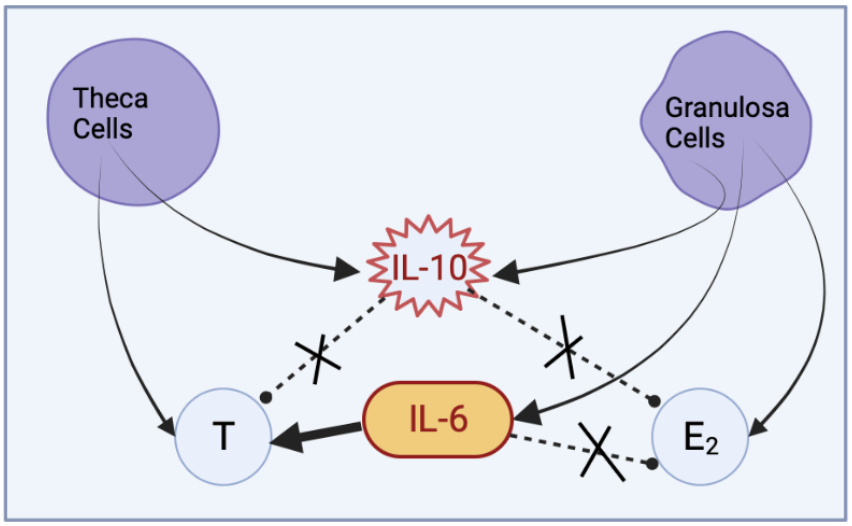
Chapter - III

Source of IL-6 and IL-10 in the ovary and their interdependency with steroid

Abstract:

Information about the role of IL-6 and IL-10 on steroid production in teleost fish is not clearly understood. In this study we try to elucidate the effect of IL-6 and IL-10 on estradiol and testosterone. Moreover we tried to establish the secretory area of IL-6 and IL-10 in oocyte.

The in vitro studies suggested that IL-6 (2 ng/ml) can modulate the secretion of T but not E2 in post vitellogenic follicle whereas there was no significant correlation between IL-10 and steroid production in oocyte. Furthermore, isolated theca and granulosa cell treatment established that IL-6 is secret from granulosa cell whereas IL-10 can be secreted from both theca and granulosa cells.



Introduction:

E2, P and T, these three are the major sex steroid present in female teleost fish. They are the key regulator of gonadal development, vitellogenesis, maturation, ovulation and spawning. In teleosts, ovarian function is regulated by a variety of factors in addition to steroids and gonadotropins. Numerous recent studies have shown that cytokines are essential for teleost fish reproduction (Crespo et al., 2012,2015). IL-6 is a hormonally regulated cytokine. Its production is suppressed by glucocorticoids (Breuninger et al., 1993) and estrogens (Girasole et al., 1992) and stimulated by catecholamines (van Gool et al., 1990, Papanicolaou et al., 1996). In turn, it has many endocrine effects. It is one of the major cytokines that stimulate the hypothalamic-pituitary-adrenal axis during inflammatory stress. A study in goldfish also showed that macrophage-derived factors (e.g. recombinant murine TNF-α and IL-1 and supernatants from a fish macrophage cell line) are important in the regulation of testicular androgen biosynthesis: regulatory features of TNF-α occur at multiple sites within the steroid biosynthetic pathway, but the major effect appears to be related to cholesterol availability in the mitochondria (Lister and Van Der Kraak, 2002). IL (i.e., IL-1β and IL-lα) have been found to inhibit FSH-induced estrogen production by 20% to 80% in cultures of rat (Gottschall et al., 1989, Kasson and Gorospe 1989), porcine (Yasuda et al., 1990), bovine (Spicer and Alpizer 1993), and human (Fukuoka 1992) granulosa cells. IL-6 administration caused significantly acute elevations in circulating IL-6 levels, similar to those observed in severe inflammatory and noninflammatory stress, which induced prolonged suppression in testosterone levels in healthy men (Tsigos et al., 1999). When rat GCs differentiate, it has been demonstrated that IL-6 can lower 17β-estradiol (E2) production and that there is a dissociation between NO production and IL-6-induced E2 suppression (Tamura et al. 2000). Studies revealed that IL-6 decreased FSH-induced estradiol production by granulosa cells from small and large follicles by 75 and 32%, respectively. This is in agreement with the findings of another study in which IL-6

inhibited FSH- induced progesterone production by rat granulosa cells (Gorospe et al. 1992). In bovine GCs, IL-6 prevented E2 from being secreted in response to FSH (Alpizar and Spicer 1994, Gorospe et al. 1992). Earlier studies suggested that progesterone levels are typically low in patients with ovarian hyperstimulation syndrome (OHSS), where higher levels of IL-6 are found, it has been suggested that IL-6 may act in part by inhibiting FSH-induced progesterone release. (Loret de Mola et al., 1996; Geva et al., 1997; Hammadeh et al., 2001; Salmassi et al., 2001; Borman et al., 2004; Engmann and Benadiva, 2010).

