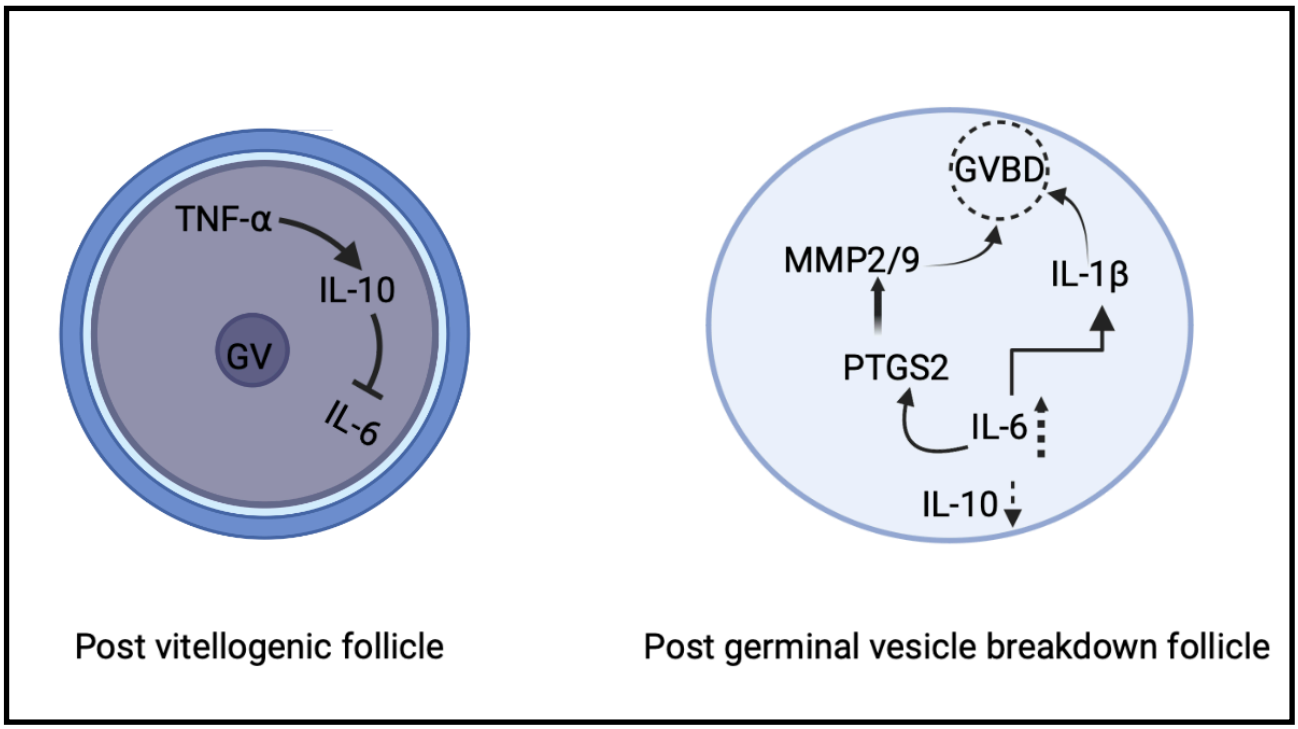
Chapter II

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

Abstract:

The interaction between the interleukins and other crucial paracrine factors in oocyte are important to understand the role of IL-6 and IL-10 during the time reproductive processes. In this study we found that IL-6 played a significant role in the time of ovulation which eventually established by the up regulation of *ptgs2* and matrix metalloproteinase and also induce the function of IL-1β, which can also enhance the LH induced ovulation. However IL-10 was not directly involved during oocyte maturation and ovulation rather it may play a significant role to maintain the homeostasis of the cytokine environment in the oocyte which is in term controlled by TNF-α.



