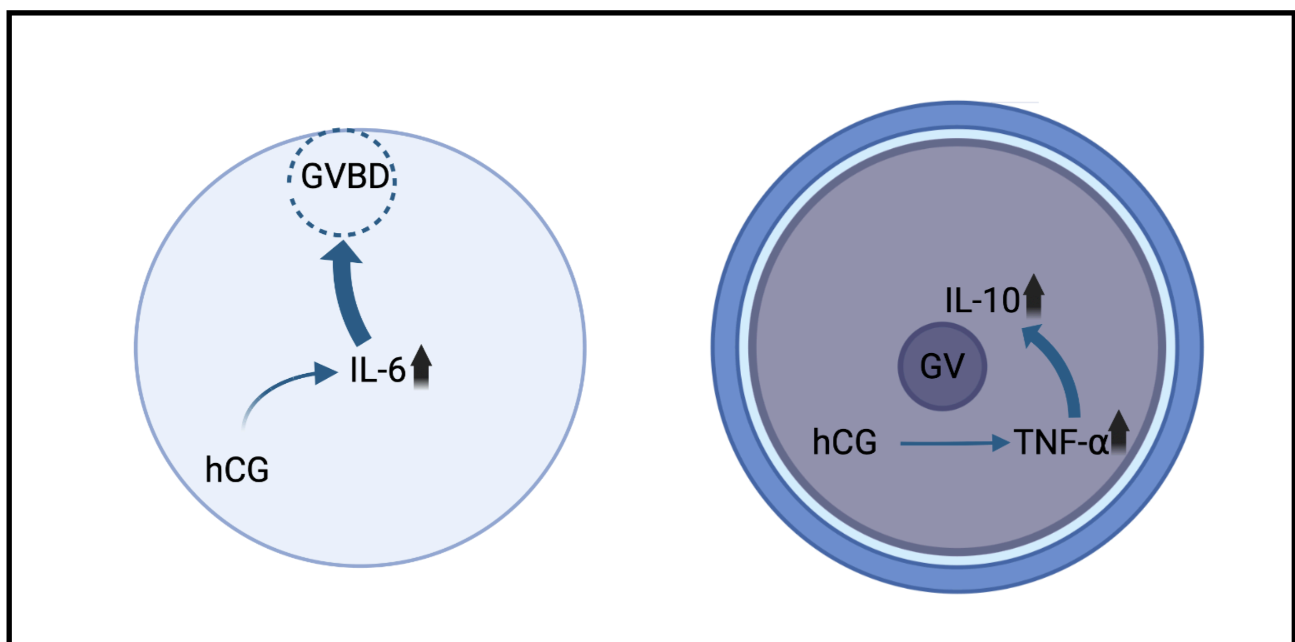
Chapter - I

Understanding the interplay between the pro- and anti-inflammatory cytokine (IL-6 and IL-10) under the influence of gonadotropin.

Abstract:

In mammals, interleukins play a vital role during the time of ovulation and also in the process of oocyte growth and maturation. However in fish things are not properly understood till now. In this chapter of this thesis we tried to find out the interplay between interleukins (IL-6, IL-10 and TNF-α) under the influence of hCG. Results showed that IL-6 have greater concentration in post-GVBD stages where as IL-10 at its peak in the post vitellogenic and post GVBD follicle and having a continuous resemblance with the secretion of TNF-α and hCG induce all of them in in vivo as well as in in vitro study. This study can established the fact that Interleukins have a significant role where IL-6 can be involved during the time of ovulation and the secretion of IL-10 may be controlled by TNF-α.



A. Post Germinal Vesicle Breakdown follicle **B. Post vitellogenic follicle**

Introduction:

In majority of the fishes reproduction is seasonal. Because of *Anabas* ovary is synchronous in nature, that is, at a time we found only one stage of the oocyte from four different stages of follicle (previtellogenic, vitellogenic, post vitellogenic and post-GVBD). The reproductive processes of *Anabas*, which include gonadal recrudescence, vitellogenic event and final maturation occur in a specific time of the year. The master of the endocrine activity is initiated by the decapeptide hormone, gonadotropin-releasing hormone (GnRH) which is secreted from brain and eventually regulate the reproductive events in all vertebrates. Chronologically GnRH then stimulate, LH and FSH acts on two cell layer in pulsatile manner which respectively induce both the theca and granulosa cell layer to synthesize 17α-hydroxy progesterone and 17β-estradiol respectively, two cell two gonadotroph hypothesis (Nagahama et al., 1995). 17β-estradiol is produced during the growth phase of the oocytes and during the time of maturation, maturation inducing hormone (MIH) has been produced (Senthilkumaran et al., 2004).

IL-6 regulate the release of hCG from human trophoblast cells and placental lactogen (Nishino et al. ,1990, Stephanou and Handwerger, 1994), as well as the anterior pituitary hormones adrenocorticotropin, prolactin, growth hormone (GH), and LH in rats (Spangelo et al., 1989, 1995). According to Kawasaki et al., 2003, the cAMP signaling pathway could be used by FSH and/or LH to regulate IL-6. LHR and CYP19 mRNA expression were both downregulated by IL-6 during the in vitro maturation of rat GCs (Tamura et al., 2001; Tamura et al., 2000). Additionally, according to Breard et al., (1998), FSH decreases the relative abundance of the IL-6 mRNA transcript in rabbits in a dose dependent manner. Salmassi et al., (2001) found that when FSH is stimulated in human granulosa cells, IL-6 inhibits the production of progesterone. The relative abundance of IL-6 mRNA transcript increases in granulosa cells and the Cumulus Cell Oocyte Complex (COC) just prior to ovulation (Liu et al., 2009).

Only hCG has the ability to change CD19+ cells into IL-10-producing B cells and to influence plasma cells' capacity to secrete asymmetrically glycosylated antibodies (AAbs) (Fettke et al., 2016). In fact, hCG and E2 receptors are expressed by B cells, highlighting the potential modulatory effects of these hormones on B cells during pregnancy (Muzzio et al., 2014; Asaba et al., 2015; Hill et al., 2015). During a normal pregnancy, hCG may promote the growth of regulatory B cells that produce IL-10, thereby reducing unwanted immune activation that might jeopardize the unborn child's health (Rolle et al., 2013). It is exciting to note that most published studies (Huck et al., 2005; Uemura et al., 2008) found that human Dendritic Cells (DC's) significantly increased their production of the IL-10 following treatment with hCG. In a murine model of autoimmune diabetes, hCG prevented the onset of the condition. Here, hCG treatment effectively reduced IFN-γ production in the splenocytes while increasing IL-10 and TGF-α production (Khil et al., 2007). The ability of hCG to increase the regulatory function of human regulatory B cells (Breg or B10), which has been shown to support foetal survival, is one of the hormone's pregnancy-protective effects. Breg produces more IL-10 in response to hCG treatment and expresses the LH/CG receptor (Rolle et al., 2013). Although in fish the relationship between cytokines and gonadotropin is still unclear, some work like Crespo et al., 2012 establishes the role of TNF-α, in the this chapter we tried to elucidate the relationship between the gonadotropin and IL-6, and IL-10 in every stages of ovarian follicle which may give us a proper view point that how this interleukins acts in fish ovary during their reproductive processes.

Materials and methods:

Animals :

Anabas testudineus, a species of climbing perch, was selected for the current study due to its high reproductive rate. In South-East Asian countries, this freshwater fish is edible and naturally breathes air (Day, 1889). Since *A. testudineus* is a seasonal breeder (with spawning season occurring from May to August in West Bengal), it only lays eggs once a year. The Departmental Aquaculture Facility (DST-FIST sponsored), Presidency University, received females of the reproductively mature *A. testudineus* species, aged 10 to 12 months, from a nearby fish farm. Before the experiments, the females were acclimated to the temperature of 28 ±1 °C.

Chemicals :

The materials used in the current study—chemicals, reagents, and antibodies—were of analytical grade. Human chorionic gonadotropin (hCG) (Sigma, CG10), IL-6 (Abcam, ab53327), TNF-α (Abam, ab9642), and TAPI-I (Abcam, ab221418) were purchased from different reliable companies mentioned in parenthesis. Recombinant human interleukin-6 (27 kDa) and tumor necrosis factor-alpha (17 kDa) proteins were produced in *Escherichia coli*. Total RNA isolation (TRI) and the Smart-PCR cDNA synthesis kit (Cat#170-8891) were purchased from Ambion (Foster City, CA, USA) and BioRad, India, respectively.

Methodology :

In vivo experiment :

The fish were acclimatized with commercial fish food (Shalimar Fish Food, Bird and Fish Food Manufacturer, Mumbai, India) for two days in a lab aquarium (100 L capacity) before the research. The fish used in the in vivo experiment was divided into different stages of maturity based on the characteristics of the ovarian follicles (follicle diameter, lipid droplets, and position of GV). From the head to the genital side, a few follicles were gently squeezed out and fixed in cleaning solution/ GVBD fixative (ethanol:formaldehyde: glacial acetic acid - 6:3:1) before being observed under a

microscope. Fish were kept in the lab for an additional 15 days after giving a single dose of hCG (100 ng/100 gm body weight) for the in vivo experiment. The fish were in the pre-vitellogenic, vitellogenic, post-vitellogenic, and pre-ovulatory (post-GVBD) stages of maturation. A total of 40 fish were kept—5 fish and 2 replicates for each group. Saline injections were given to specimens of fish at various developmental stages kept in a different aquarium for 15 days (five at each stage x two replicates: n = 40). After 15 days, by giving MS222 (150 mg/L), fish were sacrificed, their blood and ovarian tissues were collected, and they were stored at -20 °C pending further examination. Three fish were injected with hCG (100 ng/kg body weight) post-GVBD fish specimens, which were then kept in an aquarium for 0, 1, 5, 10, and 15 days. Following fish decapitation, ovarian tissue samples were obtained for further examination. To evaluate the timing of the response after treatment, this was done for kinetics studies.

