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STIMULATORY EFFECTS OF SALMON CALCITONIN ON THE SECRETION OF MATURATION-INDUCING STEROID BY THE POST-VITELLOGENIC OVARIAN FOLLICLES OF COMMON CARP *Cyprinus carpio*

ARINDAM CHAKRABORTY[a], SURAVI MAJUMDER[b], SHREYASI GUPTA[c], PAYEL GUHA[d], KOUSIK PRAMANICK[e], BUDDAHADAV MALLICK[f] AND DILIP MUKHERJEE[1g]

[abcdf]Department of Zoology, Kalyani University, Kalyani, India
[e]Department of Integrative Biology Research Unit, Department of Life Sciences, Presidency University, Kolkata, India

ABSTRACT

The effects of salmon calcitonin (sCT) on the secretion of 17α,20β-dihydroxy-4-pregnane-3-one (17,20β-P), maturation inducing steroid (MIS), were examined in female common carp, *Cyprinus carpio*. Post-vitellogenic stage fish were injected (i.p.) with saline, sCT, human chorionic gonadotropin (HCG) or HCG plus sCT for different time intervals. In the in vitro experiments, fully grown intact ovarian follicles were incubated with sCT or HCG. Injections of sCT increased the basal and HCG-stimulated 17,20β-P levels in plasma indicating sCT induced 17,20β -P secretion by the post-vitellogenic ovarian follicles of *C. carpio*. Isolated post-vitellogenic ovarian follicles incubated with increasing doses of sCT produced gradual and significant increase in 17,20β-P content in the media. Results further demonsrated that HCG-induced secretion of 17,20β -P by the ovarian follicles was increased in presence of sCT in the incubation. Taking all these in account, the result suggests that sCT can induce secretion of 17,20β-P by the ovarian follicles of carp independently or synergistically with gonadotropin.

KEYWORDS : Salmon Calcitonin (sCT), Maturation Inducing Steroid (MIS), Ovary, *Cyprinus carpio*

Calcitonin is a hypocalcemic hormone produced by the parafollicular C cells of thyroid gland in mammals. In non-mammalian vertebrates, CT is released from ultimobranchial gland (UBG) (Copp et al., 1967). Several laboratories although have been able to show the hypocalcemic action of CT in fish (Wendelaar Bonga, 1981; Chakraborti and Mukherjee 1993; Srivastava et al., 1998; Suzuki et al., 1999; Mukherjee et al., 2004a, b), conflicting results are often reported (Wendelaar Bonga and Pang, 1991; Singh and Srivastava, 1993). On the contrary, physiological role of CT on fish reproduction is consistent. Available reports indicated hyperactivity of UBG during preovulatory female and plasma calcium level are higher during spawning season in many teleosts (Yamane and Yamada 1997; Bjorsson et al., 1986; Norgberg et al., 1989). It has been reported that plasma CT level increased in response to E2 treatment in rainbow trout (Suzuki et al., 2004). Wang et al., (1994) demonstrated that human CT and sCT inhibit spontaneous and gonadotropin-stimulated testosterone secretion in rat testis. Tsai et al., (1999) reported that sCT induced inhibition of progesterone secretion in rat ovary. In contrast to these findings, recently we reported a complete opposite action of sCT in fish ovary. We have demonstrated the stimulation of 17β-estradiol (E2) secretion by the ovarian follicles of carp, *Cyprinus carpio* in response to salmon calcitonin (Paul et al., 2008).

Fish ovary, by the stimulation of pituitary gonadotropin, secretes estrogens, mainly 17β-estradiol during vitellogenic growth, and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β -P) during post-vitellogenic growth (Nagahama et al., 1995). 17,20β-P is considered to be maturation-inducing steroids (MIS) in most teleosts (see review Nagahama 1997). Although pituitary is considered to be the primary endocrine factor for the synthesis of MIS by the full grown ovarian follicles of fish, available information also reported for the involvement of other hormones and factors, namely, insulin and insulin-like growth factor for the synthesis of MIS either alone or synergistically with gonadotropin (Mukherjee et al., 2006). In our recent study, although we have demonstrated the stimulatory effect of sCT on the secretion of E2 by the ovarian follicles of carp, it is still not known whether sCT is effective for the synthesis of 17,20β-P, MIS of this fish. In the present study, an attempt has been made to evaluate the effect of sCT on the basal and HCG-stimulated in vivo release of 17,20β-P and in vitro production of this hormone by the intact post-vitellogenic follicle of common carp *C. carpio*.