Introduction:

In fish, inflammation play key physiological roles in the ovulatory processes. Fish contains all major cytokine families, which is now generally accepted as a result of earlier research and reviews. By secreting locally, they can control ovarian processes. Pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6, as well as different paralogues are found in the fish ovary of the majority of species. Prostaglandins (PGs) and the pro-inflammatory cytokine, tumor TNF-α may control key stages of the mammalian ovulatory process, according to studies of Crespo et al, 2015. Previously it was reported that TNF-α regulates ovulation in the rat ovary (Brannstrom et al., 1995). TNF-α play a pivotal role collagenolytic activity and death in preovulatory follicles (Silva et al., 2017), can also produce more PGs in preovulatory follicles (de Mola et al., 1998). The ability of IL-6 to increase oocyte competence, a key factor to promote the rate of in vitro fertilization, provides insights into the functions of cumulus oocyte complexes (OCCs) and oocyte quality (Liu et al. 2009). As a crucial regulator of ovarian cumulus cells (CCs) function and OCC expansion, IL-6 may mediate some of its effects by activating the ERK1/2, JAK/STAT, and p38MAPK pathways. According to Liu et al. (2009), IL-6 did activate in mice genes related to matrix stability and formation. Experimental evidence suggests that IL-6 and IL-6R mRNAs are present in the porcine corpus luteum (CL) throughout the estrous cycle (Sakumoto et al. 2006). Furthermore, it has been shown that the number of macrophages increased during luteolysis and that IL-6 is produced by activated T-cells and macrophages in the ovary of both porcine (Hehnke et al. 1994) and bovine (Penny et al. 1999) animals. It appears that IL-6 has the ability to attenuate steroid production during luteolysis because it inhibited hCG-induced progesterone secretion in purified porcine small and large luteal cells. Like mammals, recombinant IL-6 of fish can also promote IL-6 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 isolated culture media of rainbow trout when treated with rIL-6 (Costa et.al, 2011).

Early in the gestational period, IL-10 has been demonstrated to promote pregnancy (Brogin et al., 2012). IL-10 may play several advantageous roles during the implantation of the blastocyst and the development of the early placenta. It may prevent the release of proinflammatory cytokines like TNF-α and IFN-γ, which are harmful to pregnancy (Raghupathy and Kalinka 2008, Giannubilo et al., 2012, Haider and Knofler 2009). Another study found that IL-10 stimulates placental angiogenesis by causing trophoblastic cells to produce VEGF C and the aquaporin (AQP1) system (Thaxton and Sharma 2010). Additionally, progesterone, catecholamines, and prostaglandins are some other intrauterine regulators that IL-10 may mediate (Hanna et al., 2000). A local and systemic skew towards Th2 cytokine predominance during later pregnancy stages suggests that the environment is primarily anti-inflammatory (Chatterjee et al., 2014). IL-10, along with other mediators, may help to control harmful Th1 reactions during this phase. In fact, it has been suggested that one significant immuno-regulatory function of IL-10 during pregnancy is the maintenance of Th2 bias and the induction of a shift away from Th1 bias. Programmed cell death and the activation of catabolic enzymes during parturition, the last stage of pregnancy, result in the delivery of the baby due to a predominately pro-inflammatory environment. As parturition continues, IL-10 declines at this stage of the gestation (Chatterjee et al., 2014).

Present chapter tried to elucidate the role IL-6 and IL-10 in the process of oocyte maturation and ovulation with their interference with other genes those are critical during the time of the reproductive processes of *A. testudineus*.

Materials and method:

Animals : As same as Chapter I

Chemicals : As same as chapter I

Methodology :

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 in an aerated incubator.

Detection of oocyte maturation and ovulation:

After completion of the experiments the degree of germinal vesicle breakdown (GVBD) of the oocytes were observed under a microscope following the procedure of Pramanick et al., 2014. For this, following the in vitro tests using various effectors, follicles were picked up and placed into a tube containing GVBD fixative. After 24 hrs observation was done under microscope by placing follicles in a grooved slides with 50% glycerine solution. For the identification of ovulation, Propidium iodide (PI) staining was done after completion of the experiments. PI-negative staining indicates the ovulation has been completed in those follicles.

RNA isolation and cDNA synthesis :

The total RNA from ovarian follicles was isolated 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 $2\Delta Ct$ method after normalization using GAPDH mRNA abundance data (Rao et al., 2013). The forward and reverse primers were made using primer 3 (Table 1). in biomedical research (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.