At early time of pregnancy in human it was reported that IL-10 induces the synthesis of progesterone in cultured luteal cell. Geva et al., (1997) establish the negative correlation of IL-10 with the concentration of progesterone in follicular fluid. It suggested that progesterone production from newly luteinized cells, inhibited by IL-10. In multiple study, it has been suggested that their is numerous immunological effects of estrogen on peripheral blood mononuclear cells (PBMCs) (Hu et al., 1988, Vernon-Roberts 1969). Asai et al., (2000) reported that the secretion of IL-10 when stimulated with E2 is related with the gender (wheather the person is male or female). Study also suggested that in male the secretion of LPS induced IL-10 is lower than that of female when subjected to E2. In addition to reducing clinical EAE activity in EAE mouse models, estriol treatment decreased IFN- ϒ dependent anti-myelin basic protein IgG2a levels and increased IL-10 production. Estriol's ability to treat EAE was closely related to the MBP-specific T cells' increased in vitro IL-10 production (Kim et al., 1999).

A number of studies have examined the effects of cytokines on granulosa or thecal cells isolated from immature follicles where they could potentially affect cell differentiation and follicular development by regulation of steroidogenesis, receptor formation, and eicosanoid as well as protein secretion from undifferentiated cells. TNF-α is also an inhibitor of steroidogenesis in immature rat granulosa (Adashi etal., 1989, 1990) and thecal—interstitial cells (Andreani et al., 1991). An increasing amount of research indicates that ILs were produced at ovary and play a significant role

in reproductive processes. Granulosa and theca cells have been shown to produce ILs and their receptors, with preovulatory follicles showing the highest production levels following gonadotropin activity (Brännström, 2004; Ingman and Jones, 2008)

So, in this chapter our study elaborates the effect of IL-6 and IL-10 on the seasonal steroidogenesis in *Anabas* ovary as well as from where IL-6 and IL-10 were secreted, the theca cells or the granulosa cells. As a whole this chapter (chapter III) in combination with Chapter I gives us a concrete idea about how IL-6 and IL-10 are involved in the two cell two gonadotroph hypothesis in female *A. testudineus*.

Materials and method:

Animals : As same as Chapter I

Chemicals : As same as chapter I

Amersham Biosciences sold the labelled steroids [3H] estradiol-17 (E2, specific activity 75.0 Ci/ mmol) and [3H] testosterone (T, specific activity 95.0 Ci/mmol). Gordon Niswender gave a substantial contribution of the E2 antibody (Colorado State University, Fort Collins, Colorado, USA).

Methodology :

In vitro experiment to find out the effects of IL-6 and IL-10 on T and E_2 production by ovarian follicles at various developmental stages:

100 mg of each of the follicles at the pre-vitellogenic, vitellogenic, post-vitellogenic, and post-GVBD stages were incubated with varying concentrations of IL-6, IL-10 and hCG for 16 hours. To measure T and E2, aspirated medium samples were put through a radioimmunoassay.

Extraction and assay of steroids :

Phosphate buffer saline (PBS) was used to homogenise the experimental samples, and steroids were then extracted from the homogenate by vortexing with diethyl ether (DEE: pH 7.4) at least twice. The DEE fraction was then transferred using a Pasteur pipette in another set of test tubes, dried by air, and then dissolved in steroid assay buffer (SAB). The procedure previously reported by Guchhait et al., (2018a, b) was used to conduct the radioimmunoassay (RIA) for steroid hormones. The samples were incubated with a particular steroid antibody and [3H]-steroid overnight at 4 °C in order to quantify the amount of extracted steroids. A liquid scintillation counter was used to count the free [3H]-steroid after it had been separated using dextran-coated charcoal (0.6% activated charcoal, Norit A, and 0.06% dextran). 3 mL of scintillation fluid (Cocktail T) had then been added. The assay had a sensitivity of 14 pg/mL for T and 12 pg/mL for E2. For the RIA, the inter- and

intra-assay variations for T and E2 were 5% and 9%, respectively. Experiments regard to RIA has been done in University of Kalyani, under the supervision of Prof. Dilip Mukherjee.

