Effects of gonadotrophin *in vivo* and 2-hydroxyoestradiol-17 β *in vitro* on follicular steroid hormone profile associated with oocyte maturation in the catfish *Heteropneustes fossilis*

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Abstract

An HPLC method was used to tentatively identify progesterone (P_4) and its metabolites (17-hydroxyprogesterone (17- P_4) and 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P)), corticosteroids (cortisol and corticosterone) and testosterone in ovary/follicular preparations of the catfish *Heteropneustes fossilis* associated with *in vivo* or *in vitro* oocyte maturation/ovulation. A single i.p. injection of human chorionic gonadotrophin (100 IU/fish, sampled at 0, 8 and 16 h) induced oocyte maturation and ovulation, which coincided with significant and progressive increases in 17,20 β -P, and P_4 and 17- P_4 , the precursors of the former. Both cortisol and corticosterone also increased significantly. Conversely, testosterone decreased significantly and progressively over time. Under *in vitro* conditions, incubation of post-vitellogenic (intact) follicles or follicular envelope (layer) with 2-hydroxyoestradiol (2-OHE₂, 5 μ M for 0, 6 and 24 h) elicited a sharp significant increase in 17,20 β -P, the increase being higher in the follicular envelope incubate. P_4 and 17- P_4 also registered significant increases over the time with the

peak values at 24 h. Cortisol and corticosterone increased significantly in the intact follicle, but not in the follicular envelope incubate. Testosterone decreased significantly in the intact follicle, but increased significantly (24 h) in the follicular envelope incubate. Coincident with these changes, the percentage of germinal vesicle breakdown (GVBD) increased over the time in the intact follicle incubate (48.9% at 6 h and 79.8% at 24 h). Denuded oocytes on incubation with 2-OHE₂ (5 μ M) did not produce any significant change in the percentage of GVBD or in the steroid profile. While corticosterone and 17,20 β -P were undetected, P_4 , 17- P_4 , cortisol and testosterone were detected in low amounts. The results show that the 2-OHE₂-induced GVBD response seems to be mediated through the production of 17,20 β -P and corticosteroids. It is suggested that hydroxyoestrogens seem to be a component in the gonadotrophin cascade of regulation of oocyte maturation/ovulation in the catfish.

Journal of Endocrinology (2006) **189**, 341–353

Introduction

In teleosts, meiotic resumption is initiated by the production of an ovarian maturation-inducing substance (MIS) or hormone under a luteinizing hormone (LH) surge. In a majority of fishes, the most potent MIS is the progesterone (P_4) derivative 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) (Goetz 1983, Scott & Canario 1987, Jalabert *et al.* 1991, Nagahama 1997) and its synthesis has been demonstrated during oocyte maturation both *in vivo* and *in vitro*. In perciform fishes, another C₂₁ steroid, 17,20 β ,21-trihydroxy-4-pregnen-3-one, has been identified as the MIS (Trant *et al.* 1986, Thomas 1994, Garcia-Alonso *et al.* 2004). The catfish *Heteropneustes fossilis* has been used as a model for oocyte maturation studies since the 1960s (Sundararaj & Goswami 1977). In a series of investigations, Sundararaj and his coworkers have demonstrated that corticosteroids (11-deoxycortisol,

11-deoxycorticosterone, 21-deoxycortisol and cortisol) of interrenal (adrenal) origin are the MIS in the catfish and proposed a pituitary–interrenal–ovarian control of oocyte maturation and ovulation. The MIS activity of corticosteroids has been demonstrated *in vitro* in other teleosts as well (Goetz 1983, Jalabert *et al.* 1991). Subsequently, it has been demonstrated *in vitro* that, as in other teleosts, 17,20 β -P is the most potent MIS in this species (Sundararaj *et al.* 1985). However, its occurrence in the ovary has not been demonstrated in this species up until this time.

We reported earlier that catfish ovary synthesizes catecholestrogens, which show both seasonal and peri-ovulatory changes (Mishra & Joy 2006a). Further, hydroxyoestrogens have been shown to stimulate oocyte maturation (Senthilkumaran & Joy 2001, Mishra & Joy 2006b). However, the mechanism of induction of oocyte maturation, direct or indirect, was not clearly understood.

