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Full Length Research Paper

Relative *in vitro* effectiveness of several gonadal steroids on oocyte maturation in freshwater teleost *Barilius vagra*

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The potency of various estrogens, androgens and progestogens at different concentrations and incubation periods on inducing final oocyte maturation *in vitro* was investigated. At the highest concentration (1000 ng/ml), all of the C_{21} steroids tested, progesterone (P_4), 17α-hydroxyprogesteron (17-OHP), 17α,20ß-dihydroxy-4-pregnen-3-one (17, 20ßP), as well as C_{19} steroids, testosterone (T) and androsteindione (AD) were able to induce germinal vesicle breakdown (GVBD), whereas C_{18} steroids, estrogens (E_1 and E_2) were found totally ineffective in inducing oocyte maturation at all the concentrations and exposure time. The relative potency of steroids were progestogens > androgens > estrogens. Among the steroids tested, 17,20βP appeared as the most potent oocyte maturation inducing steroid, causing 46% GVBD at the lower concentration of 10 ng/ml at 72 h exposure. The next most potent steroid was 17-OHP, inducing 25% GVBD at 72 h. The relative response among progestogens were 17, 20βP > 17-OHP > P_4. These data suggest that 17,20βP is the major maturation-inducing steroid (MIS) in *Barilius vagra* and both the duration of exposure and the hormonal concentration had a positive influence on the percentage of germinal vesicle breakdown.

Key words: *In vitro*, oocyte maturation, germinal vesicle breakdown, estrogens, androgens, progestogens.

INTRODUCTION

Progressive development of ovarian follicles in teleost involves several steps (Nagahama et al., 1994; Patino and Sullivan, 2002; Yaron and Levavi-Sivan, 2005), which are regulated by steroids that are synthesized therein under the direct influence of gonadotropins (Patino et al., 2001; Rehman et al., 2001; Matsuyama et al., 2002; Skoblina, 2009). Generally, oocyte growth (vitellogenin uptake) is followed by a process called final oocyte maturation (FOM). In the period of oocyte growth, C_{18} steroid estradiol 17β regulates the synthesis of hepatic yolk that is then sequestered into the oocyte. The gonadotropin activates the enzyme P_{450} aromatase that converts testosterone (T) and androstenedione (AD) to estrogens (E2 and to E1); the entire process occurring, respectively, in the thecal cells and granulosa cells of the follicle (two-cell model) (Nagahama et al., 1994), where the theca cells supply the precursor steroids, and the granulosa cells produce the steroidal mediators under the direct influence of gonadotropin. On completion of vitellogenesis, previtellogenic oocyte change to the vitellogenic oocyte up to the tertiary yolk globule stage and involves a distinct shift in steroidogenesis, that is, from estradiol to maturation inducing steroid (MIS). The steroidogenic enzyme genes from ovarian cytochrome P_{450} aromatase to 20 ß-hydroxysteroid dehydrogenase (20 ß-HSD), also occurs in the granulosa layers of ovarian follicles prior to oocyte maturation (Senthilkumaran, 2004). FOM, the shift of post vitellogenic follicle to pre-ovulated oocyte, is necessary for fertilization by sperm and the entire process involves germinal vesicle migration and breakdown (GVBD), coalescence of lipid droplets and yolk globules, release of the 1st polar body (Nagahama et al., 1983) and surge in gonadotropin secretion that induce FOM by the production of MIS (Nagahama et al., 1994; Rehman et al., 2001).

Current information about oocyte maturation indicates

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that gonadotropin regulates the process in two stages. During the first stage of maturation, the follicle (somatic) cells acquire the ability to produce MIH and the oocyteresponse to MIH [that is, oocyte maturational competence, (OMC)], whereas in the second stage, the follicle cells produce MIH and consequently the oocyte is released from meiotic arrest (Patino et al., 2001). In teleost species, progestogens induced oocyte maturation and the grade of induction is related to the position of specific functional groups on the steroid nucleus (Canario and Scott, 1988). Progesterone derivatives 17,20βP and 20β-S has been demonstrated as a MIS (Nagahama, 1997; Matsuyama et al., 2002; Garcia-Alonso et al., 2004) in several teleost, whereas 17,20βP was identified *in vivo* and *in vitro* as MIS in Salmonids and Cyprinids (Nagahama et al., 1983; Haider and Inbaraj, 1989; Nagahama and Yamashita, 2008; Lankford and Weber, 2010).

