Changes in nitric oxide (NO) synthase isoforms and NO in the ovary of *Heteropneustes fossilis* (Bloch.) during the reproductive cycle

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Abstract

The purpose of the study was to demonstrate the presence of nitric oxide (NO) synthase (NOS) isoforms (neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS)) and the role of NO in the ovary of *Heteropneustes fossilis*. In one half of the ovary collected during different reproductive stages, NOS isoforms were localized immuno-histochemically in paraffin sections whereas the other half was processed for NOS and NO quantification using western blot followed by densitometry and nitrate/nitrite assay respectively. The role of NO on oocyte maturation was studied by examining the effect of NO donor (sodium nitroprusside; SNP) and NOS inhibitor (*N* ω -nitro-L-arginine methyl ester) on 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -P)-induced germinal vesicle breakdown (GVBD) in the cultured oocyte collected during prespawning phase. NOS immunostaining was predominantly localized in previtellogenic follicles, with nNOS detected in the nucleus and cytoplasm of oocytes whereas iNOS and eNOS localized in granulosa,

theca cells, and cytoplasm of oocytes. The NOS expression was higher in previtellogenic phase when compared with vitellogenic phase. The nitrate/nitrite concentrations in ovary showed gradual increase from recrudescence (4.9 ± 0.19 nM/mg protein) to late previtellogenic phase (7.02 ± 0.53 nM/mg protein), but showed a sharp decline during the vitellogenic phase (0.41 ± 0.053 nM/mg protein). Serum and ovarian nitrate/nitrite level showed a close association during the reproductive cycle. The results showed an increase in NOS activity and nitrate/nitrite concentrations as the follicle grow suggesting involvement of NO in follicular development. SNP significantly inhibited 17 α ,20 β -P-induced GVBD in fish oocytes. Thus, it is concluded that the fish ovary possesses NOS/NO system and a possibility that NO has a role in follicular development and regulation of oocyte maturation in fish, *H. fossilis*.

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Introduction

Nitric oxide (NO) is a biologically active-free radical molecule well recognized for diversity of its physiological function and general ubiquity (Dixit & Parvizi 2001). It is produced from the L-arginine by the action of enzyme NO synthase (NOS; Dixit & Parvizi 2001). NOS requires co-factor including NADPH in order to produce NO and L-citrulline (Bush *et al.* 1992). NOS enzyme exists in three isoforms that have been classified depending on tissue of origin as well as functional properties. Two of them, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutive and seem to be responsible for the continuous basal release of NO; the third one is inducible NOS (iNOS) and is expressed in response to inflammatory cytokines and lipopolysaccharides (Morris & Billiar 1994). Estimation of nitrate/nitrite in biological samples is used to provide indirect means of estimating endogenous NO (Archer 1993, Schulz *et al.* 1999). Because NO plays an important role in the function of the hypothalamus–pituitary–gonadal axis, its involvement in

mechanisms regulating the reproductive processes is quite obvious (Rosselli *et al.* 1998, Dixit & Parvizi 2001).

In ovary, expression and activity of eNOS and iNOS varies greatly in different animal species and throughout the different ovarian processes (Rosselli *et al.* 1998). In rat ovary, expression of eNOS increases after LH surge (Zackrisson *et al.* 1996, Jablonka-Shariff & Olson 1997), and eNOS-derived NO stimulates ovulatory process (Jablonka-Shariff & Olson 1998, Mitsube *et al.* 1999). The changes in iNOS expression during ovulatory process are controversial (Jablonka-Shariff & Olson 1997, Matsumi *et al.* 2000). The expression and functional activity of nNOS during follicular development and ovulation are not demonstrated in any mammalian species (Tamanini *et al.* 2003). The impairment of steroid production by NO has been demonstrated in human and rat (Tamanini *et al.* 2003). NO synthesis is shown to be important for oocyte maturation because eNOS knockout mice exhibited a reduced number of oocytes in metaphase II – a high percentage of oocytes remained in metaphase I or were atypical compared with controls (Jablonka-Shariff & Olson 1998, 2000).

Furthermore, sodium nitroprusside (SNP), a NO donor, has been demonstrated to stimulate meiotic maturation to metaphase II stages in cumulus-enclosed oocytes of mice (Sengoku *et al.* 2001). The studies available so far in mammals thus provide convincing evidence that NO is involved in many of the ovarian functions and plays a crucial role in reproductive processes. The information regarding the role of NO in the ovary of lower vertebrates is surprisingly lacking, although the presence of NOS has been demonstrated in the hypothalamo-hypophyseal complex of some fish species (Holmqvist *et al.* 2000, Oyan *et al.* 2000, Saiej *et al.* 2000, Cox *et al.* 2001).

The present study in the fish, *Heteropneustes fossilis*, aimed to: a) detect the presence and distribution of the NOS isoforms in the ovary by means of immunohistochemistry, b) determine the molecular weight of the NOS isoforms by western blot analysis, c) estimate changes in NOS activity and NO level in the ovary during reproductive cycle, and d) examine the role of NO in oocyte meiotic maturation. Finally, the fish *H. fossilis* was chosen because there is no data available in this vertebrate group and because of recent interest in the ovary of fish to improve aquaculture technology.

Materials and Methods

Animal

All experiments were conducted in accordance with the principles and procedures approved by the Departmental Research Committee of Banaras Hindu University, Varanasi, India. A total of 72 adult female *H. fossilis* (40–50 g) were used in the study. The fishes were collected from the pond in Varanasi (28° 18' N; 83° 1E) during different reproductive phases of annual reproductive cycle. They were maintained in laboratory conditions under natural photoperiod and temperature for a week and fed with minced goat liver which fish had access to *ad libitum*. In each reproductive stage, fish ($n=5$) were weighed and blood was collected by caudal cut for serum. Serum was separated by centrifugation at 1500 g and stored at -70°C . Fish were weighed and killed by decapitation and ovaries were dissected out and weighed. One lobe of ovary was fixed in Bouin's fixative for 8–10 h at room temperature, dehydrated in ethanol, cleared in xylene, embedded in paraffin, and serially sectioned at 6 μm . The other lobe of ovary was stored in -70°C for western blot and nitrate/nitrite assay.

