Role of Neurokinin B in gametogenesis and steroidogenesis of freshwater catfish, *Clarias batrachus*

Ankur Singh[1], Bechan Lal[1,*], Pankaj Kumar[2], Ishwar S. Parhar[3], Robert P. Millar[4]

[1] Fish Endocrinology Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, 221005 Varanasi, India

[2] Department of Zoology, Rajiv Gandhi University, Rono Hills, Doimukh, Itanagar, Arunachal Pradesh, India

[3] Brain Research Institute, School of Medicine and Health Sciences, Monash University, Sunway Campus, Malaysia

[4] Centre for Neuroendocrinology, Department of Immunology, University of Pretoria, Pretoria and Department of Integrative Biomedical Sciences, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa

*Correspondence to Bechan Lal. Email: lalbhu@yahoo.co.in

Abstract

Neurokinin B (NKB), a recently discovered neuropeptide, plays a crucial role in regulating the kiss-GnRH neurons in vertebrate's brain. NKB is also characterized in gonadal tissues; however, its role in gonads is poorly understood. Therefore, in the present study, the effects of NKB on gonadal steroidogenesis and gametogenesis through in vivo and in vitro approaches using NKB antagonist MRK-08 were evaluated. The results suggest that the NKB antagonist decreases the development of advanced ovarian follicles and germ cells in the testis. In addition, MRK-08 further reduces the production of 17β-estradiol in the ovary and testosterone in the testis under both in vivo and in vitro conditions in a dose-dependent manner. Furthermore, the in vitro MRK-08 treatment of gonadal explants attenuated the expression of steroidogenic marker proteins, i.e., StAR, 3β-HSD, and 17β-HSD dose-dependently. Moreover, the MAP kinase proteins, pERK1/2 & ERK1/2 and pAkt & Akt were also downregulated by MRK-08. Thus, the study suggests that NKB downregulates steroidogenesis by modulating the expressions of steroidogenic marker proteins involving ERK1/2 & pERK1/2 and Akt/pAkt signalling pathways. NKB also appears to regulate gametogenesis by regulating gonadal steroidogenesis in the catfish.

Keywords: NKB; Steroids; *Clarias batrachus;* Gonads (ovary and testis); Steroidogenesis

Introduction

Neurokinin B (NKB), belonging to the tachykinins family of peptides, has been recognized as an essential regulator of reproductive function in vertebrates, including fishes by regulating the kiss-GnRH axis (Hu et al. 2014). NKB is comprised of 10–11 amino acid residues and shares a common carboxy-terminal amino acid sequence (Phe-X-Gly-Leu-Met-NH2) with other members of the tachykinin family (Substance P, Neurokinin A, Neurokinin B, endokinins, and hemokinin) (Page et al. 2006). The NKB exerts its effect through its cognate receptor, NK3R (Almeida et al. 2004). Mutation in NKB and NK3R genes leads to hypogonadotropic-hypogonadism (Topaloglu et al. 2009).

NKB was first isolated and characterized from the porcine spinal cord (Kimura et al. 1983) and later from many other regions of the central nervous system (see review, Rance et al. 2010). NKB is co-expressed with a kiss and dynorphin in the neurons, termed as KNDy, in the arcuate nucleus of the mammalian brain (Lehman et al. 2010; Navarro et al. 2012). It is generally agreed that the KNDy system of the arcuate is involved in the direct negative steroidal regulation of pulsatile secretion of GnRH. At the same time, a separate set of kiss neurons, located in the preoptic area of the hypothalamus, is associated with the positive steroidal regulation of GnRH secretion in mammals. Thus this system operates upstream of GnRH neurons and serves as the gatekeeper of reproductive activities (Navarro et al. 2009; Lehman et al. 2010; Goodman et al. 2013, 2014; Hu et al. 2014; Moore et al. 2018). The regulation of GnRH secretion by NKB-kiss is not fully understood and is still debatable. The stimulatory effect of kiss1 on GnRH is widely accepted in mammals (Navarro et al. 2004, 2009, 2011, 2012, 2015; Wakabayashi et al. 2010; García-Galiano et al. 2012; Ruiz-Pino et al. 2012); conversely, the inhibition and stimulation of the reproductive axis by NKB are still contentious (Sahu and Kalra 1992; Navarro et al. 2011; García-Galiano et al. 2012; Grachev et al. 2012; Kinsey-Jones et al. 2012; Ruiz-Pino et al. 2012; Sandoval-Guzmán et al. 2014).

In fishes, the kiss exists in two forms kiss1 and kiss2 (Singh et al. 2021a, b, 2022). kiss1 and NKB are produced from a separate set of neurons in the habenula, while kiss2 and NKB from the ventral and dorsal hypothalamus (Servili et al. 2011; Biran et al. 2012; Ogawa et al. 2012; Zhou et al. 2012; Zmora et al. 2017). In fishes, NKB and kiss act as a steroid mediated-up-stream regulators of GnRH secretion. Thus, fishes show different brain dispositions of NKB and kiss neurons. Consistent with the reproductive actions of the NKB/NK3R system in mammals, NKB is effective in activating zebrafish NK3R-mediated reproductive functions. Recently, independent pathways for kiss and NKB in zebrafish have been suggested (Ogawa et al. 2012). The stimulatory effect of NKB on reproductive activities has been demonstrated in some fishes; zebrafish (*Danio rerio*) (Biran et al. 2012; Qi et al. 2016), European eel (*Anguilla anguilla*) (Campo et al. 2018), tilapia (*Oreochromis niloticus*) (Biran et al. 2014), and in orange-spotted grouper (*Epinephelus coioides*) (Chen et al. 2018), suggesting strongly that NKB peptides possibly act via kiss on GnRH (Campo et al. 2018). However, NKB has a negative effect on kiss2 but not on GnRH-I in the striped bass (Zmora et al. 2017).

NKB and kiss have been reported in numerous non-neuronal peripheral tissues, like the heart, retina, liver, fat, intestine, gill, muscle, kidney, stomach, and pancreas, including gonads of the vertebrates (see review, Biran et al. 2014; Hu et al. 2014). In addition, NKB transcript and peptide are widely expressed in various components of the reproductive system of rats, like the placenta (Page et al. 2000), uterus (Cintado et al. 2001; Patak et al. 2003), ovary (Lasaga and Debeljuk 2011), prostate gland and testis (Pinto et al. 2010).

In zebrafish, NKB transcripts are widely expressed in tissues like the ovary (Biran et al. 2012; Qi et al. 2016). Qi et al. (2016) have demonstrated NKB expression in the ovary of zebrafish, and also established that NKB stimulates estradiol production by directly acting at the ovarian level in an autocrine/paracrine manner (Qi et al. 2016). Further, NKB through NK3R stimulates the production of the P450cc and aromatase via MEK/ERK and PKA-CAMKII-CREB pathways to promote steroidogenesis in follicular cells of zebrafish ovary (Qi et al. 2016). However, due to scanty reports, the situation in fishes is very perplexing, and studies are mostly limited to the brain.

Thus, the present study was undertaken to decipher the role of NKB in gonadal steroidogenesis and gametogenesis in the catfish, *Clarias batrachus*. The *C. batrachus* is a widely consumed food fish in India and fetch a good market value.

Materials and methods

Chemicals

NK3R antagonist (MRK-08) was provided by Prof. Robert P. Millar (Millar and Newton 2013). Kiss1 antibody and its respective peptides (Code-PAS3809 and Code-AS1560) (against zebrafish kiss1) were provided by Prof. I.S. Parhar (Brain Research Institute, School of Medicine and Health Sciences, Monash University, Sunway Campus, Malaysia). The specificity of the antibody has been validated (Singh et al. 2021a). Biotinylated goat anti-rabbit-IgG secondary antibody was procured from GeNei (Cat No. 1110280011730) Bangalore, India. ABC Kit (elite kit PK-6100) was purchased from Vector Laboratories, Inc, Burlingame, CA, USA. Triton X-100, 3.3′-diaminobenzidine tetrachloride (DAB) was obtained from Sigma-Aldrich, India. Steroids estimation kits (17β-estradiol, Cat No. DKO003 and testosterone, Cat No. DKO002) were purchased from DiaMetra, Segrate, Milan, Italy. Medium 199 (Cat No. AL094A) and Streptomycin sulphate (Cat. No. RM220) were procured from Himedia Laboratories Pvt. Ltd., India. At the same time, other routine AR Grade laboratory chemicals were acquired from Qualigens, Merck, SRL and HiMedia. The details of antibodies, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), extracellular signal-regulated kinase (pERK1/2 and ERK1/2) have already been validated in the present catfish and published elsewhere (Yadav and Lal 2017; Singh et al. 2021b). While, Protein kinase B, AKT (Cat #9272), and pAkt (Cat #9271) were purchased from Cell Signaling USA, and their specificities were also validated for their use in *Clarias batrachus* before routine experiments. The immunoblot analyses of pAkt and Akt revealed a single band of 60 kDa in the catfish (ovary and testis) like a mouse (ovary and testis), serving as a positive control (Fig. 8).

