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OOCYTE MATURATION IN PROTANDROUS BLACK PORGY, *ACANTHOPAGRUS SCHLEGELI*, STIMULATED BY ENCLOMIPHENE AND LUTEINIZING HORMONE RELEASING HORMONE ANALOGUE

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Ching-Fong Chang and Wen-Shiun Yueh (1990) Oocyte maturation in protandrous black porgy, *Acanthopagrus schlegeli*, stimulated by enclomiphene and luteinizing hormone releasing hormone analogue. *Bull. Inst. Zool., Academia Sinica* **29**(3): 173-179. Black porgies, *Acanthopagrus schlegeli*, are the marine protandrous hermaphordite. The objective of this study was to investigate the oocyte maturation and the negative feedback effects of estrogen in black porgy by treatment with an anti-estrogenic enclomiphene (cis-clomiphene) based on oocyte maturation and plasma sex steroid levels. The effects of enclomiphene were compared to those of a luteinizing hormone releasing hormone analogue (LHRH-A). Eighteen mature female black porgies were equally divided into three groups and treated with enclomiphene, LHRH-A or saline. Oocyte maturation was stimulated by both enclomiphene and LHRH-A. Enclomiphene failed to increase the levels of plasma estradiol-17β (E_2) and testosterone (T) but stimulated a high level of progesterone. Plasma E_2 and T levels increased significantly in the LHRH-A treated group. Neither enclomiphene nor LHRH-A could stimulate the response of 17α hydroxyprogesterone.

Key words: Oocyte maturation, Enclomiphene, Estrogen, Black porgy, LHRH-A.

Black porgies, *Acanthopagrus schlegeli*, the marine protandrous hermaphrodite, are widely distributed in the China Sea. This species is the object of considerable attention by both commercial fishery and culture industries. During the first and second spawning seasons, black porgies exhibit male characteristics, but begin to sexually reverse themselves during or after the third year. In Taiwan black porgies have an annual reproductive cycle with a multiple spawning pattern occurring in late winter and spring. However, the control of maturation in this species still requires further characterization.

Direct evidence of negative feedback from gonadal steroids on the hypothalamo-hypophyseal axis has been demonstrated in castrated rainbow trout (Billard *et al.*, 1977). The anti-estrogen, clomiphene, blocks the negative feedback system for estrogens (Adashi, 1984). This compound has been used to induce ovulation in gonochoristic teleosts (Pandey and Hoar, 1972; Pandey *et al.*, 1973; Singh and Singh, 1976; Kapur and Toor, 1979). Further support for this phenomena has also been provided by the implantation of an anti-estrogen in goldfish (Billard and Peter, 1977). However, enclomiphene (cis-clomiphene) is

considered to exhibit more potent anti-estrogenic properties in comparison to either clomiphene or zuclomiphene (trans-clomiphene) in rodents and domestic animals (Nagel *et al.*, 1970; Huang and Miller, 1983; Chang and Reeves, 1987). The effects of enclomiphene upon the latter stages of reproductive cycles in fish have not yet been studied. In addition, the negative feedback system of estrogen in marine protandrous hermaphrodites has not been reported. Thus, we examined the effects of enclomiphene upon plasma steroids and the maturation of oocytes in black porgies. The stimulatory effects of enclomiphene were also compared to those of a luteinizing hormone releasing hormone analogue (LHRH-A).

MATERIALS AND METHODS

Fish: Wild mature female black porgies were captured by gill nets in the coastal waters of Taiwan in January of 1988. The captured fishes were transported to the holding facilities within 24 hours. Following 3 days of acclimation, mature females (body weight 510-1,470 g) with oocyte diameters of about 0.5 mm were selected for experimentation. All fishes were fed with raw oysters and reared in tanks with 2.5 tons of sea water at 20-24°C at the Universty's fish hatchery.

Experimental Design: Eighteen female black porgies were randomly divided into three equal groups and treated with LHRH-A, enclomiphene or saline. The fishes were injected twice (days 0 and 2) with doses of 5 μg/kg wt and 25 μg/kg wt in the LHRH-A group (D-Ala^6, des-Gly^{10} LHRH ethylamide, Sigma Chemical Co.), 4 mg/kg wt in the enclomiphene group and 0.5 ml saline/kg in the control group. The fishes were anesthetized in a bath of 0.4 ml/liter 2-phenoxyethanol prior to handling. Blood samples were collected in heparinized tubes from the caudal vasculature on days 0, 2, 3, 4 and 6. The plasma was separated by centrifugation and stored at −20°C for later hormone analysis. The intra-ovarian oocytes were aspirated with a plastic tubing on days 0, 3 and 6. The dimensions of at least 10 of the fresh and largest class of oocytes were measured with an ocular micrometer on a microscope.