In vitro experiment :

After sacrifice ovaries were taken from female *A. testudineus* immediately after dissection. Idler's medium with streptomycin (100 g/mL) and penicillin (100 IU/mL) that had been pH-adjusted to 7.4 and ice cold used as a medium to kept the dissected ovary (Pramanick et al., 2014; Guchhait et al., 2018a, b). Individual ovarian follicle is gently separated with the help of fine forceps which were then further use for separate in vitro experiment. Follicles were initially placed for 2 hours in each well of a 24-well culture plate (Tarson, India) that contained 1.0 mL of Idler's media for each trial in order to lessen surgical shock. After two hours, the medium was swapped out for a new one that contained the effectors. Inhibitors were added an hour before the test chemicals were added. In an orbital shaker, cultures were placed at 28±1 °C with air.

Enzyme linked immunosorbent assay:

The manufacturer's recommended procedures were followed to measure the concentrations of IL-6, TNF-α, and IL-10 using an ELISA kit designed specifically for fish. Before the assays were performed, plasma samples were made by centrifuging blood for 20 min. at 3000 g. One milliliter of phosphate buffer (pH 7.4) was added to homogenise 100 mg of follicle tissue for ovarian samples. The centrifuge was then operated for 20 minutes at 3000 g. 50 L of the medium and supernatant from each model were used for the IL-6, TNF-α, and IL-10 assays. In addition to the test sample, the sensitivity of ELISA was evaluated using known concentrations of recombinant IL-6, IL-10, and TNF-α (those used in the in vitro investigation). The concentrations of recombinant IL-6, IL-10, and TNF-α were compared to the concentrations of antigen in the ELISA kit. The variations were no more than 5%.

RNA isolation and cDNA synthesis :

The total RNA from ovarian follicles was separated using the TRI reagent (Promega), as directed by the manufacturer. Using Revert Aid M-MuLV reverse transcriptase (MBI; Fermentas, USA), mixed oligo (dT), and random primers (Promega), 2.0 g of DNase-treated RNA was subjected to cDNA synthesis after the extracted RNA was quantified using Nanodrop techniques. The RNA and cDNA were stored at -80°C and -20°C before use, respectively.

Realtime-PCR for relative abundance of mRNA transcripts :

The relative abundance of mRNA transcript was calculated for every testing group using quantitative real-time PCR (Bio-rad PCR system) with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the template. 50°C for 2 min, 96°C for 10 min, and 45 cycles of 95 °C for 30 s and 59 °C for 1 min were the reaction's operating temperatures. The total reaction volume was 20 L of SYBR Green qPCR SuperMix (Invitrogen), 10 L of cDNA, 5 L of cDNA, and 500 nM of forward and reverse primers. The abundance of GAPDH mRNA transcripts

was constant across samples ($P > 0.05$). The threshold values were calculated using fluorescence data. The relative abundance of the mRNA transcript was calculated using the 2ΔCt method after normalization using GAPDH mRNA abundance data (Rao et al., 2013). The forward and reverse primers were made using primer 3 (Table 1) software (Whitehead Institute). The "no template control" (NTC) samples lacked a primer-dimer or an amplification component. Statistics were applied to melting curve data with a single peak. According to Bustin et al. (2009)'s MIQE recommendations, Δct values, regression slope, PCR efficiency, Y-intercept, and correlation coefficient (R2) were calculated. Three times each sample was examined.

Experiments to find the effects of hCG and TNFα on relative abundance of IL-6 and IL-10 mRNA transcript and production :

Post-vitellogenic and post-GVBD follicles (100 mg) were incubated with Idler's medium for 16 hours while being treated with hCG (100 ng/mL), TNF-α (50 ng/mL), and hCG+TAPI-I to examine the relative abundance of IL-6 and IL-10 mRNA transcript and the in vitro regulatory effects of hCG and TNF-α on IL-6 and IL-10 production in *A. testudineus*. A control group was also used in this experiment. IL-6 and IL-10 levels in the medium were assessed following the incubation period, and follicles were used for RNA separation and subsequent real-time PCR analysis. A kinetics study was conducted using follicles in the PtV and PtGVBD phases over a variety of periods. 100 mg of follicular tissues were incubated in triplicate for 0, 8, 12, 16, and 24 hours. Following each endpoint, medium samples were aspirated, centrifuged (at a speed of 5000 g), and then stored at -20 °C in preparation for the ELISA.

Table 1

Primers used in realtime-PCR (primer designed using primer 3 interactive primer design software)

Gene Product	Forward primer (5'-3')	Reverse primer (5'-3')	Size of amplicon (bp)
IL-6 (NM_001261449.1)	GCAGTATGGGGGAACTATCCG	CTGACCCCTTCAAATGCCGT	193
GAPDH (DQ107520.1)	CTGAGGCATCTCACAAACGA	TCACCCTCAACCTTGACCTC	230
IL-10 (AY887900.1)	ACGCTTCTTCTTTGCGACTG	CACCATATCCCGCTTGAGTT	210

Table 2:

Stage detection of *Anabas testudineus* depending on the oocyte characteristics.

Stage	Microscopic characteristics of oocyte
Previtellogenic	Diameter 0.21±0.82 mm; light orange in colour; lipid vacuoles appear with developing germinal vesicle (GV).
Vitellogenic	Diameter 0.33±0.12 mm; yellowish colour; densely packed lipid granules with centrally located GV.
Postvitellogenic	Diameter 0.64±0.16 mm; yellowish colour; centrally or migratory GV with the presence of peripheral lipid droplets.
post-GVBD	Diameter 0.71±0.06 mm; colour pale-yellowish; no GV observed in most oocytes; oocytes comparatively transparent with peripherally concentrated lipid droplets.

Statistics:

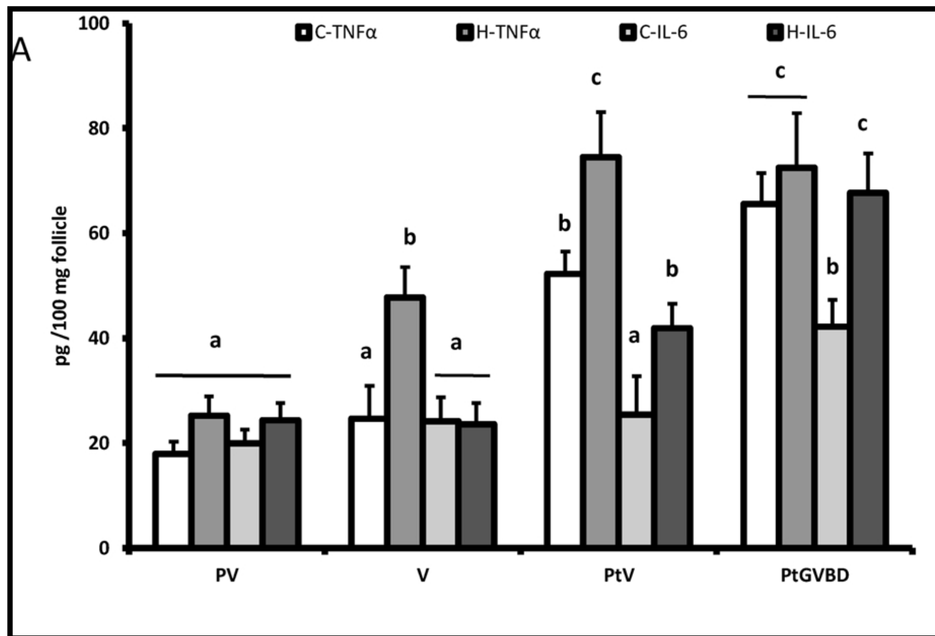
Three replicate incubations of ovarian follicles obtained from a single donor fish were used to collect the data. The three replicates data were comparable, thus the mean values were taken to represent results from a single experiment. For samples from five separate fish, the experiment's results were 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 :

Natural and hCG-induced in vivo release of ovarian IL-6 and TNFα :

By this in vitro study, we found out that the post-GVBD stage had higher levels of IL-6 (saline injection; 19.96±2.4 pg/mL) than the other developmental stages (42.13±5.72 pg/mL) (Fig. 1.A). TNF-α, however, was more common in post-vitellogenic than in earlier developmental stages (52.24±4.36 pg/mL), with post-GVBD having the highest concentration (65.55±8.13 pg/mL). Gonadal IL-6 concentrations were higher when hCG was given at the post-vitellogenic (41.87±6.2 pg/mL) and post-GVBD (67.66±8.73 pg/mL) stages as opposed to the earlier developmental stages (P 0.05). The secretion of ovarian IL-6 during the previtellogenic and vitellogenic stages, however, was unaffected by hCG therapy. In comparison to when saline was added at the Post-GVBD stage, IL-6 and TNF-α concentrations in the media were higher. When hCG was used as a treatment and they were higher in the post-GVBD stage. It's interesting to note that hCG administration only stimulated TNF-α release during the vitellogenic stage and had no impact on TNF-α production during the post-GVBD stages (P 0.05).

The kinetics study (Fig 1.B), which assessed effects over time, showed that levels of IL-6 were higher the day after receiving hCG treatment (76.85±10.43), but there was no further increase on subsequent days of incubation. The concentration of IL-6 in the control group increased after 10 days in culture ($P \leq 0.05$).