Zymography :

Gelatine zymography was performed following the methodology of Crespo et al. 2015. After the incubation period, follicles were quickly homogenized in 1 mL of an extraction buffer containing

apotinin (2 g/ml) and 1 mM PMSF (20 mM ZnCl2, 1.5 mM NaN3, 0.01% Triton X, and distilled water (dH20). 20 L of the supernatant and 20 L of a loading dye (without 2-mercaptoethanol) were added to each well of a 10% SDS-PAGE that contained 0.1% porcine gelatine (SRL, India) after centrifuging the sample for 3 min (5000 x g) at 4°C. After the electrophoresis in the cold room, the gel was washed twice in 2.5% Triton X for 30 minutes, then twice in dH2O at room temperature. The gel was then incubated for 48 hours at 37°C with incubation buffer (50 mM Tris-HCl, 5 mM CaCl2, 0.05% Brij 35, 0.02% NaN3, and dH2O). After 30 minutes of destaining (50% methanol, 10% glacial acetic acid, and 40% dH2O), a clear band could be seen against a blue background. Coomassie blue staining was done for two hours in an orbital shaker using coomassie blue (Commasie blue 0.5%, methanol 40%, 10% glacial acetic acid, and 50% distilled water).

Follicular cell isolation and TUNEL analysis:

After termination of in vitro incubation for GVBD assay, ovarian follicles treated with 4 ng/ml IL-6 were collected and were placed in 5 ml beakers and digested with Type-I collagenase (0.3%) for 10 min with continual gentle mixing on a rotating shaker. Then follicles were rotated over 100 µm nylon mesh for 5 min. Oocytes were digested again for the same dose of collagenase and repeat the same procedure. Medium was collected after each rotation, pooled, centrifuged (2000×g for 5 min) and the pellet was resuspended in fresh Idler's medium. One drop of cellular suspension placed in a glass slide, air dried, fixed with chilled methanol and performed TUNEL analysis according to the manufacturer's protocol in order to determine the apoptotic cells in IL-6-induced follicular cells. For this purpose, commercial kits (Promega), were used.

Table 3: 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
TNF-α (AB183467.1)	AAGCCAAACGAAGAAGGTCA	AACCCATTTCAGCGATTGTC	168
NFκβ (NM_001001839)	CAACGACACCACGAAAACG	CGTCAGGAATCTTGAATGGGT	188
Cpla2 (BC162082.1)	TGCTCTTGGAAGTTTGCG	TCTGCGTGTCTGCATGAACAG	189
MMP2 (NM_198067.1)	GCCTTAATGGTGATGGTCACA	GGTCTGTCGATGTTCAGCAG	205
MMP9 (AY151254.1)	TGGGCACCTGCTCGTTGA	TTGGAGATGACCGCCTGC	195
MIF (AB298722.1)	TGCCGATGTTTGTAGTGAACA	CAAAGGTGCTGTTGTTCCAG	198
IL-1β (AY340959)	TGGACTTCGCAGCACAAAATG	GTTCACTTCACGCTCTGGATG	124

Effects of IL-6 and IL-10 on oocyte maturation

Post-vitellogenic follicles were treated with recombinant IL-6 and IL-10 at the doses of 0, 0.05, 0.1, 0.5, 1, and 2 ng/ml for 16 hours. After incubation, oocytes were overnight fixated in GVBD fixatives while the percentages of GVBD were computed under a microscope.

Functions of IL-6 and IL-10 in ovulation

The post-GVBD follicles were exposed to increasing concentrations of IL6 and IL-10 (0.05, 0.1, 0.5, 1, and 2 g/mL), while a control sample was incubated for 16 hours with hCG, TNF-α, and hCG plus TAPI-1. This was done to investigate how IL-6 and IL-10 function during the ovulation process. To determine the percentage of ovulations that actually happened, oocytes were washed in phosphate buffer saline after being stained with DAPI for 20 minutes after the incubation process was finished. The DAPI-negative oocytes were assumed to be follicles from which ovulation had occurred. In a related experiment, oocytes at the same developmental stages were incubated with IL-6 at a concentration of 2 ng/ml. A control group was also used in this experiment. One replication (follicles) was fixed with TRI reagent for RNA separation before being homogenised in ice-cold zymography extraction solution and subjected to gelatin zymography.

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 on oocyte maturation

Only post-vitellogenic follicles were selected for the maturational study. The effects of various IL-6 concentrations (0.05, 0.1, 0.5, 1.0, and 2.0 ng/mL) and hCG concentrations (0.0, 0.1 g/mL) on the induction of GVBD are shown in Figure 5.A. IL-6 had no effect on the induction of GVBD at any concentration after 16 hours of incubation.