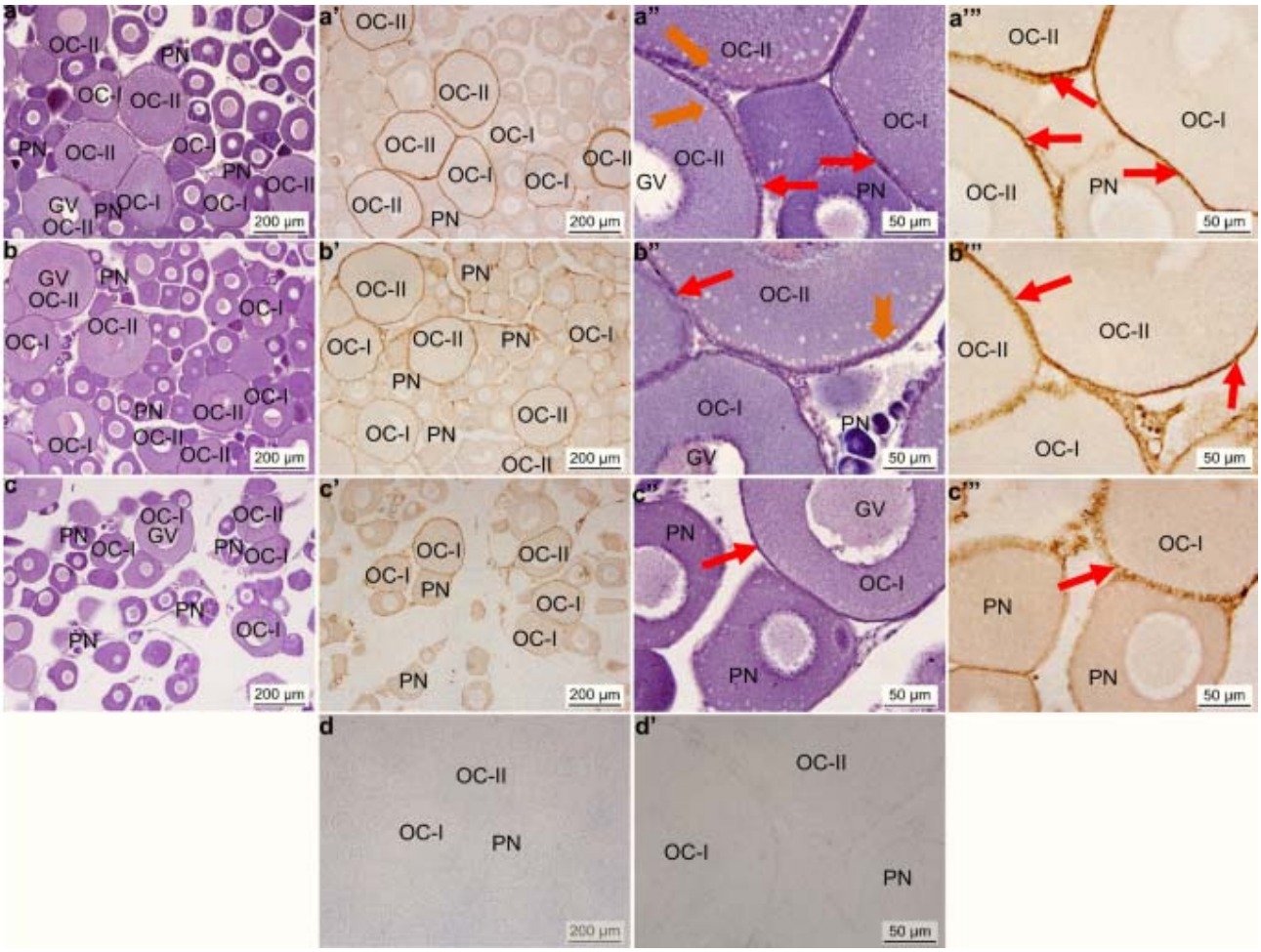

Fig. 1. Representative images of hematoxylin/eosin stained and kiss1 immunostained sections of the ovary of *Clarias batrachus*, treated with MRK-08 (0.5 µg & 5 µg/100 g b.wt.) during the mid-recrudescence (April) (**a**, **a'**, **b**, **b' c**, **c'** & **d** at 10 × and **a"**, **a"'**, **b"**, **b"'**, **c"**, **c"'** & **d'** at 40 × magnification). Note- Perinucleolar oocytes (PN), Oocytes-I (OC-I), Oocytes-II (OC-II), Oocytes-III (OC-III), Ovulated Oocytes (OO), Germinal vesicle (GV), Cortical Alveoli (➡) and Follicular cells (➡)

Procurement of catfish and in vivo treatment with NK3R antagonist

Clarias batrachus, weighing 90-95gm, were collected in the first week of the mid-recrudescence (April) and were acclimated to laboratory conditions in cemented tanks with 200L capacity. Catfish were kept for two weeks under ambient photoperiod and temperature and fed with chopped goat liver ad libitum. Following acclimatization, catfish were sorted into three groups (n = 15/group). Group-I served as control and was administered with fish saline. At the same time, group-II and III were injected with 0.5 µg and 5 µg per 100 g body weight of MRK-08, separately intra-muscularly near the lateral line. The saline, as well as the antagonist treatment, were given daily for fourteen days. After 24 h of the last injection, ten catfish were cold anaesthetized by immersing in ice-cold water and sacrificed. The blood was collected by caudal puncture in a glass tube. The serum was separated from the blood by spinning and stored at -80 °C until further use. The ovaries and testes were quickly dissected aseptically and blotted free of blood. The one lobe of the ovaries and testes was fixed in Bouin's

solution for histological examination and immunohistochemistry. The other lobe was stored at -80 °C for the estimation of steroids. All experiments were conducted in accordance with the guidelines of Institutional Animal Ethics and Care of Banaras Hindu University, India (approval letter No. F.Sc./IAEC/2016–17/1136).

Gonadal histology

Haemotoxylin and eosin (H&E) staining was performed following standard procedures described elsewhere (Priyadarshini and Lal 2018; Singh et al. 2021a). Briefly, the catfish ovaries and testes after 22 h of Bouin's fixation were dehydrated using a series of graded ethanol solutions (30%–100%), cleared in xylene, and embedded in paraffin. Then, six-micron-thick sections of ovarian and testicular tissues were cut for morphometric analysis and immunohistochemical studies. The sections were rehydrated using a series of graded ethanol solutions (Xylene–70%) and were stained with Ehrlich's haematoxylin and eosin. The morphometric study of ovaries and testicular sections was done using similar procedures described elsewhere using Motic Software (Singh and Lal 2016; nee Priyadarshini and Lal 2018; Singh et al. 2021b, a). Briefly, the stained sections of ovaries were examined under Leica DM2000 light microscope, and 10 × and 40 × images were clicked. Following the calibration of images, different oocytes, namely perinucleolar oocytes, oocytes-I and II in the ovarian sections, were counted manually from representative fields (10^2 mm/area). From a single ovarian section, three random areas were selected for counting. A similar procedure was adopted for three fishes. The final counting was expressed as Mean ± SEM in per cent. In the case of the ovary, the different types of oocytes were counted and their relative frequency out of their total number of oocytes was calculated in terms of per cent. While, in the case of testes, no such counting was done. Rather, the measurement of the total area and perimeter of the seminiferous tubules was undertaken in testes. For the area (sq mm) and perimeter (mm) of the seminiferous tubules of the testis, images were captured at 100 × of each catfish and final results were expressed as Mean ± SEM (n = 5).

