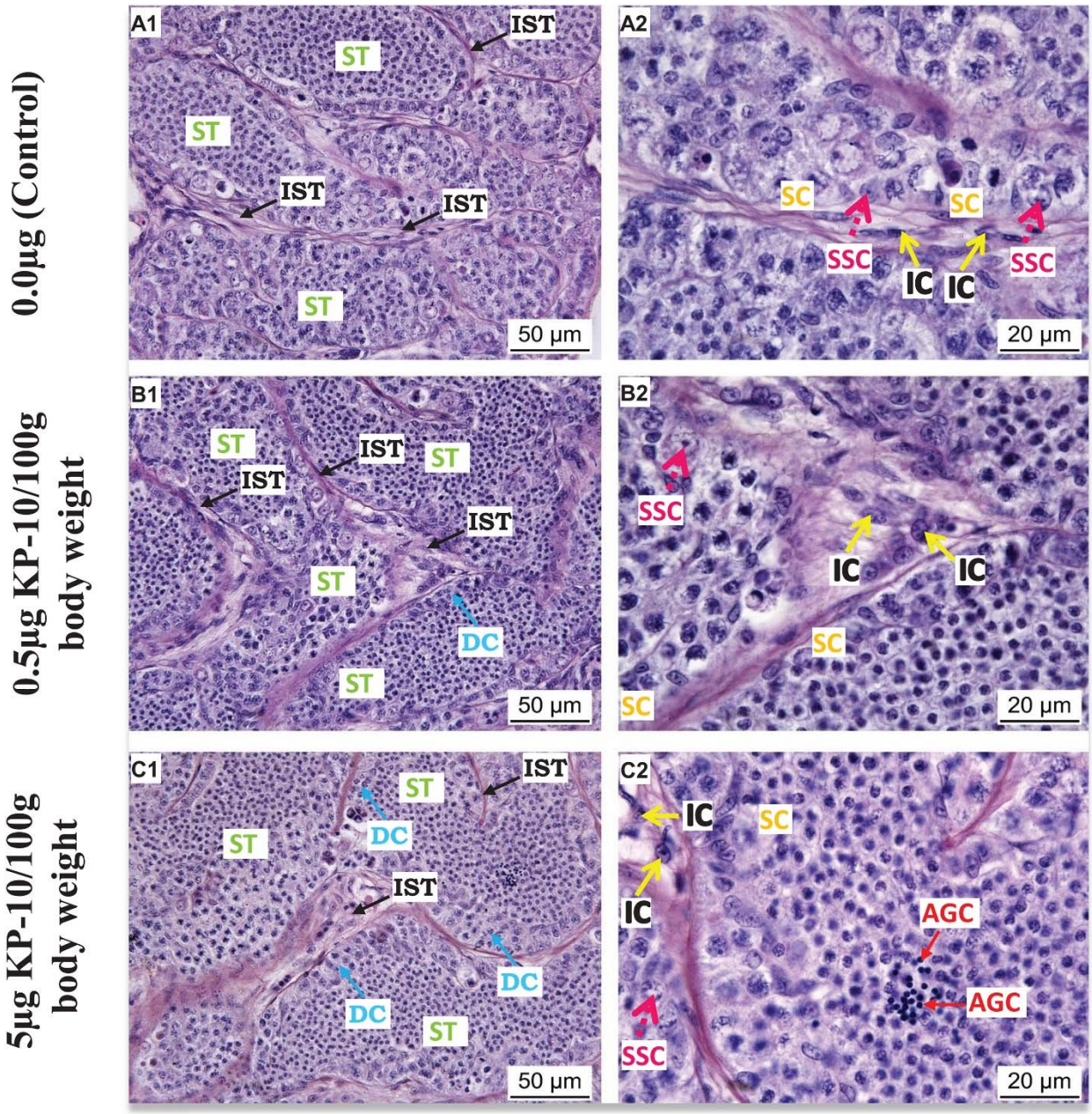


Fig.3. Representative images of hematoxylin/eosin stained transverse sections of the testis of *Clarias batrachus,* during the late-quiescence phase (February) after seven days *in vivo* treatment with KP-10 (A1 to C1 at 40x and A2 to C2 at 100x magnifications). Note- interstitium (IST), seminiferous tubule (ST), spermatogonial stem cells (SSC), interstial cells (IC), Dividing Cyst (DC), Advance germ cells (AGC), Sertoli cells (SC).

