Nitric oxide mediated kisspeptin regulation of steroidogenesis and gametogenesis in the catfish, *Clarias batrachus*

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Abstract

Nitric oxide (NO) is a gaseous molecule that regulates various reproductive functions. It is a well-recognized regulator of GnRH-FSH/LH-sex steroid secretion in vertebrates including fish. Kisspeptin is a recently discovered neuropeptide which also regulates GnRH secretion. Nitrergic and kisspeptin neurons are reported in close physical contact in the mammalian brain suggesting their interactive role in the release of GnRH. The existence of kisspeptin and NOS is also demonstrated in vertebrate gonads, but information on their reciprocal relation in gonads, if any, is obscure. Therefore, attempts were made to evaluate the functional reciprocal relation between nitric oxide and kisspeptin in the catfish gonads, if any, by administering the nitric oxide synthase (NOS) inhibitor, L-NAME {N(G)-nitro-l-arginine methyl ester}, which reduces NO production, and kisspeptin agonist (KP-10) and assessing their impacts on the expressions of kisspeptin1, different NOS isoforms, NO and steroid production in the gonadal tissue. The results revealed that L-NAME suppressed the expression of kiss1 in gonads of the catfish establishing the role of NO in kisspeptin expression. However, KP-10 increased the expression of all the isoforms of NOSs (iNOS, eNOS, nNOS) and concurrently NO and steroids in the ovary and testis. In vitro studies also indicate that kisspeptin stimulates the production of NO and estradiol and testosterone levels in the gonadal explants and medium. Thus, in vivo results clearly suggest a reciprocal interaction between kisspeptin and NO to regulate the gonadal activity of the catfish. The in vitro findings further substantiate our contention regarding the interactive role of kisspeptin and NO in gonadal steroidogenesis.

Keywords: NO; Kisspeptin; Steroids; *Clarias batrachus* ; Gonads (ovary and testis); Steroidogenesis

Introduction

Nitric oxide (NO) is a biologically active free radical gaseous molecule and is well-known to play a role in the regulation of a variety of reproductive functions. NO is catalytically produced from L-arginine in biological tissues by one of the three isozymes (iNOS, eNOS, and nNOS) (see nee Pathak and Lal 2008, 2010; Weissman et al. 2005). NO plays a vital role in controlling the HPG axis (Dixit and Parvizi 2001). Bhat et al. (1995) have reported NOS and GnRH neurons in close contact within the hypothalamus, suggesting the regulatory role of NO in the release of GnRH. Some other studies have also shown the anatomical closeness of NOS synthesizing neurons with GnRH neurons in the mammalian brain (Bedenbaugh et al. 2018; Hanchate et al. 2012). In addition, in vivo and in vitro studies on the mammalian brain have established that NO has a significant role in GnRH regulation (Brann et al. 1997; Jadhao and Malz 2003; Kohsaka et al. 1999). The physiological relationships between NOS-synthesizing neurons and NO with the kisspeptin (kiss)-synthesizing neurons and its kiss expression have also been established in mammalian species (Bedenbaugh et al. 2018; Clasadonte et al. 2008; García-Galiano et al. 2012; Hanchate et al. 2012). nNOS-synthesizing neurons surround the GnRH neurons in mice hypothalamus, which expresses kiss1 receptors, suggesting that nNOS may regulate GnRH secretion through activation of kiss neurons (Bedenbaugh et al. 2018; Hanchate et al. 2012). Hanchete et al. (2012) have reported an interaction between kiss and nNOS neurons playing a central role in regulating the hypothalamus-pituitary–gonadal axis in mice. Two types of kiss receptors (kiss1 and kiss2) are shown on the nNOS-positive neurons in the brain of European sea bass (*Dicentrarchus labrax*) (Escobar et al. 2013).

Kiss transcripts are also demonstrated in the gonads of various fishes (Bakshi and Rai 2019; Chaube et al. 2020; Saha et al. 2016; Selvaraj et al. 2012; Shahi et al. 2017). Recently, the expressions of kiss and NOSs have also been demonstrated in the gonads of the catfish, *Clarias batrachus* in the author's laboratory (nee Pathak and Lal 2008, 2010; Singh et al. 2021a, b; Singh and Lal 2017, 2016). Additionally, GnRH1 expression too has been demonstrated in the gonad of the catfish (Singh et al. 2019).

Despite these reports on the existence of kiss, GnRH, and NOSs in the fish gonads, however, questions regarding their local interactions in gonads remain unanswered. It is likely that gonadal kiss, GnRH, and NO may interact locally with each other in the gonad and regulate their activities to fine-tune the gonadal activity akin to their counterpart in the brain. Therefore, the present study was undertaken to examine (i) the effects of nitric oxide synthase (NOS) inhibitor, L-NAME {N(G)-nitro-l-arginine methyl ester}, on kiss1 expression and (ii) the effects of kiss1 agonist (KP-10) on expressions of different isoforms of NOS (iNOS, eNOS, nNOS) along with on NO and steroid production in the gonads of *Clarias batrachus*.

Clarias batrachus, or the walking catfish, is prized in aquaculture for its hardiness, rapid growth, and versatility in various farming systems. Its high consumer demand, especially in Southeast Asia, attributes to its economic importance, providing livelihoods and cultural significance. Additionally, it serves as a natural pest controller in aquaculture ponds. The flesh of *Clarias batrachus* is appreciated for its taste and texture in many parts of Asia. It is a popular food fish in countries like India, Thailand, Indonesia, and Malaysia, where it is consumed fresh or processed into various products such as fillets, smoked fish, and fish balls. Its cultivation provides employment opportunities, generates income for farmers, and contributes to the economy through trade and export. It is also important in fish reproduction due to its prolific spawning, adaptability to various environments, and parental care behaviors. Thus,

understanding its reproductive biology is essential for successful breeding programs and conservation efforts.

Material and methods

Animal collection and maintenance

Adult catfish, *Clarias batrachus*, weighing 85–90 g were collected from nearby ponds in Varanasi in the first week of the mid-recrudescence phase (April) of the annual reproductive cycle of the catfish. Before the experiment, fish were acclimated to the laboratory environment for 2 weeks, as described previously (Singh et al. 2023). All experiments were conducted in accordance with the guidelines of Institutional Animal Ethics and Care of Banaras Hindu University, India, and as per the CCSEA Guidelines of the Government of India.

In vitro treatments with KP-10

To evaluate the direct effect of KP-10 on NO production in gonadal tissues, an in vitro study was performed using ovarian and testicular explants during the mid-recrudescence phase of the reproductive cycle of the catfish (see Singh et al. 2021a, b). Briefly, male and female fish were sacrificed, and ovaries and testes were removed aseptically and washed with medium199. The ovarian and testicular fragments (approximately 10 mg/fragment) were dropped in culture medium199 supplemented with 0.2% NaHCO3, penicillin 100 IU/mL, streptomycin 100 µg/mL, and 40 µg/mL gentamycin. Before exposing to various doses of KP-10 (0.5, 5, and 50 nM), the fragments were pre-incubated (three fragments/well) for about 3 h at 25 °C. Thereafter, the culture medium was aspirated, and gonadal explants were incubated with fresh medium199 (2 mL) containing different doses of KP-10. The control gonadal fragments were incubated with a medium without agonist simultaneously. All the in vitro treatments were performed in triplicate for each dose. At the end of the 24 h, ovarian and testicular explants and their respective medium were collected separately and stored at − 80 °C for further analyses of steroids in gonadal fragments and culture medium. The in vitro experiments were repeated five times.

In vivo treatments of catfish with the NOS inhibitor (L-NAME) and kiss1 agonist (KP-10)

The acclimated fish were divided into five groups ($n = 15$) for L-NAME and KP-10 treatments. Group I served as control and received fish saline, groups II and III were administered with L-NAME (50 and 100 µg/100 g body weight) for 14 days, whereas groups IV and V were treated with KP-10 (0.5 µg and 5 µg/100 g body weight) for 7 days. The tested doses of L-NAME and KP-10 were standardized and validated in the earlier study (see Singh et al. 2021a, b). All the injections were administered intramuscularly near the lateral line (see Singh et al. 2023).