Immunohistochemical localization of kiss1 and histo-morphometric analyses of the ovary and testis

The method described elsewhere was adopted to evaluate the immunohistochemical expression of kiss1 (Singh et al. 2021a). Briefly, the ovarian and testicular sections were washed in 0.05 M PBS (pH 7.5) several times after de-paraffinization and hydration. Then, the slides were incubated overnight in a humidified chamber at 4 °C with the kiss1 primary antibody (1:1200) after blocking (5% normal goat serum, 10% Triton X (TX)-100 and 1% H_2O_2). Parallelly, the negative control sections were incubated with kiss1 antibodies pre-adsorbed with kiss1 peptide (25 μg/mL). No immunoreaction was observed in the negative controls. The following day the sections were washed four times in PBS (15 min each) and subsequently incubated with biotinylated secondary antibody (1:100) for 2 h at room temperature. Next, sections were washed and processed for ABC elite staining for two hours. After washing, the sections were immersed in a solution containing 0.025% DAB and 0.066% hydrogen peroxide. Thereafter, sections were washed in PBS and processed for permanent mounting in DPX. After proper drying, slides were examined under a Leica microscope with a High-Resolution Camera and images were captured under a bright field. The immunoreactivity of follicular cells in the ovarian oocytes and interstitial cells in the testis was analyzed by spot densitometry tool, Alpha EaseFC software (Alpha Innotech Corp., USA) after deducting the value of negative control (Singh and Lal 2016; nee Priyadarshini and Lal 2018; Singh et al. 2021a). Briefly, the densitometric analyses of immunoreactive kiss1 in the gonadal sections were performed by

Alpha EaseFC software (Alpha Innotech Corp., CA, USA). Images of immunoreactive kiss1 in gonadal sections were captured at 40 × magnification using a microscope (LEICA DM 2000, Leica Microsystems, Germany) attached to a CCD camera (Model No. Leica DFC295). The images were saved in jpeg format for further analysis of immunoreactive signals. The images of the ovary and testis immunoreactive for kiss1 were opened in the Alpha EaseFC software (Alpha Innotech Corp., CA, USA) and were converted into grayscale with the help of a conversion tool. Thereafter, spot denso analysis tool was selected which had an option of the square object feature. A box of the size 1 × 1 cm and or 0.5 × 0.5 cm for the ovary and testis, respectively, was chosen using the square object feature of the software. Thereafter, the square box was randomly placed over kiss1 positive cells to obtain the final readings. The readings thus obtained were exported to an excel sheet. The above method was used for a minimum of 4 slides (belonging to one ovary or one testis; each slide with 4 ovarian sections or 8 testicular sections). 48 readings of the single ovary and 96 of single testes were taken to calculate the average. Such a process was repeated for three ovaries or testis in each group. Finally, the average of the three ovaries or testis was considered for further statistical analyses to show an increase or decrease in the immunoreactive signal of kiss1 in the ovary and testes in the present study as also described elsewhere (Singh et al. 2021a, b). The method described above provides semi-quantitative data on the immunoreactive signals in terms of IDV (Integrated Density Value) and is very frequently used by peer researchers quantifying the immunoreactive signals in immunohistochemistry (Kumar and Thakur 2012; Priyadarshini and Lal 2018; nee Priyadarshini and Lal 2018; Singh and Lal 2016, 2017).

In vitro effect of NK3R antagonist (MRK-08) on gonadal explants

In order to evaluate the local effect of MRK-08 (independent of extra-gonadal factors) on gonadal steroidogenesis, an in vitro study was performed using ovarian and testicular explants. The in vitro experiments were conducted during the mid-recrudescence phase (April). First, the adult catfish (n = 5) were sacrificed, and their ovaries and testes were dissected out aseptically and cleaned of any adhered fat tissues. Then gonadal tissues were diced into small pieces of 8-10 mg and were seeded in 24well culture plates containing culture medium199 supplemented with $NaHCO_3$(0.2%), penicillin (100 IU/ml), streptomycin (100 μg/ml) and gentamycin (40 μg/ml). After preincubation at 25 °C for 2 h, the medium was aspirated and replaced with fresh medium containing similar doses of MRK-08, 0.0 nM, 0.5 nM, 5 nM and 50 nM/ml for females and males. The entire experiment was run in triplicate and was repeated three times. Gonadal fragments and culture medium were harvested separately after 24 h and then stored at -80 °C for subsequent estimation of steroids and western blot analysis. For the Western blots analysis of StAR, 3β-HSD, 17β-HSD, pERK1/2, ERK1/2, pAkt and Akt the gonadal explants incubated with 5 and 50 nM/ml of NK3R antagonist were selected.

Immunoblot analysis

Tissue homogenate 10% (w/v) of ovaries and testis were processed as described elsewhere (Singh et al. 2021b). Following sonication, the ovarian and testicular tissues were centrifuged at 12,000 g for 30 to 40 min to collect the supernatant. After determining protein concentration in the supernatant, an equal amount of protein (50 μg) was resolved on a 12% sodium dodecyl sulphate-polyacrylamide (SDS) gel. Proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Merck Millipore, Bangalore, India) for overnight at 4 °C. The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. The membranes were then incubated with primary antibodies against StAR, 3β-HSD, 17β-HSD, pERK1/2, ERK1/2, pAKT, Akt and β-actin for overnight at 4 °C.

All the antibodies used in the present study have been previously validated in the author's laboratory and published recently (Singh et al. 2021b). Next day, membranes were washed (3x) with TBS (0.1% Tween-20) for 30 min in total and incubated with appropriate antirabbit/goat-horseradish peroxidase-conjugated secondary antibodies dissolved in TBS for 3 h at room temperature. The membranes were then washed three times for 10 min each in TBS with 0.1% Tween-20, and lastly, signals were detected using an ECL kit using X-ray films. The Western Blots in the present study were quantified through densitometry analyses using Image J software, NIH, in terms of IRDV (Integrated Relative Density Value) as described elsewhere (Singh et al. 2021a, b). The procedure of densitometric quantification of bands in terms 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, Yadav and Lal 2017).

17β-estradiol and testosterone assay

17β-estradiol and testosterone levels were measured in serum and gonadal homogenate by enzyme-linked immunosorbent assay using a commercial kit (DiaMetra, Segrate, Milan, Italy) as per the manufacturer's instructions. As per manufacturers, the sensitivities of the kit were 0.008 ng/ml and 0.10 ng/ml, which is well within the detectable limits of fish estradiol and testosterone levels. The intra- and inter- assays variations reported in the kit are 5.4% and 6.8% for17β-estradiol and 6.4% and 7.2% for testosterone, respectively. A total of 25 µl of gonadal homogenate and serum/culture medium were added to the ELISA plate separately. Subsequently, estradiol conjugate (200 µl) and testosterone conjugate (100 µl) was added and incubated at 37 °C for 2 h for estradiol and 1 h for testosterone. Following incubation, the content of each well was removed, and each well was washed thrice with wash buffer (300 µl). After washing, TMB (3, 3', 5, 5'- tetramethylbenzidine) substrate (100 µl) was added, and the plates were incubated at room temperature for 30 min and 15 min, respectively, in a dark room. A stop solution (100 µl) was added to terminate the reaction. The ELISA plates were then analyzed at 450 nm wavelength against a blank on ELISA Reader (Multiskan, Thermo Labsystem). For blank, only TMB substrate was added.

Statistical analyses

Data pertaining to 17β-estradiol, testosterone concentrations, the intensity of kiss1, area and perimeter of seminiferous tubules of testes are presented as Mean ± SEM (n = 5). While percentage counts of ovarian oocytes and Western blot analyses in terms of IRDV are presented as Mean ± SEM (n = 3). The levels of 17β-estradiol or testosterone are expressed as ng/g ovary or testis and ng/ml serum. For in vitro studies, the mean of five ovaries and testes or culture medium was calculated based on incubation in triplicates for every treatment conducted three times, independently. All the data were analyzed through ANOVA followed by Duncan's multiple range test ($p < 0.05$) in SPSS16 software (SPSS Inc., Chicago, IL, USA).

Results

Effect of MRK-08 on kiss1 immunoreactivity and ovary

The control ovarian sections revealed a strong positive kiss1 immunoreactive signal largely in the growing oocyte-II (Fig. 1a' & a"' and Fig. 2a). The low dose of MRK-08 treated ovary revealed IDV value of kiss1 expression similar to that of control (Fig. 1b' & b"' and Fig. 2a). However, high-dose treatment of MRK-08 significantly reduced the expression of kiss1 in the

oocytes (Fig. 1c' & c"' and Fig. 2a). No immunoreaction was observed in the pre-adsorbed control (Fig. 1d and d'). The gonadosomatic index in the control fish was significantly more (p,0.05) in control (0.222 ± 0.009) than in low-dose treated (0.17 ± 0.005) and high-dose treated (0.056 ± 0.006) fish.