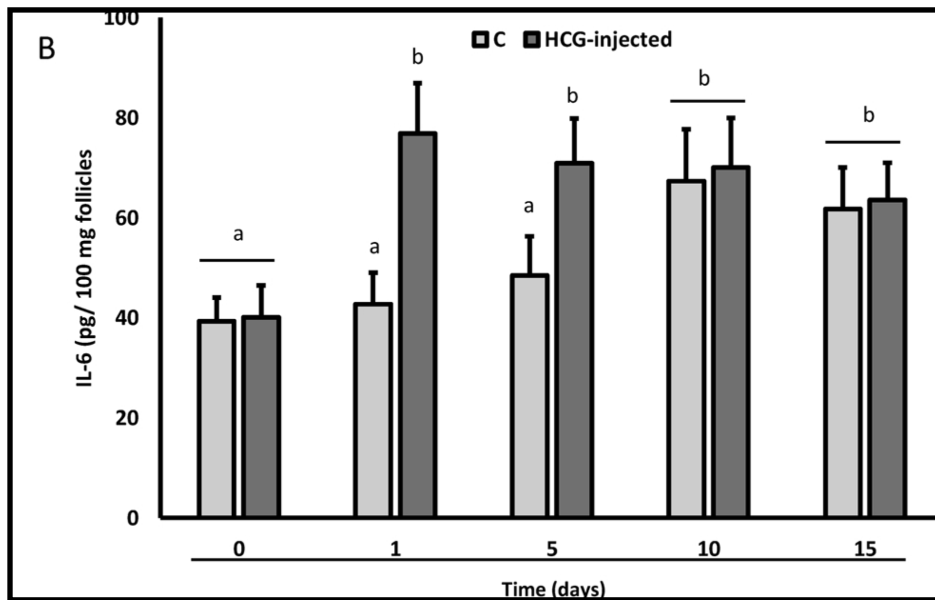


Fig 1. Ovarian IL-6 in vivo concentration at different maturational stages of _Anabas_ *respectively after treating with gonadotropin (HCG) in respect to the control (A) . Kinetics study of in vivo ovarian IL-6 respectively in fish treated with HCG (B). Data was analysed using ELISA with 100 mg of ovarian tissue. Results are the mean ± SEM of five fish for each stage; Different letters indicate mean differences between the treatment groups ($P \leq 0.05$).*

Natural and hCG-induced in vivo release of ovarian IL-10:

The post-vitellogenic (50.09 ± 10 pg/ml) and post-GVBD (55.98 ± 10.2 pg/ml) stages were when IL-10 concentrations were higher. In addition, when treated with hCG, IL-10 secretion is increased in the post-GVBD stage of oocytes as well as the vitellogenic (43.22 ± 8 pg/ml) and post-vitelligenic (71.43 ± 10.5 pg/ml) stages (Fig 2.A). The data showed a consistent similarity to the data of TNF-α.

The time kinetics study (Fig 2.B) showed that levels of IL-10 were higher in the 5th day of incubation (76.85±10.43), which is then further increase on 10th days of incubation. The concentration of IL-10 in the control group not further increased in 15th day. Whereas when treated with hCG from 1st day to 10th day it increased than control but remain almost same concentration during 1st, 5th and 10th day but there is significant low concentration in 15th day which is similar to the control in 15th day.

Effects of hCG and TNFα on in vitro IL-6 mRNA transcript abundance and IL-6 production

In the in vitro experiment (Fig. 3.A), when PtV and post-GVBD follicles were treated with hCG, the release of IL-6 was higher than the values for control samples, at 58.67±4.32 and 71.65±5.41 pg/mL, respectively (P ≤ 0.05). TAPI-I (50 mM), a TNF-α secreting inhibitor, treatment, however, decreased the hCG-induced secretion of IL-6. IL6 was only identified in its highest concentrations at the post-GVBD stage.

The relative abundance of the IL-6 mRNA transcript (Fig 3.B) increased by 4 and 5 fold respectively in PtV stages of the follicle when the follicles were treated with hCG or TNF-α (P ≤ 0.05), peaking at the GVBD stage by 5 and 5.5 fold, respectively. On the other hand, the relative abundance of this transcript increased when the follicles were treated with hCG and TAPI-1.

Results from the kinetics study (Fig.3.C) with post-vitellogenic and post-GVBD follicles showed that when hCG (100 ng/ ml) treatment was administered after 16 h of incubations, IL-6

concentrations (58.67± 7.84) were higher than they were at the 0 h (18.03±3.23; Fig. 3); in contrast, the concentrations

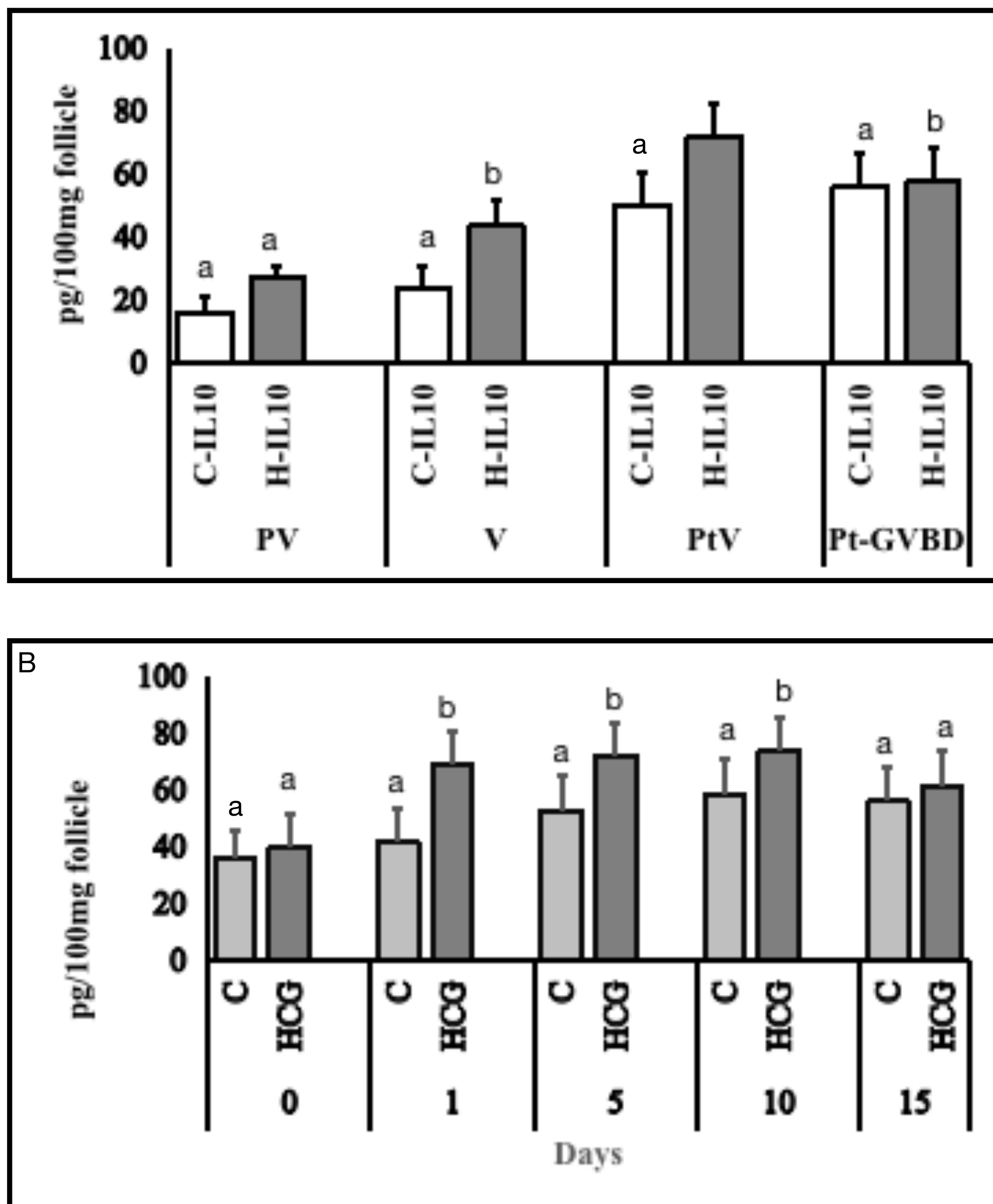


Fig 2. Ovarian IL-10 in vivo concentration at different maturational stages of Anabas respectively after treating with gonadotropin (HCG) in respect to the control (A) . Kinetics study of in vivo ovarian IL-10 respectively in fish treated with HCG (B). Data was analysed using ELISA with 100 mg of ovarian tissue. Results are the mean ± SEM of five fish for each stage; Different letters indicate mean differences between the treatment groups ($P \leq 0.05$).

