Effects of Steroid Hormones on *In Vitro* GVBD and Oocyte Steroidogenesis in Blacktip Grouper, *Epinephelus fasciatus*

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ABSTRACT : To verify the sex steroids which are involved in oocyte maturation of the blacktip grouper, *Epinephelus fasciatus*, we incubated vitellogenic oocytes (0.41 and 0.50 ㎜ in average diameter) in the presence of exogenous steroid precursor ([^{3}H]17α-hydroxyprogesterone). Steroids were extracted, separated and identified by thin layer chromatography. The major metabolites produced were androstenedione, estradiol-17β, estrone and progestogens. Progestogen metabolites in the oocytes of 0.50 ㎜ were more abundant than those of 0.41 ㎜. Also, we investigated the *in vitro* effects of human chorionic gonadotropin (HCG; 5, 50 and 500 IU/㎖), 17α,20β-dihydroxy-4-pregnen-3-one (17α20βP) and 17α,20β-trihydroxy-4-pregnen-3-one (17α20β21P; 5, 50 and 500 ng/㎖, respectively) on oocyte maturation. In the oocytes of 0.41 ㎜, treatment with 50 IU HCG stimulated GVBD (55.30±1.20%) compared with controls (32.41±3.13%, p<0.05). In the oocytes of 0.50 ㎜, treatment of 17α20βP (50 and 500 ng/㎖) stimulated GVBD (50.13±2.52 and 51.77±5.91%, respectively) compared with controls (36.81±2.89%, p<0.05). Treatment with 500 IU HCG also stimulated GVBD (49.59±5.15%) compared with controls (p<0.05). Taken together, these results suggested that both HCG and 17α20βP were effective on *in vitro* oocyte maturation and 17α20βP may act as a maturation inducing hormone in blacktip grouper.

Key words : Blacktip grouper, Germinal vesicle breakdown, Maturation inducing hormone, Oocyte maturation, Steroid metabolite

INTRODUCTION

Teleost oocyte maturation is regulated by hypothalamus-pituitary-gonad axis and sex steroid hormones produced from gonad play an important role in this process (Nagahama et al., 1994; Nagahama & Yamashita, 2008). Generally, estradiol-17β (E_2) induces vitellogenin uptake from liver into oocytes during vitellogenesis. After then, it has been reported that final oocyte maturation including germinal vesicle break down (GVBD) and ovulation are stimulated by maturation inducing hormone (MIH). Two kinds of progestogen, 17α,20β-dihydroxy-4-pregnen-3-one (17α20βP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (17α20β21P) were reported as MIH in most salmonids and some sciaenid fishes (Migaud et al., 2003; Patino et al., 2003; Baek, 2008; Nagahama & Yamashita, 2008). Until now, extensive studies described above with identification of MIH and the effects of various hormones on the induction of oocyte maturation have been performed in a number of teleost species for aquaculture. However, the molecular nature and physiological function of MIH is remained unclear yet in various teleost species.

Among the various species for aquaculture, groupers are the protogynous species which have high commercial value in East and Southeast Asia (Fukuhara, 1989; Marte, 2003). The blacktip grouper *Epinephelus fasciatus* is also commercially important in the China, Japan, Taiwan and

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Korea but the catch continues to decline (Annalie et al., 2000). So far, spawning characteristics, embryo/larval development, growth for this species were studied (Kawabe et al., 1997; Kawabe et al., 2000; Kawabe & Kohno, 2009). However, there is no information about steroid production related to oocyte maturation of blacktip grouper.

The purpose of this study was 1) to verify the steroid metabolites from vitellogenic oocytes and 2) to compare the effects of exogenous progestogens, 17α20βP and 17α20β21P on *in vitro* final oocyte maturation using isolated oocytes of blacktip grouper.