The objective of the present study was to determine whether the hydroxyoestrogen-induced *in vitro* oocyte maturation was mediated through follicular steroidogenesis. The changes in the steroid profile were then compared with those that occurred during *in vivo* human chorionic gonadotrophin (hCG)-induced oocyte maturation and ovulation. Through the latter paradigm, we also demonstrated that catfish ovary appeared to synthesize both 17,20 β -P and corticosteroids, like other teleosts.

Materials and Methods

Chemicals

1,3,5,(10)-oestratriene-2,3,17 β -triol (2-hydroxyoestradiol, 2-OHE₂), 11 β ,17,21-trihydroxy-4-pregnene-3,20-dione (cortisol), 11 β ,21-dihydroxy-4-pregnene-3,20-dione (corticosterone), 17 β -hydroxy-4-androsten-3-one (testosterone), 17-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone (17-P₄)), 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P), 4-pregnene-3,20-dione (progesterone, P₄) (steroid nomenclature after Kime 1987) and hyaluronidase (type IV) were purchased from Sigma Chemical Company. hCG (Corion; IBSA, Switzerland) was purchased from a local medical store. Other chemicals were of analytical grade and purchased locally. Methanol (HPLC grade) and degassed and filtered nanopure diamond water (Barnstead International, Dubuque, IO, USA) were used throughout chromatography.

Animal collection and maintenance

The experiments were performed in accordance to local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

Mature female *Heteropneustes fossilis* (30–40 g) were purchased from local fish markets in the pre-spawning phase (June) of the annual reproductive cycle. They were maintained in the laboratory under normal photoperiod (13 h light:11 h darkness) and temperature (25 $\pm$ 2 °C) until used for experiments. The fish were freely fed goat liver daily. Sample fish specimens were randomly checked for spontaneous ovulation by mild hand stripping. A few fish were sampled for checking the maturation stage of the ovary. Post-vitellogenic dark green rounded follicles (1.034 $\pm$ 0.01 mm, average diameter) were used for incubation studies.

Induction of ovulation

Thirty acclimatized fish were divided into two groups of 15 each. One group was injected i.p. with hCG at a dose of 100 IU/fish, the second was injected with normal saline (0.6% NaCl). Five fish each from both groups were killed at 0, 8 and 16 h and ovaries were weighed and immediately processed for extraction of steroids.

Preparation of incubation medium and test compounds

The incubation medium was prepared as follows (g): NaCl 3.74, KCl 0.32, CaCl₂ 0.16, NaH₂PO₄·2H₂O 0.10, MgSO₄·7H₂O 0.16, glucose 0.40 and phenol red 0.008 were dissolved in 1 litre of triple-distilled water. The pH was adjusted to 7.5 with 1 M sodium bicarbonate and autoclaved. Penicillin (2,00,000 U) and streptomycin sulphate (200 mg) were added and filtered. The medium was stored at 4 °C and prepared fresh every week.

2-OHE₂ was weighed and dissolved in 50 μ l ethanol in a dark bottle and kept at 0 °C. Just before the incubation, the stock solution was diluted with the incubation medium to make a working concentration of 5 μ M.

In vitro incubation of follicular preparations with 2-OHE₂

The acclimatized, gravid female catfish were killed by decapitation and ovaries were transferred to a sterile Petri dish containing freshly cooled incubation medium. Post-vitellogenic follicles were separated from each other with the help of a fine brush and watchmaker's forceps. The following preparations were used for the incubation study:

Intact follicles About 120 follicles were incubated in duplicate (group size=5) with the medium containing 5 μ M of 2-OHE₂ for 0, 6 and 24 h. The incubation was further continued in fresh plain medium for 30 h. As control, the follicles were incubated with plain medium or the medium containing vehicle (ethanol). Percentage of germinal vesicle breakdown (GVBD) was scored. The follicles and incubation medium were collected for steroid extraction.