Classification of ovarian follicle

The fishes were classified into the following reproductive phases, which were based on classification given by Vishwanathan & Sundararaj (1974) with some modification. The ovarian follicles, in different reproductive phases, were classified on the basis of follicle size, presence of vacuoles in the periphery, and amount and type of yolk accumulation.

a) Recrudescence: ovaries during this stage are small and translucent and contain primordial follicles lacking granulosa and theca cells (January). b) Early previtellogenic phase: primary follicles appear in the ovary and vacuole start appearing in cytoplasm of oocyte of primary follicle. In primary follicles granulosa cells appear and theca cells remain undifferentiated (February). c) Mid previtellogenic phase: the ovary increases in size and contains mainly stage-I secondary follicles. These follicles contain medium sized oocytes with opaque cytoplasm and varying amount of peripheral vacuoles. Follicles also contain granulosa cells and differentiating theca cells (March). d) Late previtellogenic phase: the ovary mainly contains medium sized secondary stage-II follicles. Oocyte has opaque cytoplasm containing pigmented granules. These follicles contain both granulosa and theca cells (April). e) Vitellogenic phase: the size of ovary increases and color of ovary turns greenish. The ovary mainly contains tertiary follicles with large oocytes and highly differentiated granulosa and theca cells. The cytoplasm of oocytes contains large yolk platelets (May–June). f) Spawning phase: the ovary contains fully developed follicles with maturational competence, ready for ovulation (July–August). g) Post-spawning phase/Resting phase: during the period, gradual re-absorption of unovulated follicles and regression of ovary occur (September–December). The reproductive stages were determined by histological examination of ovary and changes in gonado-somatic index (GSI). The GSI was calculated as (ovarian weight/body weight) $\times 100$ and expressed as gram percent.

Antibodies

Immunohistochemistry and western immunoblotting was performed using rabbit polyclonal antibodies against nNOS (SC-1025, Lot # L1903), iNOS (SC-651, Lot # F0204), and eNOS (SC-8311, Lot # L1505) purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The nNOS and iNOS antigen was peptide mapping at the N-terminus of human origin and eNOS was 2–160 peptides of human origin. The primary antibodies specifically recognize either nNOS (155 kDa), iNOS (130 kDa), or eNOS (140 kDa) proteins and has no cross-reactivity with other NOS isoforms (technical notes).

Immunohistochemistry

The ovarian sections were processed through standard protocol of immunohistochemistry (Singh *et al.* 2007). After deparaffinization and rehydration, endogenous peroxidase was quenched with 0.3% H_2O_2 , equilibrated in 0.05 M Tris-Cl to 0.15 M NaCl (TBS (pH 7.4)). The sections were treated with normal goat serum equilibrated (1:100 v/v) in 0.05 M phosphate buffer to 0.15 M NaCl (PBS (pH 7.4)) for 30 min to reduce background staining. The tissue sections were incubated with primary antibody (dilution 1:50 in PBS (pH 7.4)) for 45 min at room temperature. The control was performed by replacing the primary or secondary antibody

with PBS. The detection system used was ABC staining kit (sc-2018; Santa Cruz Biotech. Inc). The peroxidase activity was revealed by staining with 0.03% of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) in 0.01 M Tris-Cl (pH 7.6) and 0.1% H₂O₂. Nucleus was counter-stained with Elrich's hematoxylin.

Western immunoblot

The ovarian tissue protein was extracted as described elsewhere (Chanda *et al.* 2004). Equal amounts of protein (100 µg) as determined by Folin's method was loaded on 8% SDS-PAGE for electrophoresis. Proteins were transferred to nitrocellulose (NC) membrane (Sigma). Non-specific sites were blocked with 5% nonfat dried milk in TBS containing 0.02% Tween 20. Membranes were then incubated with rabbit anti-human nNOS, or iNOS, or eNOS antibodies (at dilution of 1:500). Immunodetection was performed with anti-rabbit IgG-conjugated horseradish peroxidase (at dilution of 1:500). Finally, a membrane was developed with DAB. The protein loading was checked by Ponceau S staining. The same procedure was used to validate the specificity of antibodies (NOS isoforms) using the positive control samples, rat cerebellum for nNOS, arterial endothelial tissues for eNOS, and lipopolysaccharide-induced peritoneal murine macrophages for iNOS, and compared with samples from fish ovary in each case. The tissue for positive control was selected as described previously (Nassauw *et al.* 1999; Technical notes, Santa Cruz). NC membrane was developed with chemiluminescent reagent (ECL; Bio-Rad) for the purpose of detecting bands of weak intensity. Blot for each protein was repeated thrice. The densitometric analysis of blots was performed by scanning and quantifying the band for density value by using computer assisted image analysis system (Image J 1.38X, NIH, Bethesda, MD, USA). The densitometry data were presented as the mean of the integrated density value $\pm$ S.E.M.

Determination of nitrate/nitrite

The nitrate/nitrite concentrations were measured together in the serum and tissue homogenates as described by Sastry *et al.* (2002). Briefly, 100 µl sample or standard (potassium nitrate) was mixed with 400 µl of 0.55 M carbonate buffer (pH 9.0). Approximately, 100 mg activated copper-cadmium alloy filings were added to the samples and the mixture was incubated at 30 °C for 1 h with gentle shaking. Then, 100 µl of 0.35 M sodium hydroxide and 400 µl of 0.12 M zinc sulfate were added and incubated for 10 min at room temperature. The tubes were centrifuged at 1600 g for 10 min and the supernatant (150 µl) was transferred to wells of microtiter plates in triplicate. Then, 75 µl of 1% (w/v) sulfanilamide and 75 µl of 0.1% (w/v) N-naphthalenediamine were gently added, incubated for 10 min, and the absorbance was measured at 545 nm in a micro plate reader (ECIL MS5605A, India).