Effects of IL-10 on oocyte maturation

A similar experiment was performed to find out the role of IL-6 on oocyte maturation. Only post-vitellogenic follicles were selected. The impact of different IL-10 concentrations (0.05, 0.1, 0.5, 1.0, and 2.0 ng/mL) and hCG concentrations (0.0, 0.1 g/mL) on the induction of GVBD is depicted in Figure 5.B. Like IL-6, IL-10 also had no effect directly on the induction of GVBD after 16 hours of incubation, at any concentration.

Function of IL6 in ovulation processes

Treatment with IL-6 at all doses induced ovulation in comparison to hCG and TNF-α (Both positive regulator of ovulation) treatments (Fig 6.A). When TAPI-I was administered, the hCG-induced ovulations was attenuated significantly. The MMP activity and relative abundances of the ptgs2 mRNA transcript in follicles treated with IL-6 (2 g/mL) were shown to be significant markers for the ovulation processes in teleost when the roles of IL-6 in the ovulating processes were further assessed. As shown in Fig. 6.B and C, after treatment with IL-6, MMP2 was activated, and there were higher relative abundances of the ptgs2 mRNA transcript. This suggests that upon translation, there will likely be higher abundances of the ptgs2 protein (which is necessary for the synthesis of PGF2), indicating that IL-6 has ovulatory functions in fish.