Follicular envelope (layer) Batches of about 120 follicles each were treated with 0.03% hyaluronidase (Fan *et al.* 2002) in plain incubation medium for 2 min under mild agitation. (Hyaluronidase dissolves hyaluronic acid in extracellular matrix present between corona radiata and zona pellucida of mammalian oocytes (Talbot 1984)). The follicular envelope containing granulosa and thecal cells was separated *in toto* by the enzyme treatment and was collected by forceps. They were washed with fresh medium and incubated with 2-OHE₂ (5 μ M) for 0, 6, or 24 h in duplicate, as described above. The denuded oocytes were also collected for incubation, as described below. The incubation continued further up to 30 h in fresh plain medium. As control, the follicular envelopes were incubated in plain medium or the medium containing the vehicle. The tissues and medium were collected for steroid extraction.

Denuded oocytes Batches of about 120 denuded oocytes (see above) were washed in fresh medium and incubated with 2-OHE₂ for 0, 6 or 24 h, as described

above. The incubation continued further up to 30 h in fresh plain medium. As control, the denuded oocytes were incubated in plain medium or the medium containing the vehicle. GVBD was scored, and the oocytes and medium were collected for steroid extraction.

Extraction of steroids

The tissues (ovaries and follicular preparations) from different experiments were homogenized separately or group-wise in 4 volumes of cold PBS (0.02 M, phosphate-buffered saline, pH 7.4) with an ultrasonic homogenizer (XL-2000 Microson; Misonix, New York, NY, USA) at 0 °C for 5–10 s. The homogenate was centrifuged at 5000 *g* for 20 min at 4 °C and extracted with 3 volumes of diethyl ether, three times. The ether phase was collected and pooled, evaporated and dried under N₂ and stored at –20 °C until chromatography. The incubation medium was directly extracted with diethyl ether, as described above. The ether phase was collected and pooled group-wise, evaporated and dried under N₂ and stored at –20 °C until chromatography.

Chromatography

A Shimadzu (Kyoto, Japan) HPLC system with two pumps (LC-10 ATVP), system controller (SCL-10 AVP) and an ultraviolet detector (SPD-10 AVP) with a variable wavelength (190–370 nm) range was used for the chromatographic analysis of steroids. The system was operated with Shimadzu Class VP Series software. The analysis was made with a reversed phase C₁₈ column (150 × 4.5 mm, i.d., 5 µm; Luna; Phenomenex, Torrance, CA, USA) and absorbance was taken at 240 nm. The mobile phase was 60% methanol in water at a flow rate of 1.5 ml/min (Nagahama & Adachi 1985). The run time was 30 min.

Preparation of standards and determination of retention time

Steroids (cortisol, corticosterone, testosterone, 17-P₄, 17,20β-P, P₄) were dissolved in methanol separately to prepare stock solutions. From the stock solutions, serial dilutions were made with methanol. The diluted solutions were filtered (0.2 µm) and injected into the 20 µl loop of the HPLC system with the help of a Hamilton microlitre syringe. The standards were tested individually at different concentrations to record detection limit, retention time and peak area under isocratic conditions. This was repeated three times with each standard to verify the concurrence of the assay parameters.

Validation of the assay

Response-linearity Different concentrations of the standards in triplicate were injected into the column to set up a concentration vs peak area curve. The

response was linear with the concentration ranges used (10–1000 ng/ml).

Recovery and sensitivity Known concentrations of the standards in different dilutions were processed in the same manner as tissue samples (described below) and were injected into the column, after filtration. The recovery study was repeated three times for each dilution. Percentage recovery was calculated from the concentrations of the standards injected directly and that measured after the extraction. Recovery was 89–93%. The values were not corrected for loss. The minimum detection limit was 1 ng/ml of the standards in individual runs.

Inter- and intra-assay variations Inter- and intra-assay variations were determined from five chromatograms each, using the same set or different sets of diluted standards. The inter- and intra-assay variations were, respectively, 10 and 8%.