Chemicals

Anti-zebrafish Kiss1 antibody and its peptide were provided by Prof. I. Parhar. The mammalian KP-10 (Kisspeptin agonist) was provided by Prof. R.P Millar and is described elsewhere (Millar and Newton 2013). Anti-iNOS (19 amino acid immunogen sequence—FSYGAKKGSALEEPKATRL of mice iNOS—cat. no. N7782), eNOS (21 amino acid sequence—RHLRGAVPWAFDPPGPDTPGP of bovine eNOS—cat. no. N3893), nNOS (21 amino acid sequence—RSESIAFIEESKKDADEVFSS of rat nNOS—cat. no. N7280) were purchased from Sigma-Aldrich Chemicals, USA. Vanadium trichloride (VCl_3) (cat. no.

208272), 3,3′-diaminobenzidine tetra-hydrochloride hydrate (DAB), and triton X-100 was obtained from Sigma-Aldrich, India. ABC Kit (elite kit PK-6100) was procured from Vector Laboratories, Inc., Burlingame, CA, USA. Other routinely used chemicals of analytical grade like salts and solvents were obtained from Merck.

Gonadal histology

The sectioning and hematoxylin and eosin staining of the ovarian and testicular tissues were performed according to the standard procedures described elsewhere (Singh et al. 2023). The morphometric observations of ovarian (oocyte count) and testicular (area and perimeter) sections were performed according to the method described in Singh et al. (2023).

Immunohistochemical localization of NOSs and kiss1 in ovary and testis

Immunolocalization of various NOS isoforms and Kiss1 was achieved by adopting the procedure described elsewhere (see Singh et al. 2021a, b). In brief, ovarian and testicular sections after de-paraffinization and hydration, were thoroughly washed in PBS (0.5 M, pH 7.5) several times. The sections were then incubated in a cocktail of blocking solution (5% normal goat serum, 10% TX-100, and 1% H_2O_2), at room temperature for 1 h. The slides were then incubated with polyclonal antibodies iNOS (1:700), nNOS (1:300), eNOS (1:500), and Kiss1 (1:1200) for 36 h at 4 °C. The control experiment included the (1) omission of the primary antibody from the reaction, and (2) Kiss1 antibodies were adsorbed by preincubation with KP-10 (25 µg /mL), which resulted in complete loss of immunoreactivity.

The slides were then washed with PBS for 1 h, followed by incubation with a secondary antibody (1:100) for 2 h at room temperature. Sections were then washed and processed with the ABC staining for 1 h followed by washing. These washed sections were subjected to chromogen (0.025% DAB and 0.066% hydrogen peroxide in PBS) and kept in the dark for 5–10 min to develop color. To stop the reactions, slides were washed with PBS and then finally with water for 30 min. Thereafter, slides were mounted using DPX and coverslips. The sections were viewed under a Leica DM2000 light microscope, and images were captured at 10 × , 40 × , and 100 × . The immunoreactivity was analyzed by Alpha EaseFC software (Alpha Innotech Corp., CA, USA) using a spot densitometry tool, after subtracting the values of negative control (see Singh et al. 2023).

Total nitrate-nitrite estimation

Estimation of NO was achieved by following the original method of Miranda et al. (2001). In brief, serum/culture medium199 and ovarian and testicular homogenate (10%) were firstly deproteinized by ethanol and thereafter centrifuged at 10,000 g for 15 min. The supernatant collected was used for NO estimation. KNO_3 standard, 100 µl of various concentrations (0, 20, 40, 60, 80, and 100 µM), serum/culture medium199, and ovarian and testicular supernatant were added in the wells of a microtitre plate, separately and in triplicate. Subsequently, 100 µl of VCl_3 solution (0.8% VCl_3 in 1 M HCl) was supplemented to each well followed by rapid addition of Griess reagent {50 µl 0.1% *N*-naphthyl ethylenediamine (NEDD) in double-distilled water and 50 µl 2% sulphanilamide in 5% HCl}. The plates were then incubated at 37 °C for 45 min, and the absorbance was measured at 540 nm. The concentrations of total nitrate and nitrite were interpolated from the standard graph plotted using absorbance against KNO_3.

Estimation of steroids (testosterone and estradiol) in testes and ovary

The levels of 17β-estradiol and testosterone were measured using an enzyme-linked immunosorbent assay (ELISA) with a commercial kit from DiaMetra (Singh et al. 2023). The kit has sensitivities of 0.008 ng/mL for 17β-estradiol and 0.10 ng/mL for testosterone, which is suitable for detecting fish estradiol and testosterone levels. Intra-assay variations were 5.4% for 17β-estradiol and 6.4% for testosterone, while inter-assay variations were 6.8% and 7.2%, respectively. For analysis, 25 µl of gonadal homogenate and serum/culture medium were separately added to the ELISA plate. Then, estradiol conjugate (200 µl) and testosterone conjugate (100 µl) were added separately and incubated at 37 °C for 2 h and 1 h, respectively. After incubation, each well was washed three times with 300 µl wash buffer. Subsequently, TMB substrate (100 µl) was added, and the plates were incubated at room temperature for 30 min for estradiol and 15 min for testosterone in a dark room. The reaction was stopped by adding 100 µl stop solution, and the plates were analyzed at 450 nm wavelength against a blank on an ELISA Reader (Multiskan, Thermo Labsystem), where the blank consisted of only TMB substrate.

Statistical analyses

Data related to 17β-estradiol, testosterone concentrations, NO, intensity of NOSs and Kiss1, area and perimeter of seminiferous tubules of testes are presented as mean ± SEM ($n = 5$), while the percent counts of ovarian oocytes are presented as mean ± SEM ($n = 3$). For in vitro studies, the mean of five separate in vitro experiments on ovaries and testes or culture medium was calculated, based on incubation in triplicates for every treatment, independently. Data were analyzed by one-way ANOVA followed by Duncan's multiple range tests ($P < 0.05$). All statistical analyses were done with the help of the SPSS16 software (SPSS Inc., Chicago, IL, USA).

Results

In vivo effects of KP-10 on ovarian morphology and 17β-estradiol

The present catfish displays asynchronous ovarian activity having oocytes at different stages of development arranged in the ovigerous folds. The control ovary during the mid-recrudescence phases (April) is filled with perinuclear oocytes, oocytes-I, and oocytes-II (Fig. 1a, d, and g). The ovary of this phase is largely characterized by the presence of oocytes-II with distinct cortical alveoli which are larger than oocytes-I (Fig. 1a, d, and g). The low-dose (0.5 µg) kisspeptin agonist (KP-10)-treated ovary exhibited a high number of oocytes-II as compared to the control (Fig. 1b, e, and g). The high-dose (5 µg) kisspeptin agonist (KP-10)-treated ovary showed oocytes-III, in addition to oocytes-II. The number of oocytes-I was substantially low as compared to the control (Fig. 1c, f, and g).