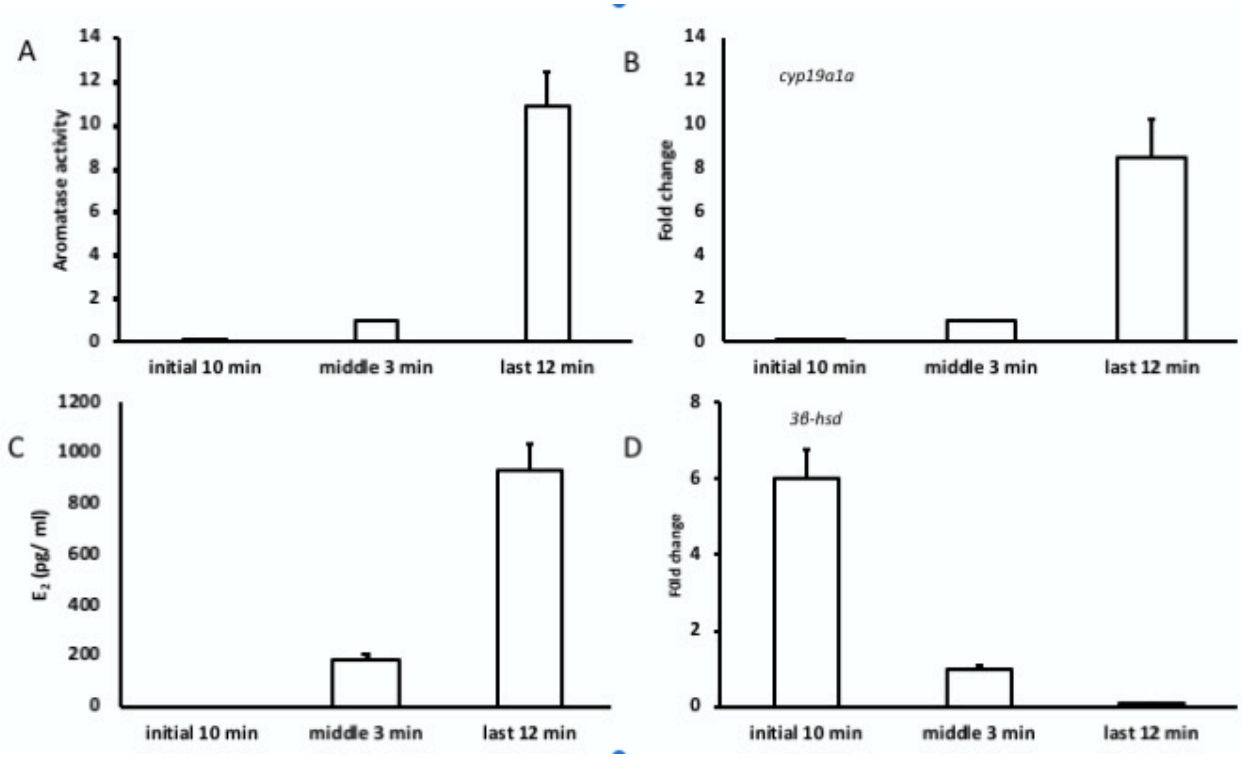
Histology and Immunofluorescence Imaging:

4% paraformaldehyde was used to fix the ovarian sample in phosphate buffer (pH 7.4). Fixed tissues were dehydrated in a series of ethanol solutions with increasing ethanol concentrations, cleared in xylene, and embedded in blocks of paraffin. A rotary microtome was used to slice the blocks into sections that were 8 m thick. Following standard procedures, tissues were stained with Hematoxylin and Eosin and examined under a bright field microscope. Ovarian sections were deparafinized in xylene, rehydrated, washed in PBS, and blocked in NP 40 for an hour at room temperature for immunofluorescence staining to localise IL-6 (protocol adapted from Majumder et al. (Majumder et al., 2015)). After that, sections were incubated with a primary antibody (rabbit anti IL-6) at a dilution of 1: 200 for an entire night at 4°C. Phalloidin was combined with a secondary antibody that was fluorescein-conjugated. Nuclei were stained using DAPI (0.05 g/ml). Imaging was performed in a Zeiss upright microscope was used to capture the image and analyzed with provided software.

Theca and granulosa cell isolation, purity evaluations and incubation with effectors

With some modest changes, the techniques of Benninghoff and Thomas (2006) and Paul et al. (2013) were used to isolate the theca and granulosa cells. After the ovarian tissues were cut apart, the follicles were placed in 50 mL beakers and thrice rinsed with ice-cold Idler's media containing streptomycin (100 g/mL) and penicillin (100 IU/mL) that had been pH-adjusted to 7.4. The follicle tissues were digested for 10 min with moderate mixing in 5.0 mL of Idler's medium containing collagenase Type-I (0.3%). Theca cells and surface epithelium were removed after digestion by rotating the follicles over a fine (100 m mesh) nylon screen for 10 min. at 23±1 °C. The follicles were then incubated with the same collagenase concentration for an additional five minutes, rotated

over a mesh of 100 micron nylon suspension in Idler's medium for three minutes, and the collected media was discarded due to the mixed cell population (both theca and granulosa). For another 12 minutes, the rotational operations were carried out. For the purpose of separating granulosa cells from other cells and detritus, the material was collected and centrifuged at 2000 g for 5 min using a Percoll. Hemocytometer counts and cell viability, which was typically greater than 90% as assessed using the trypan blue exclusion method, were used to determine cell density.