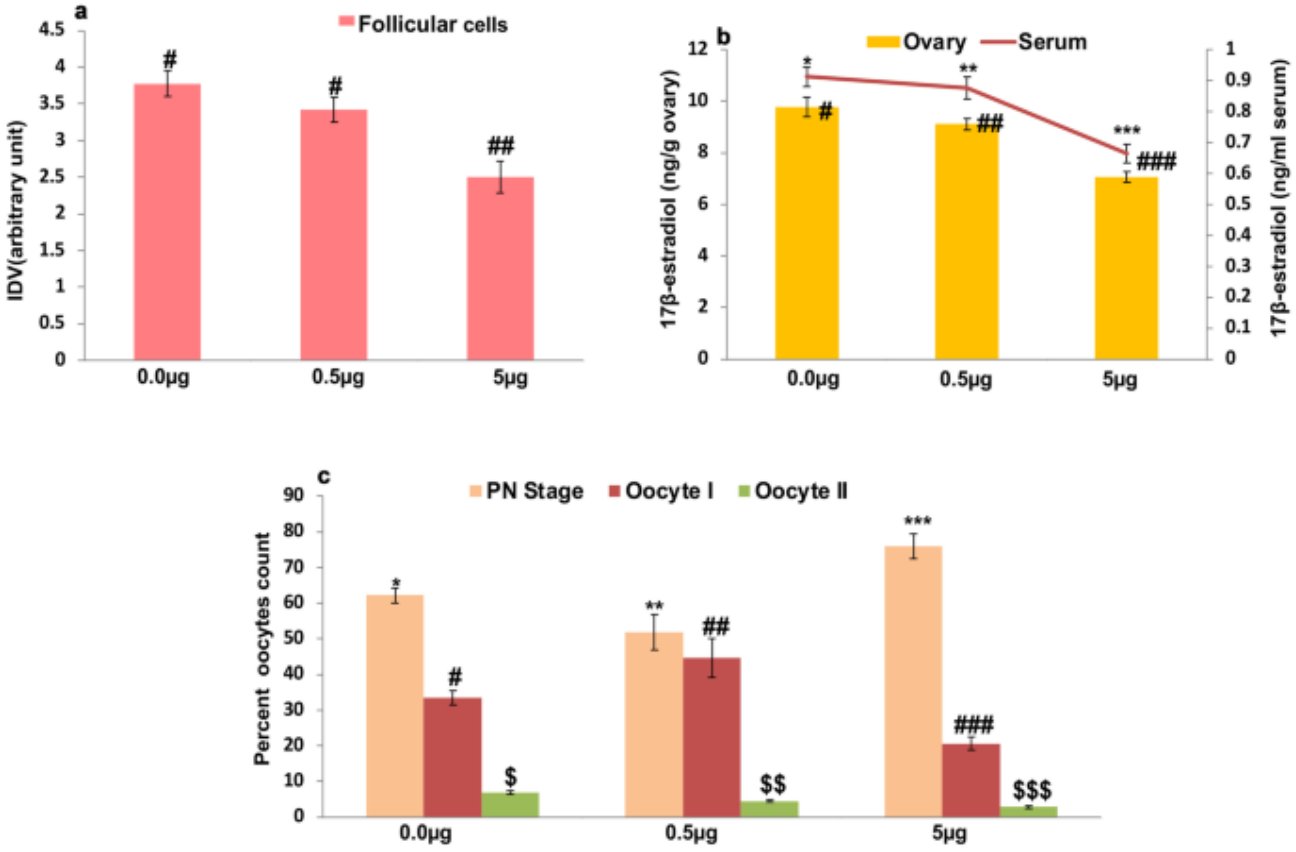

Fig. 2. Effect of different doses of MRK-08 (0.5 µg & 5 µg) per 100 g b.wt. on kiss1 immunoreactivity (**a**), 17β-estradiol levels in ovary & serum (**b**), and percentage oocyte count (**c**) of *Clarias batrachus*. The expression was quantified in term of integrated density value per unit area (IDV) and is denoted in Fig. 2a. Each bar represents Mean ± SEM (n = 5). Means bearing the same superscripts 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). Figure 2a (superscripts * and #) shows a comparison of kiss1 immunoreactivity at different doses of MRK-08 (p < 0.05), while Fig. 2b (superscripts *, # and $) shows the levels of 17β-estradiol in ovary and serum at different doses of MRK-08 (p < 0.05). Figure 2c shows the percentage of oocyte count i.e. PN Stage (superscripts *, ** and ***), Oocyte-I (superscripts #, ## & ###) and Oocyte- II (superscripts $, $$ and $$$) in the catfish treated at different doses of MRK-08 (p < 0.05)

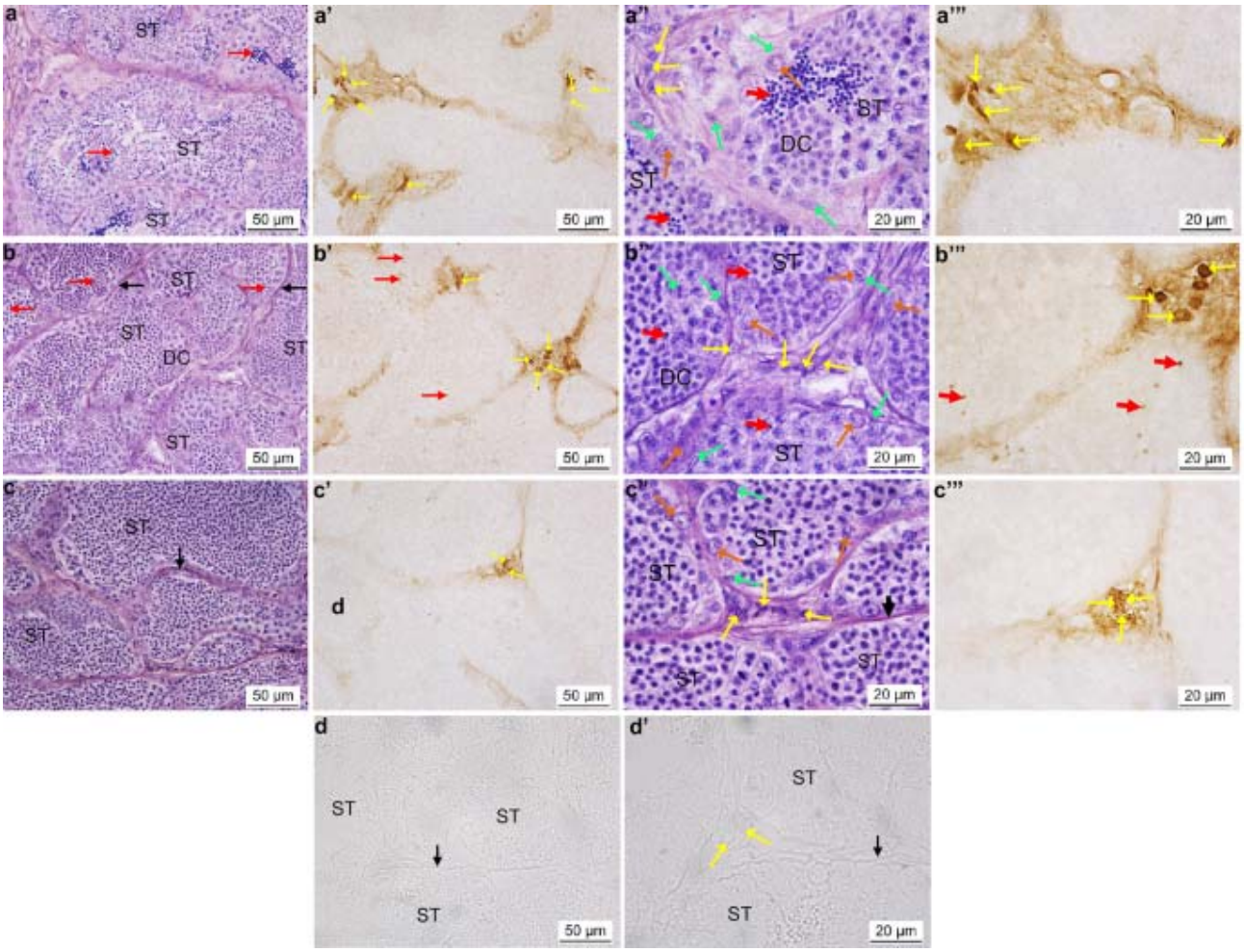

Fig. 3. Representative images of hematoxylin/eosin stained and kiss1 immunostained sections of the testis of *Clarias batrachus* treated with MRK-08 (0.5 µg & 5 µg/100 g b.wt.) in the mid-recrudescence (April) (**a**, **a'**, **b**, **b'**, **c**, **c'** & **d** at 40 × and **a"**, **a""**, **b"**, **b"'**, **c"**, **c"'** & **d'** at 100 × magnification). No immunoreaction was observed in pre-adsorbed control (d and d'). Note-interstitium ( ), seminiferous tubule (ST), dividing cysts (DC), spermatogonial stem cells ( ), Interstitial cells ( ), advance germ cells ( ), and Sertoli cells ( )