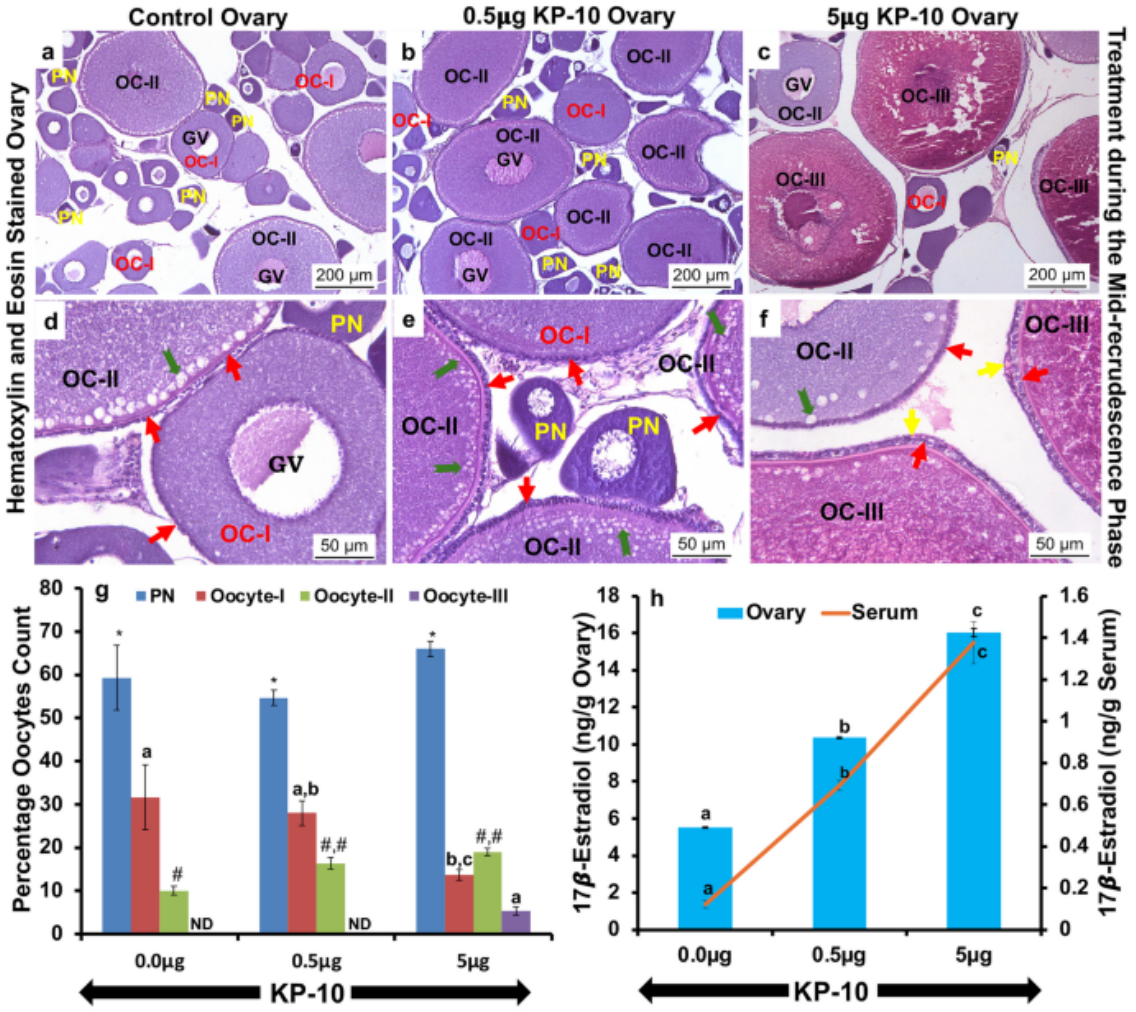


Fig. 1. Representative images of hematoxylin/eosin-stained transverse sections of the ovary of *Clarias batrachus* during the mid-recrudescence phase (April) after 7 days in vivo treatment with KP-10 (**a** to **c** at 10 × and **d** to **e** at 40 × magnification). Perinucleolar oocytes (PN), oocytes-I (OC-I), oocytes-II (OC-II), oocytes-III (OC-III), germinal vesicle (GV), cortical alveoli (green arrows), granulosa cell (red arrows), and thecal cell (yellow arrows). Histogram of percentage oocyte count (**g**) and ovarian and serum levels of 17β-estradiol (**h**) in female *Clarias batrachus* after treatment with KP-10 (0.5 and 5 µg/100 g). Each bar represents mean ± SEM ($n = 5$). Means bearing the same superscript do not differ from each other, while means bearing different superscript are different from each other statistically at $P < 0.05$ (Duncan's multiple range test). Superscripts *, a, b, #, and ## are used for percentages of oocyte counts. Superscripts a, b, and c are used for 17β-estradiol in ovarian tissue and serum both

During the mid-recrudescence phase, KP-10 treatment increased the levels of ovarian and serum 17β-estradiol in a dose-dependent manner (Fig. 1h).

In vivo effects of KP-10 on testicular histology and testosterone

The control testis of the mid-recrudescence phase (April) showed well-developed seminiferous tubules displaying active spermatogenesis. The interstitium had a large number of interstitial cells. A few seminiferous tubules with dividing cysts filled with advanced stages of the germ cell were clearly visible in the lumen (Fig. 2a, d, and h). The low-dose (0.5 µg) kisspeptin agonist (KP-10)-treated testes displayed evacuation of the advanced germ cells from some of

the seminiferous tubules (Fig. 2b, e, and h). The high-dose (5 µg) kisspeptin agonist (KP-10)-treated testes showed enlarged seminiferous tubules with more advanced germ cells and evacuating seminiferous tubules as compared to the low-dose-treated group (Fig. 2c, f, and h). The treatment with KP-10 enlarged the area and perimeter of seminiferous tubules in a dose-dependent fashion (Fig. 2h).