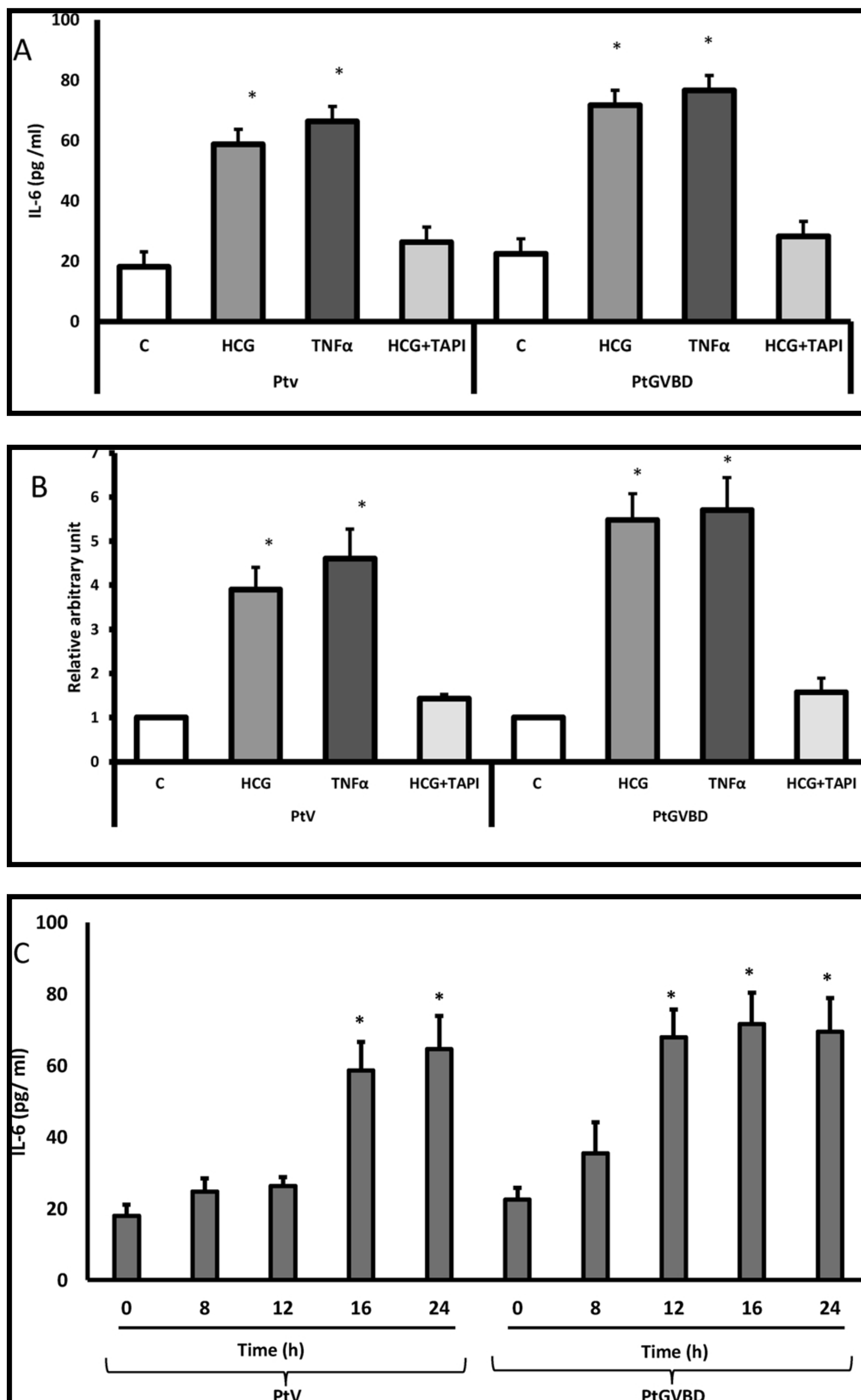


*Fig 3. (A) IL-6 In vitro production and (B) IL-6 expression of mRNA transcripts in response to effector molecule hCG, TNFα and TAPI-1 in post vitellogenic and post GVBD follicles of <u>Anabas</u>, normalized by GAPDH, (C) in vitro kinetics study of PtV and Pt-GVBD follicles for IL-6 production. Experiments were conducted using tissues from five fish with triplicate incubations at each developmental stage; Standard bar is the mean ± SEM; *denotes the values that are different in comparison to other treatments ($P \leq 0.05$).*

(67.96±8.67 pg/ml) of IL-6 were greater in Pt-GVBD follicles after incubation of 12h and were subsequently consistent.

Effects of hCG and TNFα on in vitro IL-10 mRNA transcript abundance and IL-10 production

In the in vitro experiment (Fig. 4), the release of IL-10 was higher when PtV and post-GVBD follicles were treated with hCG than the values for control samples. In terms of IL-10, these numbers were 53.25±9.5 and 55.25±10.9 pg/mL, respectively ($P \leq 0.05$). The same increased secretion of IL-10 was also found when treated with TNF-α However, administration of the TNF-α secreting inhibitor TAPI-I (50 mM) decreased the release of IL-10 induced by hCG. Both post-vitellogenic and post-GVBD follicles exhibit an increase in IL-10.

The same treatment also increased IL-10 expression, which was significantly reduced when combined with hCG and TAPI-1. In post vitellogenic follicles, IL-10 transcript expression is at its highest level.

The kinetics study with post-vitellogenic and post-GVBD follicles revealed that the concentrations of IL-10 were significantly higher in post-vitellogenic follicles from 12 hours to 24 hours (40.56± 8.6 pg/ml to 65.76±11.5 pg/ml), while the concentration of IL-10 in post-GVBD follicles was higher in 12 hours but did not change significantly after that.

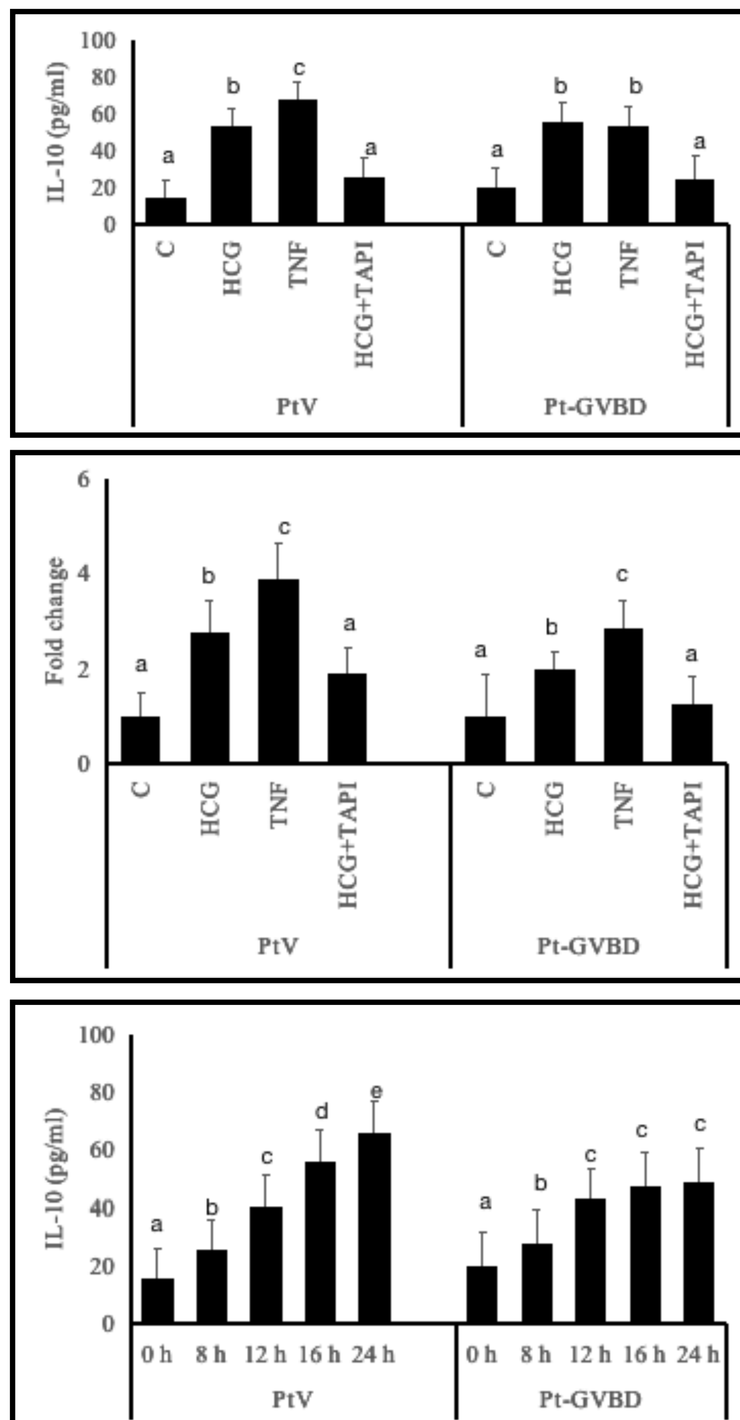


*Fig 4. (A) IL-10 In vitro production and (B) IL-10 expression of mRNA transcripts in response to effector molecule hCG, TNFα and TAPI-1 in post vitellogenic and post GVBD follicles of Anabas, normalized by GAPDH, (C) in vitro kinetics study of PtV and Pt-GVBD follicles for IL-10 production respectively. Experiments were conducted using tissues from five fish with triplicate incubations at each developmental stage; Standard bar is the mean ± SEM; *denotes the values that are different in comparison to other treatments (P ≤ 0.05).*

Discussion:

Gonadotropins are known to control ovarian growth, maturation, and ovulation in vertebrates. The functions of the ovary are also modulated by a number of additional ovarian-derived factors, such as growth factors and cytokines (Adashi et al., 1990; Ben-Rafael and Orvieto, 1992; Araki et al., 1996; Hoek et al., 1998; Salmassi et al., 2001; Richards et al., 2008; Liu et al., 2009). Immuno-endocrine factors and mammals have long been known to interact (Tsushima, 1995; Russell et al., 2001; Bagavant et al., 2002). Fish have been used to study the functions of IL-1, CSF, IL-8, and TNF as well as how these molecules interact with endocrine molecules (Chaves-Pozo et al., 2003, 2005a; Liarte et al., 2007, Chaves-Pozo et al., 2008; Crespo et al., 2012, 2015; Zou and Secombes, 2016). The concentration of ovarian IL-6,TNF-α and IL-10 at various phases of *A. testudineus* maturation was quantified in the in vivo experiment of the current investigation, both in untreated control fish and fish that had been given hCG. In the process of this investigation present study found that, only in the post-GVBD stage has the potentiality to secret IL-6 which indicating that IL-6 may play a role in *A. testudineus* ovulatory processes. At the post-vitellogenic and pre-ovulatory (post-GVBD) stages, gonadal IL-6 secretions were higher in fish treated with hCG; this could be attributed to the oocytes developing sensitivity to gonadotropins. At the Pt-GVBD stage, there were higher IL-6 concentrations after hCG treatments, and these higher amounts persisted. Because *A. testudineus* will not reproduce unless it was placed with a gravid male, IL-6 may trigger other signalling pathways that cause oocyte shrinkage. The inhibition of hCG-induced gene expression, as shown by abundances of pertinent mRNA transcripts, and IL-6 secretion when there was treatment with TAPI-I in an in vitro investigation, validates the regulatory function of gonadotropin-mediated TNF-α secretion. The studies also revealed that their was an significant difference in pattern of TNF-α secretion in different stages of follicles. We observed that the secretion of TNF-α secretion was high only in the vitellogeneic follicle when treated with hCG but the impact of hCG did not change the

secretion of TNF-α in post vitellogenic follicle indicates their involvement in the maturational phase in *Anabas* oocytes but not during the time of the ovulation.