In vitro bioassays have been widely used in evaluating the relative effectiveness of steroids in inducing final oocyte maturation, hormonal regulation and ovulation in a variety of teleost (Nagahama et al., 1983; Garcia-Alonso et al., 2004; Skoblina, 2009), where GVBD was used as an indicator of final oocyte maturation. It can be induced *in vitro* by many gonadal steroids, but potencies may vary with structure of the steroids (Goetz, 1983; Trant and Thomas, 1988), the species under investigation (Goetz, 1983), and number of modulatory effectors (Moreau et al., 1985). At the highest concentration, that is, 1000 ng/ml or above, except estrogens, all C21 steroids, as well as C19 induced GVBD (Nagahama et al., 1983; Webb et al., 2000; Mojazi Amiri et al., 2001; Zuberi et al., 2002). Although in majority of teleost, 17,20βP appeared as the most potent MIH (Nagahama et al., 1983; Haider and Inbaraj, 1989; Patiño and Sullivan, 2002; Matsuyama et al., 2002; Garcia-Alonso et al., 2004), but number of other ovarian steroids including estradiol 17 β, corticosteroids and deoxycorticosteroids have also been found to have similar and in some cases even greater potency as MIS *in vitro* in several species (Hirose, 1976; Nagahama and Yamashita, 2008; Goetz.1983; Milla et al., 2009).

This observed diversity of MIH activity on species basis provided sufficient grounds to investigate which of the ovarian steroids may be the final MIH in the cyprinids selected for this study.

This study was designed to investigate the efficacy of several estrogens, androgens and progestogens in inducing oocyte maturation in *B. vagra* and also to determine the role of concentration and incubation period on germinal vesicle breakdown.

MATERIALS AND METHODS

Chemicals

The steroids used in this study were estrone (E_1: estra-1,3,5 (10)-triene-3-ol-17-one), estradiol 17β (E_2 : estra-1,3,5 (10)-triene-3,17β-diol), testosterone (T:17β-hydroxy-4-androsten-3-one), androstenedione (AD: 4-androstene-3, 17-dione), progesterone (P_4: pregn-4-ene-3, 20-dione), 17-α-hydroxyprogester one (17-OHP: 17α-hydroxypregn-4-ene-3, 20-dione) and 17α-20β-dihydroxypregn-4-ene-3-dione (17,20βP: 17, 20β-dihydroxypregn-4-ene-3-one). These were purchased from Sigma Chemical Co (St. Louis Missouri, USA). 2 mg of each steroid were dissolved in 2 ml of 96% ethanol to make stock solution, from which three dilutions of 1000, 100 and 10 ng /ml of incubation media were achieved.

Fish

During early breeding season, that is, late March to early April, sexually matured females of *B. vagra* were captured by cast net from Ramli stream and transported live to the experimental fish laboratory of the Department of Biological Sciences, Quaid-i-Azam University, Islamabad (Pakistan). They were allowed to acclimatize to the laboratory conditions for at least one week in stocking tanks, already aerated with air pumps for oxygen supply. The fish were fed daily on commercial fish feed.

Preparation of culture medium

The culture medium (BSS) was prepared by the method described previously by Zuberi et al. (2002). Briefly, 3.7 g NaCl, 0.32 g KCl, 0.16 g $CaCl_2$, 0.1 g NaH_2PO_4, 0.16 g $MgSO_4.7H_2O$ and 0.8 g glucose were dissolved in 1 L of double distilled water. The medium was autoclaved, cooled and its pH was adjusted to 7.5 with sterilized 1.0 N sodium bicarbonate ($NaHCO_3$). Bacterial and fungal contamination was controlled with penicillin-streptomycin (100 IU/ml) and fungizone (2.5 µg/ml). The medium was sterile filtered through a millipore filter of GS 0.22 µm (Bedford, Mass, USA).

In vitro bioassay

For the *in vitro* study, gravid female *B. vagra*, which possesses full-grown vitellogenic oocytes were sacrificed, the ovaries placed in ice-cold BSS, and then reduced to small pieces. Individual ovarian follicles were separated using fine forceps without disrupting the integrity of the thecal and granulosa layers. To check the position of germinal vesicle, a sub sample of the 20 largest ovarian follicles was treated with oocyte clearing fixative (ethanol, formalin, and glacial acetic acid mixed 95:10: 5 by volume) (Zhoa and Wright, 1985). All of the follicles contained oocytes with central to nearly central germinal vesicle (CGV).