Purity detection of granulosa and theca cells. (A) Aromatase activity, (B) cyp19a1a gene expression and (C) E2 production was observed in the cells isolated in last 12 mins, which confirms that these cells are 'granulosa'; cells isolated in middle 3 mins are mixed population of both theca and granulose and D - 3beta-hsd gene expression in initial 10 min confirm that these fellas were 'theca' cells.

By calculating the amount of 3-hydroxysteroid dehydrogenase (3-HSD), the purity of theca cells was ascertained. However, cyp19a1a protein quantity, E2 release, and aromatase activity were examined in order to identify granulosa cells. It is generally known that the cyp19a1a protein is active in granulosa cells, which contain 3-HSD activity, and that granulosa cells secret E2 as a result.

Idler's media supplemented with 0.2% BSA, streptomycin (100 g/mL), and penicillin (100 IU/ml) was used to culture theca and granulosa cells in six-well culture plates for 16 hours at a density of 2.5 ×10^4 theca cells and 2×10^5 granulosa cells in a humidified shaker bath at 23±1 °C (80 rpm). Five donor fish served as the data source for each experiment. The media were taken out and prepared for the ELISA test after 16 hours.

Statistics

Three replicate incubations of ovarian follicles obtained from a single donor fish were used to collect the data. The three replicates data were comparable, thus the mean values were taken to represent results from a single experiment. For samples from five separate fish, the experiment's results were presented as the mean and SEM. Data were assessed using SPSS (version 20: Chicago, USA) software for normality of distribution and homogeneity of variances, followed by the execution of Bonferroni multiple comparison tests. When a $P \leq 0.05$ was present, mean differences were deemed to exist.

Results:

Effects of IL-6 and IL-10 on T and E2 production by ovarian follicles at various developmental stages:

T and E2 concentrations in the medium were compared following either no treatment ((0)) or treatment with doses of 0.05, 0.1, 0.5, 1.0, and 2.0 ng/mL IL-6 and IL-10. Only the 2.0 ng/mL dose of IL-6 had a significant impact on T (836.56 ± 78.57 pg/ ml) production (but not E2 production), while there was no significant correlation had been between IL-10 and the control of steroids in any doses ($P > 0.05$). The concentrations of T (1248.5 ± 112.76, 1406.5 ± 168.87, and 1230.69 ± 118.28, 1410 ± 27.98, respectively, in vitellogenic and post-vitellogenic follicles for both set of experiment) and E2 (1520.75 ± 122.35, 1230.69 ± 118.28 and 1560 ± 25.39, 1350 ± 29.87 respectively for both set of experiment), increased when hCG was administered. hCG had no impact on the levels of either steroid in pre-vitellogenic stage or post-GVBD stage follicles. (Fig 12-13).

Release of IL-6 from theca and granulosa cells

After the isolation of theca and granulosa cells, to measure the levels of IL-6 in the media, theca and granulosa cells from *A. testudineus* post-GVBD follicles were treated for 16 hours with the ideal concentration of hCG, TNF-α, and hCG combined with TAPI-I, along with a control group. The theca cells that had been isolated lacked IL-6. High levels of IL-6 were present in granulosa cells treated with hCG (62.9 ± 8.39) and TNF-α (66.35 ± 9.84), but these values were decreased by TAPI-I (26.29 ± 4.03) (Fig.14-15).

Localisation study of IL-6 in ovary:

Immune-histochemical study of to understand the localisation of IL-6 in different stages follicle showed that the localisation of IL-6 was limited to the surrounding cellular layer of preovulatory follicle (Fig. 16).