[1]**Corresponding author**

MATERIALS AND METHODS

Sexually mature female carp *C. carpio* (300-400 g. body wt.), collected from local fish farm in the month of November were maintained in normal tap water in laboratory concrete tank (300 L capacity) at 23^{0}C for five days. Fish were fed with fish food. During the month of November in the plains of West Bengal, ovary of common carp comprises mostly post-vitellogenic follicles (0.5-0.7 mm in diameter) with oocytes containing centrally located germinal vesicle and lipid droplets in the cytoplasm were found to initiate coalescence. Details of ovarian follicular stage determination and collection of follicles from ovarian mass have been described previously (Mukherjee et al., 2006; Paul et al., 2010).

In *vivo* exposure of sCT on plasma 17,20β-P levels

Post-vitellogenic stage fish were given a single injection (i.p) of increasing dose of sCT in such way that each fish received 0.1, 0.5, 1.0 or 2.0 µg sCT/100 g body wt. Controls were injected with vehicle (0.6% aqueous saline and 1% gelatin preparation) and sampling of fish was done 12 h after injection. In another set of experiment, a group of fish was given single injections of sCT (0.5 µg/100 g body wt.) or HCG (0.5 µg/ 100 g body wt) or sCT + HCG (each 0.5 µg/100 g body wt) at 0800 h in the morning. Controls were injected with vehicles. Sampling of fish was done at 0, 2, 4, 8, 12, and 16 h after injection. The volume of vehicle in both the experiment was 15 µl per fish. Fish were lightly anesthetized with MS 222 (1: 10000, pH 7.4) before injections. Immediately after sampling, fish were subjected to light anaesthesia and blood from each fish was collected from the caudal vein and processed for plasma separation. Plasma were kept at -20^{0}C until steroid assay.

In *vitro* Exposure of sCT on Secretion of 17,20β-P by the Intact Follicles

Ovaries obtained from post-vitellogenic fish were excised and immediately placed in 50 ml sterile glass beakers that contained 5 ml ice-cold-oxygenated Idler's medium supplemented with 100 µg/ml streptomycin and 100 IU/ml penicillin adjusted to pH 7.4. Fully grown ovarian follicles were then separated from pre-vitellogenic (0.2-0.3 mm diameter) and vitellogenic (0.3 -0.4 mm diameter) follicles by sieving them through stainless steel mesh (i.d. 0.5 mm).Follicles thus obtained were directly utilized for in vitro incubation experiments.

Approximately, 100 intact follicles (~60 mg) were transferred into individual wells of a 24-well culture plate (Tarsons, India) containing 1 ml ice-cold BCS-DMEM supplemented with streptomycin (100 µg/ ml) and penicillin (100 IU/ml) adjusted to pH 7.4. They were then incubated with either increasing concentration of sCT or HCG for 12 h in a metabolic shaker bath at 23 ± 1^{0}C under air. At the end of incubations, media were aspirated and stored at -200C for steroid assay.

Extraction and Assay of Steroids

The method of extraction and assay of 17,20β-P from plasma and incubation medium was similar to the previously described procedure (Sen et al., 2002; Mukherjee et al., 2006; Pramanick et al., 2014). The antisera of 17,20β-P was highly specific and cross reacted with 17,20β-P; 5β-pregnane-3α,17β-triol; 5α,3α,17α,20β-P; progesterone; 17α- hydroxyprogesterone; testosteroneand E2 at 100%, 42%, 4%, 0.001%, 0.01%, 0.25%, and 0.01% respectively. Intra- and inter-assay coefficient of variation was 9% and 10% respectively.

Statistical Analysis

Data of in vivo experiments were obtained from four donor fish for each concentration and time point. For in vitro experiment, data obtained from three replicate incubations of intact follicles from a single donor fish showed a similar tendency and therefore a mean of all three data was considered as one experiment. All data were expressed as mean ± S.E.M. of three such experiments taking intact follicles from three donor fish. After the test of normality and homogeneity, the significance of treatment effects was determined by the one way ANOVA within and across different effectors. Individual comparisons between treatments were made by adopting Bonfgerroni's multiple comparison tests using SPSS (Chicago, IL, USA). The level of significance chosen were $p<0.05$ and $p<0.01$.