Further, the ovary of the control catfish showed a considerable number of enlarged early yolky oocytes, i.e. the oocytes-II with distinct cortical alveoli (Fig. 1a & a" and Fig. 2c). The catfish treated with a low dose of MRK-08 caused a significant reduction in numbers of oocytes-II & perinucleolar oocytes and increased the number of oocytes-I (Fig. 1b & b" and Fig. 2c). While in the ovary of high-dose treated catfish, a further reduction in the oocyte-II was observed, along with a decline in oocyte-I additionally when compared to the control ovary (Fig. 1c & c" and Fig. 2c). In such ovaries, perinuclear oocytes were remarkably increased (Fig. 1c & c" and 2c).

Effect of MRK-08 on ovarian and serum17β-estradiol

The dose-dependent decrease in the serum and ovarian 17β-estradiol was observed in fishes after the MRK-08 treatment (Fig. 2b).

Effect of MRK-08 on kiss1 immunoreactivity and testicular morphology

A significant, dose-dependent decrease in the expression of kiss1 in the interstitial cells was observed after the treatment of MRK-08 in the testis (Fig. 4a). No immunoreaction was observed in the pre-adsorbed control (Fig. 3d and d').

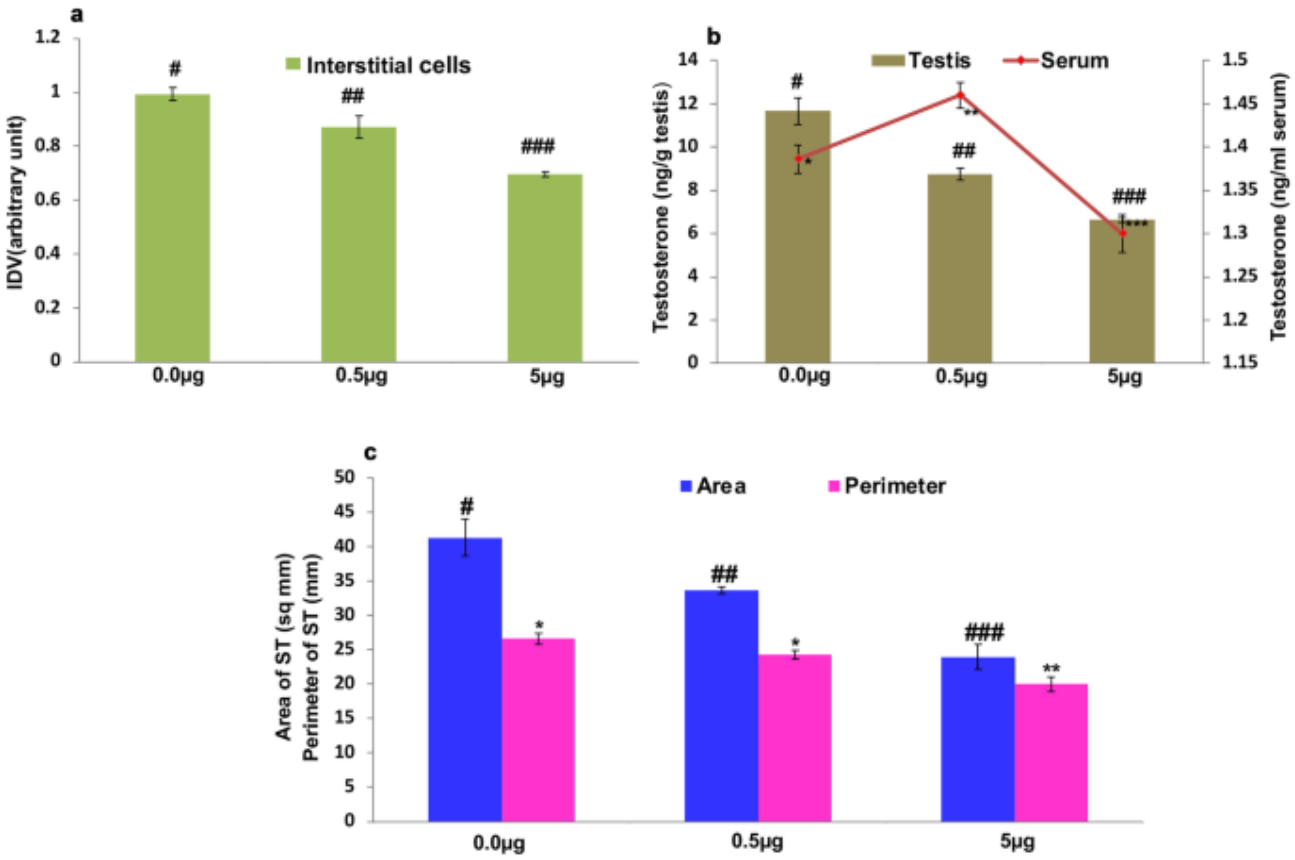

Fig. 4. Effect of different doses of MRK-08 (0.5 µg & 5 µg) per 100 g b.wt. on kiss1 expression (**a**), testosterone in testis & serum (**b**), and area & perimeter of the seminiferous tubules (**c**) of *Clarias batrachus*. The expression was quantified in term of integrated density value per unit area (IDV) and is denoted in Fig. 4a. Each bar represents Mean ± SEM (n = 5). Means bearing the same superscripts 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). Figure 4a (superscripts #, ## & ###) shows a comparison of kiss1 immunoreactivity at different doses of MRK-08 ($p < 0.05$), while Fig. 4b shows the levels of testosterone in the testes (superscripts #, ## & ###) and serum (superscripts *, ** and ***) at different doses of MRK-08 ($p < 0.05$). Figure 4c shows the area (superscripts #, ## & ###) and perimeter (superscripts *and **) of testes of catfish treated at different doses of MRK-08 ($p < 0.05$)