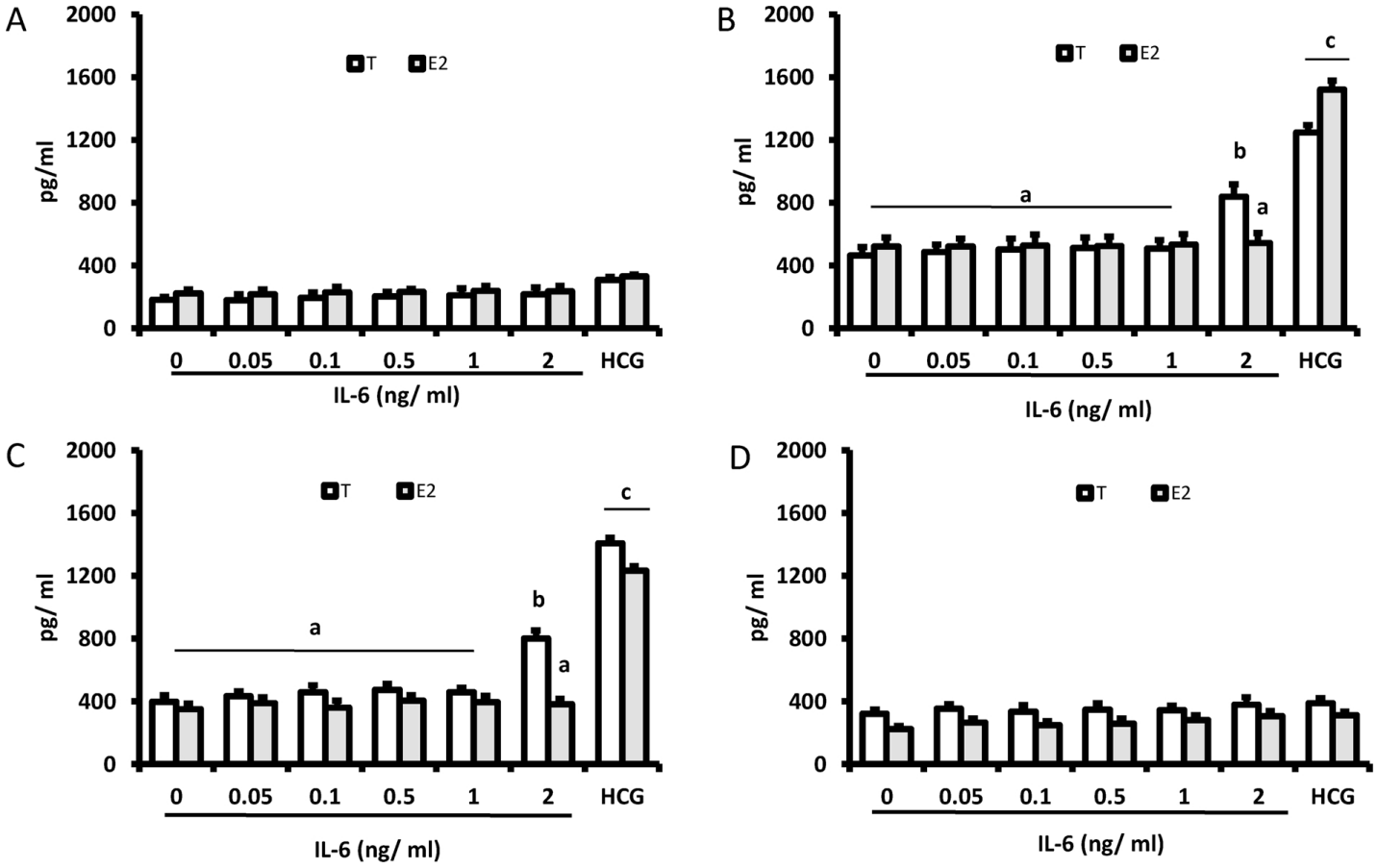


Fig 12. In vitro steroid production at different stages of ovarian follicle development when there was treatment with different doses of IL-6 (A-D); Testosterone (T) and 17β-estradiol (E2) were quantifified in previtellogenic (A), vitellogenic (B), postvitellogenic (C) and post-GVBD (D) stages of ovarian follicle after cessation of the incubation period; Each bar represents the mean ± SEM of five fish; Different letters indicate differences in mean values between treatments ($P \leq 0.05$).