Sample analysis

The ether-evaporated and dried ovary samples were reconstituted separately in 200 µl methanol. Similarly, the samples of the follicular preparations and incubation medium were pooled group-wise with 200 µl methanol. The reconstituted samples were filtered (0.2 µm) and 20 µl each of the samples were injected into the system and eluted for 30 min. The samples were analysed in triplicate. The samples were also co-chromatographed with known concentrations of the standards in a mixture and the elution pattern was compared with that of the respective standards in the mixture for identification of the steroids. Chromatograms for blanks were run with the vehicle (methanol and the mobile phase) to check any interference in the elution of the steroids. The blank eluted before the steroid peaks appeared. The differences in the peak area between the standard and the standard with sample in the chromatograms were recorded with the help of Class VP Series software and the concentrations were calculated.

Statistical analysis

The data were expressed as means ± S.E.M. and were analysed by one-way ANOVA, followed by a Newman-Keuls test (*P* < 0.05) for multiple group comparisons.

Results

Steroid separation and suggested identities

With the chromatographic conditions we adopted, cortisol eluted first, followed by corticosterone, testosterone, 17-P₄, 17,20β-P and P₄ (Fig. 1A). The retention times

Figure 1 HPLC chromatograms showing separation of steroid standards on a C₁₈ reversed phase column (Luna, 150 × 4.5 mm i.d., 5 µm); mobile phase: 60% methanol in water. Absorption was taken at 240 nm. (A) Separation profile of the standards applied in a mixture: peak 1, cortisol (retention time (Rt)=3.7 min); peak 2, corticosterone (Rt=5.5 min); peak 3, testosterone (Rt=9.9 min); peak 4, 17-P₄ (Rt=11.7 min); peak 5, 17,20β-P (Rt=13.4 min); and peak 6, P₄ (Rt=23.0 min). (B) Elution profile of an ovarian sample extract (8 h after hCG injection) in the presence of known concentrations of the standards in a mixture. (C) Elution profile of an intact follicle sample extract (6 h after *in vitro* 2-OHE₂ treatment) in the presence of known concentrations of the standards in a mixture.

were 3.7, 5.5, 9.9, 11.7, 13.4 and 23.0 min respectively. However, in the sample runs, the retention times showed minor shifts (Figs 2 and 4). Therefore, the steroid peaks were authenticated by co-running the samples with known concentrations of the standards in a mixture (Fig. 1B and C), and compared with those of the samples or standards run alone. In this manner, the steroids were tentatively identified. The chromatograms of the ovary, intact follicles and follicular envelope showed three elution peaks each, the identity of which could not be confirmed because of the lack of standards (Figs 2 and 4).

Effects of hCG on ovulation and ovarian steroid dynamics

The administration of 100 IU hCG/fish induced 100% ovulation and eggs could be stripped out from 12 h onwards. The gonadotrophin treatment produced overall significant changes in the concentrations of P₄, 17-P₄, 17,20β-P, testosterone, cortisol and corticosterone (Figs 2 and 3; $F=385.18, 846.37, 21636.43, 90.00, 71.23$ and 41.66 respectively; $P<0.001$, one-way ANOVA). The steroid concentrations did not vary significantly in the vehicle groups at 0, 8 and 16 h intervals. The administration of hCG produced a strong stimulation of the progestin pathway involving P₄, 17-P₄ and 17,20β-P production associated with oocyte maturation and ovulation. P₄ increased significantly about 2-fold at 8 h and 5-fold at 16 h ($P<0.05$, Newman–Keuls test). 17-P₄ increased significantly at 8 h of the hormone treatment but decreased to the control level at 16 h. The most dramatic change was noticed in the concentrations of 17,20β-P. The concentration was very low in the vehicle groups (1.5 pg/mg ovary) and increased sharply registering about an 88-fold increase at 8 h and about 125-fold at 16 h. Testosterone decreased consistently with the lowest concentration at 16 h ($P<0.05$). Cortisol showed a significant and progressive increase at 8 and 16 h but corticosterone increased only at 16 h ($P<0.05$).