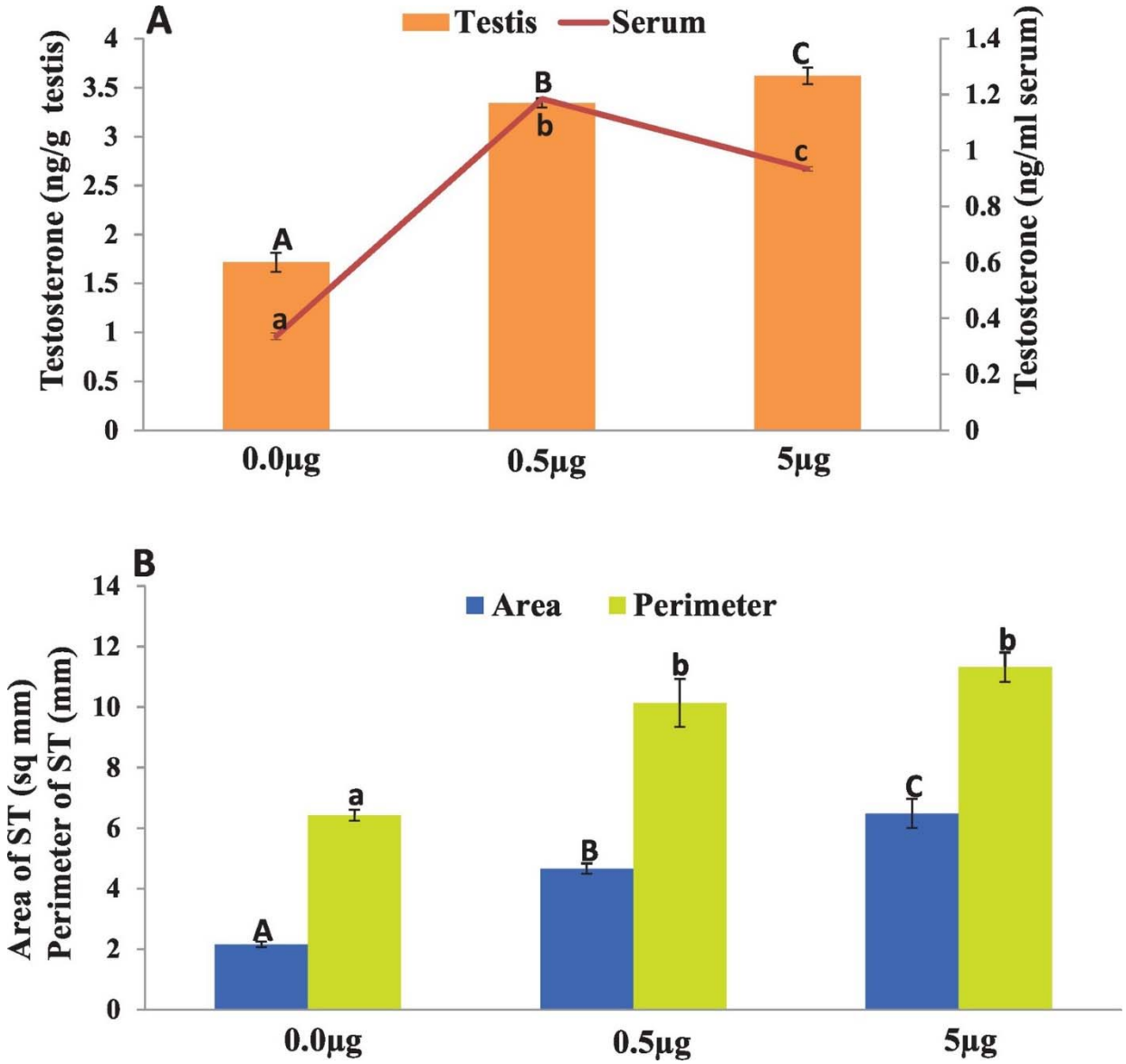


Fig.4. Testosterone concentration in testis and serum (A) and area and perimeter of seminiferous tubules (B) of *Clarias batrachus* after seven days *in vivo* treatment with KP-10 during late-quiescence phase (February). Each bar represents Mean±SEM (n=5). Means with same superscript do not differ from each other while means with different superscript are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts A, B & C are used for testicular testosterone and a, b & c for serum, respectively. For area of seminiferous tubules, superscripts A, B & C are used and a, b & c for perimeter superscripts.

The levels of testosterone were very low in the testis and serum of the control male catfish. The KP-10 treatment sharply increased testicular testosterone in dose-dependent manner.

Serum testosterone was also raised by both the doses of KP-10, but the low dose of KP-10 increased the testosterone more effectively than the higher dose (Fig.4A).

3.3. In vitro effects of KP-10 on steroids of the gonadal explants

The ovarian explants treated with various doses of KP-10 revealed dose-dependent increase in the 17β-estradiol levels (Fig. 5A). No significant change in the 17β-estradiol level of culture medium was observed. Similarly, levels of testosterone were also significantly increased in the testicular fragments and medium in a dose-dependent manner by KP-10 *in vitro* (Fig. 5B).