Assays: Estradiol-17β (E_2), testosterone (T), 17α hydroxyprogesterone (17-OH P) and progesterone (P_4) were measured in solvent extracted plasma samples without chromatographic separation as previously described by Chang and Yueh (1990). The mean extraction efficiencies for E_2, T and 17-OH P extracted with ethyl ether were 88.1%, 93.6% and 92.5%, respectively. For P_4 extracted with petroleum ether, the mean was 90.8%. Bound steroids were separated from free steroids with dextran coated charcoal solution and centrifuged following overnight incubation of extracted steroid with antiserum. The radioactivity of the bound ^{3}H-steroid in supernatant was counted in a Beckman liquid scintillation counter (Beckman 5801) with counting fluid (NE 266, Nuclear Enterprises, Edinburgh, Scotland). Standards were processed in the same manner as the unknown samples. The sensitivity of the assays for E_2, T, 17-OH P and P_4 were 10, 12.5, 12.5 and 12.5 pg per assay, respectively. Intra-assay and inter-assay variations for four measured steroids were 12.6-19.0% and 15.5-23.4%, respectively. Cross-reactions of the antisera against E_2, T, 17-OH P and P_4 were evaluated by England *et al.* (1974), Belanger *et al.* (1980), Flint *et al.* (1978),

and Wimpy *et al.* (1986), respectively. [2, 4, 6, 7-^{3}H] estradiol-17β (85-110 Ci/mmol), [1, 2, 6, 7-^{3}H] testosterone (80-105 Ci/mmol), [1, 2, 6, 7-^{3}H] progesterone (80-110 Ci/mmol) and 17α hydroxy [1, 2, 6, 7-H] progesterone (55-85 Ci/mmol) were purchased from Amersham Co. (Arlington Heights, IL).

Data Analysis: The results were examined by Duncan's multiple range test (Steel and Torrie, 1980) and presented as mean with a standard error of means (SEM).

RESULTS

The average diameter of oocytes increased significantly ($P<0.01$) within 3 days after the first injection of either enclomiphene or LHRH-A (Fig. 1). Oocyte dimensions did not change in the control group during the experimental period. Oocytes in 3 of 6 fishes in the enclomiphene group and in all of the fishes in the LHRH-A group showed maturity with transparent ooplasma and ovulation. Plasma levels of E_2, T and 17-OH P did not increase after injection of enclomiphene (Figs. 2 and 3). However, plasma levels of P_4 increased significantly ($P<0.05$) in the enclomiphene group (Fig. 3). Plasma E_2 and T reached significantly higher levels ($P<0.01$) after the injection of LHRH-A (Figs. 2 and 3). Plasma profiles for 17-OH P and P_4 in the LHRH-A group remained relatively stable (Fig. 3). The levels of measured steroids were constant in the control group (Figs. 2 and 3).

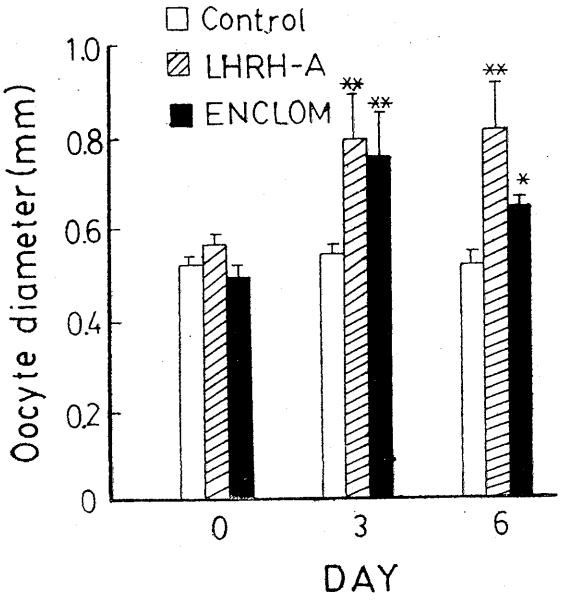

Fig. 1. Average diameter (mm) of intra-ovarian oocytes on days 0, 3, and 6 in mature female black porgies which were treated with enclomiphene (ENCLOM) or LHRH-A on days 0 and 2. The vertical bars represent the mean±SEM. Symbols ** and * represent significant levels at 1% and 5%, respectively.