Effect of NO on meiotic maturation

Gravid female fish ($n=3$) in late prespawning phase (June) were killed and healthy oocytes were collected for incubation. The oocyte culture medium contained 3.74 g NaCl, 0.32 g KCl, 0.16 g CaCl₂, 0.1 g NaH₂PO₄·2H₂O, 0.16 g MgSO₄·7H₂O, 0.8 g D-glucose, 0.008 g phenol red dissolved in one liter of triple distilled water (pH 7.5), adjusted with 1 N sodium bicarbonate and supplemented with antibiotics, penicillin (200 IU/ml), and streptomycin sulfate (0.2 mg/ml). About 40–50 oocytes were incubated for 1 h in 3 ml medium containing different concentrations of SNP, which is metabolized to donate NO in medium (SNP; 10, 50, 100, 250, and 500 µM; Sigma). Subsequently, the oocytes were incubated in fresh medium containing different concentrations of SNP together with, 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -P; 0.05 µg/ml; Sigma) for 12 h. Based on our preliminary study, a dose of 17 α ,20 β -P was selected which was inducing optimum percentage (about 60%) of germinal vesicle breakdown (GVBD), as an index of oocyte maturation in 12 h. The experiment was extended for 24 h to study the effect of SNP. The control oocytes were incubated with only 17 α ,20 β -P, since GVBD do not occur spontaneously, *in vitro*, without any treatment in this fish. A similar experiment design was set with NOS inhibitor and N ω -nitro-L-arginine methyl ester (L-NAME; 10, 50 and 100 mM; Sigma) instead of SNP. The dose of SNP and L-NAME was based on the study in mice (Nakamura *et al.* 2002). Each incubation was done in triplicate at 25 $\pm$ 1 °C. After 12 h and 24 h of incubation, the oocytes were fixed in mixture of ethanol:formalin:acetic acid (6:3:1) for scoring the percentage of GVBD. GVBD stage was determined by microscopic observation of the disappearance of germinal vesicles in the oocytes.

Data analysis

All the data were analyzed by student *t*-test or one-way ANOVA followed by Scheffe's test. Differences were considered to be significant at $P < 0.05$. We performed each experiment at least twice using different batches of animals to confirm the results.

Results

Immunolocalization of NOS isoforms in ovary

The presence of all three isoforms of NOS was demonstrated immunocytochemically in the ovary of *H. fossilis* (Fig. 1; Table 1). The sections from three ovaries were evaluated with a minimum of ten follicles of each stage for immunoreactivity. The immunoreactivity of nNOS was observed predominantly in the nucleus of smaller primordial follicles and faint immunoreactivity can also be seen in the cytoplasm, whereas larger primordial follicles showed immunostaining, both in the nucleus as well as in cytoplasm (Fig. 1A). As the follicle grow

Figure 1 Representative images of transverse sections of the ovary of *H. fossilis* during different reproductive phases showing immunolocalization of (A–F) nNOS, (G–L) iNOS, and (M–R) eNOS. Ovarian sections were counterstained with hematoxylin. Recrudescence phase: note a positive (A) nNOS immunoreactivity predominantly in nucleus, but not (G) iNOS or (M) eNOS staining, in smaller primordial follicles. Early previtellogenic phase: (B) nNOS localized in cytoplasm and nucleus but (H) iNOS and (N) eNOS in cytoplasm of primary follicle. Mid previtellogenic phase: nNOS detected in cytoplasm but not in (C) granulosa and theca cells, and weak staining of iNOS in (I) granulosa and cytoplasm but intense staining of eNOS in (O) granulosa, theca and cytoplasm in secondary-I follicles. Late previtellogenic phase: a positive (D) nNOS in nucleus, and faint immunoreactivity of (J) iNOS and (P) eNOS in theca cells with moderate staining in cytoplasm in secondary-II follicles. Vitellogenic phase: note that nNOS (E), iNOS (K), and eNOS (Q) were not detected in tertiary follicles. Resting phase: positive (F) nNOS immunoreactivity, but not (L) iNOS or (R) eNOS in the follicles of resting phase ovary. Negative control showed (S) no staining. n, nucleus; cp, cytoplasm; gc, granulosa cells; tc, theca cells; and pc, primordial. Scale bar, 20 μ m in figure C, I, J, K, O, P; 50 μ m in figure B, D, E, H, M, N, Q; and 100 μ m in figure A, F, G, L, R. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-07-0509>.

from small primordial follicle to secondary stage, nNOS immunoreactivity spread from the nucleus of the oocyte to the cytoplasm (Fig. 1A–C). From stage I to stage II of the secondary follicle, nNOS is not expressed in the cytoplasm of

the oocyte (Fig. 1D). Finally, nNOS immunoreactivity was not detected in the nucleus and cytoplasm of oocyte of tertiary follicle during vitellogenic phase (Fig. 1E). The primary follicle during early recrudescence showed more intense

Table 1 Immunoreactivity of various nitric oxide synthase (NOS) isoforms in different stages of follicles

	nNOS	iNOS	eNOS
Follicle			
Primordial			
Nucleus (GV)	+++	—	—
Cytoplasm	+/-	+/-	+/-
Granulosa	*	*	*
Theca	*	*	*
Primary			
Nucleus (GV)	++	—	—
Cytoplasm	+++	+	++
Granulosa	—	+	+
Theca	*	*	*
Secondary stage-I			
Nucleus (GV)	++	—	—
Cytoplasm	++	+	++
Granulosa	—	+	+++
Theca	—	+/-	++
Secondary stage-II			
Nucleus (GV)	++	—	—
Cytoplasm	+/-	+	+
Granulosa	—	+/-	+/-
Theca	—	+/-	+
Tertiary			
Nucleus (GV)	—	—	—
Cytoplasm	—	—	—
Granulosa	—	—	—
Theca	—	—	—
Resting			
Nucleus (GV)	+/-	—	—
Cytoplasm	++	—	—
Granulosa	*	*	*
Theca	*	*	*