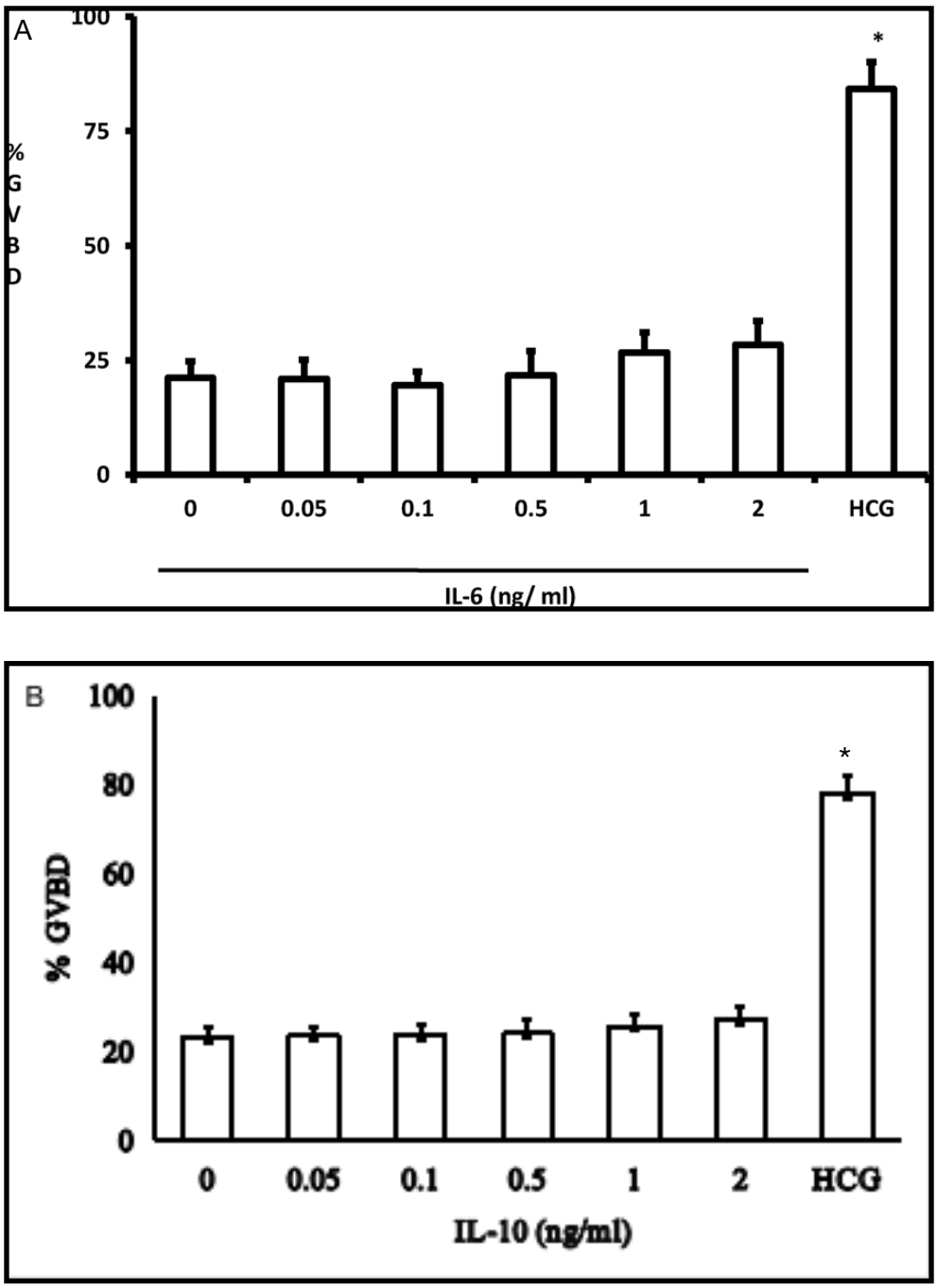


*Fig 5. Effect of IL-6 and IL-10 (A-B) on oocyte maturation; Postvitellogenic follicles were incubated with different doses of IL-6, IL-10 and hCG (100 ng /mL) and there was an untreated control group; After termination of experiment, GVBD classifications were made using a microscope and percentage of oocyte maturation is depicted in the bar graph; Results are the mean ± SEM of five fish; *denotes a change ($P \leq 0.05$).*

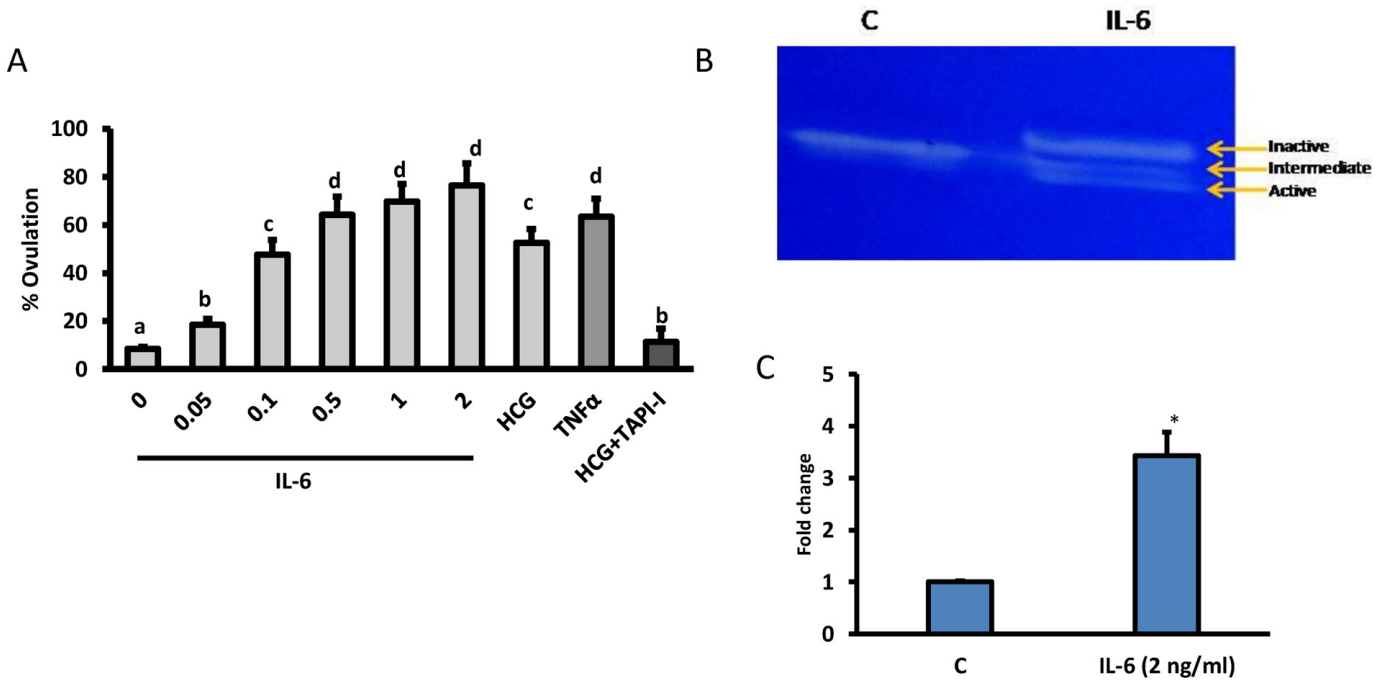


*Fig 6. (A) Regulation of ovulation in vitro with IL-6, TNFα and hCG in A.testudineus ovarian follicles; Ovarian follicles at post-GVBD stages were incubated for 16 h with different doses of IL-6, hCG, TNFα and hCG combined with TAPI-I; Percentage of follicles from which there were ovulations was assessed using a fluroscence microscope by staining with DAPI; (B) Activation of MMP. (C) Expression analysis of ptgs2. Each bar represents the mean ± SEM of five fish; Different letters and *indicates there were differences in man value among treatment groups ($P \leq 0.05$).*