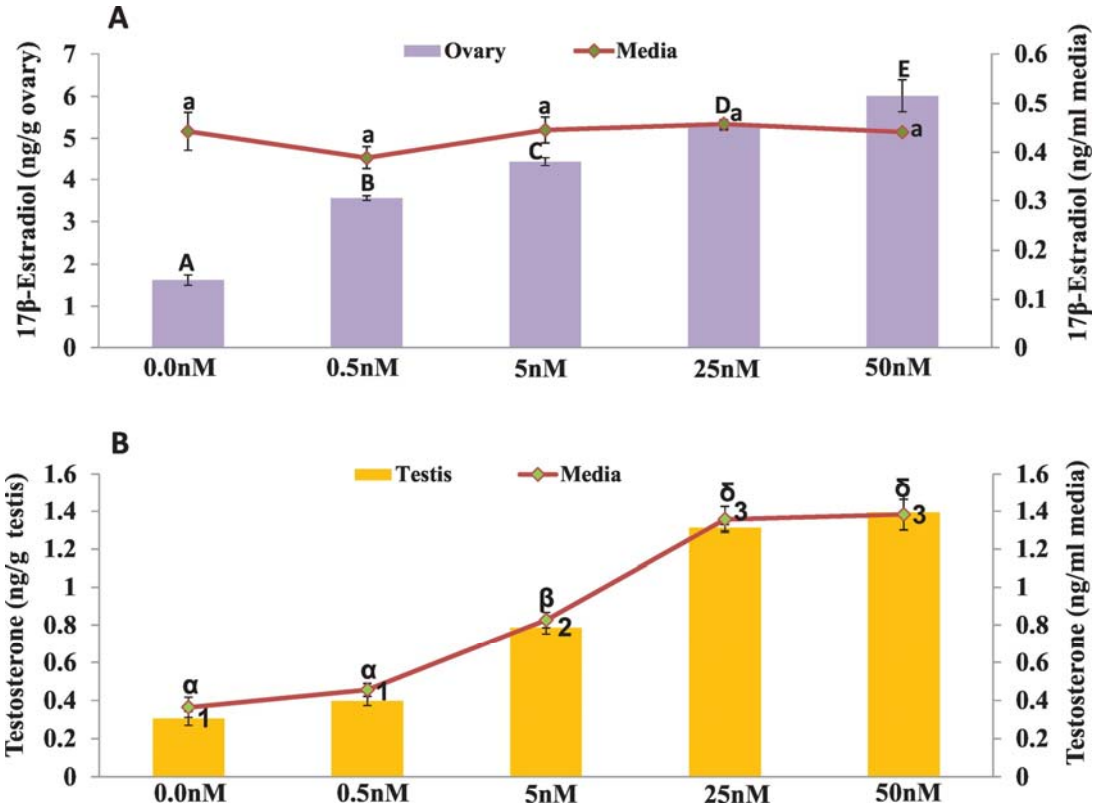


Fig.5. 17β-estradiol and testosterone levels in ovarian (A) testicular explants (B) and culture medium199 (A& B) after 24hr incubation with 0.0, 0.5, 5, 25, 50nM KP-10 *in vitro*. Each bar represents Mean±SEM (n=3). Means with same superscript do not differ from each other, while means with different superscripts are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts A, BC & D are used for 17β-estradiol level in ovarian explants and "a" is used for culture medium. Superscripts α, β and δ are used for testosterone level in testicular explants and 1, 2 & 3 for culture medium.

3.4. *In vitro* effects of KP-10 on the expressions of steroidogenic marker proteins in gonadal explants

The KP-10treatment significantly stimulated the expression of steroidogenic enzymes like 3β-HSD, 17β-HSD and StAR in ovarian (Fig. 6A) and testicular fragments (Fig. 7A). The expression of the extracellular signal regulated kinase (pERK1/2 and ERK1/2) proteins in ovarian (Fig. 6B) and testicular fragments were also increased by KP-10 (Fig.7B).