In vitro effects of 2-OHE₂ on follicular steroid profile

Intact follicle The incubation of the intact follicles with 5 μM 2-OHE₂ produced an overall significant effect on GVBD (48.9% at 6 h and 79.8% at 24 h) and steroid hormone levels: P₄, 17-P₄, 17,20β-P, testosterone, cortisol and corticosterone (Figs 4 and 5A; $F=401.74, 282.04, 518.86, 12.00, 102.50$ and 65.25 respectively; $P<0.001$, one-way ANOVA). The concentrations of both P₄ and 17-P₄ increased significantly at 6 and 24 h giving the peak levels at 24 h ($P<0.05$). 17,20β-P was not detected at 0 h, but its level increased sharply giving the peak at 24 h ($P<0.05$). Testosterone decreased significantly at 6 and 24 h ($P<0.05$). On the other hand, cortisol registered a significant increase with the peak level at 24 h but corticosterone increased at 6 h and declined to the control level at 24 h.

Follicular envelope The hyaluronidase treatment could easily separate the follicular envelope from the oocyte. The incubation of the follicular envelope with 2-OHE₂ registered overall significant effects on P₄, 17-P₄ and 17,20β-P levels (Fig. 5B; $F=117.01, 144.68$ and 2093.80 respectively; $P<0.001$, one-way ANOVA). The levels of P₄ and 17-P₄ followed a similar pattern of changes as in the intact follicle incubates but the concentrations were low at 6 and 24 h. On the other hand, the production of 17,20β-P was higher than that of the intact follicle at 6 and 24 h. Testosterone level was significantly high only at 24 h, while cortisol and corticosterone levels did not show any significant change.

Denuded oocytes The incubation of denuded oocytes with 2-OHE₂ did not produce any significant effect on GVBD. The denuded oocytes contained low concentrations of 17-P₄, testosterone and cortisol, which did not vary significantly (Fig. 5C). P₄ level was higher compared with the other steroids but did not vary either. 17,20β-P and corticosterone were not detected at any of the intervals.

Discussion

We identified tentatively steroid hormones in the ovary/follicle incubates and measured changes in their levels associated with oocyte maturation and/or ovulation by an HPLC method. The study also provided evidence for the ovarian origin of the MIS (17,20β-P and corticosteroids), which was a point of contention in this catfish species (Sundararaj & Goswami 1977).

hCG is used extensively to induce ovulation in fishes, including the catfish (Joy & Tharakan 1999). Administration of 100 IU of hCG induced 100% oocyte maturation and ovulation, fertilizable eggs could be stripped out at 12 h and fish ovulated spontaneously from 16 h onwards. The hCG administration resulted in the stimulation of the progestin pathway, as has been reported in other species (Fostier *et al.* 1983, Nagahama *et al.* 1994), and the significant increases in P₄, 17-P₄ and 17,20β-P levels can be explained by the precursor–product relationship. Of all the steroids measured, 17,20β-P, which co-migrated with the authentic standard, registered a sharp increase from 1.5 pg/mg ovary at 0 h to 89 (8 h) and 126 (16 h) pg/mg associated with the maturational activity and ovulation. The control fish, which did not ovulate maintained a basal level of the hormone throughout. Thus, the rise in 17,20β-P could be correlated with its MIS activity, as has been reported in a number of teleosts (references reviewed in Introduction).

In the catfish, P₄ that co-migrated with the authentic standard did not elicit any MIS activity *in vitro* (Sundararaj & Goswami 1977) although it elicited a moderate to strong activity in other species (Goetz 1983, Jalabert *et al.*

Figure 2 Effects of a single i.p. injection of hCG (100 IU/fish) on ovarian elution profile of steroids at 0 (A), 8 (B) and 16 (C) h. Note the increased peak profile and production of 17,20β-P during the maturational process. Note also the increase in the peak characteristics of three unidentified (UI) compounds that increased with the maturational process.