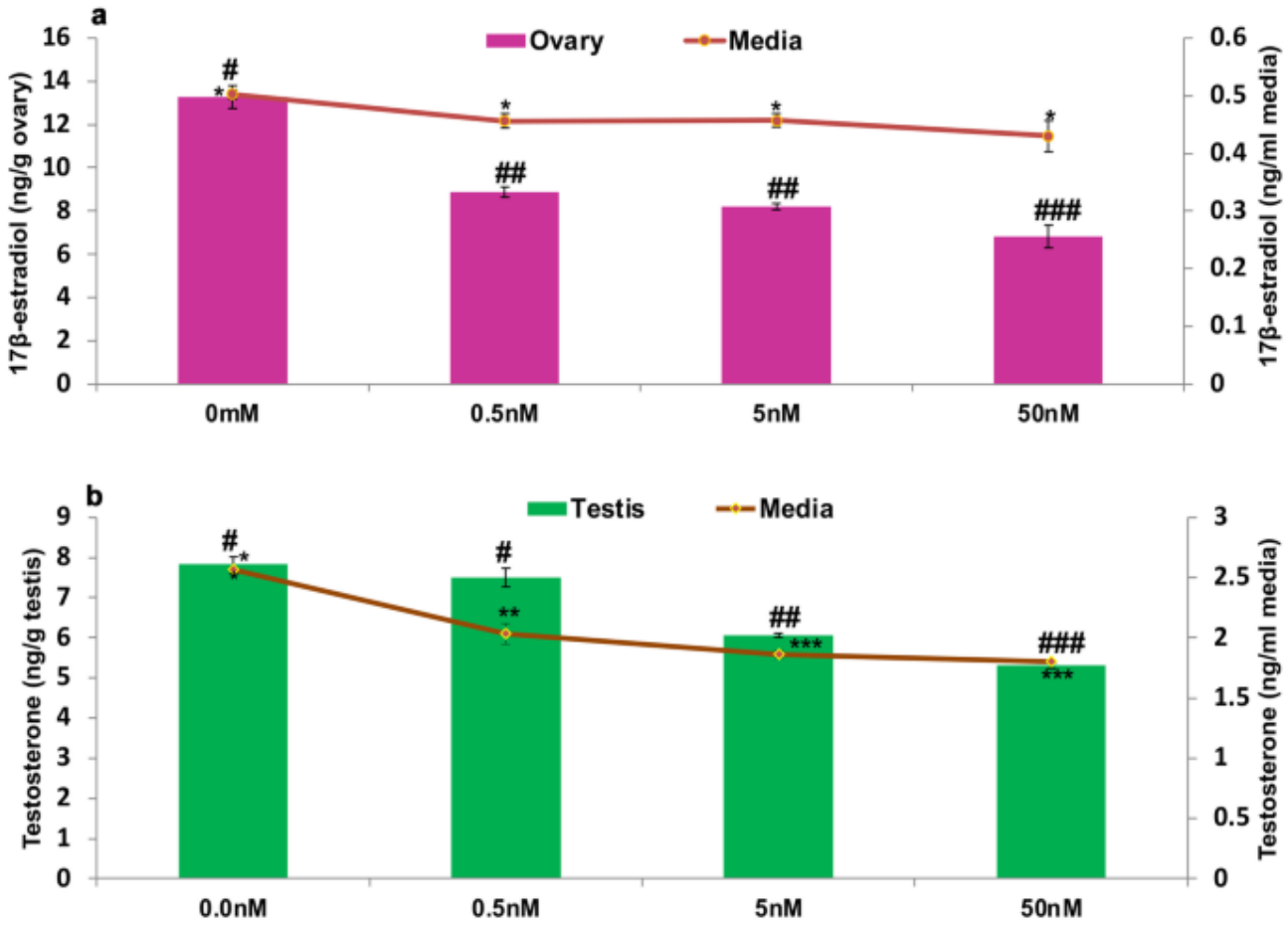

Fig. 5. Effect of MRK-08 on 17β-estradiol and testosterone levels in ovarian (**a**), testicular explants (**b**), and culture medium199 (**a** & **b**) after 24 h incubation with 0.0, 0.5, 5 & 50 nM MRK-08 in vitro. Each bar represents Mean ± SEM (n = 3). Means with the same superscript do not differ from each other, while means with different superscripts are different from each other statistically at $p < 0.05$ (Duncan's multiple range test). Figure 5a shows the 17β-estradiol levels in ovarian explants (superscripts #, ## & ###) and culture media (superscript *) at different doses of MRK-08 ($p < 0.05$), while Fig. 5b shows the testosterone levels in testicular explants (superscripts #, ## & ###) and culture media (superscripts *, ** and ***) at different doses of MRK-08 ($p < 0.05$)

The control testis displayed a large area and perimeter of the seminiferous tubules during April (Fig. 3a & a" and Fig. 4c). The interstitium was expanded with distinct interstitial cells. Some seminiferous tubules were also having advanced germ cells (Fig. 3a & a" and Fig. 4c). However, low dose treated testis showed less number of advanced germ cells in the lumen. The area & perimeter of the seminiferous were also reduced (Fig. 3b & b" and Fig. 4c). The high-dose treatment further reduced the area & perimeter of the seminiferous tubules and showed no advanced germ cells (Fig. 3c & c" and Fig. 4c).

Effect of MRK-08 on testicular and circulating testosterone

MRK-08 decreased the testicular testosterone in a dose-dependent fashion (Fig. 4b). The serum testosterone level was also lowered in the high-dose treated catfish (Fig. 4b). Surprisingly, a significant rise in serum testosterone was observed in the low-dose fish (Fig. 4b).

In vitro effect of MRK-08 on steroids in the gonadal explants and media

The treatment of NK3R antagonist caused a dose-dependent decrease in the 17β-estradiol levels in ovarian explants (Fig. 5a). No significant change in the 17β-estradiol was noted in the culture medium (Fig. 5a). A dose-dependent decrease in the testosterone levels was also noted in the testicular fragments as well as medium (Fig. 5b).

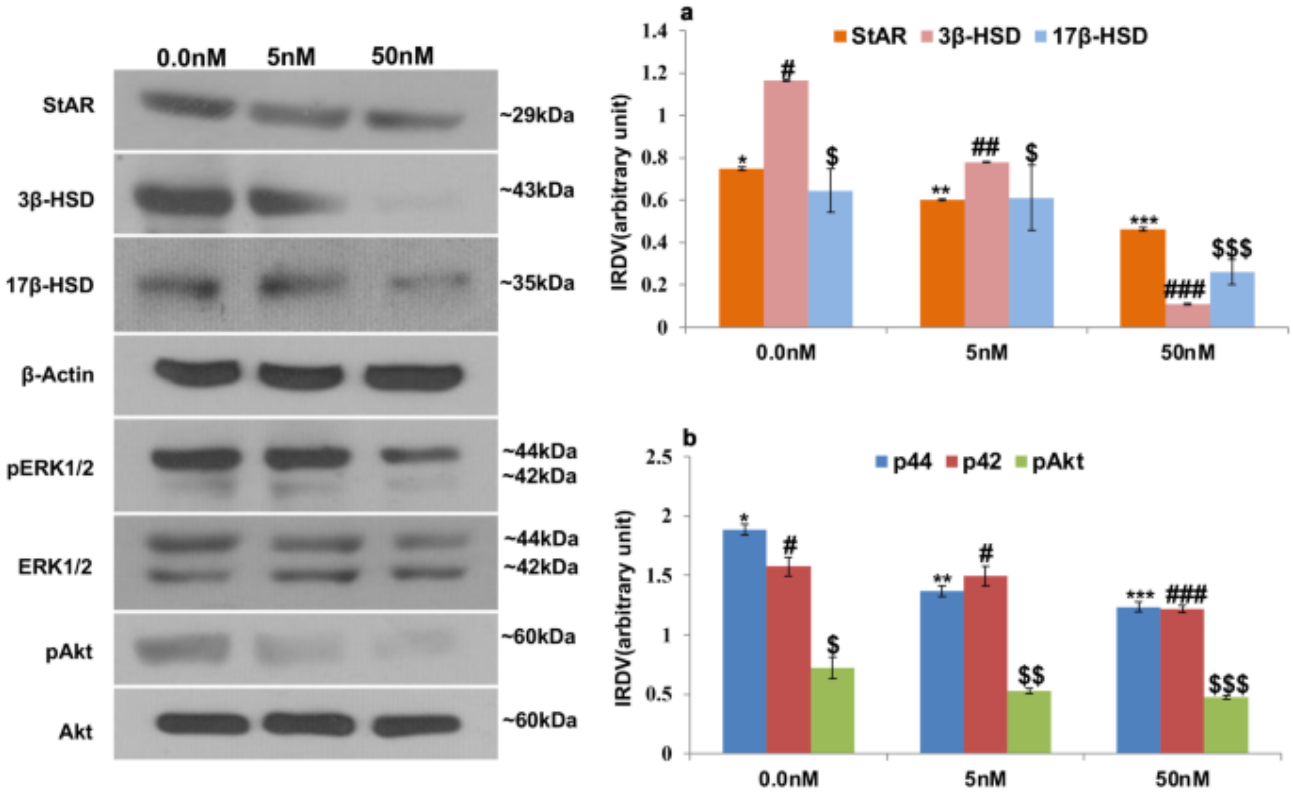

Fig. 6. Western blots analysis of StAR, 3β-HSD, 17β-HSD (**a**) and pERK1/2, ERK1/2, pAkt & Akt proteins (**b**) in the ovarian explants of *Clarias batrachus* incubated with 5 nM and 50 nM of MRK-08 for 24 h in vitro. The expression is presented as an integrated relative density value (IRDV) as an arbitrary unit. All the values are expressed as Mean ± SEM (n = 3). Means with the 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 *, ** and *** are used for StAR, while superscripts #, ## & ### are used for 3β-HSD and superscripts $ and $$$ for 17β-HSD, respectively (Fig. 6a). Superscripts *, ** and *** are used for p44 and superscripts # & ### for p42 while superscripts $, $$ and $$$ δ for pAkt (Fig. 6b)

In vitro effect of MRK-08 on the steroidogenic marker proteins, MAP kinase (pERK1/2 & ERK1/2), and protein kinase B (pAkt & Akt) in the gonadal explants

The antagonist treatment significantly reduced the expression of steroidogenic marker proteins such as StAR and 3β-HSD &, 17β-HSD in ovarian (Fig. 6a) and testicular fragments (Fig. 7a). The expressions of the extracellular signal-regulated kinase (pERK1/2 and ERK1/2) and protein kinase B proteins (pAkt and Akt) in ovarian (Fig. 6b) and testicular fragments were also suppressed in the treated catfish, as compared to controls fish (Fig. 7b).