Relation of IL-6 with genes involved in ovulation

In Fig 6. Results indicating the fact that genes involved in ovulation (*cpla2, mmp2, mmp9*) directly showing a significant up regulation when treated with IL-6 and down regulated when treated with TAPI-1. In a similar set of experiment, Fig. 7A-C, two of the important cytokine regulator of ovulatory (*il-1β* and *mif*) genes were also significantly up regulated Fig.8 when treated with IL-6 but the expression was down regulated when treated with TAPI-1.

Function of IL-10 in ovulation process

Treatment with IL-10 in different different dosses (0.05, 0.1, 0.5, 1.0, and 2.0 ng/mL) unable to induces the ovulation like hCG and TNF-α (Fig.9), not even in the highest doses of the IL-10 (2 ng/ ml). But ovulation rate was slightly lower when hCG was combined with IL-10 (2 ng/ml).

Function of IL-10 in reproductive process

Pro inflammatory cytokines, *nfkb*, *tnf* and *il6* significantly upregulated by 8.25, 6.21 and 4.93 fold when treated with HCG to post vitellogenic follicle but down regulated (4.98, 2.75 and 1.06 fold respectively) in the sample treated with recombinant IL-10 protein Fig 8.A-B. In a similar set of experiment where expression of *il-10* was measured in respect of recombinant IL-6 in post vitellogenic *Anabas* follicles, the result showed a drastic down regulation of *il-10* mRNA transcript when treated with IL-6 but there no inhibition observed when treated with hCG (Fig. 10).