Figure 3 Periovarulatory changes in ovarian steroid levels after a single i.p. injection of hCG (100 IU/fish). Values are means $\pm$ S.E.M. of five fish in each group. Data were analysed by one-way ANOVA ($P < 0.001$) and a Newman-Keuls test ($P < 0.05$). Asterisks show significant difference from the 0 h group. P₄, progesterone; 17-P₄, 17-hydroxyprogesterone; T, testosterone; F, cortisol; B, corticosterone; 17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one.

1991). In *Clarias gariepinus*, 17-P₄ has been shown to induce oocyte maturation and ovulation apparently due to its conversion into 17,20β-P (Richter *et al.* 1987). In the present study, P₄ level peaked at 16 h apparently due to reduced conversion to 17-P₄, which itself decreased to low values. Likewise, the pattern of changes in 17-P₄ levels at 8 and 16 h suggests its metabolite status rather than a hormone function.

The ovarian concentration of testosterone that co-migrated with the standard decreased significantly after the hCG treatment with the lowest level detected at 16 h. A decrease in testosterone may imply a downregulation of the enzymes involved in the conversion of 17-P₄ to androstenedione (17,20-lyase) or androstenedione to testosterone (17β-HSD), or its further conversion into ketotestosterone or glucuronide (Lambert & Van Den Hurk 1982, Scott & Baynes 1982). Scott & Baynes (1982) reported that 17,20β-P inhibited C₂₁–C₁₉ desmolase (lyase) activity, inhibiting testosterone production. The decrease in testosterone production might be a mechanism to inhibit oestradiol-17β (E₂) synthesis, prior to oocyte maturation and ovulation. According to Nagahama *et al.* (1994), E₂ synthesis is arrested by the inhibition of aromatase activity. The present data show that E₂ inhibition could be achieved upstream of the aromatization step by limiting the supply of testosterone as the

substrate (Sakai *et al.* 1988). In the catfish, the decrease in testosterone (present data) and E₂ (Mishra & Joy 2006a) at 8 and 16 h suggests a comprehensive downregulation of the C₁₉–C₁₈ pathway, suggesting a shift towards the C₂₁ steroid pathway. This results in the lowering of E₂ to the nadir, a prerequisite for the resumption/sustenance of meiosis (Mishra & Joy 2006a, 2006b).

This study reports the occurrence of corticosteroids in the ovary of the catfish. The previous investigation by Ungar *et al.* (1977) did not identify any ovarian corticosteroids, strengthening the hypothesis that corticosteroids of interrenal origin alone acted as the MIS in this species (Sundararaj & Goswami 1977). In the present study, low levels of cortisol and corticosterone that co-migrated with the respective standards could be measured in the control fish. The administration of hCG increased corticosteroid production at 8 and/or 16 h associated with maturational activity. Teleost ovary has been demonstrated to synthesize corticosteroids such as 11-deoxycorticosterone and 11-deoxycortisol (Colombo *et al.* 1973, Theofan & Goetz 1983, Scott & Canario 1990, Kime *et al.* 1992). Cortisol production by ovary has been reported in human (Yong *et al.* 2000), frog (Gobbetti & Zerani 1993) and sturgeon (Webb *et al.* 2002). Colombo *et al.* (1973) reported that ovarian corticosteroids might act as local hormones mediating pituitary gonadotrophin-induced oocyte

Figure 4 Steroid production by intact follicle incubated with 2-OHE₂ (5 μM) *in vitro*. (A) Chromatographic elution profile of a sample extract at 0 h. Note the absence of 17,20β-P peak. (B) Chromatographic elution profile of a sample extract at 6 h. (C) Chromatographic elution profile of a sample at 24 h. UI, unidentified compounds whose peak characteristics increased with the progress of maturational activity.