The experiment was conducted in replicate for each treatment with a 3×3 factorial design (3 concentrations ×3 incubation periods). Three replicate groups were assigned to one of three incubation period (24, 48 and 72 h) and one of three concentration of steroid (10, 100 and 1000 ng/ml). Samples of 30 to 35 ovarian follicles for each treatment group were incubated at 22 ± 0.2°C for one of three incubation period and hormonal concentration in 15 ml BSS (pH = 7.5). The control cultures received 96% ethanol, equal to volume in the treatment groups. Viability of the oocytes was checked in preliminary tests by Trypan Blue (Zuberi et al., 2002) in samples of cultured oocytes at various time intervals. Upon completion of the incubations, the follicles were treated with egg clearing solution and examined under an inverted microscope (Nikon, Diaphot TMD). The percentages of GVBD were recorded.

Statistical analysis

Percentage of GVBD was calculated and expressed as mean ± S.E. in the text and tables. The percentage of GVBD for the treat

Table 1. Results of the three-way analysis of variance examining the effect of different steroids on the GVBD at different concentrations and incubation periods.

Source	df	F	Significance
Concentration	2	1920.988	0.000
Time	2	1529.619	0.000
Treatment	7	1846.436	0.000
Concentration × time	4	78.444	0.000
Concentration × treatment	14	211.837	0.000
Time × treatment	14	43.932	0.000
Concentration × time × treatment	28	15.666	0.000

ment with various estrogens, androgens and progestogens at different concentrations and incubation periods were analyzed by appropriate ANOVA models using SPSS for Windows (Soft ware version 10.0 Chicago, IL).

RESULTS

The germinal vesicle breakdown was influenced significantly by treatment, hormone concentrations and incubation periods (Table 1). Pairwise comparison by using GVBD as dependable variable revealed that there was no significant difference between the control and estrogens (E1 and E2) treated group as compared to androgens (AD and T) and progestogens (P4, 17-OHP and 17,20βP) (Table 2). The relative potency of steroids were progestogens > androgens > estrogens. The incubation period had a greater influence on GVBD and it was manifold higher at 72 h as compared to 24 h. The significant interaction between two fixed factors treatment and time (T × I) indicated that percentage of GVBD in hormone treated groups increased with increase in incubation periods (Table 1 and 2). ANOVA also revealed significant effect of the concentration of hormone on the percentage of GVBD. At high concentration (1000 ng/ml), except estrogens, both androgens and progestogens showed the significant effect on germinal vesicle breakdown, whereas at low concentration (10 ng/ml) and at 72 h, 17,20βP showed 45% of GVBD as compare to17-OHP and P_4, where GVBD were 24.7 and 18%, respectively (Table 2). The relative potency of progestogens were 17,20βP >17-OHP> P_4. However, 17, 20βP appeared as the single outstanding steroid causing ~ 20% GVBD in 24 h at the concentration of 10 ng/ml where no other gonadal steroids showed any response.

DISCUSSION

In teleost fish, FOM involves the complex set of morphological and biochemical mechanisms associated with the progression of meiotic cycle and breakdown of the nuclear envelope (GVBD). GVBD is usually regarded as a hallmark of the progress of the oocyte maturation. It is triggered by MIH, which act on receptors located on the oocyte membrane and induces the activation of MPF in the oocyte cytoplasm. In a number of teleost species, C21 steroids appear as potent initiators of GVBD *in vitro*, whereas testosterone, as well as other C19 steroids induces GVBD only at high concentration. Estrogens are usually not effective inducers of oocyte maturation in fish (Nagahama et al., 1983; Webb et al., 2000; Mojazi Amiri et al., 2001; Zuberi et al., 2002; Yueh and Chang, 2000).

The result of this investigation revealed that GVBD is influenced significantly by the exogenous steroids, their concentration and incubation periods (Table 1). The exogenous E_1, E_2, AD, T, 17-OHP, P_4 and 17, 20ßP are capable of influencing the position of the germinal vesicle *in vitro*, but only the androgens and the progestogens are the most effective steroids causing its breakdown (GVBD) (Table 2). The response of the follicles is, however, both dose dependent and time dependent. Thus, at the dosage of 1000 ng/ml, except for estrogens, all androgens and progestogens elicited maturational response that was maximal when the follicles were exposed for 72 h. The response was diminishing with decrease in dosage and time. However, under such conditions (10 ng/ml and 24 h), only 17,20ßP brought about significant GVBD (Table 2). Similar findings were reported in *Cyprinion watsoni* (Zuberi et al., 2002), white sturgeon (*Acipenser transmontanus)* (Lutes, 1985) and black porgy (*Acanthopagrus schlegeli)* (Yueh and Chang, 2000). 17,20βP appeared as one of the three most effective progestogens for oocyte maturation in *B. vagra* and was partially effective even at the lower concentration (10 ng/ml).