Scores for intensity of immunoreactivity are as follows: —, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; *absence of the cells in the sections. The sections from three ovaries were evaluated with minimum ten follicles of each stage for immunoreactivity, ($n=3$).

immunoreactivity than the primary follicles during post-ovulatory stage (Fig. 1A and F). The iNOS and eNOS immunoreactivity was not detected in the nucleus of primordial follicles and a faint immunoreactivity was observed in the cytoplasm of oocytes (Fig. 1G and M). The iNOS and eNOS immunoreactivity was detected in oocytes cytoplasm of primary follicle (Fig. 1H–N). The differentiating granulosa and theca cells of the secondary stage-I follicles showed weak iNOS immunoreactivity (Fig. 1I) and intense eNOS immunostaining (Fig. 1O). The iNOS and eNOS immunoreactivity was faint in theca cells of secondary stage-II follicles and not detected in granulosa cells (Fig. 1J and P). The iNOS and eNOS expression was not detected in tertiary follicles (Fig. 1K and Q) and resting phase follicles (Fig. 1L and R).

The presence of all three NOS isoforms in the ovary of *H. fossilis* was confirmed with western blot (Fig. 2). The expression levels of nNOS, iNOS, and eNOS from the ovarian homogenate of the *H. fossilis* (March) were assessed semi-quantitatively by western blotting followed by densitometry. Immunoreactivity for all three NOS isoform was

Figure 2 Representative western immunoblot for validation of antibody specificity of NOS isoforms (nNOS, iNOS, and eNOS) in the ovary of fish (lane; b, d and f), which is compared with known mammalian positive control (lane; a, cerebellum; c, macrophage; and d, endothelial cell lysate respectively). The main band of nNOS protein is shown at 150 kDa, while iNOS and eNOS protein at 130 kDa. Additional weak immunoreactive bands were also recognized at 80 and 48 kDa against nNOS, and 60 kDa against eNOS and iNOS.

recognized by staining with antibodies of respective NOS isoforms. The major band of each of these NOS isoforms in ovarian samples corresponds closely with the major band of their respective positive control tissue (mammalian cerebellum for nNOS, macrophage for iNOS, and endothelial cells for eNOS). In the fish ovary, both iNOS and eNOS showed a minor band also at ~60 kDa. Whereas immunostaining with anti-nNOS showed two minor bands at ~80 and ~48 kDa (Fig. 2). An identical NC membrane incubated in the absence of primary antibody showed no immunoreactive bands.

Changes in expression of NOS isoforms during reproductive cycle

The changes in the intensity of nNOS, iNOS, and eNOS immunostaining were measured semi-quantitatively by densitometry following western blotting in the ovary during different phases of the reproductive cycle and shown in Fig. 3. Ovarian expression of each NOS isoform gradually increases ($P<0.05$) from recrudescence to late previtellogenic phase when it showed the highest concentration. Immunostaining of all three NOS isoforms showed only a moderate increase ($P>0.05$) from early to mid previtellogenic phase, however, showed a sharp decline ($P<0.001$) from late previtellogenic to vitellogenic phase. Immunostaining of all three NOS isoforms showed an increase ($P<0.05$) from vitellogenic to post-spawning phase.

Changes in NO concentrations during reproductive cycle

The ovarian and serum nitrate/nitrite concentrations changes significantly ($P<0.05$) during reproductive cycle (Fig. 4). The nitrate/nitrite concentrations gradually increased from recrudescence to attain a peak during late previtellogenic stage.

Figure 3 Representative western immunoblot analysis of NOS isoforms in the ovary of *H. fossilis* during different reproductive phases. Histogram ((A) nNOS, (B) iNOS, and (C) eNOS) represents densitometric analysis of the immunoblots, and values are expressed as mean $\pm$ S.E.M. Each value is represented by superscript (a–e) indicating similarities and differences between specific values. Values with similar letter are statistically not different ($P > 0.05$), whereas the values with dissimilar superscript are significantly different ($P < 0.05$) from each other (based on Scheffe's multiple range test, $n = 3$). Vg, vitellogenic.

This increase showed close association with ovarian growth (presented as GSI) during previtellogenic phase. However, the greater GSI value corresponds with very low value for nitrate/nitrite concentration during vitellogenic phase. The nitrate/nitrite concentration remained low during post-spawning phase ($P < 0.05$; Fig. 4). The serum NO level was higher during mid- and late previtellogenic phase (March and April) in comparison with the rest of the reproductive phases of *H. fossilis*.

Effect of NO on oocyte maturation in vitro

The oocytes were cultured with different concentrations of SNP in the presence of $17\alpha, 20\beta$ -P showed significant decrease in incidence of GVBD only at higher concentrations of SNP (250 and 500 μ M) after 12 h culture as compared with

the control (Table 2). The incidence of GVBD in oocytes induced with $17\alpha, 20\beta$ -P was taken as control. No effect of SNP on oocyte maturation was observed after 24 h incubation (data not shown). In addition, the NOS inhibitor L-NAME did not affect oocyte maturation (data not shown).