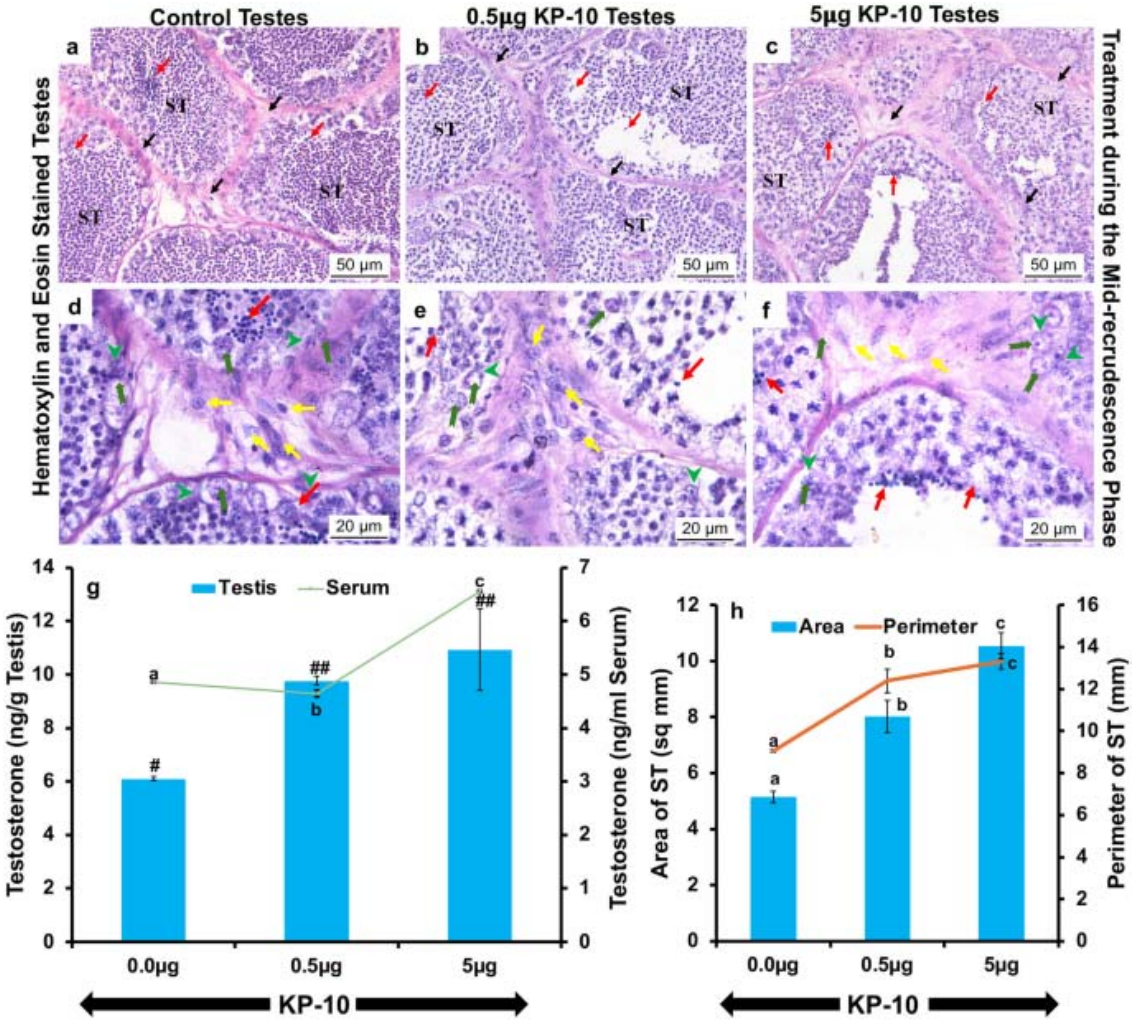


Fig. 2. Representative images of hematoxylin/eosin-stained transverse sections of the testis of *Clarias batrachus* during the mid-recrudescence phase (April) after 7 days in vivo treatment with KP-10 (**a** to **c** at 10 × and **d** to **f** at 40 × magnification). Interstitium (black arrows), seminiferous tubule (ST), spermatogonial stem cells (green arrows), interstitial cells (red arrows), advance germ cells (yellow arrows), and Sertoli cells (green arrowheads). Histogram of serum and testicular testosterone (**g**) and area and perimeter of seminiferous tubules (**h**) in male *Clarias batrachus* during mid-recrudescence phase (April) after treatment with KP-10 (0.5 and 5 µg/100 g). Each bar represents mean ± SEM ($n = 5$). Means bearing the same superscript do not differ from each other, while means bearing different superscript are different from each other statistically at $P < 0.05$ (Duncan's multiple range test). Superscripts # and ## and a, b, and c are used to compare variation in testosterone in the testis and serum. Superscripts a, b, and c are used for both area and perimeter

KP-10 treatment also increased the level of testosterone in serum and testes in a dose-dependent manner (Fig. 2g).

In vivo effects of KP-10 on total nitrate-nitrite in gonad and serum

During the mid-recrudescence phase, KP-10 treatment significantly increased ovarian and serum NO levels in a dose-dependent fashion (Fig. 3a). The high dose of KP-10 elevated the circulating levels of NO while the low dose was ineffective (Fig. 3a).

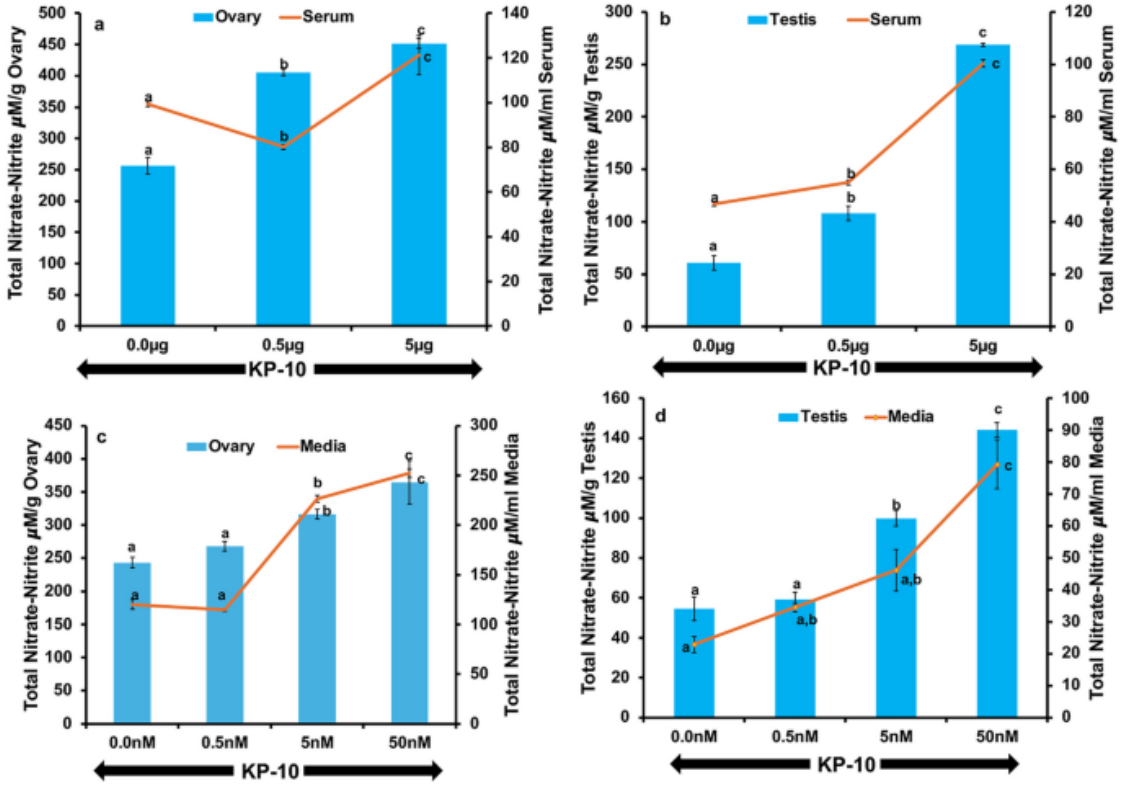


Fig. 3. Effect of different doses of KP-10 (0.5 and 5 µg) per 100 g body weight on total nitrate-nitrite in the ovary and serum (**a**) and testis and serum (**b**) of *Clarias batrachus* during mid-recrudescence phase (April). Each bar represents mean ± SEM ($n = 5$). Means bearing the same superscripts 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). Superscripts a, b, and c are used for NO levels in the ovary and serum in females and also for NO levels in the testis and serum in males. In vitro effects of different doses of KP-10 (0.5, 5, and 50 nm) on total nitrate-nitrite concentration in the ovary and medium (**c**) and the testis and medium (**d**) of *C. batrachus* during mid-recrudescence phases of the reproductive cycle. Each bar represents mean ± SEM ($n = 5$). Means bearing the 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, and c are used for total nitrate-nitrite levels in the ovary and medium as well as for total nitrate-nitrite levels in the testis and medium

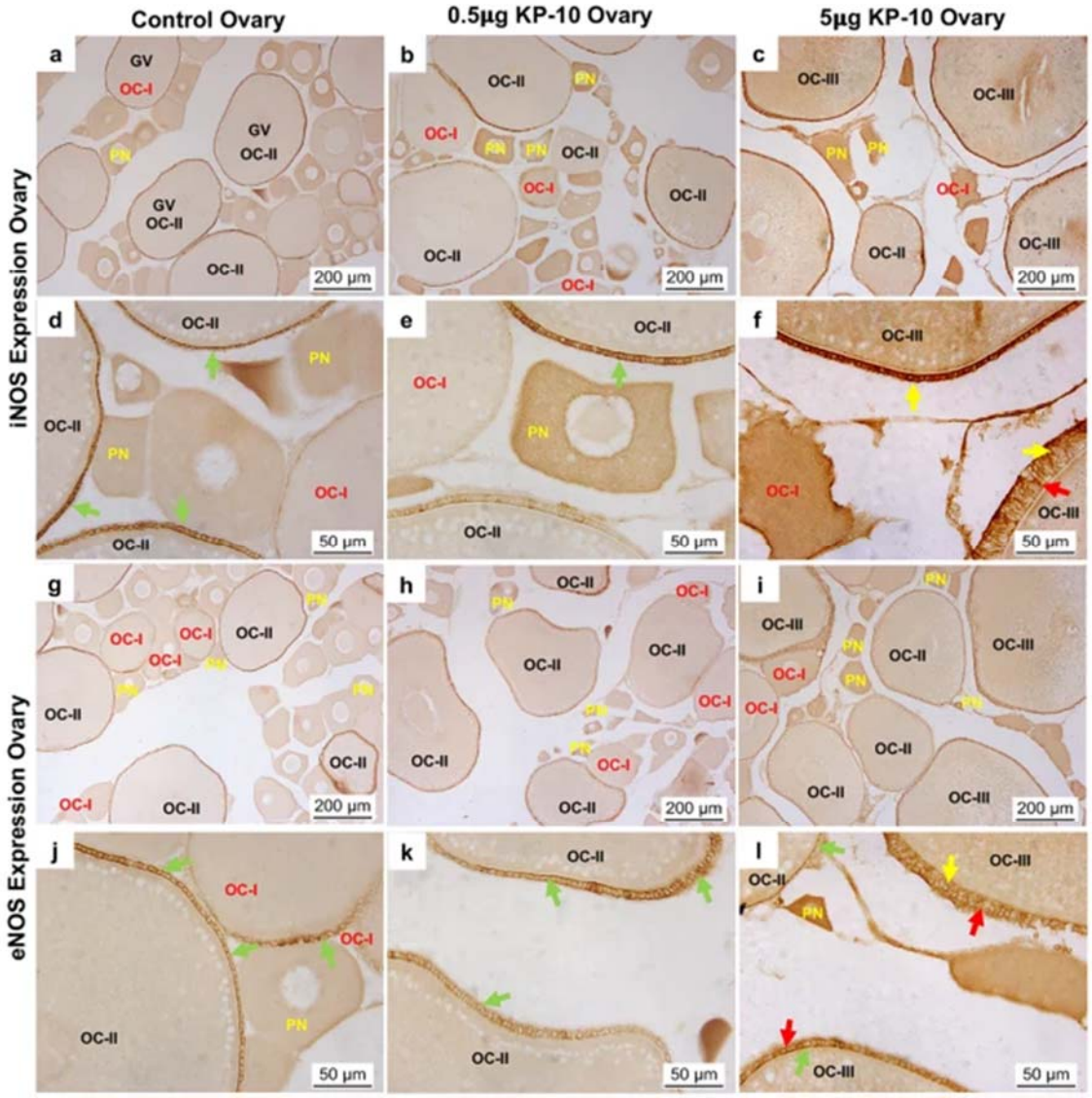
Similarly, KP-10 increased the levels of NO in testes and serum as well in a dose-dependent manner. High doses of KP-10 were more effective in increasing the level of serum and testicular testosterone significantly (Fig. 3b).