RESULTS

Effects of sCT and HCG on plasma 17,20β-P levels

Figure 1A shows that sCT, like that of HCG, at increasing doses (each at 0.2, 0.5, 1.0, or 2.0 µg/100 g body

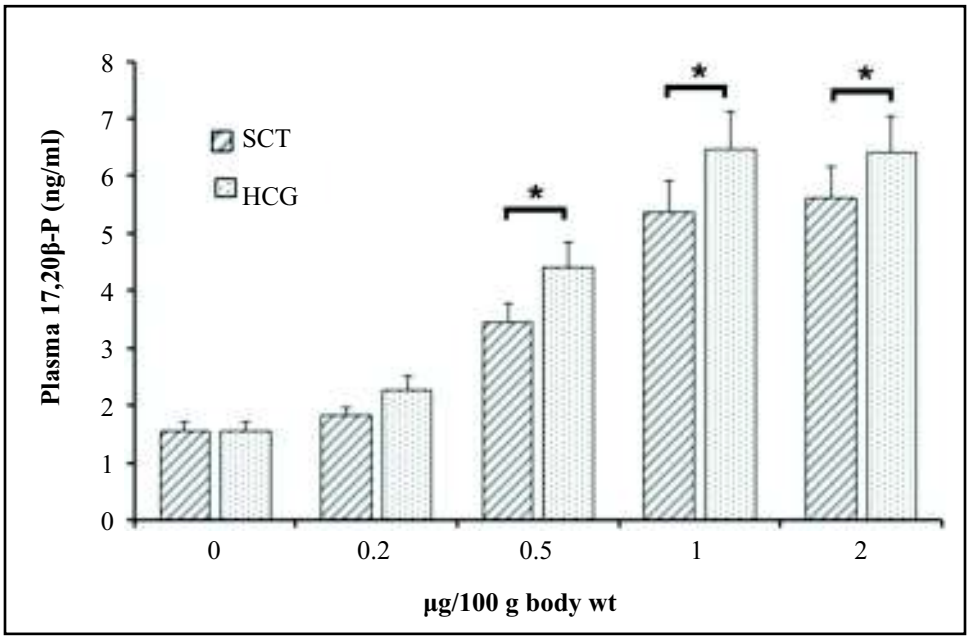

Figure 1 A

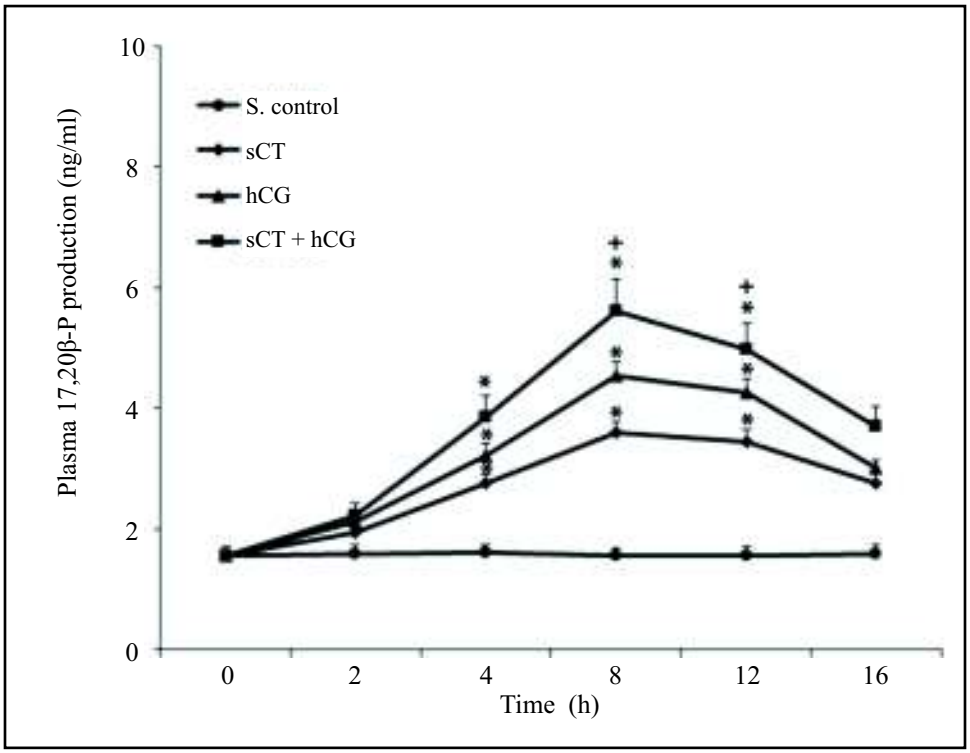

Figure 1 B

Figure 1 : Dose-response (A) and time-course effects (B) of salmon calcitonin (sCT), HCG and HCG + sCT on plasma 17,20b-P levels in post-vitellogenic stage common carp *Cyprinus carpio*. (A) Fish were injected i.p., with increasing doses of sCT or HCG as indicated and sacrificed 12 h after injection. (B) Fish were injected i.p., with a single dose of sCT or HCG (each 0.5 ug/100 g body wt.) and sCT + HCG (each 0.5 µg/100 g body wt.). Blood samples were collected from the caudal vein at the time indicated after hormone challenge. Each value (A and B) is mean four observations. (A) * p<0.05sCT versus 0 µg/ 100 g body wt. (B) * p <05 versus S. control at each time point and +p<0.01versus HCG at 0.05 µg/100 g body wt.