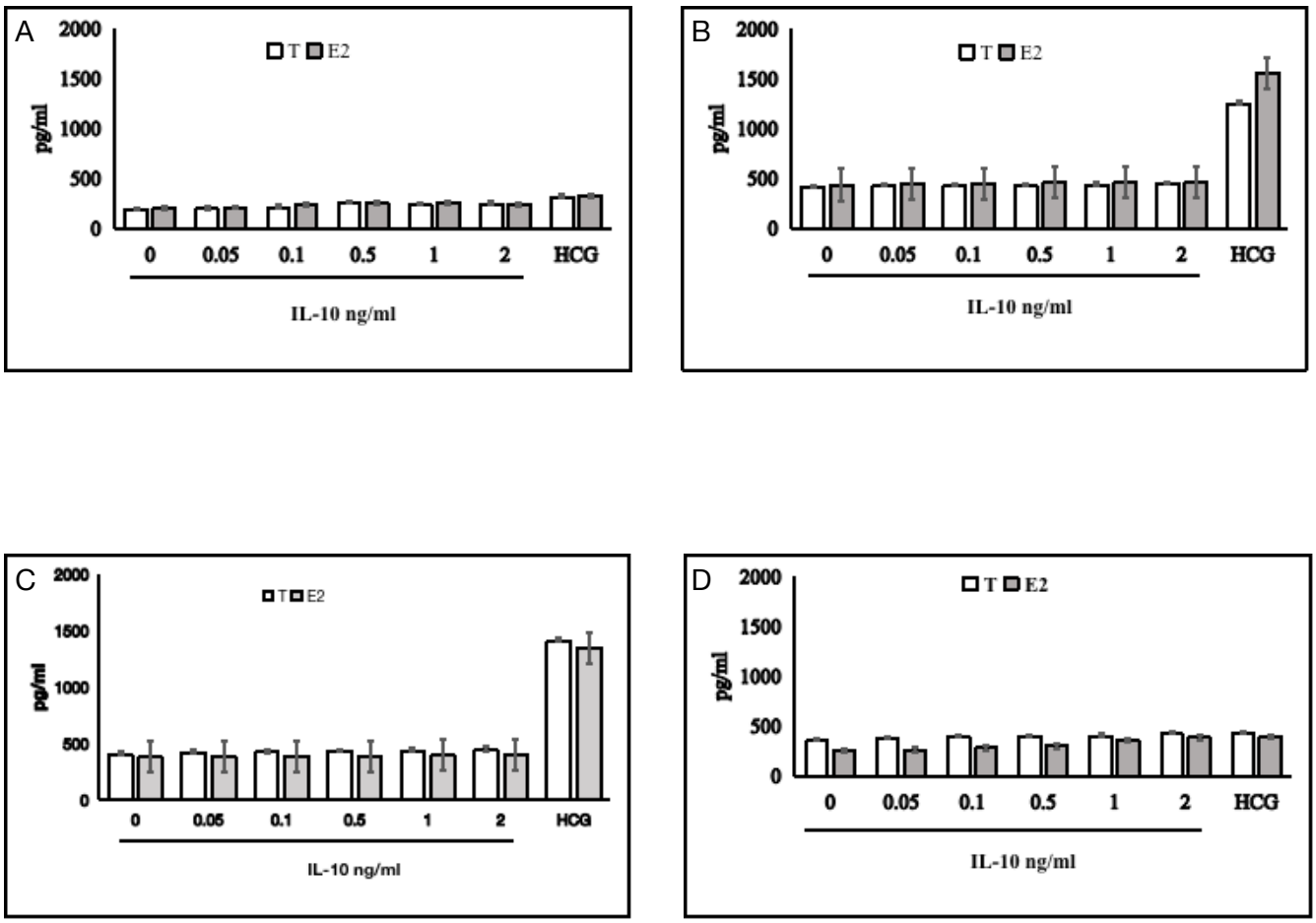


Fig 13. In vitro steroid production at different stages of ovarian follicle development when there was treatment with different doses of IL-10 (A-D); Testosterone (T) and 17β-estradiol (E2) were quantifified in previtellogenic (A), vitellogenic (B), postvitellogenic (C) and post-GVBD (D) stages of ovarian follicle after cessation of the incubation period; Each bar represents the mean ± SEM of five fish; Different letters indicate differences in mean values between treatments ($P \leq 0.05$).

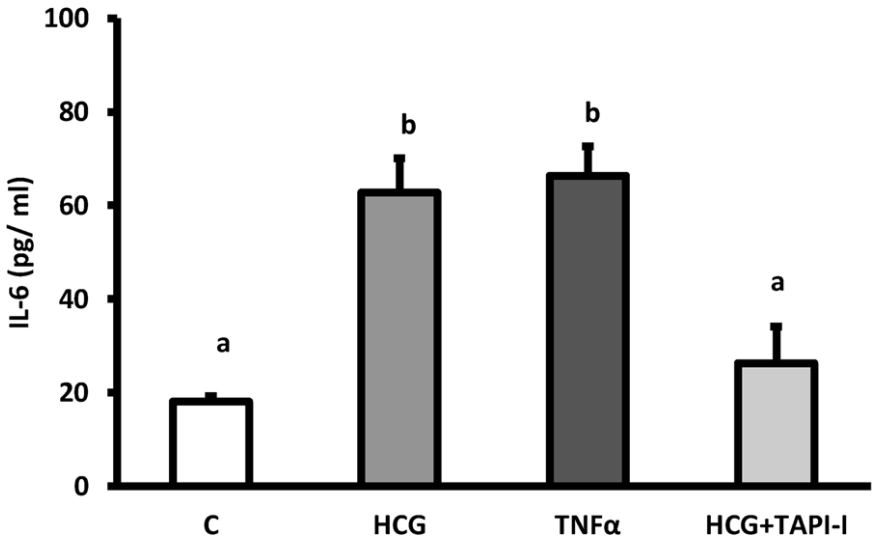


Fig 14. In vitro release of IL-6 from granulosa cells isolated from post-GVBD follicles; Isolated granulosa were incubated in DMEM for 16 h and data analysed by ELISA. Experiments were repeated in three different fish and the bar represents the mean ± SEM; Different letters indicate differences in means among treatment groups (P ≤ 0.050)