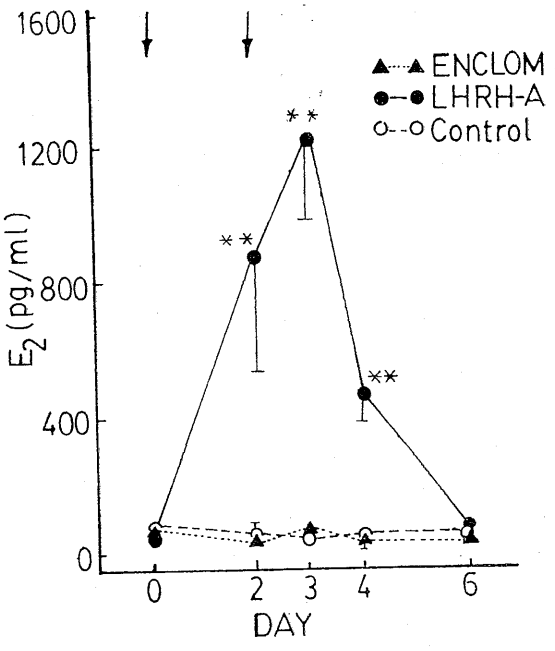

Fig. 2. The profiles of plasma estradiol-17β (E_2) in mature female black porgies which were treated with enclomiphene (ENCLOM) or LHRH-A. Arrows (↓) indicate injection schedule. Also see legend Fig. 1 for details.

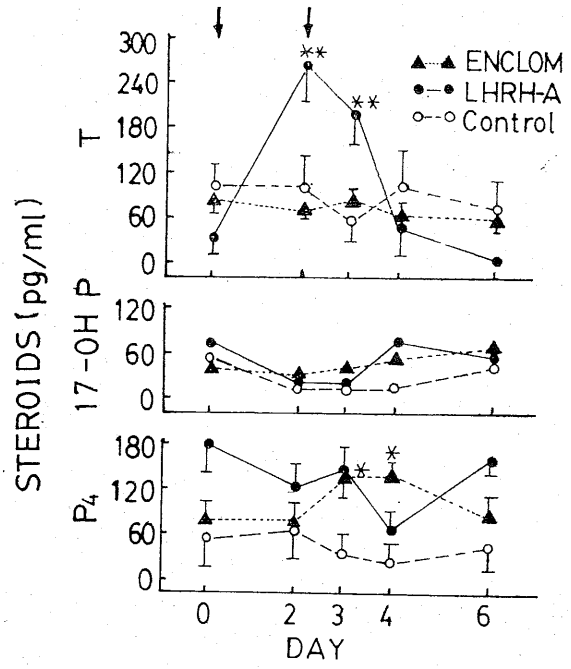

Fig. 3. The profiles of plasma testosterone (T), 17α hydroxyprogesterone (17-OH P) and progesterone (P_4) in mature female back porgies which were stimulated with enclomiphene (ENCLOM) or LHRH-A during the spawning season. Also see legend Fig. 1 for details.

DISCUSSION

Clomiphene is an important member of the family of synthetic triphenylethylene derivatives. It has been used to study the stimulation of ovulation and negative feedback system of estrogen in fishes. The results presented indicate that there was a stimulation of reproduction in black porgy following enclomiphene treatment (Fig. 1). These results are consistent with the results of treatment with clomiphene in gonochoristic teleosts such as goldfish (Pandey and Hoar, 1972; Pandey *et al.*, 1973), catfish (Singh and Singh, 1976), carp (Breton *et al.*, 1975; Kapur and Toor, 1979) and coho salmon (Donaldson *et al.*, 1981).