Some of the published studies (Huck et al., 2005; Uemura et al., 2008) found that human DCs significantly increased their production of IL-10 following treatment with pregnancy hormones. Previous study also showed that IL-10 in response to hCG treatment and expresses the LH/CG receptor (Rolle et al., 2013). Though their is no work till date, that what is the role of IL-10 during the time of reproductive processes in fish. In this investigation to find out how they play their role in the reproductive processes, we observed in the in vivo studies that the secretion of IL-10 in untreated fish was gradually increase pre-vitellogenic follicle to post-vitellogenic follicle in *Anabas*. The same pattern of secretion was also observed when the fish treated with hCG which also corroborated the results of previous studies. The in vitro study also confirmed the result of in vivo experiment. Whenever the follicles were treated with TAPI-1 secretion level of IL-10 was lower and also down regulates the mRNA transcripts in oocyte. The interesting thing observed in this experiment is that secretion pattern of TNF-α and IL-10 was similar in post vitellogenic and post-GVBD follicle. This result establish the fact that the production of IL-10 was directly controlled by the secretion of TNF-α which is similar what have earlier study done by Secombes 2003 in Fugu. So, we can clearly say that not only the pro- inflammatory but also the anti-inflammatory cytokine like IL-10 play a pivotal role in the reproductive processes of the teleost.

Furthermore, to clear out the role of IL-6 and IL-10 in reproductive processes we have done an in vivo kinetic study followed with an in vitro one. The experiment were held to understand the secretion of both IL-6 and IL-10 over the time period of follicular maturation and ovulation. In case of the in vitro study we selectively perform this experiment over post-vitellogenic and post-GVBD follicle. Our result showed that the secretion IL-6 was highest in the day after injection but after that their was no such significant changes occur in later period of time. Whereas in case of fish who were left untreated the secretion of IL-6 was increased in day 5 and so on upto the 15th day where

the secretion of IL-6 was almost similar to that of the treated one. By more closer look to that fact, the in vitro study provide the data which supporting the involvement of IL-6 during the time of ovulation where we can see the secretion of IL-6 was higher in later phase (16th and 24th hour of incubation) of post-vitellogenic follicle and the gradual increase of IL-6 secretion in pos-GVBD follicle. In a similar set of experimental set up for IL-10 showed a different pattern of secretion than IL-6. The secretion of IL-10 was highest a day after hCG treatment while in the 15th day after treatment the level of IL-10 secretion was lower down a bit in comparison to the 10th day. When the fish left untreated, in 10th day we observed the highest secretion of IL-10. In vitro study was also showed that the secretion of IL-10 was highest in post-vitellogenic follicle and it was increased gradually during the each hour of experiment. In the post-GVBD follicle their was a significant halt in secretion IL-10 during the later period of incubation suggested the possible insolvent of IL-10 during the time of oocyte maturation but not in the ovulation.

Review of literature

Introduction:

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. In some ways, inflammation and the ovulation process in fish are similar. As a result of earlier studies and reviews, it is now generally acknowledged that fish contain all major cytokine families. They can manage the ovarian functions by secreting locally. In the fish ovary of the majority of species, pro-inflammatory cytokines like interleukin-1β (IL-1β), tutor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as various paralogues, are present. According to studies, prostaglandins (PGs) and the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) may regulate critical phases of the mammalian ovulatory process (Crespo, 2015). Studies suggested that ovulation in rat ovary regulated by TNF-α. Collagenolytic activity and death in preovulatory follicles can also be conducted by TNF-α itself. TNF-α can also stimulate the production of PGs in rat preovulatory follicles. There is growing evidence that IL-6 regulates a number of critical reproductive processes in a paracrine or autocrine manner. IL-6 regulated the secretion of placental lactogen (Stephanou and Handwerger, 1994) and human chorionic gonadotropin (hCG) in human trophoblast cells (Nishino et al. 1990), as well as the release of anterior pituitary hormones such as adrenocorticotropin, prolactin, growth hormone (GH), and Luteinizing hormone (LH) in rats (Spangelo et al. 1989, 1995). The anterior pituitary cells produce IL-6 in response to elevated intracellular cAMP and the neuropeptide vasoactive intestinal peptide, as shown by Spangelo et al. (1990a,b). Similar to this, Kawasaki et al., (2003) hypothesised that IL-6 could be controlled by follicle-stimulating hormone (FSH) and/or LH via the cyclic AMP signalling pathway. According to Kawasaki et al.'s 2003 study, human follicular fluid (FF) contained a higher concentration of IL-6 than serum. In comparison to fluid-containing immature oocytes, the FF-containing mature oocytes had significantly higher IL-6 and IL-6 sR concentrations. The human oocytes also contained the protein gp 130 (van Eijk et al. 1996).

Many species of Granulosa cells (GCs) are active sites of IL-6 biosynthesis (Gorospe et al., 1992; Machelon et al., 1994), and cultured rat GCs have been found to contain both IL-6R components (Tamura et al., 2000). According to Liu et al. (2009), mice GCs of ovulating follicles expressed and released IL-6. The role of cytokines of the IL6 type in the development of the human ovary, specifically in controlling the differentiation of GCs, has been established by Eddie et al. (2012). During the in vitro maturation of rat GCs, IL-6 downregulated the expression of LHR (Tamura et al. , 2001) and decreased the expression of CYP19 mRNA (Tamura et al., 2000). When rat GCs differentiate, it has been demonstrated that IL-6 can lower 17β-estradiol (E2) production and that there is a dissociation between nitric oxide (NO) production and IL-6-induced E2 suppression (Tamura et al. , 2000). In bovine GCs, IL-6 prevented E2 from being secreted in response to FSH (Alpizar and Spicer, 1994, Gorospe et al., 1992). According to the research of Maeda et al. (2007), IL-6 produced in the GCs of healthy follicles may help to prevent apoptotic cell death and thereby aid in the selection of follicles by up-regulating the expression of intracellular apoptosis inhibitors. According to Kawasaki et al. (2003), IL-6 may be crucial for the angiogenesis of human follicles, which speeds up the development of oocytes. According to Van der Hoek et al. (1998), IL-6 decreased LH and IL-1 increased ovulatory rates in the perfused rat ovary. Inhibiting IL-6 by itself does not seem to have an effect on ovarian function in mice (Poli et al., 1994). Gp130 disruption, however, resulted in abnormal oocyte zygotic development, suggesting that this pathway may be important before, during, or after fertilisation (Molyneaux et al., 2003). In addition to being a pivotal factor to be used to increase the rate of in vitro fertilisation, capacity of IL-6 to increase oocyte competence also offers insights into the roles of oocyte-cumulus complex (OCCs) and oocyte quality (Liu et al., 2009). Through activating the extracellular signal-regulated kinase 1/2 (ERK1/2), Janus kinase/signal transducers and activators of transcription (JAK/STAT) and Mitogen-activated protein kinase (p38MAPK) pathways, IL-6 may mediate some of its effects as a key regulator of ovarian cumulus cells (CC) function and OCC expansion. In mice, IL-6 did

activate genes involved in matrix stability and formation (Liu et al., 2009). According to evidence presented (Sakumoto et al., 2006), IL-6 and IL-6R mRNAs are present in the porcine corpus luteum (CL) throughout the estrous cycle. Furthermore, it has been demonstrated that IL-6 is produced by activated T-cells and macrophages in the porcine (Hehnke et al., 1994) and bovine (Penny et al., 1999) ovary, and that the macrophage count rose during luteolysis. Given that IL-6 inhibited hCG-induced P4 secretion in purified porcine small and large luteal cells, it appears that IL-6 has the function of reducing steroid production during luteolysis. In contrast, Pitzel et al. (1993) found that IL-6R gene expression was higher in the regressed CL than in the other stages. According to research by Telleria et al. (1998), a decrease in P4 increased the expression of the IL-6R in rat CL. The results mentioned above imply that IL-6 regulates CL function, particularly during luteolysis, via autocrine and/or paracrine mechanisms (Sakumoto et al., 2006). In addition, FSH dose-dependently reduces the relative abundance of IL-6 mRNA transcript in rabbits (Breard et al., 1998). According to Salmassi et al. (2001), IL-6 prevents progesterone from being produced when FSH is stimulated in human granulosa cells. In granulosa cells and the Cumulus Cell Oocyte Complex (COC), the relative abundance of IL-6 mRNA transcript rises just before ovulation (Liu et al., 2009).

Although there is almost no significant study had been done on interleukin-10 (IL-10) in fish gonad, in 1999, Roth and Fisher suggested that cytotrophoblastic matrixmetalloprotienase-9 (MMP9) activity is inhibited by IL-10. Kothari et al., 2014 suggested that IL-10 induced by JAK-dependent pathway control the MMP9 expression induce by IL-6. Previously it was reported that IL-10 suppress the activity of TNF-α converting enzyme. IL-10 also inhibit the activity of cyclooxygenase 2 (COX2) and prostaglandin E_2 (PGE_2) (Mosmann and Moore, 1991). At early time of pregnancy in human it was reported that IL-10 induce the synthesis of progesterone in cultured luteal cell. Also from Secombes 2003, by activating the macrophages IL-10 can inhibit the inflammatory response via the suppression of nitric oxide production. According to Kube et.al., 1995, TNF-α can enhance