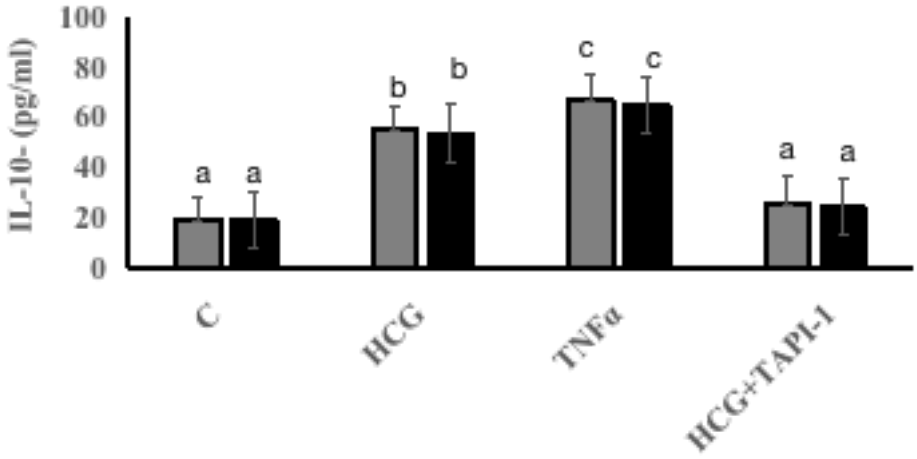


Fig 15. In vitro release of IL-10 from granulosa and theca cells isolated from post-GVBD follicles; Isolated granulosa and theca cells were incubated in DMEM for 16 h and data analysed by ELISA. Experiments were repeated in three different fish and the bar represents the mean ± SEM; Different letters indicate differences in means among treatment groups (P ≤ 0.050)