Clomiphene was first used by Greenblatt (1961) to induce ovulation in anovulatory women. In mammals, it is generally assumed that this anti-estrogen displaces endogenous estrogens from estrogen receptor sites on target organ such as the hypothalamus or pituitary (reviewed by Adashi, 1984; Cushing *et al.*, 1985). This interaction brings about the alleviation of the negative feedback exerted by endogenous estrogens and results in increasing the release of luteinizing hormone releasing hormone and luteinizing hormone (reviewed by Adashi, 1984; Chang and Reeves, 1987). Serum gonadotropin levels were stimulated in mature female goldfish after implantation of clomiphene in the hypothalamus and pituitary (Billard and Peter, 1977). Clomiphene also induced the levels of plasma gonadotropin in common carp (Breton *et al.*, 1975). It has been reported that decreasing plasma E_2 levels precede the rise of gonadotropin in female rainbow trout during the final stage of the reproductive cycle (Scott *et al.*, 1983). Therefore, it was suggested that there exists a negative feedback effect of estrogen at the hypothalamo-hypophyseal level in fish (Billard and Peter, 1977; Scott *et al.*, 1983). Timmers and Lambert (1989) suggested that the inhibition of the methylation of dopamine by the catecholestrogens may involve the negative feedback of gonadal steroids. Different degrees of feedback inhibition between gonadal steroids and gonadotropin at various phases in the reproductive cycle of male rainbow trout have also been reported (Billard *et al.*, 1977).

Because purified black porgy gonadotropin and anti-gonadotropin serums were unavailable, only the responses of oocyte dimension and sex steroids were measured in the present study. Oocyte size and maturation were clearly stimulated by enclomiphene (Fig. 1). This stimulation was probably mediated from

the secretion of gonadotropin. However, increases in plasma E_2 and T were not observed in the enclomiphene group. The hypothalamus is also considered the target of enclomiphene in black porgies. The direct action of enclomiphene on ovarian steroidogenesis or some unidentified indirect action on maturation can not be excluded. The possible target organ and the mechanism of action of enclomiphene on the reproduction of black porgies need further study.

The dose (4 mg/kg wt) of enclomiphene administered to black porgies was calculated from the dose of clomiphene clinically administered to women (Murad and Haynes, 1980) and cows (Chang and Reeves, 1987). The possibility of enclomiphene increasing the levels of plasma E_2 and T at 2 mg and 20 mg/kg wt dosages in ayu, *Plecoglossus altivelis*, was considered (Hu, 1989). The effects of dosages on different reproductive stages in different species were also considered.

The diameter of oocytes in black porgies after the stage of the yolk accumulation was about 0.5 mm. Oocytes became mature and transparent when their dimensions increased to at least 0.8 mm. This characteristic was observed in both the enclomiphene and LHRH-A groups. The decrease in the oocyte diameter of the enclomiphene group on day 6 (Fig. 1) was most likely due to the depletion of the mature oocytes after oviposition or sampling variation.

The use of LHRH-A as a positive control to compare the effects of enclomiphene in this experiment demonstrated that a dose of LHRH-A (5 μg/kg) was sufficient to induce maturation and also increase the concentrations of plasma E_2 and T. However, both LHRH-A and enclomiphene failed to increase plasma levels of 17-OH P in our sampling schedules. 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-diOH P) and 17-OH P have been reported to induce final oocyte maturation in the hermaphroditic red sea bream, *Pagrus major* (Adachi *et al.*, 1988). Due to the unavailability of 17α, 20β-diOH P assay in this experiment, only 17-OH P and P_4 levels were measured although 17α, 20β-diOH P was very important in teleosts. The physiological role of the slight increase in P_4 levels in the enclomiphene group is not known. It could be due to a cross-reaction between the antiserum and an unidentified steroid.

In conclusion, through the inducement of enclomiphene and LHRH-A, similar stimulatory results on oocyte maturation were demonstrated in protandrous female black porgies; however, the effect of these hormones on sex steroid profiles were different. Enclomiphene failed to increase plasma E_2 and T but stimulated P_4 profiles. In contrast, plasma E_2 and T levels increased significantly in the LHRH-A group.

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抗雌性激素與 LHRH-A 促進黑鯛卵細胞成熟

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本研究之目的爲探討抗雌性激素 (enclomiphene) 對黑鯛生殖之影響及研究雌 性激素的負迴饋作用。18尾黑鯛平均分爲三組，分別注射 LHRH-A、抗雌性激素或生理食鹽水。LHRH-A 與抗雌性激素均可促進卵細胞增大與成熟。 LHRH-A 並可促使黑鯛血液雌二醇與睪固酮濃度增高。而類黃體激素 (17α hydroxyprogesterone) 並無改變。但抗雌性激素却促使黃體激素濃度升高，而雌二醇與睪固酮濃度則無改變。本研究的結果無法提供直接證據支持雌性激素對黑鯛的負迴饋作用。