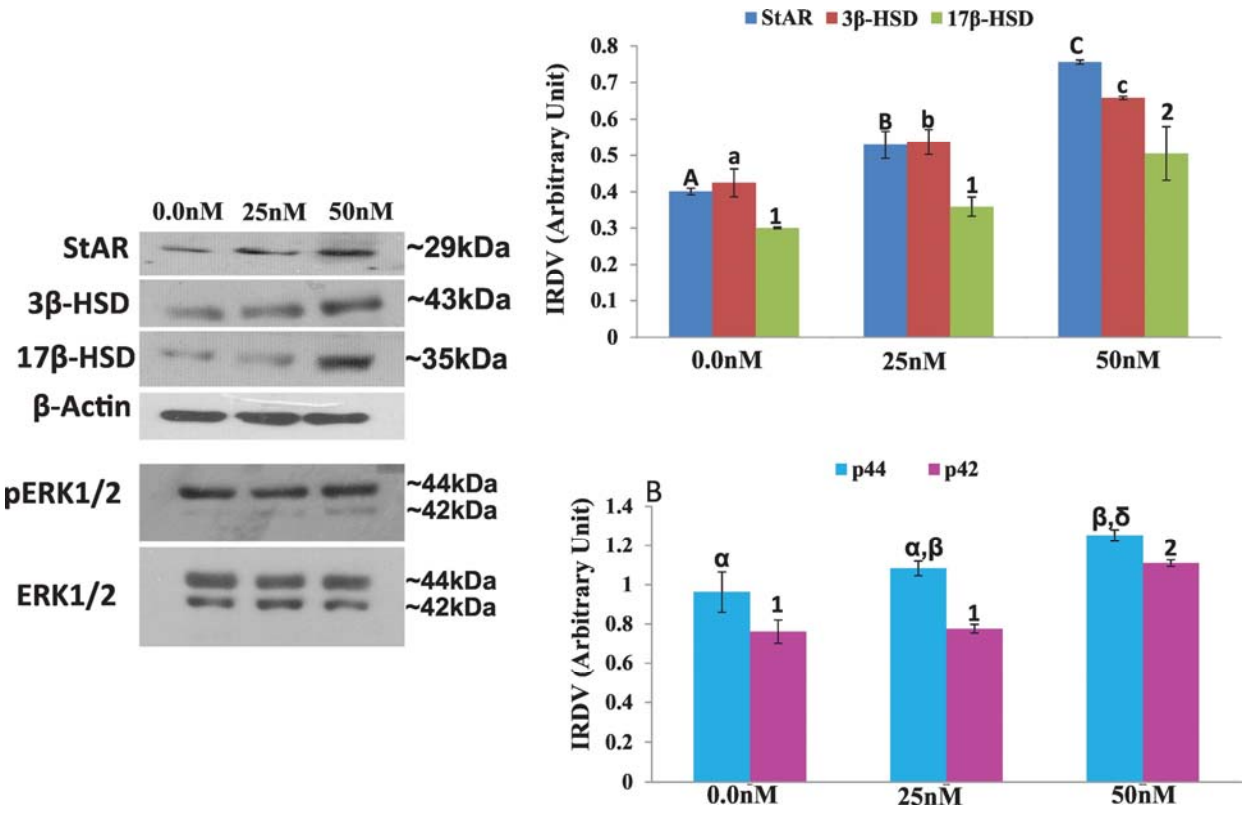


Fig.6. Western blots analysis of StAR, 3β-HSD, 17β-HSD (A) and pERK1/2 and ERK1/2 proteins (B) in the ovarian explants of *C.batrachus* incubated with 25nM and 50nM of KP-10 for 24hr *in vitro* .The expression is presented as integrated relative density value (IRDV) as arbitrary unit. All the values are expressed as Mean±SEM (n=3). Means with same superscript do not differ from each other, while means with different superscripts are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts A, B and C are used for StAR while superscripts a, b & c are used for 3β-HSD and 1and 2 for 17β-HSD respectively. Superscripts α, β & δ are for used p44 and 1 and 2 for p42.

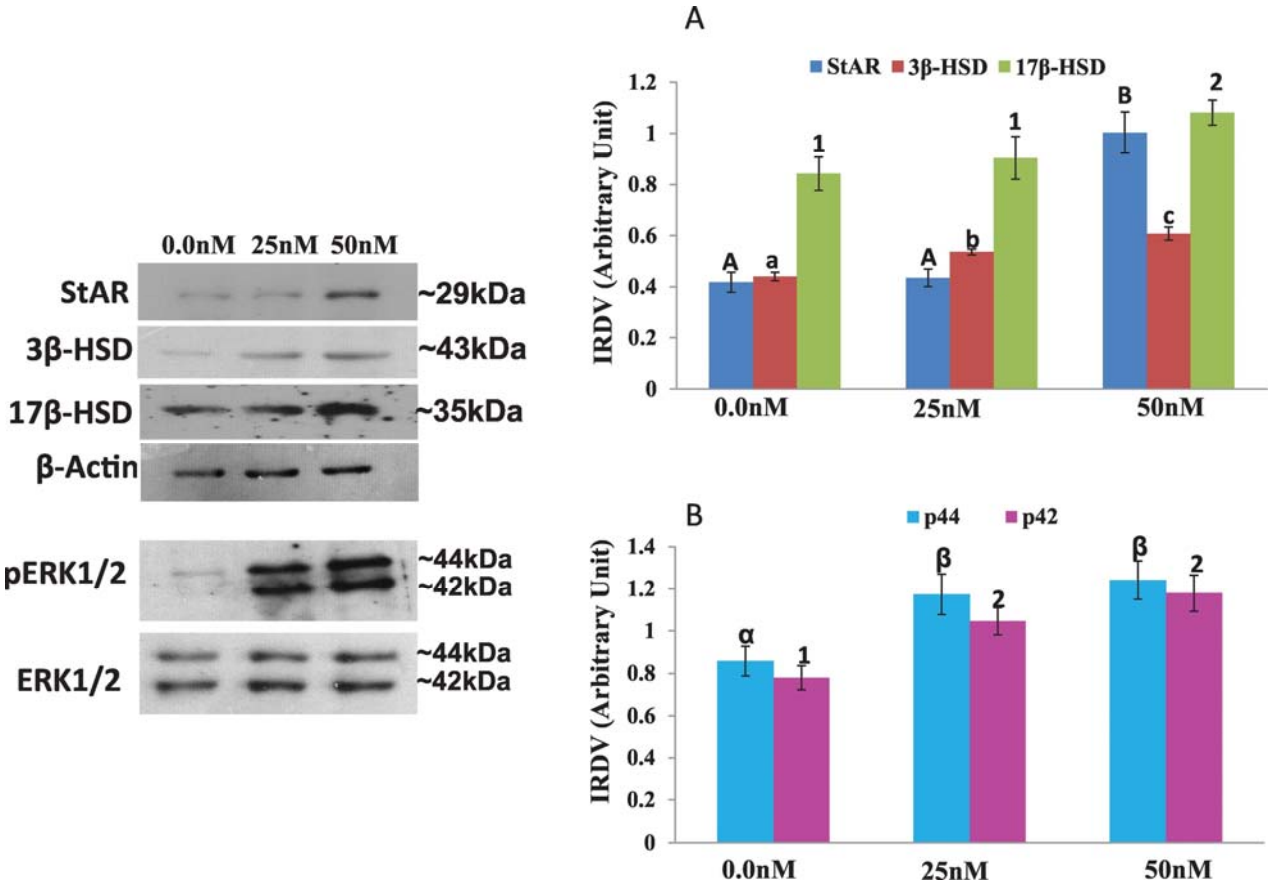


Fig.7. Western blots analysis of StAR, 3β-HSD, 17β-HSD (A) and pERK1/2 and ERK1/2 proteins (B) in the testicular explants of *C.batrachus* incubated with 25nM and 50nM of KP-10 for 24hr *in vitro*. The expression is presented as integrated relative density value (IRDV) as arbitrary unit. All the values are expressed as Mean±SEM (n=3). Means with same superscript do not differ from each other, while means with different superscripts are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts A, B and C are used for StAR while superscripts a, b & c are used for 3β-HSD and 1and 2 for 17β-HSD, respectively. Superscripts α, β & δ are used p44 and 1 and 2 for p42.