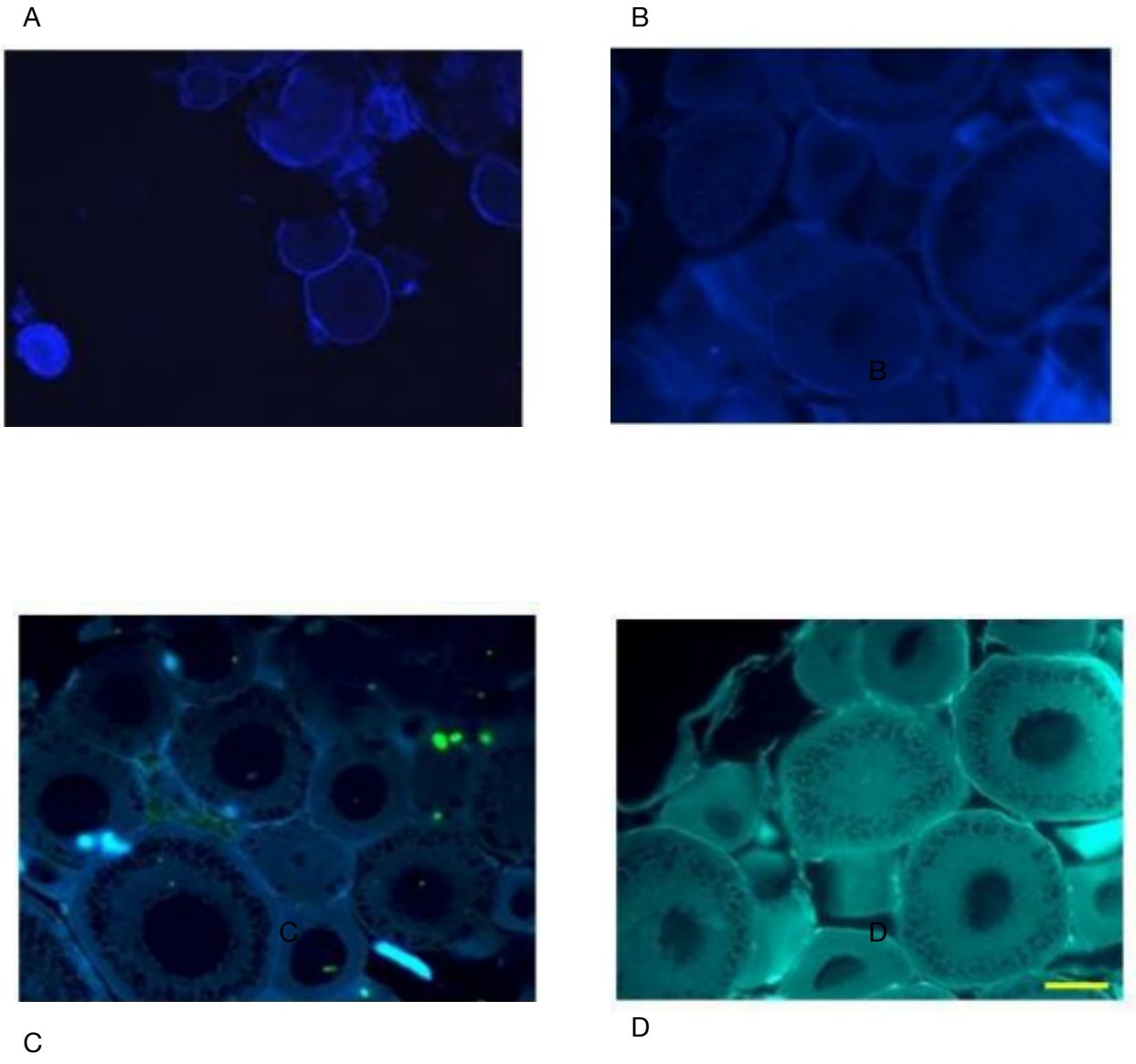


Fig 16. Immunofluroscence imaging to detect the source of IL-6 in different stages of follicle (A) previtellogenic, (B) vitellogenic, (C) post vitellogenic and (D) post GVBD follicle of the ovary. The scale bar is 200μm.

Discussion:

Fish oocyte growth is caused by the process of vitellogenesis. This process was controlled by 17β-estradiol, which the ovary produces when GtH is present (Wallace, 1895; Paddle et al., 1989, 1991; Nagahama, 1994; Devlin and Nagahama, 2002). Thus, the control of 17β-estradiol, which is in turn controlled by FSH, is necessary for vitellogenesis, and cytochrome P450 aromatase is thought to be a key regulator in this process (Tanaka et al., 1992; Gen et al., 2001; Kagawa et al., 2003). On the other hand, LH, the primary regulator of maturation-inducing hormone (MIH) production, which in turn stimulates the production of maturation-inducing steroid hormone (MIS) from follicular cell layers, is in charge of regulating the final maturation of gametes. Next, MIS helps to create maturation promoting factor (MPF), which triggers GVBD (germinal vesicle breakdown). Theca (the outer layer) and granulosa (the inner layer) are the two cell layers that make up the follicular cell layer of the fish oocyte, just like in other vertebrates (Nagahama, 1983). Two cell two gonadotroph hypothesis: LH and FSH act on this two cell layer in a pulsatile manner, causing the theca and granulosa cell layers to synthesise 17α-hydroxy progesterone and 17β-estradiol, respectively (Nagahama et al., 1995).

According to earlier research findings, cytokines have an impact on mammals and teleost fish during the development of their oocytes (Liu et al., 2009; Crespo et al., 2012; Crespo et al., 2015). The effects of gonadotropin on IL-6 showed that IL-6 does not have any significant role during the time of maturation of oocytes. In this experiment we considered the role of IL-6 on steroid production in different doses also establish the fact that IL-6 does not change the level of E2 in vitellogenic and post vitellogenic follicle while it changes production of T in higher doses follicles were at vitellogenic and post vitellogenic stages. Results from mammalian studies show that IL-6 stimulates T production at the vitellogenic and post-vitellogenic stages, highlighting the importance of androgens in the stages preceding ovulation (Walters et al., 2008).

The production of several cytokines by monocytes, including IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, GM-CSF, and G-CSE, is effectively blocked by IL-10 (Malefyt et al., 1991).The preovulatory Follicular Fluid (FF) concentration of IL-10, E2, oocyte number, oocyte fertilization rate, embryo quality, and pregnancy rate were all found to be unrelated in 1997 by Geva et al. However, it was discovered that there was a negative correlation between P concentration and IL-10 levels. In our recent study when the four different follicle were isolated and treated with different doses of rIL-10, no significant correlation has been found between the IL-10 (in any doses) and steroid production in any stages of follicle. Which supported the previous work done by Geva et al., 1997 and establish the fact that IL-10 alone cannot make any significant differences during the time of the oocyte maturation and ovulation.

Production of IL-6 by isolated granulosa (not theca) cells supports its ovarian cellular origin. This conclusion is supported by Liu et al.'s (2009) investigation into mammals. Brown trout theca cells secrete TNF-α , not the granulosa cells of the follicles, as previously thought (Crespo et al., 2012). Therefore, it is likely that TNF-α plays a role in granulosa cells production of IL-6, which in turn causes apoptosis and ovulation. Although there hasn't been much research on the cellular origin of IL-10 in fish, our findings suggest that both the theca and the granulosa cells may be capable of producing it. The fact that TNF-α controls the production of IL-10 could support our assertion (Kobe et al., 1995).