In vitro effects of KP-10 on NO level in gonadal fragments and culture medium

Treatment of KP-10 significantly increased the NO levels in ovarian and testicular explants and the respective medium199 in a dose-dependent fashion (Fig. 3c and d).

In vivo effects of KP-10 on the expressions of different isoforms of NOS in the ovary

KP-10 treatment during the mid-recrudescence phase stimulated the expression of iNOS, eNOS, and nNOS in the follicular layers (granulosa and theca cells) of the treated catfish in a dose-dependent manner, evident from its immunoreactive levels and IDV values (Fig. 4a–u).



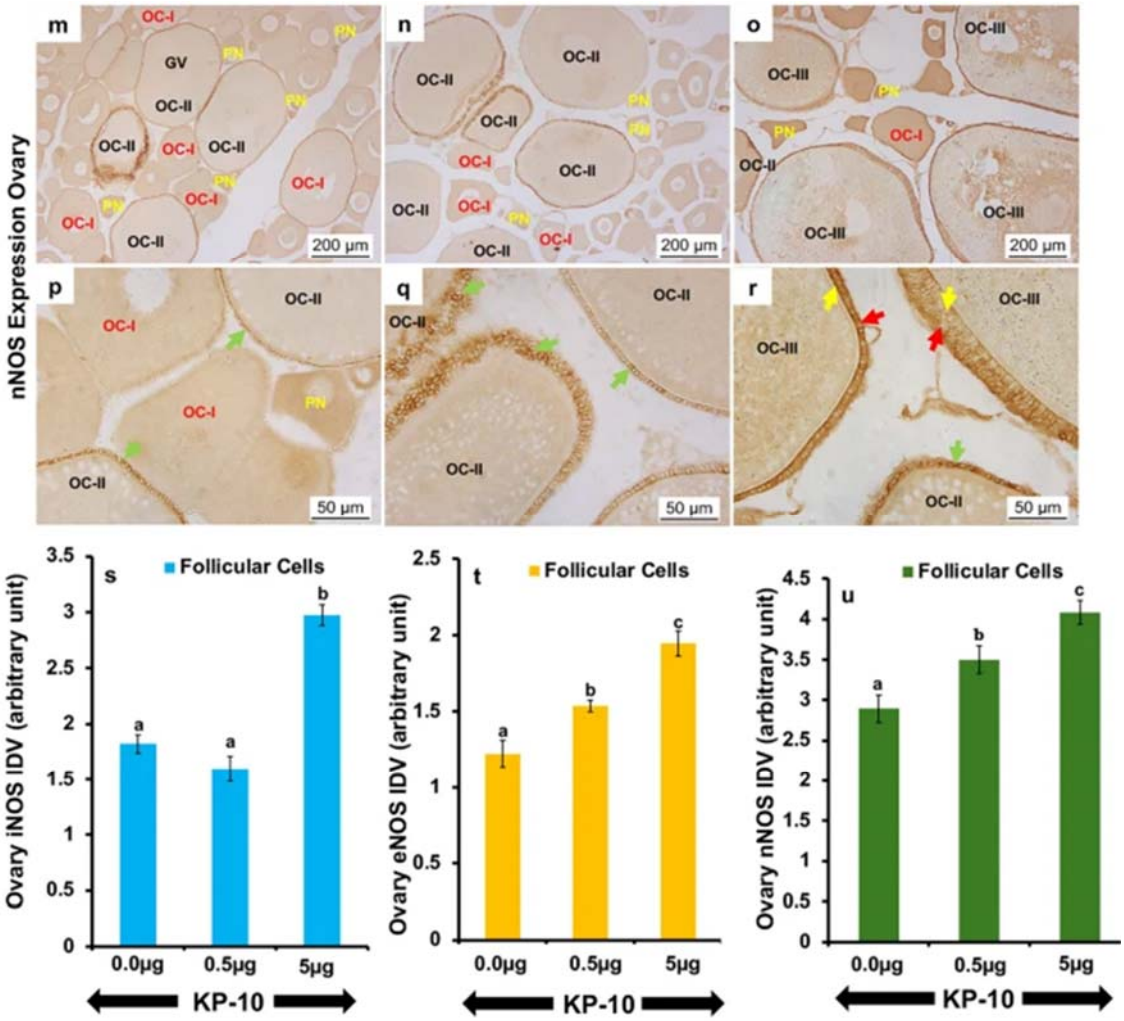
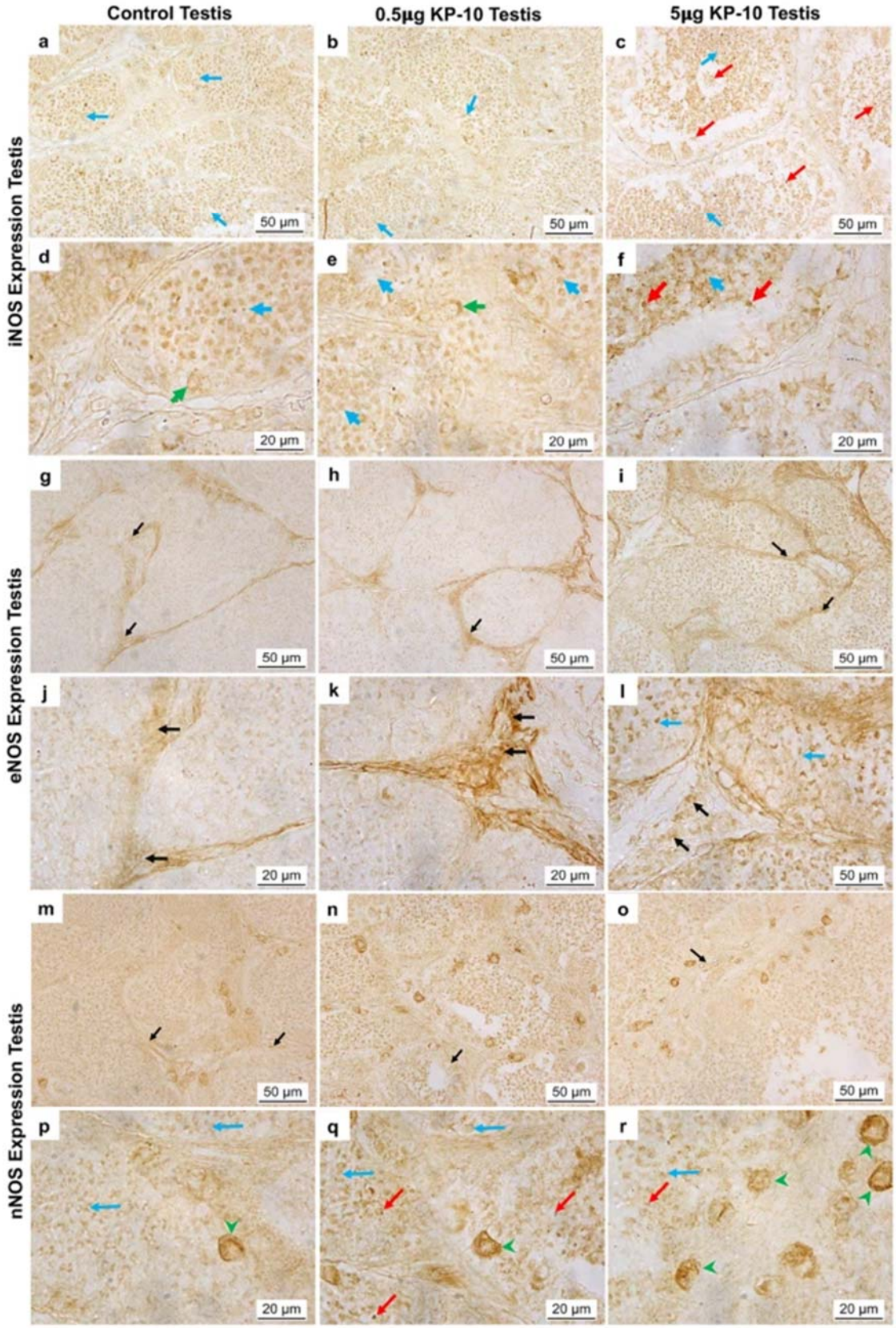