MATERIALS AND METHODS

1. Chemicals

Steroid standards were purchased from Sigma Chemical (St. Louis, Missouri, USA) or Steraloids Inc. (Wilton, NH, USA). Stock solutions (㎎/㎖) were prepared by dissolving the compounds in ethanol. These were further evaporated to dryness and diluted in incubation media. The ethanol concentration in the incubation medium was maintained at less than 0.1%. Radioactive [^{3}H]-17α-hydroxyprogesterone ([^{3}H]-17αP) was obtained from Amersham Life Science (London, England).

2. Experimental Fish and Incubation Protocols

The experimental fish were reared and adjusted to 14L:10D and 25℃ in recirculating aquaria (500 ℓ) from Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. Oocytes were obtained by cannulation after anesthetization of mature females. The cannualted oocytes were put into cold balanced salt solution (BSS; 132.96 mM NaCl, 3.09 mM KCl, 0.28 mM $MgSO_4 \cdot 7H_2O$, 0.98 mM $MgCl_2 \cdot 6H_2O$, 3.40 mM $CaCl_2 \cdot 6H_2O$, 3.65 mM HEPES). Oocytes were separated into groups using fine forceps. Vitellogenic oocytes with average diameters of 0.41 and 0.50 ㎜ were used for the *in vitro* studies.

We performed two separate experiments. In experiment I, we incubated oocytes with a radiolabeled exogenous precursor for separation of potential MIH. Oocytes were separated into small pieces in ice-cold BSS, and approximately 20 follicle enclosed oocytes of 0.41 and 0.50 ㎜ were incubated in each well of 24-well culture plates containing 1 ㎖ of Leibovitz L15 medium (Gibco, Grand Island, NY, USA). Incubations were initiated by adding 55 kBq of [^{3}H]-17αP as the radiolabeled precursor. The pH and osmolality of the media were adjusted to 7.85 and 370 mOsm, respectively. The plates were incubated for 24 h at 18℃ with constant gentle shaking. In experiment II, we evaluated the effects of various steroids on *in vitro* oocyte maturation. Oocytes of 0.41 and 0.50 ㎜ were incubated with 17α20βP, 17α20β21P (5, 50 and 500 ng/㎖) and human chorionic gonadotropin (HCG; 5, 50 and 500 IU/㎖). The pH and osmolality of the media were adjusted as described above. The plates were incubated for 24 h at 18℃ with constant gentle shaking.

3. Analysis of Steroid Metabolism and GVBD Assay

At the end of the incubation (experiment I), steroids were extracted three times from the media and oocytes using 4 ㎖ dichloromethane. The extracts were concentrated and applied to a thin-layer chromatography (TLC) plate ($60F^{254}$; Merck, Darmstadt, Germany) with non-radioactive standard steroids as carrier steroids, and developed in a mixture of benzene : acetone (4 : 1) and benzene : ethyl acetate (4 : 1). Retention factor (Rf) values for each steroid standard are listed in Table 1. Radioactive steroid metabolites were analyzed using a BAS 1500 bio-imaging analyzer (Fuji Film, Tokyo, Japan), and estrone (E_1) and E_2 standards were visualized by exposure to iodine vapor. Other standard steroids were detected by UV absorption at 254 nm.

In experiment II, the oocytes were fixed with clearing solution (ethanol : formalin : glacial acetic acid = 6 : 3 : 1) after incubation. The location of the germinal vesicle (GV) was observed under low-power magnification using a dissecting microscope. The number of oocytes that had completed GVBD, i.e., dissolution of the nucleus or had ovulated was counted in each well.

Table 1. The retention factor (Rf) values of steroid metabolites on the TLC plates

Steroids	Rf values[a]
E_1	0.79
A_4	0.64
E_2	0.54
17αP	0.44
T	0.39
17α20βP	0.19
17α20αP	0.14
17α20β21P	0.04

Rf is defined as the distance migrated by the compound divided by the distance traveled by the solvent (150 ㎜).

[a]Solvent system of mobile-phase was 3 times of benzene : ethyl acetate = 8 : 2 (v/v) after benzene : acetone = 8 : 2 (v/v). E_1, estrone; A_4, androstenedione; E_2, estradiol-17β; 17αP, 17α-hydroxyprogesterone; T, testosterone; 17α20βP, 17α,20β-dihydroxy-4-pregnen-3-one; 17α20αP, 17α,20α-dihydroxy-4-pregnen-3-one; 17α20β21P, 17α,20β,21-trihydroxy-4-pregnen-3-one.