3.5. *In vitro* effects of KP-10 on the activities of ovarian steroidogenic enzymes

In addition to the stimulation in expressions of ovarian 3β-HSD & 17β-HSD, the KP-10 also enhanced their activities concurrently in dose-dependent manner (Fig.8).

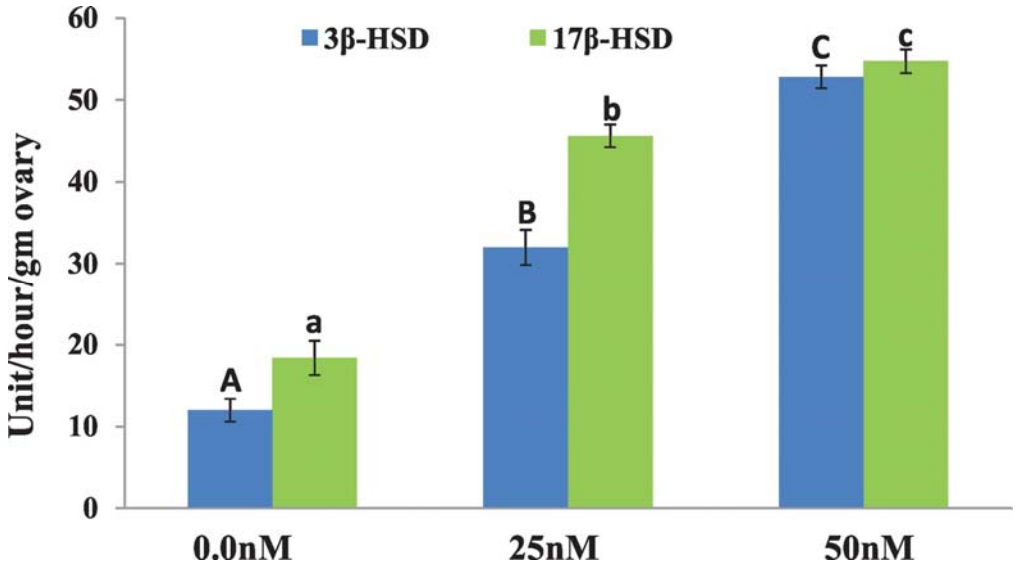


Fig.8. Activities of steridogenic enzyme 3β-HSD and 17β-HSD in female catfish, *Clarias batrachus* after *in vitro* treatment with KP-10 (25 and 50nM) for 24hr. Each bar represents Mean±SEM (n=5). Means with same superscript do not differ from each other while means with different superscript are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts A, B and C are used for 3β-HSD while superscripts a, b and c are used for 17β-HSD, respectively.