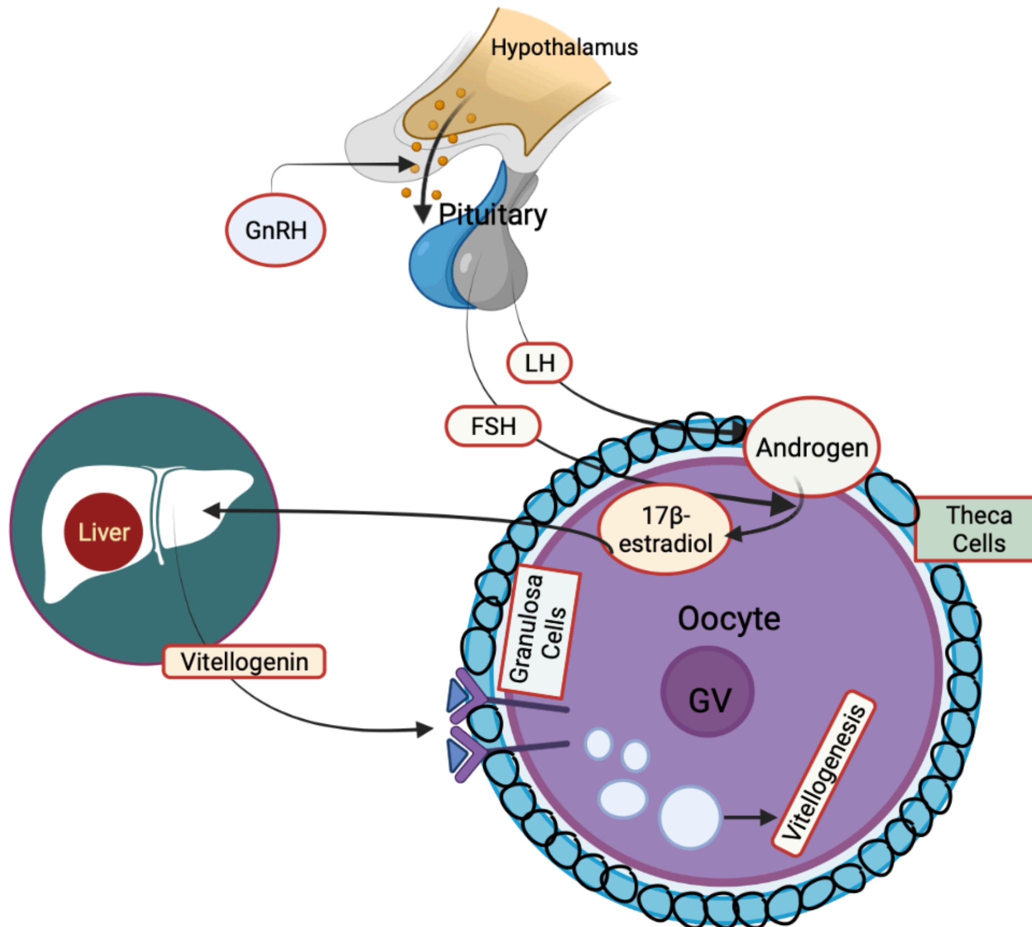
the production of IL-10 in human peripheral blood mononuclear cells. In our study we tried to explore the probable involvement of IL-6 and IL-10 in the reproductive processes of teleost fish. By activating nuclear factor of activated T cells (NFAT) pathway IL-10 may be upregulated by TNF-α in Fugu (Secombes, 2003).

Fish oocyte, gonadotropin and and steroid secretion:

Burzawa-Gerard in 1982 suggested, by a biochemical studies, that, a single LH like gonadotropin regulates gametogenesis in fish. In 1983, Nagahama and Idler join out the existence of another gonadotropin which is responsible for the uptake of vitellogenin into oocyte. Kawauchi in 1988 finally establish the presence of two gonadotropins (GtH), GtH I and GtH II in salmon (Suzuki et al., 1988 a,b,c; Itoh et al., 1990). In further study it has been established that GtH I and GtH II are orthologue of tetrapod FSH and LH, respectively (Li and Ford, 1998; Queart et al., 2000).

Vitellogenesis, the process which is responsible for growth of fish oocyte. 17β-estradiol which is synthesised in ovary induced by GtH, regulated this process (Wallace, 1895; Paddle et al., 1989, 1991; Nagahama, 1994; Devlin and Nagahama, 2002). Vitellogenesis is thus depended on the regulation of 17β-estradiol which in turn regulated by FSH and in this process cytochrome P450 aromatase is considered as key regulator (Tanaka et al, 1992; Gen et al., 2001; Kagawa et al., 2003). On the other hand final maturation of gametes is controlled by LH which is the key regulator for the production of Maturation-inducing hormone (MIH), which is then stimulate the production of maturation-inducing steroid hormone (MIS) from follicular cell layers. After that, MIS facilitate the formation of maturation promoting factor (MPF) which induce germinal vesicle breakdown (GVBD). MPF is constituted by the complex of cyclin B and cdc2 (Nagahama and Yamashita, 2008). So, like other vertebrates, the follicular cell layer of fish oocyte also composed by two cell layer named theca (the outer one) and granulosa (the inner one) (Nagahama, 1983). LH and FSH acts on this two cell layer in pulsatile manner which respectively induce both the theca and granulosa cell layer to synthesize 17α- hydroxy progesterone and 17β- estradiol respectively, two

cell two gonadotroph hypothesis (Nagahama et al., 1995). In conclusion, 17β- estradiol is produced during the period of oocyte growth and during the time of maturation MIH has been produced (Senthilkumaran et al., 2004).



A schematic representation of two cell two gonadotroph hypothesis.

Cytokine in general

Cytokines, which are protein-based molecules, are typically produced under inflammatory conditions.This process starts a cascade of events, with the production of subsequent cytokine amplifying and multiplying the initial stimulus (Kennedy and Jones, 1991). Generally categorised under small protein (~5-21 kDa) have been found to be involved in autocrine, paracrine and endocrine signalling as immunomodulating agents. In 1965, interferon gamma was first identified lymphocyte derivative mediator (Wheelock, 1965). John David and Barry Bloom simultaneously identified Macrophage migration inhibitory factor (MIF) in 1966. Stanley Cohen in 1974 experimentally established that production of MIF is not only limited to the immune cells but also from other cells which led the term cytokine (Cohen et. al, 1974). Cytokines are generally produced by all nucleated cell, especially endo/epithelial cell and resident macrophages (Boyle, 2005). Lymphokine, interleukin and chemokine are generally classes of cytokines and often divided into two classes, class I and class II cytokines.

According to Huising et al. (2006), class 1 cytokines frequently have a role in the survival, maturation, recruitment to inflammatory/infection sites, and proliferation of cells. They can also affect other physiological systems like reproduction, food intake, and metabolism because of their pleiotropic nature. They both utilise class 1 cytokine receptors and have a similar 4 helical 2 sheet 3D shape. These cytokines are thought to have evolved from a single ancestor, despite having little in common in their basic sequences (Bazan, 1990).

Interferons, which facilitates antiviral responses, and IL10, considered to be anti-inflammatory in nature, are examples of Class II cytokines, which, in contrast to Class I, generally act on minimising damage to host following noninfectious and infectious insult (Krause and Pestka, 2005). Their sheets have been replaced by -helices, which is another difference between their structure and that of Class 1 cytokines (Langer et al. 2004). Interferons, which facilitates antiviral responses, and IL10, considered to be anti-inflammatory in nature, are examples of Class II cytokines, which, in

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Cytokines in fish:

At the transcript level it is now well established that all of the major cytokine family have been present in fish (Secombes et.al, 2011, Wang and Secombes, 2013, Secombes et.al., 2016). Particularly in case of teleost fish from a specific gene multiple copies (paralogues) of that gene occur. IL-1β, TNF-α and IL-6, classical pro inflammatory cytokines, are one of those examples in most species having multiple paralogues and it is also similar to that of the cytokines responsible for the adaptive immune responses like IL-4/13, IL-10, transforming growth factor beta-1 (TGF-β1) amongst others (Zou and Secombes , 2016). Best known for their role in the time of inflammation, Interleukins, the polypeptides produced from immunocompetent cells primarily involved in immune system. CD helper T-lymphocyte is the major synthetic area of interleukin, and also through monocytes, macrophages and endothelial cells (Vasiliou et.al., 2011). Amongst all, IL-1β is of the first family member discovered in mice and humans which is also widely present in other non mammalian vertebrates. In our study, we specifically focused on the role of IL-6 and IL-10 which are respectively categorised as Type I and Type II helical cytokines. Il-6 is the family comes under IL-6/12 super family and IL-10 from the IL-10 family. Type I helical cytokine family are generally signal through type I cytokine receptor family as their name suggested and as same as for the type II helical cytokine family via type II cytokine receptor family.

IL-6 sub family:

Generally pleiotropic in nature, having both pro and anti-inflammatory properties, having four members in this family, IL-6 itself, IL-11, a molecule termed CNTF-like and M17 (Fuji et.al., 2003, Wang and Secombes, 2009). The first two studies describing IL-6 date back to 1980, when

two laboratories independently discovered and named interferon β2, a new mRNA that appeared after interferon b activated fibroblasts.Worldwide research teams identified numerous proteins during the ensuing years, including 26 kDa protein, B cell stimulatory factor 2 (BSF-2), hybridoma growth factor (HGF), and hepatocyte-stimulating factor (HSF), which ultimately turned out to be one and the same protein known as IL-6 (Weissenbach et al., 1980, Sehgal and Sagar 1980, Content et al., 1982, Haegeman et al., 1986, Hirano et al., 1985, Van Damme et al., 1987, Bauer et al., 1986, Yasukawa, 1987). The IL-6 mRNA codes for a protein of 212 amino acids, including a signal peptide with 29 amino acids, which results in a secreted protein with 184 amino acids. This would result in a protein of about 21 kDa, however because of various N-linked glycosylations, IL-6 is found in many isoforms ranging from 21 to 28 kDa (Schiel et al., 1990). The synthesis and fate of the IL-6 mRNA are regulated by several variables, either at the transcriptional level or post-transcriptionally.A cyclic AMP (cAMP) responsive element, a binding site for CCAAT enhancer binding protein b (C/EBPb, also known as NF-IL6, and a binding motif for NF-κB are all present in the IL-6 promoter region upstream of its TATA box sequence (Angel et al., 1987, Lee et al., 1987, Lee et al., 1987, Matsusaka et al., 1993, Shimizu et al., 1990, Libermann et al., 1990, Ray et al., 1988). Studies had been found that LPS can induce IL-6 and also TNF-α and IL-1 α, both are pro inflammatory cytokine which in all can responsible for (NF-κB) activation (Fong et al., 1988, Kohase et al., 1986, Kohase et al., 1987). Like mammals, recombinant IL-6 of fish can also promote antibody production (Kaneda et.al, 2012, Chen et.al, 2012). Being a multifunctuional cytokine, in mammals, produced by variety of cells which affects, B-cells, T-cells (Iliev et al., 2007), hepatocytes (Gauldie et al., 1987), hematopoietic progenitor cells (Ikebuchi et al., 1987), ovary (Iliev et al., 2007) etc. Macrophage growth has been found in the presence of rIL-6 in rainbow trout in culture (Costa et.al, 2011). Apart from pro inflammatory responses, in a study, IL-6 also suppress the production of IL-1 and TNF-α, on the contrary it facilitate the production of IL-10 (Dienz et al.,

2012). First successful cloning of IL-6 was happened in 1986 from human (Hirano et al., 1986). Later it has been identified in different teleost fishes includes, Takifugu rubripes (Fugu rubripes) (Bird et al., 2005), Dicentrarchus labrax (European seabass) (Sepulcre et al., 2007), Oncorhynchus mykiss (rainbow trout) (Iliev et al., 2007), spotted green pufferfish (Bird and Secombes, 2006 (Unpublished)), Paralichthys olivaceus (Japanese flounder) (Nam et al., 2007) and Sparus aurata (Castellana et al., 2008). Structurally mammalian and teleost species has similarity in gene with five exons and four introns. Recombinant IL-6 facilitated the phosphorylation in STAT-3 and inhibitor of jak-2 abolish the state in RTS-11 cells suggested their signalling pattern where Jak2/Stat-3 playing a significant role (Wang et.al., 2015).