Fig. 4. Representative images of iNOS-, eNOS-, and nNOS-immunostained transverse sections of the ovary of *Clarias batrachus* treated with KP-10 (0.5 and 5 µg/100 g body weight) during mid-recrudescence phases (April) of the reproductive cycle (**a** to **c**, **g** to **i**, and **m** to **o** at 10 × and **d** to **f**, **j** to **l**, and **p** to **r** at 40 × magnification). Perinucleolar oocytes (PN), oocytes-I (OC-I), oocytes-II (OC-II), oocytes-III (OC-III), germinal vesicle (GV), follicular cells (light-green arrows), granulosa cell (yellow arrows), and thecal cell (red arrows). Effect of different doses of KP-10 (0.5 and 5 µg) per 100 g body weight on iNOS, eNOS, and nNOS immunoreactivity in the ovary of *C. batrachus*. The immunoreaction was quantified in terms of integrated density value per unit area (IDV) and is denoted in **s**, **t**, and **u**. 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). Superscripts a, b, and c are used to compare the iNOS, eNOS, and nNOS immunoreactions in the follicular cells of the ovary

In vivo effects of KP-10 on the expressions of different isoforms of NOS in testis

Treatment with KP-10 augmented the expression of iNOS, eNOS, and nNOS in the germ cells (spermatogonial/advanced germ cells) and Sertoli cells of the treated catfish in a dose-dependent fashion, evidenced by the IDV values (Fig. 5a–u).


Control Testis
0.5µg KP-10 Testis
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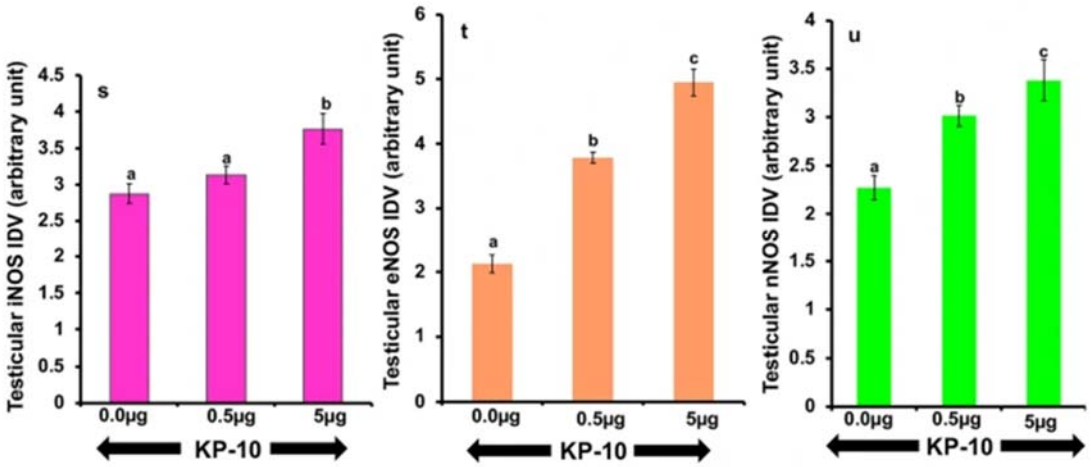

Fig. 5. Representative images of iNOS-, eNOS-, and nNOS-immunostained transverse sections of the testes of *Clarias batrachus* treated with KP-10 (0.5 and 5 µg/100 g body weight) during mid-recrudescence phases (April) of the reproductive cycle (**a** to **c**, **g** to **i**, and **m** to **o** at 10 × and **d** to **f**, **j** to **l**, and **p** to **r** at 40 × magnification). Interstitium (black arrows), seminiferous tubule (ST), spermatogonial stem cells (dark-green arrows), advance germ cells (red arrows), Sertoli cells (blue arrows), and spermatogonial cells (green arrowheads). Effect of different doses of KP-10 (0.5 and 5 µg) per 100 g body weight on iNOS (**s**), eNOs (**t**), and nNOS (**u**) immunoreactivity in the testis of *C. batrachus*. The immunoreactivity was quantified in terms of integrated density value per unit area (IDV) and is denoted in **s**, **t**, and **u**. 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). Superscripts a, b, and c are used to compare iNOS, eNOS, and nNOS immunoreactivity signals in the testes

In vivo effects of L-NAME on kiss1 expression and NO concentrations in the ovary and serum

The L-NAME suppressed the kiss1 expression in the ovary in a dose-dependent manner during the mid-recrudescence phase (Fig. 6a–l and o). Concurrent dose-dependent decline in ovarian and circulating NO levels was also recorded in response to L-NAME treatment (Fig. 6m and n).