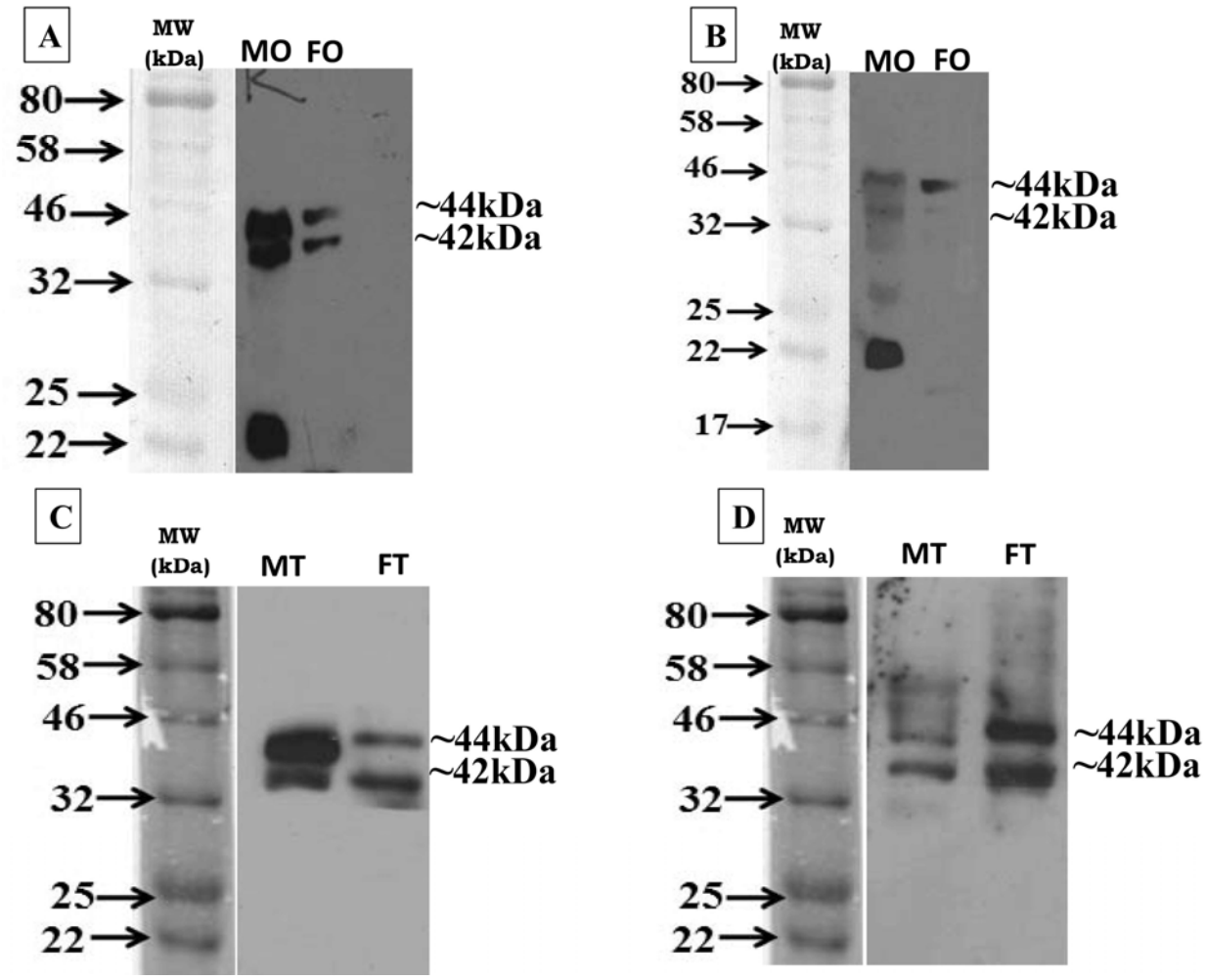


Fig. 9. Validation of pERK1/2 (A & C) and ERK1/2 (B & D) antibodies. Note: mice ovary (MO), fish ovary (FO), mice testis (MT) and fish testis (FT).

4. Discussion

The present study presents some noteworthy results. The exogenous administration of mammalian kisspeptin decapeptide, KP-10, initiates and promotes reproduction, as was evident from the KP-10-induced stimulation of gametogenesis, production of sex-steroids, and the expression of steroidogenic markers proteins such as StAR, 3β-HSD and 17β-HSD and signaling molecules like pERK1/2 and ERK1/2 proteins. The most valuable part of the study is the

demonstration of the putative mode and mechanism of steroidogenic action of KP-10 at gonadal level in fish. The *in vitro* results establish the local mechanistic cascade of regulatory action of KP-10 in gonadal steroidogenesis, independent of the extra-gonadal factors.

The mammalian KP-10 used in the present study is the native mammalian decapeptide. The doses of KP-10 used in *C. batrachus* are similar to those used in rats, mice and sheep (Roseweir et al., 2009; Pinilla et al., 2012; Sonigo et al., 2012), dogs (Albers-Wolthers et al., 2014) and humans (George et al., 2011, 2013), wherein doses are not "pharmacological". In rodents, sheep, dogs and humans, the dose was similar to the 0.5µg/100g BW, used in *C. batrachus*. This kind of dose would deliver a circulating level in the low nM range which is in the physiological range of receptor binding. The pharmacological range is in the µM. Again, in the present *in vitro* studies, there were responses at 25nM which is in the physiological range for receptor binding. Thus, the doses used in *C. batrachus* are physiological, and its actions at physiological doses indicate that there must be receptors mediating its effects. The fact that the effects are physiological is also indicated by the low doses that have been used in the present *in vivo* and *in vitro* study.

Moreover, the doses used in this fish are also much lower than the dose used in other fishes (Beck et al., 2012; Nocillado et al., 2013; Zmora et al., 2012). Beck et al. (2012) have administered 250ng/g twice a day for eight weeks in white bass, while Nocillado et al. (2013) have injected KP-10 to male yellowtail kingfish at the dose of 50µg/kg BW for four weeks to investigate the effect of kisspeptin on fish reproduction. Zmora et al. (2012) have administered a single injection of Kiss1 at a dose equivalent to 8.8, 44, and 176.1µg/BW to induce HPG axis in *Morone* species.

The stimulation of reproductive activities by exogenous kisspeptin has been well established in mammals, though such information are largely based on its impact on hypothalamo-hypophyseal level of the HPG axis (Irwig et al., 2004; Navarro et al., 2004; Plant et al., 2006). However, gonado-stimulatory role of kisspeptins in fishes is not unequivocally claimed because of highly varied and contrasting observations in teleost fishes (Ohga et al., 2018).

Unlike mammals, multiple kisspeptins genes namely *kiss1* & *kiss2* and their receptors have been identified in brains of zebrafish, goldfish, sea bass, chub mackerels, European eel, pejerrey and medaka (Escobar et al., 2013a, 2013b; Felip et al., 2009; Kanda et al., 2008; Kitahashi et al., 2009; Lee et al., 2009; Li et al., 2009; Ogawa and Parhar, 2013;Ogawa et al., 2020; Tovar Bohórquez et al., 2017; van Aerle et al., 2008) with differences in their activities, while in Nile tilapia, fugu, stickleback and puffer fish only one kisspeptin i.e. *kiss2* gene has been reported (Ogawa and Parhar, 2013; Pasquier et al., 2012; Shahjahan et al., 2010). The comparative importance of Kiss1 and Kiss2 in regulation of fish reproduction have been tested by some researchers, and they have reported species-specific, gonadal-stage-dependent efficacies of these two kisspeptins (Alvarado et al., 2013; Ohga et al., 2018; Tang et al., 2015; Trudeau, 2018; Zmora et al., 2012).