Figure 5 *In vitro* effects of 2-OHE₂ (5 μM) on steroid production by intact follicle (A), follicular envelope (B) and denuded oocyte (C). Values are means ± S.E.M. of 120 follicle preparations incubated in duplicate (group size=five fish). Data were analysed by one-way ANOVA ($P<0.001$) and a Newman-Keuls test ($P<0.05$). Asterisks show significant difference from the 0 h group. ND, not detected; P₄, progesterone; 17-P₄, 17-hydroxyprogesterone; T, testosterone; F, cortisol; B, corticosterone; 17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one.

maturation and ovulation. In the catfish, cortisol, but not corticosterone, was shown to have MIS activity *in vitro* (Goswami & Sundararaj 1971). However, in *Fundulus heteroclitus*, corticosterone induced a high percentage (98%) of GVBD (Greeley *et al.* 1986). Whether the low levels of corticosteroids in the presence of high amounts of 17,20 β -P do elicit any direct maturational activity is debatable. Since high levels of corticosteroids have deleterious effects, their presence in low levels may assume physiological significance. Cortisol may enhance the sensitivity of the oocytes to gonadotrophin or MIS (Fostier & Jalabert 1982). Since corticosteroids are involved in metabolic and osmoregulatory processes, they may play an important role in metabolism and hydration of the follicles associated with maturation and ovulation (LaFleur & Thomas 1991).

In vitro incubation of the intact follicles or follicular envelope with 2-OHE₂ stimulated 17,20 β -P synthesis, as in the hCG study. Further, the significant sharp increase in the 17,20 β -P level (6 and 24 h) could be correlated with the GVBD response (48.9 and 79.8% respectively) in the intact follicle incubates. Conversely, the incubation of the denuded oocytes with 2-OHE₂ did not induce GVBD. The latter observation appears to rule out a direct effect of the steroid on oocyte maturation, although further detailed studies are required to confirm it. It is not known whether the hyaluronidase treatment had affected the oocyte membrane structure and function. The present study further shows that the follicular envelope (theca-granulosa complex) is responsible for the secretion of 17,20 β -P, since denuded oocytes did not elaborate the steroid. The follicular envelope incubate produced more 17,20 β -P than the intact follicle incubate. This suggests that the oocyte within the follicle might have inhibited the synthesis rate in some manner or the steroid might be used for maturational activity (Petrino *et al.* 1989). A similar pattern was reported by Kagawa *et al.* (1982) with regard to the *in vitro* production of E₂. However, the intact follicle incubates secreted (accumulated) high amounts of P₄ or 17-P₄ (24 h) than the follicular envelope incubates, which suggests that in the latter the steroids might have been converted to 17,20 β -P due to higher 20 β -hydroxysteroid dehydrogenase (20 β -HSD) activity.

The pattern of the *in vitro* production of P₄, 17-P₄ and 17,20 β -P in both intact follicle and follicular envelope preparations showed parallelism with that of the ovary following the hCG treatment. A similar parallelism, in the overall pattern of changes in the corticosteroids (cortisol and corticosterone) and testosterone was noticed between the *in vivo* and *in vitro* studies. The basal level of 17,20 β -P was not detected *in vitro* but a low level was detected *in vivo*, implying that the steroid induction started with the addition of 2-OHE₂. Steroid precursors and gonadotrophin have been shown to promote MIS production *in vitro* (Petrino *et al.* 1989). Nagahama *et al.* (1986) reported that 17,20 β -P could not be measured *in vitro*

unless gonadotrophin or 17-P₄ (substrate) was added exogenously. Since 2-OHE₂ is not a precursor of the steroid, it might have stimulated the activity of specific enzymes (3 β -hydroxysteroid dehydrogenase (3 β -HSD), 17 α -hydroxylase and 20 β -HSD), like gonadotrophin (LH) to elevate the steroid production (Jalabert *et al.* 1991, Nagahama *et al.* 1994). Like LH, 2-OHE₂ stimulated P₄ secretion in rat luteal cells by stimulating the cholesterol side-chain cleavage enzyme and 3 β -HSD, and inhibiting 20 α -HSD activity (Tekpetey & Armstrong 1994). A 4 day co-treatment with either follicle-stimulating hormone (FSH) or LH enhanced P₄ production in porcine granulosa cells, stimulated by 2-OHE₂ (Spicer & Hammond 1988, 1989). On the other hand, the catecholestrogen inhibited basal or LH-stimulated androstenedione and 17-P₄ production in porcine thecal cells (Morley *et al.* 1989). In the catfish, FSH β mRNA transcript is expressed but the protein is not yet demonstrated in the circulation (Swanson *et al.* 2003). LH is present throughout the reproductive cycle (Tharakan 1998) and may serve the FSH function.