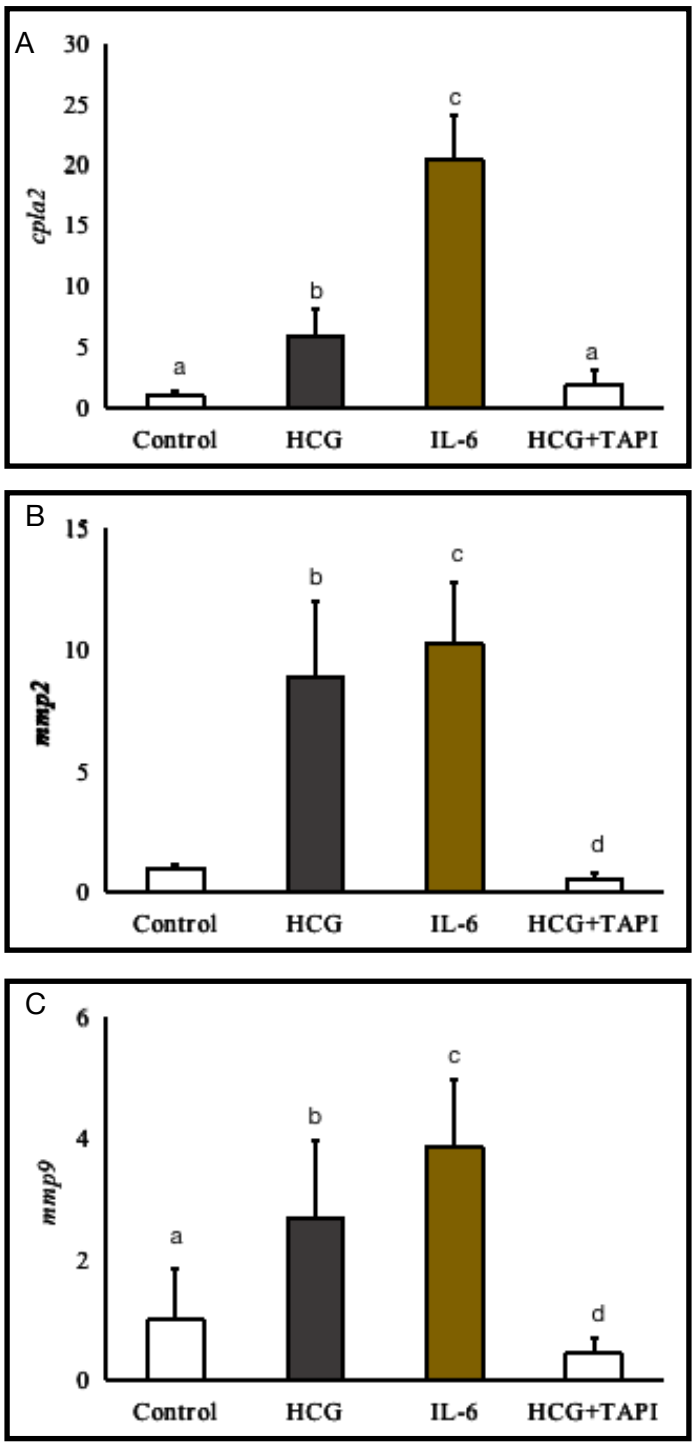


*Fig 7. Expression analysis of specific genes (A-C)involved during the time of ovulation of A. testudineus. Data were normalized by control GAPDH. Each bar represents the mean ± SEM of five fish; Different letters and *indicates there were differences in man value among treatment groups (P ≤ 0.05).*

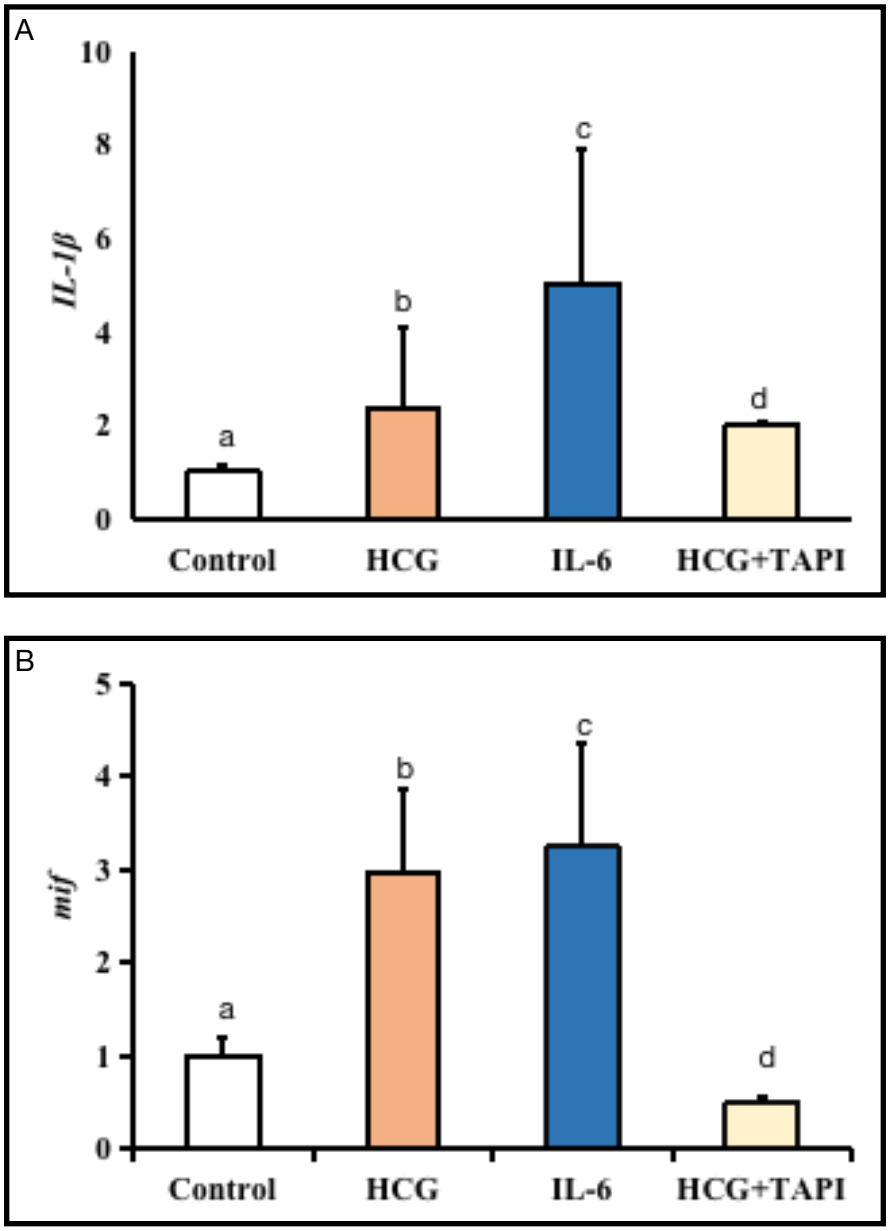


*Fig 8. Expression analysis of il-1β and mif (A-B) involved during the time of ovulation of A. testudineus. Data were normalized by control GAPDH. Each bar represents the mean ± SEM of five fish; Different letters and *indicates there were differences in man value among treatment groups (P ≤ 0.05).*