Discussion

Although several studies have described the presence and distribution of NOS–NO system in the brain of the majority of the vertebrate classes (for review, see Panzica *et al.* 1998, 2006), the studies on the distribution of NOS isoforms or the NO generating system and their physiological significance in the ovary has been mostly demonstrated in mammalian species (Tamanini *et al.* 2003, Huo *et al.* 2005). This is the first

Figure 4 The figure represents variation in ovarian and serum nitrate/nitrite concentrations during different reproductive phases (bottom part) and corresponding changes in Gonado-somatic index (top part) in *H. fossilis*. Values are expressed as mean $\pm$ s.e.m. Each value is represented by superscript (a–d) indicating similarities and differences between the specific values. Values with similar letter are statistically not different ($P > 0.05$), whereas the value with dissimilar superscript are significantly different ($P < 0.05$) from each other (based on Scheffe's multiple range test, $n = 5$). Vg, vitellogenic.

detailed study demonstrating the presence of all three isoforms of NOS using immunocytochemical localization and western blot analysis in the fish ovary. The study further showed a significant variation in the concentrations of nitrate/nitrite, suggesting the rate of NO production, in the fish ovary during different reproductive stages. The presence of three isoforms of NOS and significant seasonal variation in the production of NO in the ovary thus suggests the importance of NOS–NO system in seasonal ovarian activity of *H. fossilis*.

Table 2 Percentage of oocytes showing germinal vesicle breakdown (GVBD) following treatment with sodium nitroprusside (SNP; 10, 50, 100, 250, and 500 μ M), in $17\alpha,20\beta$ -P (0.05 μ g/ml) induced oocytes, after 12 h, *in vitro*. Each incubation well contains 40–50 oocytes collected from the ovary of three animals

Treatment group	Percentage of oocytes at GVBD stage
Control ($17\alpha,20\beta$ -P)	62.66 $\pm$ 2.96
$17\alpha,20\beta$ -P + 10 μ M SNP	63.38 $\pm$ 1.98
$17\alpha,20\beta$ -P + 50 μ M SNP	61.33 $\pm$ 2.56
$17\alpha,20\beta$ -P + 100 μ M SNP	58.66 $\pm$ 2.04
$17\alpha,20\beta$ -P + 250 μ M SNP	49.00 $\pm$ 2.3*
$17\alpha,20\beta$ -P + 500 μ M SNP	46.00 $\pm$ 1.73*

Values are the mean $\pm$ s.e.m. ($n = 3$), *Significant at $P < 0.05$ versus control.

This study demonstrates that nNOS, iNOS, and eNOS show distinct cell-specific distribution patterns in different parts of oocyte–follicle of fish ovary. Most of the earlier studies in the ovaries of human and rodents showed immunolocalization of eNOS and iNOS, but nNOS could not be detected in these species (Tamanini *et al.* 2003). Recently, in porcine ovary, nNOS immunoreactivity was demonstrated in the oocytes, theca, and granulosa cells of multilaminar and antral follicles, and stroma (Kim *et al.* 2005). In the fish ovary, NOS immunostaining is predominantly localized in developing previtellogenic follicles, with nNOS mainly located in ooplasm of primary follicles whereas iNOS and eNOS localized in granulosa, theca cells, and cytoplasm of oocytes of secondary follicles. Unlike the porcine, nNOS in fish was not detected in granulosa or theca cells. Furthermore, in the fish ovary, both iNOS and eNOS showed nearly identical pattern of immunolocalization. The NOS isoforms immunolocalized in previtellogenic follicles and not detected in the vitellogenic/preovulatory tertiary follicles. Western blot also showed a sharp decline in the NOS immunoreactivity during vitellogenic period when ovaries contains mostly tertiary follicles.

The presence of NOS isoform proteins in the ovary of fish was further detected by immunoblotting using antibodies raised against human NOS isoforms. The major band of iNOS and eNOS protein was detected at ~ 130 kDa, whereas nNOS protein was detected at ~ 150 kDa. A few additional immunoreactive bands were also observed against each NOS isoforms. Similarly, a second minor band at ~ 48 kDa immunostained with eNOS was demonstrated in human testis and epididymus (Zini *et al.* 1996). The control mammalian endothelial cells also showed a second band in staining with eNOS antibody. The molecular mass of the bands obtained in protein preparation of quail ovary was much lower than the molecular mass of the bands obtained from protein preparation of positive control tissues when stained with anti-eNOS and iNOS (Nassauw *et al.* 1999). A possible explanation could be that during homogenization, proteolysis has occurred. Proteolysis of NOS generates two fragments, the oxygenase and the reductase fragments (Yun *et al.* 1996). Nassauw *et al.* (1999) could detect only the reductase fragments of eNOS and nNOS in the quail ovary homogenates. It has been found that in mammals, the reductase fragment of nNOS has a molecular mass of 79 kDa (Yun *et al.* 1996). Nassauw *et al.* (1999) demonstrated an nNOS band at 155 kDa in protein from rat pituitary lysate and an eNOS band at 140 kDa in the protein preparation from human endothelial lysate. The molecular mass of the iNOS band in the ovarian homogenates of quail is 120 kDa. The results from quail ovary differs significantly from other studies in mammals as well as the present study on fish. The difference in the molecular masses of major and minor bands of various isoforms described in present as well as earlier studies might be due to the presence of tissue and species-specific proteases (Lloyd *et al.* 1995, Zini *et al.* 1996) or due to differences in the immunodetection method used. The present

study in fish and earlier study on birds suggest that multiple NOS isoforms exist in ovaries of non-mammalian species.