In the catfish (*H. fossilis* and *C. batrachus*), it has been reported that the LH surge stimulates oestrogen-2-hydroxylase activity resulting in the synthesis of 2-hydroxyoestrogens (Senthilkumaran & Joy 2001). The present data in conjunction with the above study suggest that 2-OHE₂ may be a part of the LH cascade stimulating progesterin synthesis during oocyte maturation and ovulation in this species. According to our hypothesis (Fig. 6), the LH surge inhibits E₂ by metabolizing it into hydroxy-oestrogens (Mishra & Joy 2006a), which stimulate alone or in synergy with the gonadotrophin the steroid pathway at multiple sites, altering the activity of key enzymes leading to the synthesis of 17,20 β -P. Further work is required to demonstrate the effects of 2-OHE₂ alone or in synergy with LH on the activity of the key enzymes involved in steroid metabolism in fish ovary/follicles.

The denuded oocytes presented a steroid hormone content with a relatively high level of P₄ compared with testosterone, cortisol or corticosterone but lacked 17,20 β -P. The occurrence of steroid hormones (androgens, oestrogens, progestins and corticosteroids) and thyroid hormones has been reported previously within fish oocytes (Schreck *et al.* 1991, Tagawa 1996). The egg (yolk) hormones may represent a continuum between the mother and offspring and have a role in sexual differentiation, early development, metabolism or behaviour (Schreck *et al.* 1991).

The chromatograms showed the elution peaks of three unidentified compounds, the peak area of which increased with the progress of maturational activity. Ungar *et al.* (1977) characterized a major ovarian steroid, 3 α -hydroxy-5 β -pregnan-20-one (pregnanolone), that sensitized oocytes for increased maturational response by cortisol (Sundararaj *et al.* 1979). Further, 11-deoxycorticosterone, 11-deoxycortisol and 21-deoxycortisol were shown to have MIS activity *in vitro* (Sundararaj & Goswami 1977).

Figure 6 A schematic diagram showing multiple sites of action of hydroxyoestradiol (OHE₂) on progesterin pathway during oocyte maturation. (+) stimulation, (−) inhibition, (?) a direct action of OHE₂ on maturational activity is suspected. C, cholesterol; P₅, pregnenolone; P₄, progesterone; 17-P₄, 17-hydroxyprogesterone; 17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one (MIS); MPF, maturation promoting factor; ME₂, methoxyoestradiol. (1) oestrogen-2/4-hydroxylase; (2) catechol-O-methyltransferase; (3) 3β-hydroxysteroid dehydrogenase; (4) 17α-hydroxylase; (5) 20β-hydroxysteroid dehydrogenase; (6) P-450 17,20-lase; (7) 17β-hydroxysteroid dehydrogenase. R, receptor; A, androstenedione; OM, oocyte maturation.

The chemical identity and functional significance of the compounds may lead to the concept that multiple MISs may exist in the catfish ovary.

In conclusion, the present study suggested the occurrence of 17,20 β -P in the catfish, which showed a significant increase associated with oocyte maturation, both *in vivo* and *in vitro*. Testosterone level registered a significant decrease, which may account for the decrease in E₂ level (Mishra & Joy 2006a). The catfish ovary may synthesize cortisol and corticosterone, which increased with maturational activity. A comparison of the changes in the steroid profiles suggests that 2-OHE₂ may be an important link in the hormonal cascade of LH stimulation of oocyte maturation and ovulation.

Funding

The work was funded by a research project of DST, New Delhi (SP/SO/C-13/2001) to K P J. A M is grateful to

Banaras Hindu University for a research fellowship. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 18 January 2006

Accepted 6 February 2006

Made available online as an Accepted Preprint
13 February 2006