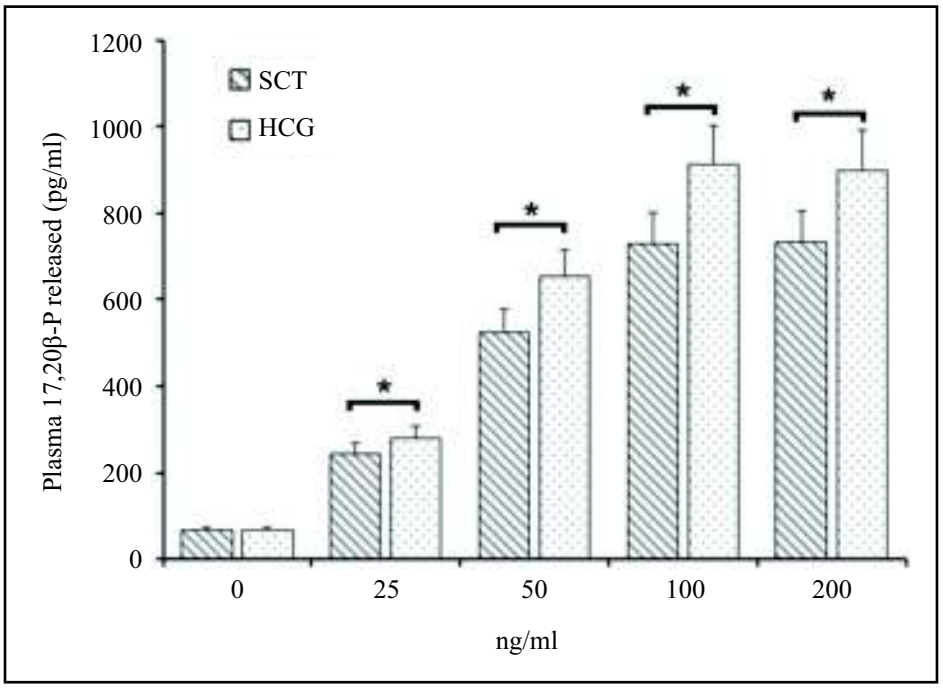

Figure 2 A

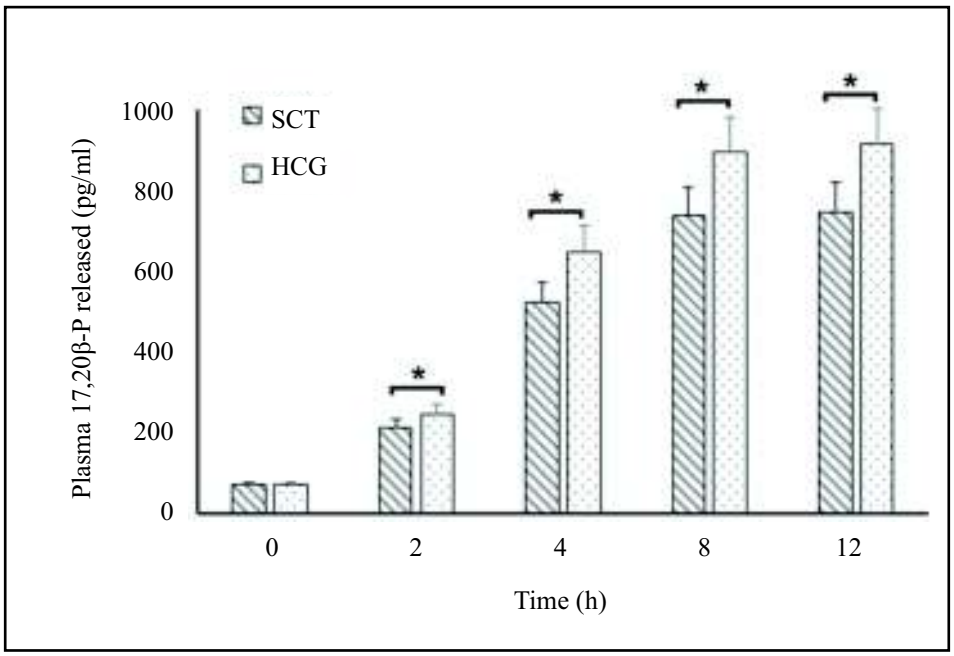

Figure 2 B

Figure 2 : Dose-response (A) and time-course (B) effects of sCT and HCG on in vitro release of 17,20b-P by the post-vitellogenic follicles of C. carpio. One hundred ovarian follicles ((~60 mg) were incubated in absence or presence of sCT or HCG at increasing doses for 12 h and at increasing time from 0-12 h with 100 ng/ ml of both the hormones. Each value represents ± S.E.M of three incubations taking follicles in triplicate from three donor fish. *p < 0.01 versus tissues incubated without hormones (0).

wt.) caused a gradual and significant increase ($p<0.01$) in plasma 17,20β-P levels in post-vitellogenic stage carp 12 h after injection compared to vehicle-injected control. The maximum effective dose of both sCT and HCG was 1.0 µg/ 100 g body wt. Minimum tried concentration at which steroid release started was 0.2 ug/ ml for both the hormones. Stimulatory effects of HCG on steroid secretion was little higher than sCT (Fig 1A).

In a time-course study, injections of vehicle for 16 h did not alter the plasma 17,20β-P levels in carp (Fig.1B). Plasma steroid levels started to increase after 2 h of sCT and HCG injection (each 0.5 µg/100 g body wt.) and maximum was recorded at 8 