IL-6 receptors:

IL-6 α-receptor (IL-6R) and the signal-transducing β-subunit glycoprotein 130 (gp130), a heterodimeric signalling complex are actually responsible by which IL-6 activated the cells and it has been shared by other cytokines (Garbers et al., 2012). There is a distinct difference between IL-6 signalling depending upon if IL-6R exists as soluble (IL-6 trans- signaling) or as membrane bound form (IL-6 classic). While IL-6R is primarily expressed in hepatocytes, megakaryocytes, and a number of leukocyte subpopulations, Gp130 expression can be found in every tissue and cell type of the human body. Recent studies has been found that the pro inflammatory activity of IL-6 was accompanied via the trans signalling, that is, the soluble IL-6 R. (Chalaris et al., 2011, Rose-Jhon , 2012, Jones et al., 2011). In fugu, IL-6R and gp130 two chains of IL-6 receptor has been expressed (Kaneda et al., 2012). In fish, homologs of IL-6 and its receptors have been identified, indicating that the IL-6 signaling pathway is conserved in fish. However, the organization of the IL-6 receptor complex in fish appears to be different from that of mammals.

In some fish species, such as zebrafish and Atlantic salmon, there is only one IL-6 receptor homolog, which contains both IL-6R and gp130 domains in a single protein. This differs from mammals, where IL-6R and gp130 are separate proteins that interact to form a receptor complex. In

other fish species, such as rainbow trout, two separate IL-6 receptor homologs have been identified, one with an IL-6R domain and the other with a gp130 domain. These two receptors can heterodimerize to form a functional receptor complex that is capable of binding and signaling in response to IL-6.

Overall, the IL-6 receptor system in fish is different from that of mammals, but appears to serve similar functions in regulating immune responses and inflammation. Further research is needed to fully understand the role of the IL-6 receptor system in fish.

IL-10 family:

IL-10 sub family is considered as type II α helical cytokines which also includes e type I and III IFNs (Lutfalla et al., 2003) and passes their signal through type II cytokine family receptor (Vosshenrich et al., 2002). In fugu genome IL-10 was first discovered (Zou et al., 2003). Generally similar to its mammalian orthologues, function of IL-10 has been characterised in gold fish and carp (Piazzon et al., 2015). IL-10 is anti inflammatory and suppress the immune response, mostly present as a single copy gene except rainbow trout where duplicated copies are present, herpesviruses of European eel and Koi carp also encode an IL-10 homologue (Piazzon et al., 2015, Sunarto et al., 2012, Van Beurden et al., 2011). In recent times in several teleost species (Piazzon et al., 2016) viz; fugu (*Fugu rubripes*) (Zou et al., 2003) and zebrafish (*Danio rerio*) (Zhang et al., 2005), and also in severale conomically important species, such as common carp (*Cyprinus carpio*) (Piazzon et al., 2015; Ram et al., 2003), goldfish (*Carassius auratus*) (Grayfer et al., 2011), grass carp (*Ctenopharyngodon idella*) (Wei et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Inoue et al., 2005), sea bass (*Dicentrarchus labrax*) (Pinto et al., 2007) and Atlantic cod (*Gadus morhua*) (Seppola et al., 2008), IL-10 had been identified and studied. Expression of IL-10 is constitutively seen in immune tissues, gills, intestine and spleen in carp (Savan et al., 2003). In in vitro study the expression of IL-10 can be up regulated by exposure of various stimulants, like lipopolysaccharides (LPS) and in in vivo by the injection of live or dead bacteria (Buonocore et al., 2007, Harun et al.,

2011, Savan et al., 2003, Glenney et al., 2007, Pinto et al., 2007, Swain et al., 2012). Studies has been found that rIL-10 can reduce the reactive oxygen species (ROS) and nitrogen oxygen species (NOS) (Grayfer et al., 2011). In the response to LPS stimulation rIL-10 can also down regulate the expression of IL-6 (pro inflammatory cytokine), p53 (Piazzon et al., 2015). In the same study Piazzon et al., also established that IL-10 play this part in association with STAT-3, which via phosphorylation eventually induce the expression of suppressor of cytokine signaling 3 (SOCS3). Incubation with IL-10 significantly reduces the production of TNF-1, TNF-2, IL-1, IL-10, CXCL_8/IL-8, and the NADPH oxidase subunit p47(phox) in goldfish monocytes stimulated with heat-killed *A. salmonicida* (Grayfer et al., 2011). In recent studies a viral homologue has been found in Koi herpesvirus (Khv) genome. The KhvIL-10 gene is substantially expressed during the acute and reactivation periods in virus-infected carp. Similar to zebrafish IL-10, the KhvIL-10 increases the number of lyz+ cells when injected into zebrafish embryos. This activity can be blocked by co-injecting IL-10R1 morpholinos (Sunarto et al., 2012).

IL-10 receptors:

IL-10 is a cytokine that plays an important role in regulating immune responses and inflammation. The IL-10R is a transmembrane protein that binds to IL-10 and transduces its signal into the cell.

In mammals, there are two types of IL-10 receptors: IL-10R1 and IL-10R2. IL-10R1 is the primary binding site for IL-10, while IL-10R2 acts as a co-receptor to enhance the binding affinity of IL-10 for IL-10R1. IL-10R1 is expressed on a variety of immune cells, including T cells, B cells, macrophages, and dendritic cells. IL-10R2 is expressed on fewer cell types, including endothelial cells, epithelial cells, and some immune cells. IL-10 signaling through its receptors leads to the activation of various downstream signaling pathways, including the JAK/STAT pathway, MAPK pathway, and PI3K/AKT pathway. These pathways ultimately lead to the regulation of gene expression and SOCS-3 (Wei eta al., 2014). Dysregulation of IL-10 signaling has been implicated in the pathogenesis of various autoimmune and inflammatory diseases, including inflammatory bowel

disease, rheumatoid arthritis, and multiple sclerosis. Understanding the role of IL-10 and its receptors in these diseases may lead to the development of new therapies for these conditions. Interleukin-10 (IL-10) is also present in fish, where it plays an important role in regulating the immune system. Fish IL-10 has been found in a variety of species, including zebrafish, rainbow trout, and Atlantic salmon. In zebrafish, IL-10 R1 gene located on chromosome 15 and the ligands are highly conserved (Grayfer et al., 2012).

The expression of IL-10R1 and IL-10R2 has been detected in various fish tissues, including the spleen, head kidney, and intestine. Studies have shown that IL-10 can regulate the immune response in fish by inhibiting the production of pro-inflammatory cytokines and promoting the differentiation of regulatory T cells.

Cytokines and JAK/STAT:

Interleukins, interferons, and hemopoietins those who are structurally related induces homodimerization and activate JAK kinases (JAK1, JAK2, JAK3, and Tyk2) by which they can modulate immunity and inflammation (Ihle, 1995). The cytoplasmic domains of the receptor are phosphorylated by the activated JAKs, generating docking sites for signalling proteins that include SH2. Members of the STAT protein family, which is a family of signal transducers and activators of transcription, are among the substrates of tyrosine phosphorylation (Ihle 1996, Darnell 1997). It was believed in beginning that interferons (IFN) was the one who take up this pathway but later it has been found that a large number of cytokines, growth factors and some hormonal factors can also activate the JAK/STAT. Pro inflammatory cytokine IL-6 activated JAK1 and STAT-3 via binding with its own receptors. Anti inflammatory cytokine, IL-10, interesting also activate STAT-3 (O’Farrell et al.,1998).

Role of cytokines in ovary:

Years of research establish that cytokine play a major role in reproductive processes. In between the immune and reproductive systems there have been multiple autocrine-paracrine-endocrine loops shown to exist. Cytokines may be significant autocrine and paracrine regulators of ovarian activities, as evidenced by their synthesis in the ovary, accumulation in ovarian FF, and effects on specific ovarian processes (Richards and Pangas, 2010; Qiao and Feng, 2011; Sirotkin, 2011). Brannstrom and Norman in 1993 showed that cytokine has been produced and regulated during menstrual cycle. In another study, Espey 1994, stated that the ovulatory process and an acute inflammatory reaction shares certain similarities to each other. An increasing amount of research indicates that the ovary is where ILs are produced and put to use. 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). TGF-β and cytokines played an important part in the communication between oocytes and its surrounding somatic cell not only these but also they played a significant role in the regulation of follicle survival and apoptosis (Kaipia and Hsueh, 1997; Drummond et al., 2003; Shimasaki et al., 2003; Bornstein et al., 2004; Bristol and Woodruff, 2004; McNatty et al., 2005; Knight and Glister, 2006). Most of the immune factor present in the ovary secreted by the macrophages, lymphocytes, neutrophils, eosinophils and mast cells, and secret both anti- and pro- inflammatory cytokines. They played wide range of function including oocyte development, ovulation, and progesterone production (Vinatier et al., 1995; Bukulmez and Arici, 2000; Brannstrom and Enskog, 2002; Wu et al., 2004). In addition to the ovulatory process, cytokines may be implicated in the control of other ovarian functions, such as ovarian cell proliferation, apoptosis, folliculogenesis, luteogenesis, oogenesis, and release of hormones, response to upstream hormonal regulators, fertility and in some cases also in the development of different ovarian disorders (Sirotkin, 2011). Earlier research has been suggested that not only the steroids, LH can also stimulate the production of cytokines