The NO synthesized in tissue is completely metabolized to nitrate and nitrite (NO_2 and NO_3). Therefore, biochemical estimation of nitrate/nitrite provides indirect means of estimating endogenous NO concentration (Schulz *et al.* 1999). The changes in nitrate/nitrite concentrations in fish ovary, suggesting rate of NO production, undergoes a gradual increase from recrudescence to active growth stages of previtellogenic phase, but showed a sharp decline during the vitellogenic phase and remained low in the fish ovary during spawning and post-spawning/resting phases. Serum and ovarian nitrate/nitrite level showed a close association during reproductive cycle. Changes in NOS activity as determined by western blotting during different reproductive phases corresponds closely with changes in nitrate/nitrite concentration in the ovary of fish. Thus suggesting that the level of NOS directly affects the rate of NO production in the ovary of fish. As nitrate/nitrite concentration in the ovary showed gradual increase, similarly three NOS isoforms in ovary showed variation in immunostaining during follicular development. The present study thus suggests that changes in NOS activity and rate of production of NO undergo changes in close association with follicular development in the ovary of fish. Similar changes in circulating nitrate/nitrite concentration during follicular development were observed in women undergoing *in vitro* fertilizations (Rosselli *et al.* 1994, Anteby *et al.* 1996). Manwar *et al.* (2006) recently demonstrated that NO regulates follicular development and hierarchy in the quail ovary. The higher serum nitrate/nitrite concentrations were found to be associated with higher egg production in quail (Manwar *et al.* 2006).

The present study showed an increase in NOS immunostaining and NO concentration in the ovary of *H. fossilis* from recrudescence to late previtellogenic stage coinciding with an increase in previtellogenic follicular growth, and decrease in their concentration during vitellogenic phase when proliferation of the follicle decreases (Patino & Sullivan 2002). Thus, it may be hypothesized that NO acts as a growth promoting factor in the ovary of fishes. A similar observation has been described earlier in mammals (Tamanini *et al.* 2003). Rosselli *et al.* (1994) demonstrated significant increase in nitrate/nitrite (NO) levels in the mammalian ovary during follicular growth. Follicular development induced by pregnant mare's serum gonadotropin in immature rat is associated with an increase in eNOS expression (Van Voorhis *et al.* 1995, Jablonka-Shariff & Olson 1997). A growth promoting effect of NO is further supported by the observation (Hattori *et al.* 1996) that NO increases epidermal growth factor receptor in rat granulosa cells, and interleukin-1 β stimulated NO production is effective in promoting muscle cell growth in the presence of basic fibroblast growth factor (Dubey *et al.* 1997). It may be hypothesized that NO regulated follicular development and these effects of NO are strongly dependent on interactions with other growth modulatory factors acting within the ovary. Members of the

transforming growth factor β (TGF- β) superfamily, such as TGF- β , activin, inhibin, growth differentiation factor 9 (GDF9), and several bone morphogenetic proteins (BMP), have been shown to have a role in controlling ovarian follicle development in mammals (Chabbert-Buffet & Bouchard 2002). By contrast, little is known about their influence on ovarian functions in fish, although TGF- β family members including TGF- β , activin β A, inhibin, GDF9, BMP4, and BMP7 have been demonstrated in the gonad of fish (Bobe *et al.* 2004, Kohli *et al.* 2005).

The observation of an increase in NOS/NO level during previtellogenic phase and a sharp decline in the level of NOS/NO during vitellogenic phase may be associated with the changes in the steroid concentrations reported in *H. fossilis* (Lamba *et al.* 1983). They have reported that the estradiol level sharply increases during vitellogenic phase. This suggests that an increase in estradiol level coincides with the period of sharp decline in NO level in the fish. This observation thus agrees with an earlier finding in rats suggesting an inhibitory effect of NO on estradiol level (Dong *et al.* 1999). The inhibitory effect of NO on estradiol production may be exerted through androstenedione production as shown in rat (Dunnam *et al.* 1999) or through an inhibitory effect of NO on cytochrome P450 steroidogenic enzyme (Van Voorhis *et al.* 1994, Peterson *et al.* 2001). However, further investigation is needed to establish this hypothesis.

The present study demonstrated that 17 α ,20 β -P induces GVBD in the cultured oocytes of fish, but treatment with increasing concentration of NO donor, SNP, partially prevented the GVBD in the 12 h culture of fish oocytes. Whereas treatment with increasing concentrations of NO inhibitor, L-NAME, did not affect the rates of GVBD in cultured oocytes. The finding in fish is supported by the recent study on bovine oocytes, where the addition of increasing concentration of SNP to the maturation medium blocked the progression from metaphase I to metaphase II after 24 h culture (Viana *et al.* 2007). Both stimulatory and inhibitory effects of NO on oocyte maturation have been demonstrated in mammals (Bu *et al.* 2003, Tao *et al.* 2004). In quail ovary, egg production increases in L-arginine-treated group whereas it was not different in SNP and L-NAME treated group (Manwar *et al.* 2006). These findings suggest that NO may have a crucial role in oocyte maturation in vertebrates. But the exact roles of NO in oocyte maturation remain unclear and need to be studied further. Several studies on mammalian species suggest an involvement of the NOS–NO system in ovulatory mechanism(s), mainly via its effects on vasculature and prostaglandins production (see review, Tamanini *et al.* 2003). Administration of iNOS inhibitor has been reported to suppress the ovulation in rat, an effect that can be reversed by SNP treatment (Shukorski & Tsafiriri 1994). On the contrary, the present study on fish showed a sharp decline in the NOS–NO system prior to ovulation. A very mild or no immunoreactivity of NOS isoforms in tertiary follicles, NOS isoforms activity, and nitrate/nitrite concentration during vitellogenic phase in the fish ovary

showed a sharp decline of NO during vitellogenic phase. Recent study on rat also showed a significant decrease in nitrate/nitrite concentration in preovulatory follicles following hCG treatment (Yamagata *et al.* 2002). Further study on rat showed that treatment with NO donor prevented oocyte maturation *in vitro* (Nakamura *et al.* 2002). The findings in fish and rat together suggest that NO may be one of the inhibitors of oocyte maturation in these species. A high concentration milieu of NO most likely plays a role in the meiotic arrest of oocytes and decline in NO may be required for oocyte maturation. The inhibitory role of NO is supported by the present experimental study demonstrating the suppressive effect of NO donor on GVBD of fish oocyte.