h followed by a gradual and significant decline at 16 h. Interestingly, injections of sCT and HCG (each 0.5 ug/100 g body wt.) in combination caused a significantly higher levels of plasma steroid at 8 h compared to that by HCG alone ($p<0.05$, Fig.1B).

Effects of sCT and HCG on 17,20β-P Production by Ovarian Follicles in *vitro*

The result of the effect of sCT and HCG ranging from 25 ng to 200 ng/ml incubation on 17,20β-P release by the intact follicles is depicted in Fig. 2A. After 12 h incubation, both sCT and HCG at 100 ng dose released maximum quantity of 17,20β-P in the medium (Fig. 2A). Higher dose over 100 ng/ ml had no additive effects. In a time-course study, ovarian follicles were incubated with sCT sCT and at 100 ng/ ml for different time intervals from 0 to 12 h and steroid contents in the media at each time point were estimated. Results depicted in Fig 2B shows that sCT at 100 ng dose induced secretion of 17,20b-P from 2 h onwards with a maximum at 8 h after incubation. No further increase in steroid production was noticed after 12 h incubation with sCT.

DISCUSSION

We, in the present study found that sCT is able to stimulate spontaneous and HCG-stimulated 17,20β-P production both in vivo and in vitro in post-vitellogenic stage carp and isolated ovarian follicles.