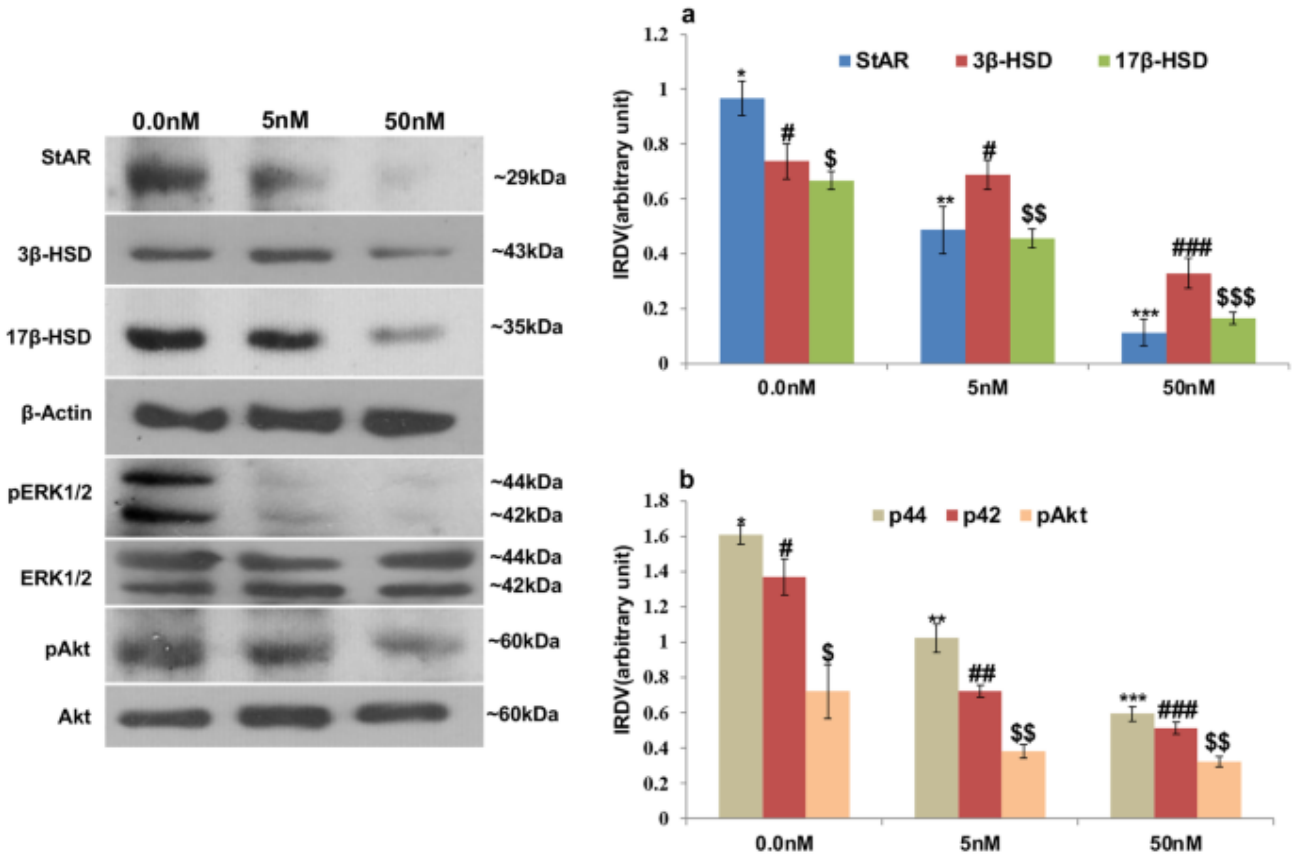

Fig. 7. Western blots analysis of StAR, 3β-HSD, 17β-HSD (**a**) and pERK1/2, ERK1/2, pAkt & Akt proteins (**b**) in the testicular explants of *Clarias batrachus* incubated with 5 nM and 50 nM of MRK-08 for 24 h in vitro. The expression is presented as an integrated relative density value (IRDV) as an arbitrary unit. All the values are expressed as Mean ± SEM (n = 3). Means with the 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 *, ** and *** are used for StAR, while superscripts #, ## & ### are used for 3β-HSD and superscripts $ and $$$ for 17β-HSD, respectively (Fig. 7a). Superscripts *, ** and *** are used for p44 and superscripts # & ### for p42 while superscripts $, $$ and $$$ for pAkt (Fig. 7b)

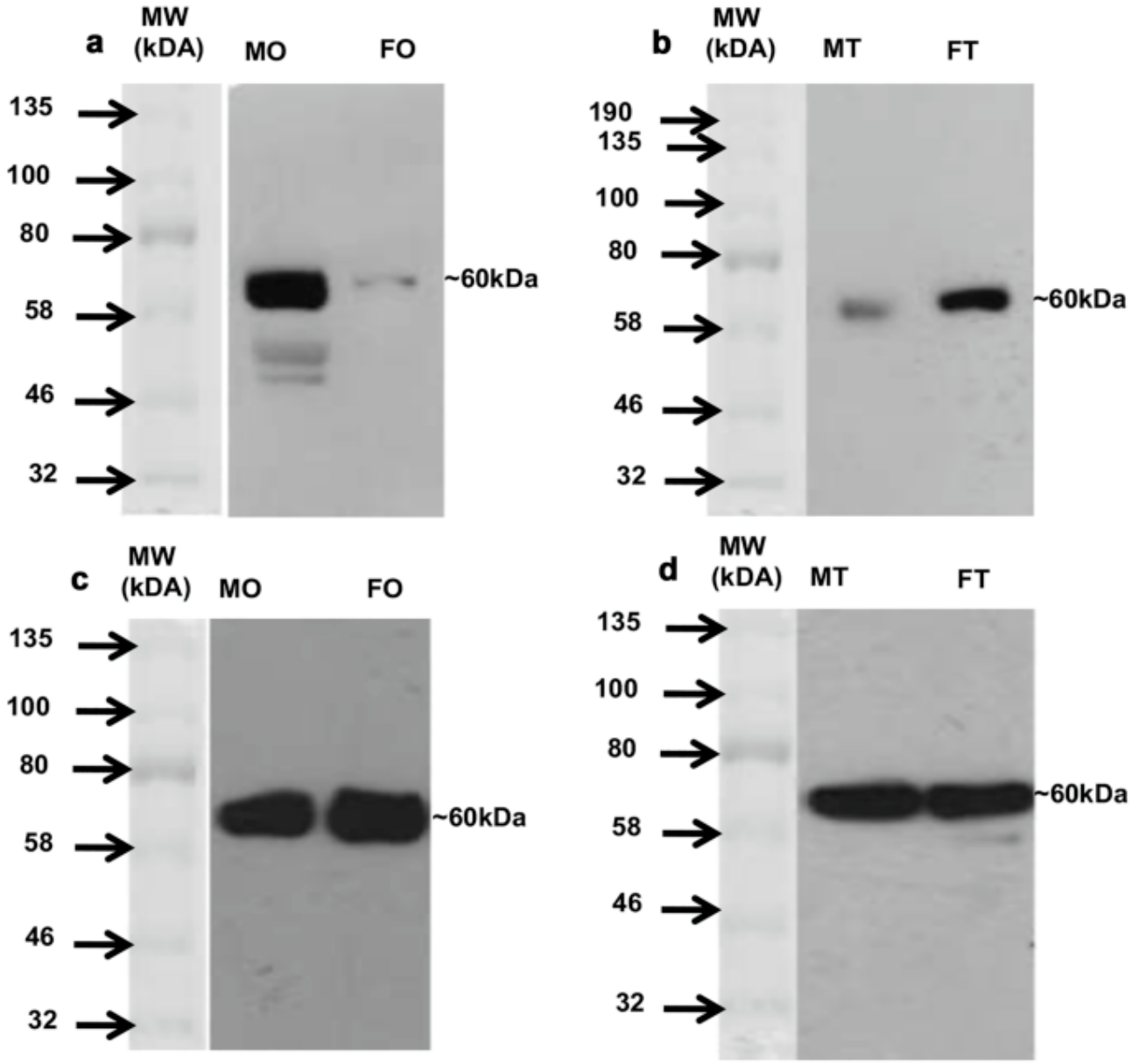

Fig. 8. Validation of pAkt (**c** & **d**) and Akt (**a** & **b**) antibodies. Note: mice ovary (MO), fish ovary (FO), mice testis (MT) and fish testis (FT)