Kitahashi et al.(2009) have noticed that Kiss2-10 is more potent in increasing *fshβ* and *lhβ* transcripts in the zebrafish than Kiss1. Kiss2-10 also elevates LH level more efficiently in European sea bass than Kiss1 (Felip et al., 2015). In contrast to this, *in vivo* administration of KP1-10 increases LH release in goldfish (Li et al., 2009). Selvaraj et al. (2013 a, b) have also shown that Kiss1 more effectively increases the *fshβ* & *lhβ* mRNA and11-ketoteststerone, and that leads onset of spermiation in male, while only *fshβ* transcript and estradiol-17β in female Chub mackerels. Decline in the expression of Kiss2 in the ovary of Chub mackerel during the

late-vitellogenic phase further suggests that Kiss2 could have very little role in gonadally active months (Selvaraj et al., 2010). In goldfish and medaka, Kiss1 is shown to play an important role in regulation of reproduction rather than Kiss2 (Li et al., 2009; Oka, 2009; Selvaraj et al., 2013a, 2013b). The administration of Kiss1 pentadecapeptide (Kiss1-15), Kiss2 dodecapeptide (Kiss2-12), Kiss1 hexadecapeptide (1-16) and Kiss2 tridecapeptide Kiss2-13) have varied potency in their receptor activation in zebrafish, European sea bass, Chub mackerel and masu salmon (Felip et al., 2015; Lee et al., 2009; Ohga et al., 2018; Osugi et al., 2013). All these variations in reproductive responses to Kiss1 and Kiss2 may be attributed to species variation (occasionally sex also), their gonadal status and reproductive strategy adopted by the species. In addition to species variation, there may be other factors such as types of kisspeptins administered, and treatment methodologies (duration and concentration), and physiological conditions could also be the possible causes of the variation. Therefore, more studies on different fish species with varied reproductive strategies are essential for our better understanding of comparative biology and role of kisspeptins in fish reproduction to validate its potentials for commercial applications. The present study establishes the gonado-stimulatory role of kisspeptin-10 in the catfish, determined by histological examination and analysis of precise mechanistic cascade of steroidogenic actions of KP-10 at the gonad level during the late-quiescence phase of the reproductive cycle of the *C. batrachus*, when it is reproductively inactive.

4.1 Gonad and gametogenesis

Ovary of the present catfish, being asynchronous, shows follicles at different developmental stages in the ovigerous folds of the ovarian matrix. The quiescent ovaries largely have perinucleolar oocytes and non-yolky oocytes-I. During this quiescent phase, the ovarian and circulating 17β-estradiol are also at the basal level. Although low-dose of KP-10 was not very

effective in causing any apparent change in follicular architecture, the high dose of KP-10 significantly stimulated the development of ovarian follicle, as the ovary revealed sufficiently high number of cortical alveoli containing developing oocyte-II and significantly low number of oocytes-I. Simultaneously, KP-10 also increased the level of ovarian 17β-estradiol. Thus, it is likely that KP-10 might have promoted folliculogenesis by stimulating the estradiol production. As it is well known that the growth and accumulation of cortical alveoli is stimulated by estradiol in all egg-laying vertebrates, including fishes (Campbell et al., 2003; Forsgren and Young, 2012; Miura et al., 2007). Earlier researchers have also attributed pro-gonadal role of kisspeptins through stimulation of hormonal secretions of the hypothalamo-hypophyseal-gonadal axis (Kim et al., 2014), while some others have suggested the pro-gonadal role of kisspeptins simply by correlating the level of kisspeptins expression in gonad and brain with the gonadal status (Filby et al., 2008). A very few have studied the impact of kisspeptins on the gonadal level. Ohga et al. (2018) have shown that Kiss1 treatment to Chub mackerel induced the development of vitellogenic oocytes while Kiss2 does not. Selvaraj et al. (2013a,b) have also reported that Kiss1 induces the onset of vitellogenic oocyte stage in ovary, but the Kiss2 fails, in immature Chub mackerels. In contrast, Beck et al. (2012) have reported that Kiss2 is more potent than Kiss1 in developing advanced stage of oocytes in genus *Morone*.

Further, the control quiescent testis exhibited underdeveloped interstitium and seminiferous tubules with smaller size and diameter. The germinal epithelium was lined by the Sertoli cells surrounding the spermatogonial stem cells. The KP-10 induced proliferation in spermatogonial cells with significant increase in area and perimeter of seminiferous tubules. It also promoted the development of interstitium in the catfish. These results suggest that kisspeptin-10 not only initiates but also causes rapid progression in spermatogenic activities, as

the high-dose treated testis displayed advanced germ cells in some of the cysts within the seminiferous tubules. The development of interstitium with distinct interstitial cells (probably Leydig cells) and increased level of testosterone in dose-dependent manner in KP-10 administered testis of *C.batrachus* suggest the putative role of androgen in accelerating the spermatogenic activity. As the androgens are well known to induce spermatogenesis and male reproductive activities in vertebrates including fishes (see review Schulz et al., 2010). Ramaswamy et al. (2007) and Irfan et al. (2013) have demonstrated the direct action of kisspeptin on Leydig cells to produce testosterone in rhesus monkey.