(Machelon and Emilie, 1997). Studies have demonstrated that steroids can decrease the production of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), while increasing the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) (Barnes 2005, Coutinho and chapman, 2011). One study published in the journal "Cytokine" investigated the effects of the steroid dexamethasone on cytokine production in human whole blood cultures (Wong et al., 2001). The researchers found that dexamethasone significantly reduced the production of IL-1β, IL-6, and TNF-α, while increasing the production of IL-10.The researchers found that dexamethasone significantly reduced the production of IL-1β, IL-6, and TNF-α, while increasing the production of IL-10. Cytokines can involve in the ovarian development, folliculogenesis and leutinization (Field et al., 2014). Macrophages, which are present in the ovary throughout the process of follicle development, may also play a role in mediating the primary-to-antral follicle transition. According to in vivo investigations on mice, intraperitoneal injections of anti-macrophage migration inhibitory factor (MIF) antibodies decrease the proliferation of granulosa and theca cells. This impact may be achieved by altering the production of macrophage cytokines (Matsuura et al., 2002). Macrophages concentrate in the theca cell layer of murine and human primary follicles, where they release a variety of cytokines (IL-1a/b, IL-6, GM-CSF, and TNF-α) that promote cell proliferation and prevent apoptosis (Wu et al., 2004, 2007a; Tingen et al., 2011). Reduced oocyte apoptosis and cell proliferation has been found in TNF-α knock out mice (Cui et al., 2011). It has been also reported that apoptosis of murine granulosa cells conducted by TNF-α which in turn stimulated by excessive nerve growth factor (NGF) (Son et al., 2004b). TNF-α is thought to be expressed in the oocyte at the primary stage and increases as the follicle approaches ovulation, according to immunohistochemical studies of human ovaries (Kondo et al., 1995). In swine, where IL-6 receptors can support granulosa cell survival (Maeda et al., 2007). According to this mechanism, which involves IL-6 and its soluble receptor, IL-6(sR), atresia and follicle growth in humans have been linked (Kawasaki et al., 2003). It has been demonstrated in

rat models that IL-8 may function as an angiogenic factor in late folliculogenesis (Arici et al., 1996; Goto et al., 2002; Yoshino et al., 2003; Hickey and Simon, 2006). Bovine theca cells are stimulated to produce IL-8 by VEGF, which enhances angiogenesis and peri-follicular blood flow and may improve oocyte viability (Murayama et al., 2010; Pancarci et al., 2012). As the follicle approaches rupture, IL-1β mRNA levels rise in follicular-fluid macrophages and granulosa cells in mice and human (Machelon et al., 1995; Adashi, 1998b; Wang et al., 2002b). Chronological variations in IL-1β production are most likely controlled by shifting gonadotrophin profiles, according to in vitro experimental data. (Loret de Mola et al., 1998; Levitas et al., 2000; Martoriati et al., 2002, 2003a; Martoriati and Gerard, 2003). IL-1β promotes the transitional switch of granulosa cell proliferation to differentiation by decreasing the expression of LH receptors in rat granulosa cells in vitro (Gottschall et al., 1988; Karakji and Tsang, 1995), suggesting that it plays a role in determining whether follicles progress to ovulation or experience atresia. Additionally, it can also increase the production of NO in intraovarian macrophages and granulosa cells, which can affect the developmental choice between growth and apoptosis (Hattori et al., 1996; Tao et al., 1997; Rosselli et al., 1998; Matsumi et al., 2000). The expression of specific genes involved in extracellular matrix formation/stability (Has2, Ptx3, Ptgs2, Tnfaip6) and immune cell function (Pdcd1, Runx1, Runx2) is induced by IL6, which is also thought to be an autocrine regulator of ovarian function (Liu et al., 2009). It has been reported that Theca and granulosa cell produce IL-8 (Runesson et al., 1996; Szukiewicz et al., 2007). IL-8 encourages the recruitment of the leukocytes necessary to mediate ovulation when levels rise with follicle size (Brannstrom and Norman, 1993; Brannstrom and Enskog, 2002; Malizia et al., 2010). It is thought that TNF-α produced by the thecal endothelium promotes ovulation by causing localised apoptosis at the ovarian surface-follicular interface. (Murdoch et al., 1999). In the time of ovulation TNF-α can also up regulate MMPs which eventually activate the extracellular matrix remodelling event (Johnson et al., 1999; Yang et al., 2004). 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). IL-6 mRNA transcript abundance increases in the granulosa cells of mice and rats following treatments with human chorionic gonadotropin (hCG) (Liu et al., 2009a) and follicle stimulating hormone (FSH) (Gorospe and Spangelo, 1993), respectively. Follicles containing mature oocytes have higher follicular fluid concentrations of IL-6, and IL-6sR concentrations has significantly higher in follicles containing mature oocytes (Kawasaki et al., 2003; Kawano et al., 2004b). Evidence suggests that IL-10 may be a significant immune-deviating cytokine during pregnancy. Cycling mice's utertus, oviducts, and ovaries as well as the uterine myometrium and cervix during pregnancy all express IL-10 (Sallinen et al., 2000). The first and second trimesters of the human placenta are when IL-10 expression is greatest (Hanna et al., 2000). Cytotrophoblast cells (Roth et al., 1996) and placental leukocytes (Sacks et al., 2001) are both thought to be involved in its synthesis. Human leukocyte antigen (HLA)-G expression is stimulated by IL-10 (Moreau et al., 1999), and matrix metalloproteinase-9 (MMP-9) activity is inhibited (Roth and Fisher, 1999). IL-10 has been discovered in the follicular fluid of IVF patients (Geva et al., 1997). Prior research suggested that IL-10 was present in controlled ovarian stimulation patients, suggesting that this protein may play a role in ovulation(Geva et al, 1987, Calogero et al., 1998).

Systematic position of

Anabas testudines

Kingdom : Animalia

Phylum: Vertebrata

Subphylum : Craniata

Series : Pisces

Class: Teleostomi

Subclass: Actinopterygii

Order: Perciformes

Family: Anabantidae

Genus: *Anabas*

Species: *testudineus*

Common names:

Koi - West Bengal, Bangladesh.

Standard name:

Climbing perch

Identifying characters:

1. Slender body.
2. Body divisible into head, trunk and tail and covered with cycloid scales.
3. Long based dorsal fin is present.
4. Scales are large and regularly arranged.
5. Beneath the eye their is longitudinal stripes.
6. On the margin of the gill cover iris golden, dark spot is present.
7. Accessory air breathing apparatus is present.
8. Presence of labyrinth organ situated above the gills.
9. On the cover of the gill long robust spines is present.
10. Tails have perfect symmetry.

Habit and habitat:

Found in fresh and brackish water (Talwar, 2001). They are bottom dweller and feeds on insect.

Breeding habit:

In the month of April breeding start with the onset of monsoon and its typically continues till July. Eggs of *Anabas* are pelagic in nature and floated over surface of the water body. At the time of hatching the optimum temperature is 28 degree C. and usually takes places within 18 hours (Rahaman, 2005). *Anabas* has high fecundity rate. Ovaries are synchronous in nature.

Distribution:

This amphibious freshwater fish is native to far eastern Asia. This fish is found in the fresh water system of Pakistan, India, Bangladesh, Srilanka, China, Japan and southeast asia west of the Wallace line in the south.

Introduction to the presented work:

In the field of reproductive endocrinology it has been a long interest on how the hormonal regulation occur during the oocyte maturation and ovulation in female teleost. Principally it has been regulated by the hormones secreted from brain, pituitary and ovary, termed as hypothalamus-hypophysial-gonadal axis (HPG axis). GnRH which is a decade-tide secreted from brain stimulate anterior part of the pituitary and facilitate the secretion of gonadotropins (LH and FSH). Furthermore, this gonadotropins reaches the ovary and respectively acts on theca cells and granulosa cells to produce the steroid hormones. These steroid hormones then control the maturation and ovulation phase of female teleost.

Apart from this traditional two cell two gonadotroph hypothesis, in recent time scientific research establish the fact that not only the steroidal regulation but the locally secreted factors like prostaglandin, different paracrine factors specially cytokines are playing a pivotal role in the reproductive processes of female teleost fish. Over the last years there are several studies has been done to indentify, characterise and distribution of different cytokines in different fish like fugu (*Fugu rubripes*), zebrafish (*Danio rerio*) common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*) , sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*). Cytokines like TNF-α, IL-6, NF-κβ, IL-10, IL-8, IL-4, IL-1β have been identified and characterised in teleost fishes. Earlier studies in mammals show that during the time of reproductive processes IL-6, a pro inflammatory cytokine involve in steroidogenesis and it significantly regulates the process of ovulation. In case of fishes, studies suggested that TNF-α plays a significant role in the oocyte maturation processes. Furthermore, participation of matrix metalloproteases and prostaglandin in the process of ovulation suggested that the overall reproductive processes of female teleost is a complex interactive scenario rather than a classical simple model. There is also some evidence that enlighten the involvement of

anti inflammatory cytokines in the reproductive process in mammals but there is not much study in fishes till date.

The present thesis enlighten the role of IL-6 and IL-10 during the reproductive processes of female teleost, *Anabas testudines* and give a concrete idea about how these paracrine factors involve in this process and interact with other pivotal factors in this microenvironment of ovary and ensure the formation of a healthy oocyte.

This present thesis contain following four chapters

Chapter - I

Understanding the interplay between the pro- and anti inflammatory cytokine (IL-6 and IL-10) under the influence of gonadotropin.

Chapter - II

Role of IL-6 and IL-10 during the reproductive processes of *A. Testudineus.*

Chapter - III

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

Chapter IV

Signal transduction pathway of IL-6 and IL-10 during the reproductive processes of *Anabas testudineus*