In summary, this study clearly demonstrates that three isoforms of NOS in the ovary of fish, *H. fossilis*, are expressed at protein level. The immunohistochemical studies showed nNOS was localized mainly in the oocytes, but iNOS and eNOS were localized mainly in cytoplasm of oocyte, granulosa, and theca cells of previtellogenic follicles. A gradual increase in NOS activity and nitrate/nitrite concentrations during follicular development suggest its role in folliculogenesis and differentiation of granulosa and theca cells. The *in vitro* study suggests an inhibitory effect of NO on meiotic maturation. It is concluded that the fish ovary possesses a NOS–NO system, and that NO may be considered as mediator involved in follicular development and regulator of oocyte maturation. Further studies on the mechanism of action of NO during oocyte maturation *in vitro* may help to improve our understanding of fish reproductive processes.

Declaration of interest

We declare that there is nothing which conflict the interest to the impartiality of the work.

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References

- Anteby EY, Hurwitz A, Korach O, Revel A, Simon A, Finci-Yeheskel Z, Mayer M & Laufer N 1996 Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. *Human Reproduction* **11** 1947–1951.
- Archer S 1993 Measurement of nitric oxide in biological models. *FASEB Journal* **7** 349–360.
- Bobe J, Nguyen T & Jalabert B 2004 Targeted gene expression 1 profiling in the rainbow trout (*Oncorhynchus mykiss*) ovary during maturational competence acquisition and oocyte maturation. *Biology of Reproduction* **71** 73–82.
- Bu S, Xia G, Tao Y, Lei L & Zhou B 2003 Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes *in vitro*. *Molecular and Cellular Endocrinology* **207** 21–30.
- Bush PA, Gonzales NE, Griscavage JM & Ignarro LJ 1992 Nitric oxide synthase from cerebellum catalyses the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. *Biochemical and Biophysical Research Communications* **185** 960–966.
- Chabbert-Buffet N & Bouchard P 2002 The normal human menstrual cycle. *Reviews in Endocrine and Metabolic Disorders* **3** 173–183.
- Chanda D, Yonekura M & Krishna A 2004 Pattern of ovarian protein synthesis and secretion during the reproductive cycle of *Scotophilus heathi*: evidence for the synthesis of albumin – like protein. *Biotechnic & Histochemistry* **79** 129–138.
- Cox RL, Mariano T, Heek DE, Laskin JD & Stegeman JJ 2001 Nitric oxide synthases in the marine fish *Stenotomus chrysops* and the sea urchin *Arbacia punctulata*, and phylogenetic analysis of nitric oxide synthase calmodulin-binding domains. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **130** 479–491.
- Dixit VD & Parvizi N 2001 Nitric oxide and the control of reproduction. *Animal Reproduction Science* **65** 1–16.
- Dong YL, Gangula PR, Fang L & Yallampalli C 1999 Nitric oxide reverse prostaglandin-induced inhibition in ovarian progesterone secretion in rats. *Human Reproduction* **14** 27–32.
- Dubey RK, Jackson EK, Rupperecht HD & Sterzel RB 1997 Factors controlling growth and matrix production in vascular smooth muscle and glomerular mesangial cell. *Current Opinion in Nephrology and Hypertension* **6** 88–105.
- Dunnam RC, Hill MJ, Lawson DM & Dunbar JC 1999 Ovarian hormone secretory response to gonadotropins and nitric oxide following chronic nitric oxide deficiency in the rat. *Biology of Reproduction* **60** 959–963.
- Hattori MA, Sakamoto K, Fujihara N & Kojima I 1996 Nitric oxide: a modulator for the epidermal growth factor receptor expression in developing ovarian granulosa cells. *American Journal of Physiology* **270** C812–C818.
- Holmqvist B, Ellingsen B, Alm P, Forsell J, Oyan AM, Goksoyr A, Fjose HC & Sen HC 2000 Identification and distribution of nitric oxide synthase in the brain of adult Zebrafish. *Neuroscience Letters* **292** 119–122.
- Huo LI, Liang CG, Yu LZ, Zhong ZS, Yang ZM, Fan HY, Chen DY & Sun QY 2005 Inducible nitric oxide synthase-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte. *Reproduction* **129** 403–409.
- Jablonska-Shariff A & Olson LM 1997 Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. *Endocrinology* **138** 460–468.
- Jablonska-Shariff A & Olson LM 1998 The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock out mouse oocytes. *Endocrinology* **139** 2944–2954.
- Jablonska-Shariff A & Olson LM 2000 Nitric oxide is essential for optimal meiotic maturation of murine cumulus–oocyte complexes *in vitro*. *Molecular Reproduction and Development* **55** 412–421.
- Kim H, Moon C, Ahn M, Lee Y, Kim H, Kim S, Ha T, Jee Y & Shin T 2005 Expression of nitric oxide synthase isoforms in the porcine ovary during follicular development. *Journal of Veterinary Science* **6** 97–101.
- Kohli G, Clelland E & Peng C 2005 Potential targets of transforming growth factor- β 1 during inhibition of oocyte maturation in zebrafish. *Reproductive Biology and Endocrinology* **3** 53–62.
- Lamba VJ, Goswami SV & Sundararaj BI 1983 Circannual and circadian variation in plasma levels of steroids (cortisol, estradiol-17 β , estrone and testosterone) correlated with annual gonadal cycle in the catfish, *H. fossilis* (Bloch.). *Reproductive Biology and Endocrinology* **50** 205–225.
- Lloyd RV, Long J, Qian X, Zhang S & Scheithauer BW 1995 Nitric oxide synthase in the human central nervous system tumours. *Cancer Research* **55** 727–730.