Some researchers have demonstrated the spermatogonic effects of kisspeptins in fishes (Nocillado et al., 2013; Selvaraj et al., 2013a, b; Ohga et al., 2018). Through chronic peripheral administration of the kisspeptin decapeptides (Kiss1-10 or Kiss2-10), Nocillado et al. (2013) have shown that Kiss1-10 causes rapid testicular development in yellowtail kingfish, *Seriola lalandi,* with most cysts having spermatids and spermatozoa, while Kiss2-10 treated testis exhibited most cysts with spermatogonia and a very few cysts had advanced germ cell. Similarly, Selvaraj et al. (2013a,b) have also demonstrated that *in vivo* Kiss1 administration to Chub mackerel induces the development of higher number of spermatids and spermatozoa in testis when compared with saline-treated control and Kiss2-treated mackerels. Ohga et al. (2018) have reported that the testis of 66.7% of Kiss1-15-treated pubertal Chub mackerels had spermatozoa, while Kiss2 treated testis showed no spermatozoa. Thus, it is clear that kisspeptin-10 triggers the gametogenesis in the reproductively quiescent catfish by augmenting gonadal steroid production.

4.2 Gonadal steroidogenesis

The KP-10 stimulated *in vivo* increase in estradiol and testosterone in ovary and testis, respectively, suggest its regulatory role in gonadal steroid production. This contention is further

supported by our earlier demonstration of Kiss1 in the steroidogenic cells in gonad i.e. granulosa and thecal cells in ovarian follicles and Leydig cells in testis of *C.batrachus* (manuscript under revision). The *in vivo* stimulation of steroidogenesis by KP-10 in *C.batrachus* may not be any new finding, as the effect of KP-10 on steroids might be mediated through hypothalamo-hypophyseal route under *in vivo* condition as reported by several researchers(Nocillado et al., 2013; Ohga et al., 2018; Selvaraj et al., 2013a, 2013b). Therefore, an *in vitro* approach was adopted to decipher i) does KP-10 influence steroid production directly at gonad level? If yes, ii) what is then underlying putative mechanistic cascade?. The *in vitro* study showed that incubation of ovarian and testicular explants with the physiological doses of KP-10 not only increases the production of sex-steroids but also their release in the medium suggesting thus, the direct steroidogenic action of KP-10 at gonadal level in fish. The KP-10 also stimulated the expression of the key steroidogenic enzymes like 3β-HSD, 17β-HSD and StAR, responsible for transfer of cholesterol from outer to the inner mitochondrial membrane of the steroidogenic cells for steroid production. No previous study is available on the effect of kisspeptins on expression of steroidogenic markers in any lower vertebrates, including fish, despite that kisspeptin-induced increase in plasma levels of steroid are reported by some researchers (Kim et al., 2012; Nocillado et al., 2013; Ohga et al., 2018; Selvaraj et al., 2013 a,b).

The KP-10-induced up-regulation of expression of the StAR and key steroidogenic enzymes and activities of 3β-HSD & 17β-HSD distinctly suggest that KP-10 enhances steroid production in fish gonad, independent of extra-gonadal steroidogenic factors. These findings prompt us to contemplate that kisspeptin-10 stimulates steroidogenesis locally in fish gonad in an autocrine/paracrine manner. However, non-availability of such studies makes us difficult to correlate and discuss the present findings. Nevertheless, few studies on mammalian species are

available (Anuradha and Krishna, 2017; Peng et al., 2013; Xiao et al., 2011). Xiao et al. (2011) have shown that mammalian kisspeptin-10 up-regulates the expressions of, P450scc and StAR in cultured chicken granulosa cells. Similarly, levels of StAR, CYP11a and 3β-HSD transcripts were also increased by KP-10 in the rat luteal cells (Peng et al., 2013). Recently, Anuradha and Krishna (2017) have also reported increased expressions of StAR and 3β-HSD in response to KP-10 in bat ovarian explants. Thus, these few studies in mammalian species support our notion of KP-10-stimulated gonadal steroidogenesis.

Further, in order to explore the underlying molecular mechanism of steroidogenic action of KP-10, we also analyzed the expression of MAPKs component (pERK1/2 and ERK1/2). The immunoblot analyses revealed that mammalian kisspeptin-10 stimulated the expression of pERK proteins suggesting the kisspeptin-10-induced phosphorylation of ERK1/2, and this might be one of the putative signaling pathways of stimulation of gonadal activities in the catfish. Lack of such information in any sub-mammalian vertebrate again handicaps us to correlate and discuss present findings in detail. Although our results are in agreement with the report of Peng et al.(2013) who have reported that kisspeptin stimulates progesterone synthesis by the cultured rat luteal cells involving the mitogen-activated protein kinase signaling pathway pERK1/2 and ERK1/2.

5. Conclusions

Thus, the present study describes the gametogenic action of mammalian kisspeptin-10 in the catfish, *C. batrachus* by stimulating the steroid production in gonad through up-regulation of expressions of StAR and key steroidogenic enzymes (3β-HSD& 17β-HSD) and their activities. The study also establishes the putative mechanistic cascade through which kisspeptin-10

regulates fish reproduction in an autocrine/paracrine factor locally in the gonad, independent of extra-gonadal factors, involving the pERK1/2 and ERK1/2 kinase signaling pathway.

Declaration of Competing Interest

Authors have no conflicts of interest.

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