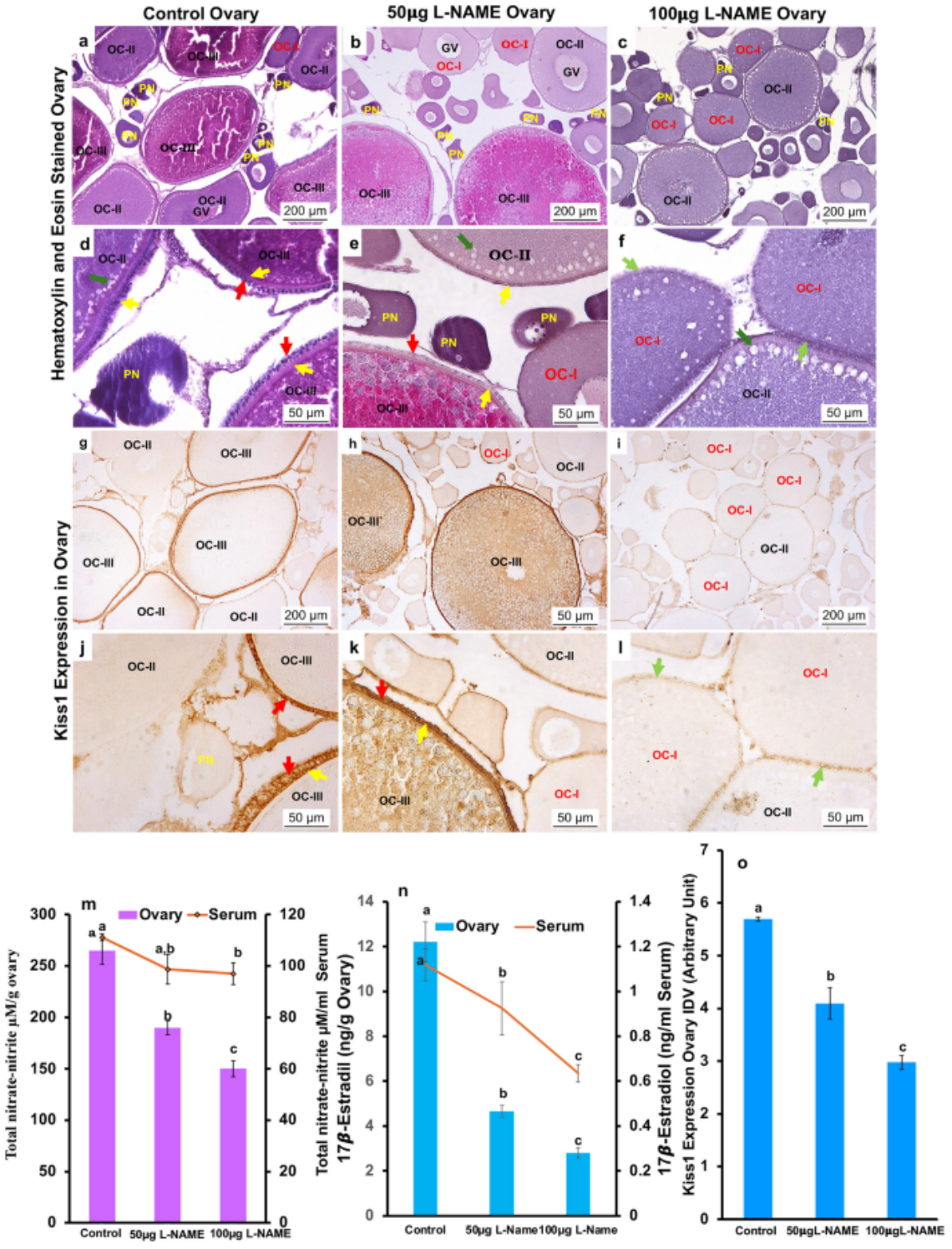

Fig. 6. Representative images of hematoxylin/eosin-stained and kiss1 immunopositive sections of the ovary of *Clarias batrachus*, treated with L-NAME (50 and 100 µg/100 g body weight) during the mid-recrudescence (April) (**a** to **c** and **g** to **i** at 10 × and **d** to **f** and **j** to **l** at 40 × magnification). Perinucleolar oocytes (PN), oocytes-I (OC-I), oocytes-II (OC-II), oocytes-III (OC-III), ovulated oocytes (OO), germinal vesicle (GV), cortical alveoli (dark-green arrows), granulosa cell (yellow arrows), thecal cell (red arrows), and follicular cells (light-green arrows). Effect of different doses of L-NAME (50 and 100 µg) per 100 g body weight on NO levels in the ovary and serum (**m**), 17β-estradiol in the ovary and

serum (**n**), and kiss1 expression (**o**) in the ovary of *C. batrachus*. The expression was quantified in terms of integrated density value per unit area (IDV) and is denoted in **m**. 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). Superscripts a, b, and c were used to compare NO expression, 17β-estradiol, and Kiss1 immunoreactivity

In vivo effects of L-NAME on kiss1 expression and NO concentrations in the testis and serum

A significant dose-dependent decline in kiss1 immunoreactivity in the testis was also observed following the L-NAME treatment during the mid-recrudescence phase (Fig. 7a–l and o). Unlike the findings in the ovary, the treatment of male catfish with a low dose of L-NAME did not alter the testicular NO level. However, its high dose significantly decreased the NO level in the testis and serum (Fig. 7m and n).

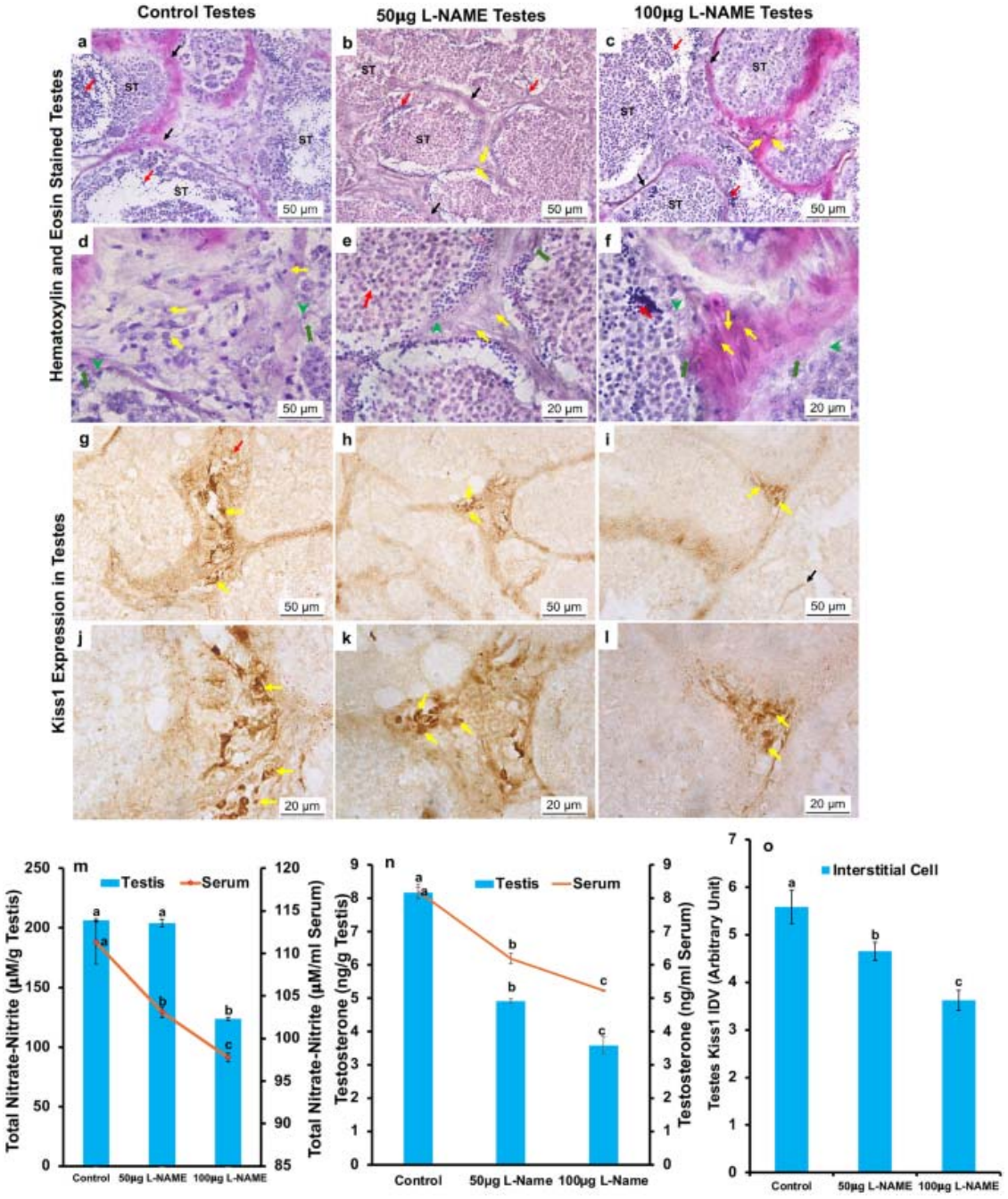

Fig. 7. Representative images of hematoxylin/eosin stained and kiss1 immunopositive sections of the testis of *Clarias batrachus*, treated with L-NAME (50 and 100 µg/100 g body weight) during the mid-recrudescence (April) (**a** to **c** and **g** to **i** at 10 × and **d** to **f** and **j** to **l** at 40 × magnification). Interstitium (black arrows), seminiferous tubule (ST), spermatogonial stem cells (brown arrows), interstitial cells (yellow arrows), advance germ cells (red arrows), and Sertoli cells (green arrowheads). Effect of different doses of L-NAME (50 and 100 µg) per 100 g body weight on NO levels in the testis and serum (**m**), 17β-testosterone in the testis and serum (**n**), and kiss1 immunoreactivity (**o**). The immunoreaction was quantified in terms of integrated density value per unit area (IDV) and is denoted in **o**. 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). Superscripts a, b, and c for NO, testosterone, and IDV were used to compare kiss1 immunoreaction

Discussion

The present study reports two novel observations: (i) NO is capable of modulating the expression of Kiss-1 in the gonads of the catfish, and (ii) kisspeptin stimulates the expression of different NOS isoforms. The study, thus, suggests the existence of a putative reciprocal interaction between the gonadal kisspeptin and nitric oxide in the regulation of gonadal steroidogenesis and, thereby, reproduction of the catfish.