Progestogens in general and 17, 20βP in particular, is now considered to be the most potent MIS in a large number of teleost (*Salmo gairdneri*, *Carassius auratus, Oncorhychus rhodurus, Plecoglossus altivelis* (Nagahama et al., 1983); *Mystus vittatus* (Upadhyaya and Haider, 1986); *Cyprinus carpio, Labeo rohita, Cirrhinus mrigala* and *Catla catla* (Haider and Inbaraj, 1989); *Fundulus heteroclitus* (Petrino et al., 1993); *Morone americana* and *Morone chrysops* (King et al., 1995); *Halichoeres poecilopterus* (Matsuyama et al., 2002); and *Danio rerio* (Seki et al., 2008). Some previous studies aimed at analysing the relative roles of the individual steroids and revealed that E_2 is capable of

Table 2. Effects of gonadal steroids on GVBD of *Barilius vagra* at three different concentrations and incubation periods.

Parameter	24 h			48 h			72 h		
	0.01 (µg/ml)	0.1 (µg/ml)	1.0 (µg/ml)	0.01 (µg/ml)	0.1 (µg/ml)	1.0 (µg/ml)	0.01 (µg/ml)	0.1 (µg/ml)	1.0 (µg/ml)
BSA	3.24±0.15	0.98±0.98	2.10±1.06	7.25±0.798	5.25±1.11	6.27±0.227	11.9±1.12	10.1±0.70	12.9±0.74
E1	3.04±1.70^{NS}	0.00±0.00^{NS}	1.19±1.19^{NS}	6.04±1.839^{NS}	6.33±1.63^{NS}	7.39±0.624^{NS}	12.75±1.80^{NS}	13.06±1.30^{NS}	14.53±0.77^{NS}
E2	0.00±0.00^{NS}	0.00±0.00^{NS}	4.17±1.14^{NS}	7.52±0.83^{NS}	8.45±1.48^{NS}	9.62±1.71^{NS}	11.2±1.02^{NS}	13.9±1.19^{NS}	14.73±0.49^{NS}
AD	2.89±1.70^{NS}	2.89±1.70^{NS}	12.47±0.91**	9.04±1.494^{NS}	7.09±0.74^{NS}	23.25±1.103***	12.97±0.67^{NS}	21.21±1.51**	39.1±1.37***
T	3.81±1.04^{NS}	5.67±0.14*	20.60±1.24***	10.14±0.399*	10.07±1.71^{NS}	42.08±2.206***	11.34±0.93^{NS}	24.11±2.87**	67.62±0.48***
P4	4.11±1.07^{NS}	12.01±1.47**	22.38±0.79***	12.97±0.67*	29.81±0.62***	52.23±0.67***	18.0±1.76*	55.2±2.79***	66.1±0.99***
17OHP	5.25±1.11^{NS}	22.32±0.67***	28.00±1.03***	10.71±0.412*	40.07±1.11***	67.99±1.438***	24.7±0.64***	65.0±0.34***	77.8±1.47***
17,20BP	19.43±1.74***	54.93±1.70***	74.47±0.53***	35.37±1.592***	58.36±3.11***	93.79±1.516***	45.80±2.33***	75.65±2.18***	100±0.00***

NS = not significant; * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

inducing oocyte maturation *in vitro* in the Japanese medaka (*Oryzias latipes*: Hirose, 1976), an observation later negated by the work of Iwamatsu (1978) on this species. It seems that conflicting observations on the effects of given hormones within a species are liable to stem from the variable culture conditions in different laboratories or on the competence of the oocytes to respond at given times (Kime, 1993). As stated earlier, in *D. rerio* (Van Ree et al., 1977), it is E_1 that has GVBD stimulatory effect, whereas both E_2 and E are ineffective. Further investigations have demon-strated that estrogens are incapable of causing maturation of oocyte *in vitro* in most species of fish (Goetz, 1983; Trant and Thomas, 1988; Skoblina, 2009; Mishra and Joy, 2006) and the present observations on *B. vagra* also affirm this view. The estrogens nevertheless have indirect involvement as inhibitors of oocyte maturation and ovulation was proposed in some studies (Goetz, 1983; Nagahama and Yamashita, 2008; Pankhurst, 2008), implying that while E_2 can regulate maturation, it does not induce it. In contrast to estrogens, T, AD, P_4, 17-OHP, 17,20βP and also 20β-S have been demonstrated to have strong but species-specific, time and dose dependent MIS effect *in vitro* (Greely et al., 1986).