4. Statistics

GVBD data were expressed as means with the standard error of the means (SEM) and tested for normality using the Kolmogorov-Smirnov test using SPSS software (version 17.0) for Windows (SPSS, Chicago, IL, USA). Non-parametric Kruskal-Wallis test followed by the Bonferroni adjustment was tested due to the assumptions of normality and equal variance were failed. A value of $P<0.05$ was considered statistically significant.

RESULTS

1. Steroid Metabolism

When vitellogenic oocytes (0.41 and 0.50 ㎜ in diameter) were incubated with [^{3}H]-17αP, the metabolites were separated and co-migrated with standard A_4, E_2, E_1 and three unknown metabolites which were assumed as progestogens although T and 17αP were overlapped slightly by TLC (Fig. 1). In the comparison of each metabolite with their photo-stimulated luminescence (PSL) values from autoradiography followed oocyte diameter (Fig. 2), the

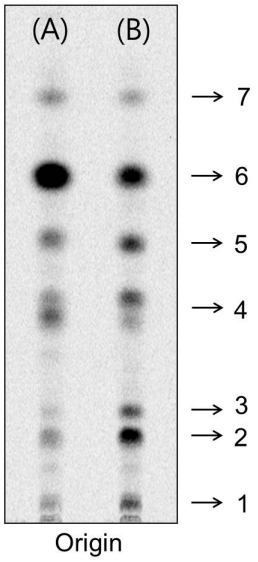

Fig. 1. Autoradiogram of radioactive steroid metabolites produced from [^{3}H]-17α-hydroxyprogesterone after 24 hours incubation with isolated oocytes (20 oocytes/㎖/well) from blacktip grouper. TLC developments were once in benzene:acetone (80:20) and 3 times in benzene:ethyl acetate (80:20). The intensity of radioactivities of metabolites was measured by using image analyzer (BAS 3000, Fuji, Japan). A, oocytes of 0.41 ㎜; B, oocytes of 0.50 ㎜; 1, progestogen 1; 2, progestogen 2; 3, progestogen 3; 4, T + 17αP; 5, E_2; 6, A_4; 7, E_1.

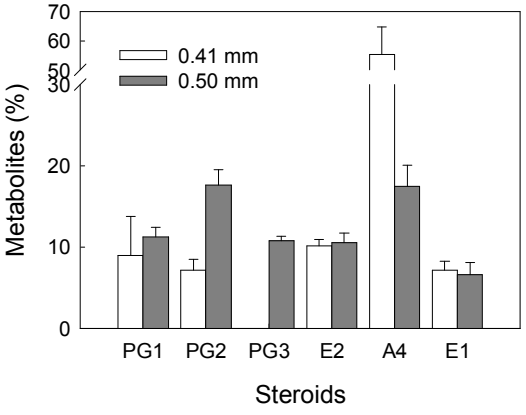

Fig. 2. Radioactivities of steroid metabolites from [^{3}H]17α-hydroxyprogesterone in blacktip grouper oocytes. The percentage of radioactivity associated with each isolated steroid was calculated based on the PSL of each metabolite from the total PSL value. Values are mean±SE (in duplicate wells, 20 oocytes/well). PG1, progestogen 1; PG2, progestogen 2; PG3, progestogen 3; E_2, estradiol-17β; A_4, androstenedione; E_1, estrone.

metabolites of progestogen 1 and 2 were increased at the oocytes of 0.50 ㎜ (11.27±1.13 and 17.62±1.93%, respectively) than those at the oocytes of 0.41 ㎜ (9.00±4.78 and 7.15± 1.40%, respectively). The metabolite of progestogen 3 was more produced in the oocytes of 0.50 ㎜ (10.77±0.59%) although it was not detected in the oocytes of 0.41 ㎜. Metabolite of E_2 was remained about 10 % in both oocytes (10.12±0.87 and 10.56±1.13%, respectively) although metabolite of A_4 was reduced in the oocytes of 0.50 ㎜ (17.49±2.57%) than in the oocytes of 0.41 ㎜ (55.21±9.50%). Metabolite of E_1 was 7.14±1.16 and 6.61±1.54% in the oocytes of 0.41 and 0.50 ㎜, respectively.