- Manwar SJ, Moudgal RP, Sastry KVH, Mohan J, Tyagi JB & Raina R 2006 Role of nitric oxide in ovarian follicular development and egg production in Japanese quail (*Coturnix coturnix japonica*). *Theriogenology* **65** 1392–1400.
- Matsumi H, Yano T, Osuga Y, Kugu K, Tang X, Xu JP, Yano N, Kuruashima Y, Ogura T, Tsutsumi O *et al.* 2000 Regulation of nitric oxide synthase to promote cytotaxis in ovarian follicular development. *Biology of Reproduction* **63** 141–146.
- Mitsube K, Mikuni M, Matousek M & Brannstrom M 1999 Effect of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the *ex-vivo* perfused rat ovary. *Human Reproduction* **14** 2537–2543.
- Morris SM & Billiar TR 1994 New insights into the regulation of inducible nitric oxide synthesis. *American Journal of Physiology* **266** E829–E839.
- Nakamura Y, Yamagata Y, Sugino N, Takayama H & Kato H 2002 Nitric oxide inhibits meiotic maturation. *Biology of Reproduction* **67** 1588–1592.
- Nassauw LV, Tao L & Harrison F 1999 Localization of nitric oxide like substance in the quail ovary during folliculogenesis. *Histochemical Journal* **31** 443–454.
- Oyan AM, Goksor A & Holmqvist B 2000 Partial cloning of neuronal nitric oxide synthase and expression in the brain of adult Atlantic salmon. *Molecular Brain Research* **78** 38–49.
- Panzica GC, Cozzi B & Viglietti-Panzica C 1998 Colocalization studies in the avian brain provide new insights on the roles of NO in higher vertebrates. *European Journal of Anatomy* **2** 63–76.
- Panzica GC, Viglietti-Panzica C, Sica M, Gotti S, Martini M, Pinos H, Carrillo B & Collado P 2006 Effects of gonadal hormones on central nitric oxide producing system. *Neuroscience* **138** 987–995.
- Patino R & Sullivan CV 2002 Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry* **26** 57–70.
- Peterson JK, Moran F, Conley AJ & Bird IM 2001 Zonal expression of endothelial nitric oxide synthase in sheep and rhesus adrenal cortex. *Endocrinology* **142** 5351–5363.
- Rosselli M, Imthurm B, Macas E, Eller PJ & Dubey RK 1994 Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for estradiol mediated NO release. *Biochemical and Biophysical Research Communications* **202** 1543–1552.
- Rosselli M, Kellar PJ & Dubey RK 1998 Role of nitric oxide in the biology and physiology and pathophysiology of reproduction. *Human Reproduction Update* **4** 3–24.
- Saiej JP, Stet RJ, Groeneveld A, Verburg-van Kemenade LB, van Muiswinkel WB & Wiegertjes GF 2000 Molecular and functional characterization of fish inducible-type nitric oxide synthase. *Immunogenetics* **51** 339–346.
- Sastry KVH, Moudgal RP, Mohan J, Tyagi JS & Rao GS 2002 Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. *Analytical Biochemistry* **306** 79–82.
- Schulz K, Kerber S & Kelm M 1999 Re-evaluation of the Griess method for determining NO/NO₂- in aqueous and protein-containing samples. *Nitric Oxide* **3** 225–234.
- Sengoku K, Takuma N, Horikawa M, Tsuchiya K, Komori H, Sharifa D, Tamate K & Ishikawa M 2001 Requirement of nitric oxide for murine oocyte maturation, embryo development, and trophoblast outgrowth *in vitro*. *Molecular Reproduction and Development* **58** 262–268.
- Shukorski L & Tsafirri A 1994 The involvement of nitric oxide in the ovulatory process in the rat. *Endocrinology* **135** 2287–2290.
- Singh P, Krishna A & Sridaran R 2007 Localization of gonadotrophin-releasing hormone I, bradykinin and their receptors in the ovaries on non-mammalian vertebrates. *Reproduction* **133** 1–14.
- Tamanini C, Basin G, Grasselli F & Tirelli M 2003 Nitric oxide and the ovary. *Journal of Animal Science* **81** E1–E7.
- Tao Y, Fu Z, Zhang M, Xia G, Yang J & Xie H 2004 Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. *Molecular and Cellular Endocrinology* **222** 93–103.
- Viana KS, Caldas-Bussiere MC, Matta SGC, Faes MR, Paes de Carvalho CS & Quirino CR 2007 Effect of sodium nitroprusside, nitric oxide donor, on the *in vitro* maturation of bovine oocytes. *Animal Reproduction Science* **102** 217–227.
- Vishwanathan N & Sundararaj BI 1974 Seasonal changes in the hypothalamo-hypophyseal ovarian system in the catfish, *Heteropneustes fossilis* (Bloch.). *Journal of Fish Biology* **6** 331–340.
- Van Voorhis BJ, Dunn MS, Snyder GD & Weiner CP 1994 Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* **135** 1799–1806.
- Van Voorhis BJ, Moore K, Strijbos PJ, Nelson S, Baylis SA, Grzybicki D & Weiner CP 1995 Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Effects of gonadotropin stimulation *in vivo*. *Journal of Clinical Investigation* **96** 2719–2725.
- Yamagata Y, Nakamura Y, Sugino N, Harada A, Takayama H, Kashida S & Kato H 2002 Alterations in nitrate/nitrite and nitric oxide synthase in preovulatory follicles in gonadotropin-primed immature rat. *Journal of Endocrinology* **49** 219–226.
- Yun HY, Dawson VL & Dawson TM 1996 Neurobiology of nitric oxide. *Critical Reviews in Neurobiology* **10** 291–316.
- Zackrisson U, Mikuni M, Wallin A, Delbro D, Hedin L & Brannstrom M 1996 Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. *Human Reproduction* **11** 2667–2673.
- Zini A, O'Bryan MK, Magid MS & Schlegel PN 1996 Immunohistochemical localization of endothelial nitric oxide synthase in human testis, epididymis, and vas deferens suggests a possible role for nitric oxide in spermatogenesis, sperm maturation, and programmed cell death. *Biology of Reproduction* **55** 935–941.

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