Androgens caused a relatively lower rate of GVBD compared with progestogens even at the higher concentration (1000 ng/ml) and at lower concentration; it showed no significant effect on maturation of oocyte. These findings in *B. vagra* were consistent with black porgy *(A. schlegeli)* (Yueh and Chang, 2000), *C. watsoni* (Zuberi et al., 2002), and white sturgeon (*A. transmontanus)* (Webb et al., 2000).

Among the progestogens, 17-OHP at the lower concentration (10 ng/ml) induced 5% of GVBD in *B. vagra* at 24 h, while it caused 9% of GVBD in *C. watsoni* (Zuberi et al., 2002), 10% in hybrid sturgeon bester (Mojazi Amiri et al., 2001) and 100% in white sturgeon (Lutes, 1985).

Although 17, 20βP is now well known to be the most potent MIS in majority of fish including *B.vagra*, other C_{21} steroids have also been found to be equally potent in some species. Among the C_{21} steroids, the effectiveness of progesterone, 11-deoxycorticosterone, as well as corticosteroids during natural oocyte maturation has been quite controversial (Goetz, 1983; Skoblina, 2009). In sterlet (*Acipenser ruthenus* L.), progesterone was

reported to be more effective than 17,20βP in inducing GVBD (Semenkova et al., 2006), where as in many other teleost fish, higher concentration of it is required to induced oocyte maturation (Webb et al., 2000). In *B. vagra*, 66% of GVBD were observed at higher concentration and exposure time (1000 ng/ml, 72 h), whereas only 12% GVBD occurred at 10 ng/ml in 24 h exposure. In the Japanese medaka, *O. latipes*, cortisol has been shown to be four times more effective than progesterone in causing maturation of the oocytes (Hirose, 1976), and in the zebra fish (*D. rerio*), it was found to be a better MIS than 17,20βP (Van Ree et al., 1977). Goswami and Sundraraj (1974) demonstrated that DOC, 11-DOC and 21-deoxycortisol are the strongest MIS in the Indian catfish, *H. fossilis*, while P4, 17-OHP and 17-dihydroxyprogesterone are poor inducers of maturation *in vitro*. Later works by Sundraraj et al. (1985) on the same species failed to confirm the earlier observation and demonstrated that 17,20βP is physiologically the most potent maturation inducing steroid in *H. fossilis,* while Mishra and Joy (2006) reported the synthesis of 17,20βP and corticosteroids in the ovary of Indian catfish. Apart from Indian catfish, involvement of corticosteroids in oocyte ovulation induction was demonstrated in medaka (Hirose, 1976), as well as in yellow perch (Theofan and Goetz, 1983). On the other hand, 11-DOC, hydrocortisone and cortisol have been demonstrated to be ineffective as MIS in ayu (*P. altivelis*), amago salmon (*O. rhodurus*), rainbow trout (*S. gairdneri*), and goldfish (*C. auratus*) (Nagahama et al.., 1983). As 17,20βP, 20βS and 11-DOC are all effective in causing GVBD in the tiger puffer (Matsuyama et al., 2001) and yellow perch (Theofan and Goetz, 1983), therefore 17,20βP remains the most potent inducer of final oocyte maturation in all these studies.

The relative potency of steroids tested in *B. vegra* were 17, 20βP > 17-OHP > P4 > T >AD > E1 and E2. All of these steroids except estrogens induced the dose related stimulation of oocyte maturation, while progentogens generally and 17,20βP particularly appeared as the potent MIS in this species. Apparently, the side chain of the pregnenes molecule is a key structural component of the MIS that interacts with the active site of the oocyte receptor controlling the final oocyte maturation (Trant and Thomas, 1988) and certain alterations of the side chain of progestogens increase the potency of the resultant steroid 1000 fold. Trant and Thomas (1988) showed that steroids with hydroxyl groups at C17, C20 (17, 20ßP), C17 (17-OHP) are more potent than P_4 (hydroxyl group at C 21 positions) particularly at concentrations below 10 ng/ml. The 17,20ß-dihydroxy configuration of P_4 (17, 20ßP) therefore results in maximal stimulation of GVBD at near physiological concentration.

In conclusion, bioassay data suggest that 17, 20βP is a prime candidate for MIS in *B. vagra,* although a role of other progestogens cannot be ruled out, including the possibility that they could be involved in chemical communication. Our results also confirm earlier sugges-tions that 20β-OH, 17α-OH and 3 = O are critical groups that contribute to the interaction with the MIH receptor. The oxygen atom is involved in making a hydrogen bond with residues in the receptor protein, whereas three oxygen atoms in 17,20βP contribute to hydrogen bonding and provide strong maturation inducing effect.

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