Unfortunately, the studies analogous to the present one are practically not available for comparison and discussion with the present results, except for a few studies that demonstrate the presence of NOS/NO in the fish gonad and their role in the gonadal activity of the fish. Nath et al. (2019) have demonstrated the expression of nitric oxide synthase (NOS) in the ovary of *Anabas testudineus* and its involvement in maintaining meiotic arrest through the nitric oxide-cyclic GMP cascade. Their findings attribute the role of nitric oxide in preventing the meiotic resumption and thus governing oocyte maturation in *A. testudineus*. The existence of NOS and NO in the testes and ovary during the different reproductive phases of the catfish, *C. batrachus*, has also been demonstrated in the authors' laboratory (nee Pathak and Lal 2008, 2010; Singh and Lal 2017, 2016). In these studies, through a series of in vivo and in vitro approaches, authors have reported the stimulatory role of NO in gonadal steroidogenesis, thereby, in the development and maturation of viable eggs and sperms.

Nevertheless, the studies showing the presence of kisspeptin and its role in the gonadal activity of the fish are very few. Kiss transcripts are shown in the gonads of various fishes (Bakshi and Rai 2019; Chaube et al. 2020; Saha et al. 2016; Selvaraj et al. 2012; Shahi et al. 2017). The authors have also demonstrated the presence of kisspeptin peptide in the gonads of *C. batrachus* in their earlier studies (Singh et al. 2021a, b; Singh et al. 2023), although enough studies have been carried out on the presence of hypothalamic kisspeptin establishing its role in vertebrate reproduction, including fishes (Clarkson and Herbison 2006; Clarkson et al. 2014; Dunham et al. 2009; Escobar et al. 2013; see reviews Dufour et al. 2020; Sivalingam and Parhar 2022; Xie et al. 2022). Wang et al. (2022) have recently provided a comprehensive review of the diversity, evolution, and regulation of kisspeptins and their receptors in teleost fish brain, underscoring their crucial role in regulating reproductive processes and spawning behavior. They delve into the intricate underlying regulatory mechanisms governing the expression and activity of kisspeptin and its receptors in fish brains, including their environmental and hormonal regulation. They have also highlighted and offered valuable insights on advancing kisspeptin research and their potential applications in aquaculture and reproductive medicine. Sivalingam et al. (2022) have also thoroughly reviewed the studies carried out on the functions of hypothalamic kisspeptin in the regulation of vertebrate reproduction and its evolutionary significance. They have suggested that the role of hypothalamic kisspeptins in vertebrate reproduction is highly conserved throughout the evolutionary lineage of the vertebrates (Sivalingam et al. 2022). They have further reported that kisspeptin is crucial for initiating puberty and regulating gonadotropin-releasing hormone (GnRH) secretion, thereby influencing fertility across various vertebrate species (Sivalingam et al. 2022; Sivalingam and Parhar 2022).

Furthermore, enough studies have been carried out to examine the anatomical and reciprocal physiological interaction between nitrergic and kisspeptin systems in the vertebrate brain. Bedenbaugh et al. (2018) have shown a very high number of kiss neurons in the preoptic area (79%) and arcuate (99%) colocalized with nNOS. Close contacts between kisspeptin and nNOS neurons are demonstrated by them in certain brain areas of female sheep. Hanchate et al. (2012)

have reported nNOS neurons with kisspeptin receptors in close association with kisspeptin fibers in the preoptic area of the mouse brain, attributing their interactive action on GnRH secretion. García-Galiano et al. (2012) have established the physiological and chemical interactions between kisspeptin and NO as well as many other aminoacidergic and peptidergic neuropeptides in female rhesus monkeys, *Macaca mulatta*, in the regulation of GnRH secretion. Petrine et al. (2020) have recently shown that NO stimulates the expression of kiss1 in the arcuate region but not in the preoptic area of the Wistar rats, confirming nitrergic effects on kisspeptin expression. In fish like European sea bass, also, a reciprocal physiological relation between kisspeptin and NO in the brain has been studied. Escobar et al. (2013) have reported the expression of kissr2 in the NOS-positive neurons in the brain of European sea bass.

However, scientific reports on the interaction between kisspeptin and NO in vertebrate gonads, including fish, are not available so far. The present study, therefore, was undertaken to establish the reciprocal physiological relation between kisspeptin and NO in the fish gonads. Wherein L-NAME induced a decrease in gonadal kiss1 expression, while KP-10 stimulated the expressions of iNOS, eNOS, and nNOS with a concurrent increase in nitric oxide in the ovary and testis, which clearly suggests a link between gonadal nitric oxide and kiss1 in regulating their expression reciprocally. It appears that NO stimulates the kiss1 expression, and KP-10 augments the NOS immunoreactive signals in the fish gonad, as the decline in NO by L-NAME-induced inhibition of NOS activity resulted in decreased kiss1 expression. Conversely, KP-10 increases NO production by up-regulating NOS expression. Thus, it seems that a positive correlation exists between NO and kiss1 in fish gonads. No such report is available in any other fish; nevertheless, the aforesaid discussion on the reciprocal relationship between hypothalamic kisspeptin and NOS indirectly supports our hypothesis on the existence of reciprocal relations of kisspeptin-NOS/NO in fish gonads, too.

Although the present findings on the interaction between kiss1 and nitric oxide in *C. batrachus* require a detailed study to investigate the underlying mode, mechanism, and signaling systems of interaction between kiss1 and NOS-expressing cells in the gonad, the suggested interaction between kiss1 and NO-NOS appears to regulate the gonadal steroidogenesis by involving GnRH physiology in the fish gonad, as in another study, we have already demonstrated the expression of GnRH in the gonad of the present catfish (Singh et al. 2019). Neurotransmitters, neuropeptides, and hormones like the gonadotropin-releasing hormone (GnRH) are established as key players in regulating reproductive processes. The intricate neuroendocrine regulation of reproduction in teleost fish has been thoroughly reviewed recently by Trudeau (2022), emphasizing the interactions between different hypothalamic neuropeptides and neurotransmitters in the regulation of gonadal activity in the fishes. The authors have also reported earlier that kisspeptin-10 stimulates gametogenesis by enhancing gonadal steroid production and may act locally to regulate gonadal activities in an autocrine/paracrine manner, independent of known extra-gonadal factors in the catfish (Singh et al. 2021a, b).

Thus, the present study clearly establishes that KP-10 increases gonadal sex steroids (estradiol and testosterone) and NO in both sexes under in vivo and in vitro conditions in the catfish. The expressions of all the isoforms of NOS (iNOS, eNOS, nNOS) in the ovary and testis were stimulated by kisspeptin. However, the exogenous administration of NOS inhibitor, L-NAME, suppresses the kiss1 immunoreactivity, total nitrate-nitrite, 17β-estradiol, and testosterone in the ovary and testis. Thus, it appears that a reciprocal physiological relationship between kisspeptin and NO exists to regulate the gonadal activities of the catfish.

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Ethical approval

The experiments were conducted as per norms and guidelines of the Institutional Animal Ethics and Care of Banaras Hindu University, India (approval letter no. F.Sc./IAEC/2016–17/1136) and as per the Guidelines of the Committee for the Control and Supervision of experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India, New Delhi, India, for the experimentation on fishes.

Competing interest

The authors declare no competing interests.

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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