Discussion

This study suggests that NKB may play role in the regulation of kiss1 in the gonads of the catfish, as the NKB antagonist, MRK-08, decreases the kiss1 immunoreactivity in the ovary and testis. The paucity of information on such studies makes comparing and discussing the present study very difficult. However, some studies have been conducted to understand the relationship between NKB and kiss expression in the mammalian brain, though results are contradictory and species-specific (Hu et al. 2014). It is reported that NKB can stimulate NK3R in KNDy neurons in sheep brains (Amstalden et al. 2010; Billings et al. 2010), thereby increasing kiss1 expression in the preoptic area to activate GnRH neuronal activity (Wakabayashi et al. 2013). NK3R receptors are adequately expressed in KNDy neurons in the

mouse brain, which are shown to be involved in NKB activation of the kiss in the arcuate nucleus of the brain (De Croft et al. 2013). Recently, it has also been demonstrated that NKB stimulates kiss to activate GnRH secretion (Fergani et al. 2018).

Unlike mammals, no KNDy neurons have been reported so far in fishes (Ogawa et al. 2012; Mizrahi et al. 2019). In fishes, two isoforms of kiss are reported, i.e. *kiss1* and *kiss2*, which are produced by separate genes located in two subsets of neurons. Further, different NKB and kiss neuron populations exist in zebrafish (Ogawa et al. 2012) and striped bass (Zmora et al. 2017). The relative frequencies of NKB and kiss neurons are also highly variable in different areas of the fish brain (Ogawa et al. 2012). It is also accepted that NKB acts upstream to kiss neurons. Recent reports suggest that NKB can directly act on GnRH neurons in the hypothalamus and pituitary gonadotroph cells (Zmora et al. 2017). Thus, it is likely that NKB may influence the expression of the kiss in fish gonads as well. The possibilities of interaction between NKB and kiss have also been reviewed for the first time in striped bass, wherein they observed that kiss does not modulate the NKB expression, but NKB alters the *kiss1* and *kiss2* expressions in the brain (Zmora et al. 2017). They have also demonstrated that NKB reduces the *kiss1* and *kiss2* transcripts in striped bass brain slices under in vitro conditions, although the effect of NKB on the expression of *kiss1* is less consistent than that on the *kiss2* transcript. Thus, the report of Zmora et al. (2017) is contrary to our findings, where we found that NKB antagonist lowers the kiss1 expression suggesting that NKB stimulates the kiss1 expression in catfish gonads, though the tissue-specific difference may exist. However, based on these limited results, it may be suggested that NKB may modulate the expression of kiss1 in fishes with species and tissue-specific differences. Nevertheless, more detailed studies are needed to elucidate the mode, mechanism, and signalling system involved in NKB stimulation of kiss1expression in the gonads of present catfish.

The present study reports the autocrine/paracrine effects of NKB on gonadal steroidogenesis and the putative mode, mechanism, and signalling system involved in the catfish. NKB appears to increase the steroid production in the gonads, as the exogenous treatment of fish with MRK-08 reduces the ovarian estradiol and testicular testosterone within serum under in vivo and in vitro conditions suggesting its role in steroid production. The in vitro treatment of MRK-08 also down-regulated the expression of steroidogenic marker proteins like StAR (responsible for the transfer of cholesterol from the outer to inner compartment of mitochondria) and steroidogenic enzymes, i.e., 3β-HSD& 17β-HSD suggesting that NKB stimulates the production of sex steroids in fish gonad directly, independent of the other known extra-gonadal regulators of the steroidogenesis. Furthermore, MRK-08 also suppressed the expressions of the pERK1/2 & ERK1/2 and Akt/pAkt signalling molecules. This further indicates that NKB probably involves these signalling molecules in regulating gonadal steroid production in fish. Previously many researchers have also suggested the role of NKB in sex steroid production. Still, their suggestions are primarily based on the correlation that NKB up-regulates GnRH-FSH/LH secretion, and so are the sex-steroid levels. However, some have measured the level of steroids after NKB agonist or antagonist treatments. However, in the present study, not only the levels of sex steroids are measured, rather the expressions of steroidogenic marker proteins and signalling molecules have also been estimated to decipher the mode, mechanism, and signalling system in the NKB-induced steroidogenic process. An attempt was made to investigate the detailed understanding of NKB-induced changes in steroid production in zebrafish (Qi et al. 2016). Qi et al. (2016) have also reported that NKB induces estradiol production by enhancing CYP11A1 and CYP19A1 expression in cultured follicles. Still, when ERK inhibitors were used in the culture system, this stimulatory effect was abolished. They

have also shown that NKB increases the CYP11A1 and CYP19A1 transcripts and aromatase protein levels and activities in a human granulosa cell line, COV434.

Further, MRK-08 decelerated gametogenesis by reducing the production of gonadal steroids in the present study. Thus, it appears that NKB stimulates steroidogenesis and, thereby, gametogenesis in fishes. The low-dose treated ovary had a relatively high number of oocytes-I than oocytes-II. While the high-dose treated ovaries showed a significant reduction of both oocytes-I and oocytes-II, although the number of perinucleolar stage oocytes was high. MRK-08 also retarded the progression of spermatogenesis as the numbers of advanced germ cells (spermatids/ sperms) were lowered, along with the decline in the area and perimeter of seminiferous tubules. The administration of the MRK-08 also decreased circulating and ovarian 17β-estradiol and testicular testosterone levels. Hence, it is likely that the NK3R antagonist arrests the folliculogenesis by reducing the estradiol production, which is essential for ovarian growth and progression of oogenesis (Miura et al. 2007; Lubzens et al. 2010; Forsgren and Young 2012) and spermatogenesis by lowering testosterone, as it is well known that estradiol and testosterone are necessary for the progression of spermatogenesis in fishes (Schulz et al. 2010). Earlier studies have also suggested that NKB stimulates gametogenesis in vertebrates, including fishes, by augmenting the secretions of GnRH-FSH/LH-gonadal sex steroids from the hypothalamo-pituitary–gonadal axis (Rance et al. 2010; Hu et al. 2014; Qi et al. 2016; Zmora et al. 2017), but so far no efforts are made to study its effects at the level of germs cells in gonads (ovarian follicles with eggs in ovary and spermatocytes/ advanced male germ cells in testis). NKB inhibition decreases serum estradiol leading to a reduction in follicular diameter in women (Skorupskaite et al. 2018). More recently, the antagonization of NK3R in PCOS women reduces LH pulse frequency and LH level, causing a decline in testosterone concentrations (George et al. 2016). In ewes, the role of NKB in increasing GnRH/LH during the follicular phase have been recognized (Li et al. 2015).

Moreover, the existence of the NKB/NK3R system in steroidogenic cells (such as thecal, granulosa, and luteal cells) of women (Cejudo Roman et al. 2012) and ovarian follicles (granulosa cells) of zebrafish (Qi et al. 2016) indicates that NKB may influence the steroid production in the gonad. Still, no detailed study on the mode and mechanism of its action on steroidogenesis has been carried out.

Conclusion

The present study suggests that NKB may act locally to regulate the gonadal activities of the present catfish. MRK-08 treatment attenuates steroid production in an autocrine/ and or paracrine fashion, thereby influencing oogenesis and spermatogenesis. The MRK-08 also downregulates the immunoreactivity of kiss1. This study, for the first time, proposes a putative mode, mechanism, and signalling system involved in NKB-induced regulation of steroidogenesis.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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