2. Effects of Exogenous HCG, 17α20βP and 17α20β21P on *in vitro* GVBD

In the oocytes of 0.41 ㎜ (Fig. 3-A), there was no significant effect on GVBD in the treatment of 17α20βP and 17α20β21P. Treatment of 50 IU HCG resulted in a significant increase in GVBD (55.30±1.20%) compared with controls (32.41±3.13%, $p<0.05$). In the oocytes of 0.50 ㎜ (Fig. 3-B), HCG stimulated GVBD dose-dependently and 500 IU treatment resulted in a significant increase in GVBD (49.59±5.15%) compared with controls (36.81±2.89%, $p<0.05$). Treatment of 17α20βP also stimulated GVBD dose-dependently. Treatment of 50 and 500 ng/㎖ 17α20β P resulted in a significant increase in GVBD (50.13±2.52 and 51.77±5.91%, respectively) compared with controls ($p<0.05$).

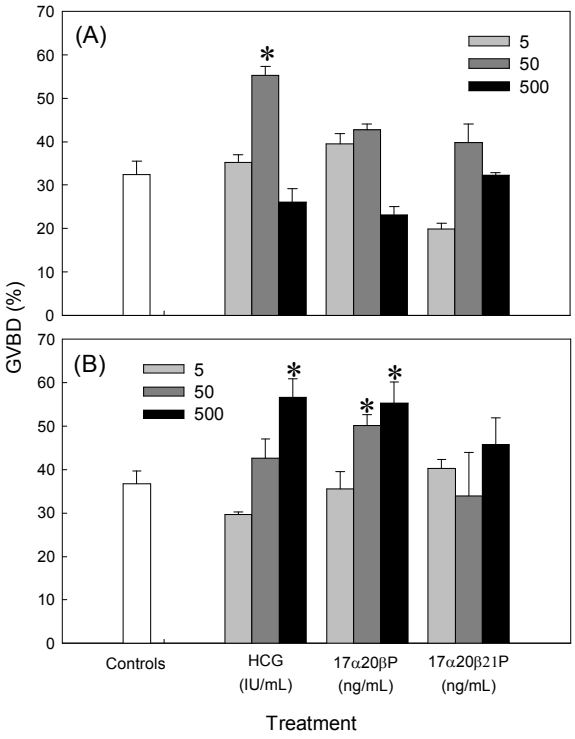

Fig. 3. Effects of HCG and progestogens on *in vitro* GVBD from oocytes of blacktip grouper. Values are the mean±SE of triplicates. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks indicate significant differences from controls ($p<0.05$). A, Oocytes of 0.41 ㎜ in diameter; B, Oocytes of 0.50 ㎜ in diameter.

DISCUSSION

In the present study, we verified the steroid metabolites from vitellogenic oocytes (0.41 and 0.50 ㎜ in diameter) and tested the effects of 17α20βP and 17α20β21P which were expected as MIH on *in vitro* final oocyte maturation.

A_4, E_2, E_1 and three kinds of unknown metabolites (these were assumed as progestogens) were produced from the oocytes of blacktip grouper. Interestingly, A_4 metabolite was the highest in both of 0.41 and 0.50 ㎜ oocytes. Androgens have been shown to induce sex change in some protogynous hermaphrodites (Cardwell & Liley, 1991). On the other hand, Montero and coworkers reported that androgens are linked to steroidogenic feedback in hypothalamus and they may act as the substrate for estrogen production (Montero et al., 1995). However, because A_4 metabolite was still high in accordance with progestogen metabolites, we considered that there is the other physiological function of A_4 or regulatory process in maturation rather than feedback or estrogen conversion in balcktip grouper. In our previous study, T and A_4 also showed high production from the vitellogenic oocytes of red lip mullet, *Chelon haematocheilus* (Baek et al., 2011). The role of androgen

including T and A_4 in oocyte maturation process should be conducted.