CT is a hypocalcemic hormone that mineralized the bone by suppressing the activity of osteoclast in mammals (Regnister 1993). In fish, although several laboratories reported for its hypocalcemic function (Wendelaar Bonga, 1981; Wates and Barrett 1983; Chakraborti and Mukherjee 1993, Srivastava et al., 1998, Mukherjee et al., 2004a,b), inconclusive results are reported from time to time and still there is no consensus on the hypocalcemic action of CT in this group of vertebrate. On the contrary, physiological role of CT on fish reproduction is consistent. Plasma levels of CT in coho salmon, Japanese eel, and rainbow trout are higher in females during spawning season and reached a peak prior to ovulation (Bjorson et al., 1986; 1989). A direct action of estrogen on the secretion of CT in this fish has also been reported (Suzuki et al., 2004). In our previous study, we have been able to show that sCT administration to carp during vitellogenic stage significantly stimulated spontaneous and gonadotropin-stimulated E2 secretion both in vivo and in vitro (Paul et al., 2008). Our preliminary study also reported for secretion of MIS after sCT administration in this fish (Mukherjee et al., 2011). In a subsequent study in grey mullet Mugil cephalas, Das et al.(2014) demonstrated that sCT administration resulted induction of oocyte maturation.

The present study demonstrate that sCT is effective in increasing plasma 17,20β-P levels in post-vitellogenic stage fish and stimulating spontaneous and HCG-induced secretion of 17,20β-P in a dose-, and time-dependent manner in isolated intact follicles in carp in vitro. Findings, thus clearly demonstrated a direct action of sCT on ovarian tissues of this fish. Our previous study also demonstrated a direct effect of sCT on ovarian tissue of this fish for E2 production in vitro by binding to specific CT receptors located on the membrane preparation of ovarian follicles (Paul et al., 2008). Binding of sCT with membrane preparation was found to be saturable with high-affinity. Therefore, sCT-induced 17,20β-P production by the intact follicles and binding of sCT with membrane preparation of ovarian follicles as shown previously (Paul et al., 2008) suggest a link between sCT binding and 17,20β-P production. We are not certain whether sCT has any direct effect on the release of pituitary gonadotropin hormone which in turn induces the production 17,20β-P by the ovarian follicles. Previous study with rat however, reported inhibition of pituitary LH release by sCT administration (Tsai et al., 1999).

Interestingly, our previous study (Paul et al., 2008), as well as the present finding on the stimulatory role of sCT on basal and HCG-stimulated E2 and 17-20β-P production by the vitellogenic and post-vitellogenic ovarian follicle are completely opposite to what observed in sCT action in rat ovary and testes on steroid production. Inhibition of progesterone secretion and GnRH-induced LH secretion in rats has been demonstrated (Tsai et al., 1999). Moreover, Cressent et al., (1983) reported for a peak level of plasma CT on the day of diestrus and reduced to the lowest levels on the day of estrus. Except human, hypocalcemic action of CT in mammal is well characterized. On the contrary, hypocalcemic action of CT in fish is not well characterized, although we have been able to show a definite action for the same in carp (Chakraborti et al., 1993; Mukherjee et al., 2004a, b). The observed stimulatory effect of CT on fish ovarian steroidogenesis therefore is not unusual. Since fish has one more hypocalcemic hormone i.e., staniocalcin, physiological action of CT in this group of vertebrate might be shifted towards its stimulatory role on steroidogenesis in addition to hypocalcemic function for its aquatic life. At present, the traditional concept of the action of a gonadotropin in the regulation of ovarian growth, steroidogenesis may no longer tenable. Available information reported for localization of several neuropeptides such as neuropeptide Y, substance P, vasoactive intestinal peptide and somatostatin in the nerves within the ovary of mammals (Ojeda et al., 1985; Ahmed et al., 1986; McDonald et al., 1987). Although function of these peptides in the ovary is not fully characterized, but stimulatory role of VIP on the synthesis of P450scc leading to production of progesterone and E2 by the cultured granulosa cells have been reported (Trzecizk et al., 1986; 1987). Clark et al., (2002) reported for the expression of CT gene in the ovary of a teleost, Fagu rubipens and suggested thatCT may acts as a potential neuropeptide in the ovary. Taking all these in consideration, it may be suggested that CT in fish has a stimulatory role on fish ovarian steroidogenesis both in vitellogenic and post-vitellogenic stages of gonadal development by acting independently or synergistically with gonadotropin.

In summary, the present findings suggests that sCT in carp ovary at post-vitellogenic stage of development acts directly on the ovarian follicle cells for the production of 17,20β-P, the maturation-inducing steroid of this fish which might have a stimulatory role on oocyte maturation.

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