Our results demonstrated that three unknown metabolites which we assumed them as progestogen were produced. Among them, progestogen 2 co-migrated with 17α20βP standard in TLC system. We considered progestogen 2 as 17α20βP and further identification is presently under investigation. In general, final oocyte maturation in teleosts is induced by C_{21} steroid hormones, especially those with 20β- and 21-hydroxylated steroids (Nagahama & Yamashita, 2008). To date, 17α20βP in salmonid fish and 17α20β21P in the Atlantic croaker, *Micropogonias undulates*, spotted seatrout, *Cynoscion nebulosus* and black porgy, *Acanthopagrus schlegeli* were reported as MIH (Trant et al., 1986; Goetz et al., 1987; Yueh et al., 2005). On the other hand, several previous studies reported that both 17α20βP and 17α20β21P may act as MIH in croaker and bass species (Trant & Thomas, 1988; Berlinsky & Specker, 1991; King et al., 1994a, b). In spite of the presence of 17α20βP metabolite of the present study, progestogen 3 was detected with high activity only in the oocytes of 0.50 ㎜. However, it did not co-migrate with any other standards in TLC analysis. This result would be of considerable interests. We hypothesize that this metabolite may play a role in regulating oocyte maturation process with 17α20βP although we have insufficient data to demonstrate it at this point.

Until now, the MIH has not been identified in grouper species (Shein et al., 2004) although Johnson and coworkers reported that both 17α20βP and 17α20β21P were measured in red grouper, *Epinephelus morio* (Johnson et al., 1998). However, we suspect 17α20βP as a MIH of blacktip grouper and then the effects of 17α20βP and 17α20β21P as representative MIH on *in vitro* GVBD were examined for inquiry of functional MIH between them on final oocyte maturation. HCG treatment was designed as positive controls.

In the comparison of *in vitro* GVBD assay, treatments of HCG and 17α20βP were more effective than that of 17α20β21P. Moreover, induced GVBD by both HCG and 17α20βP treatment were dose-dependent manner. Sorbera and coworkers also reported that 17α20βP treatment was more effective on oocyte maturation of sea bass, *Dicentrarchus labrax* than 17α20β21P treatment (Sorbera et al., 1999). More recently, Yueh and coworkers reported that both 17α20βP and 17α20β21P could induce *in vitro* oocyte maturation of black porgy although its authentic MIH was identified as 17α20β21P (Yueh et al., 2005). However, our results from both oocyte groups were different with respect to the tested concentrations of each treatment. In the oocytes of 0.41 ㎜, the highest concentrations (500 ng/㎖ and IU) of each treatment resulted in a slight decrease than their lower concentrations (50 ng/㎖ and IU). The sensitivity of oocyte to HCG and 17α20βP in the oocytes of 0.41 and 0.50 ㎜ appears to be differently regulated. We considered that the sensitivity of oocytes to certain hormone or chemical varies depending on oocyte size as reported in our previous studies (Hwang et al., 2010; Baek et al., 2011). Oocytes of 0.50 ㎜ which was suggested as fully vitellogenic stage appeared to be more sensitive than those of 0.41 ㎜ although both oocytes-groups were in the same vitellogenic stage.

In conclusion, the present study provides some evidence that 17α20βP is a major MIH in blacktip grouper. Our data also suggests that 17α20βP may act as a MIH on *in vitro* oocyte maturation. The further identification of 17α20βP and the other unknown progestogen metabolite should be investigated in the future study.

ACKNOWLEDGEMENTS

This research was supported by Development of the Suitable Systems for Aquaculture of Blacktip Grouper, *Epinephelus fasciatus* (109197-3), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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(Received 18 January 2012, Received in revised form 17 February 2012, Accepted 23 February 2012)