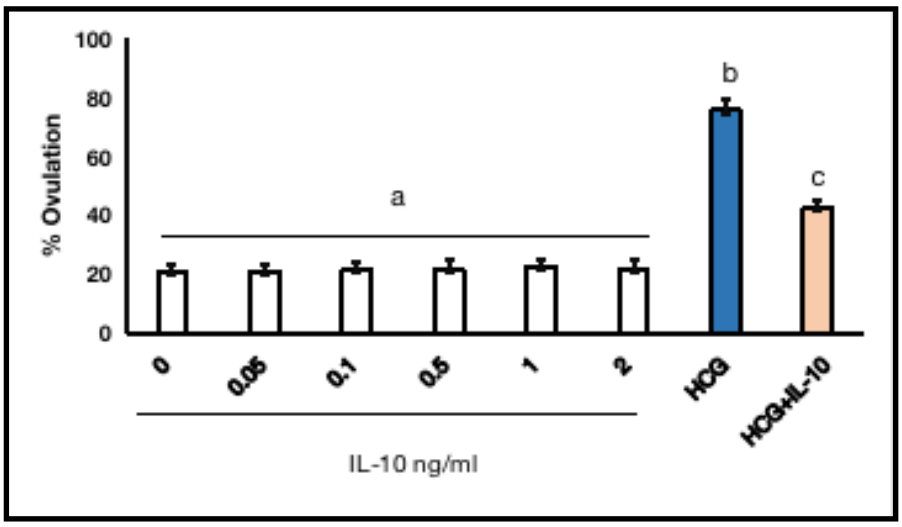


Fig 9. Regulation of ovulation in vitro with IL-10, TNFα and hCG in A.testudineus ovarian follicles; Ovarian follicles at post-GVBD stages were incubated for 16 h with different doses of IL-10, hCG, TNFα and hCG combined with TAPI-I; Percentage of follicles from which there were ovulations was assessed using a fluroscence microscope by staining with DAPI

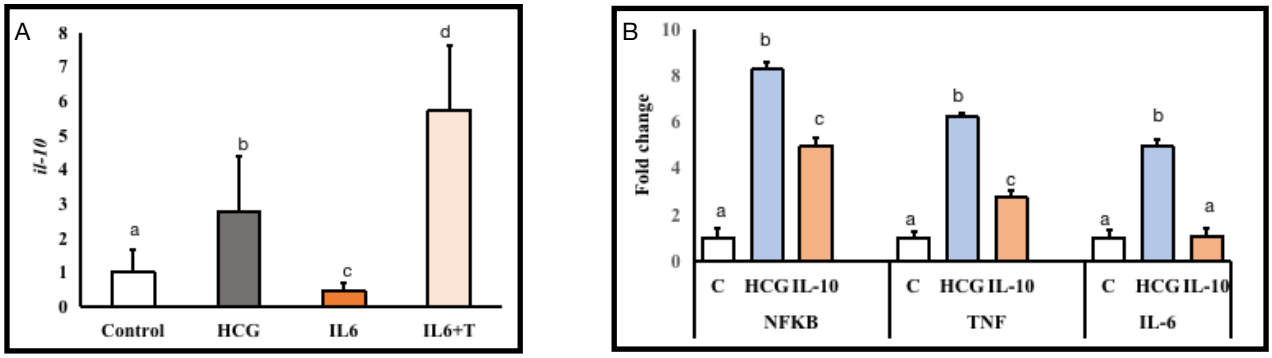


*Fig 10. (A) Expression analysis of proinflammatory cytokines when treated with anti inflammatory cytokine, IL-10 in post vitellogenic oocytes of A. testudineus.(B) expression of IL-10 when treated with IL-6. Data were normalized by control GAPDH. Each bar represents the mean ± SEM of five fish; Different letters and *indicates there were differences in man value among treatment groups (P ≤ 0.05).*

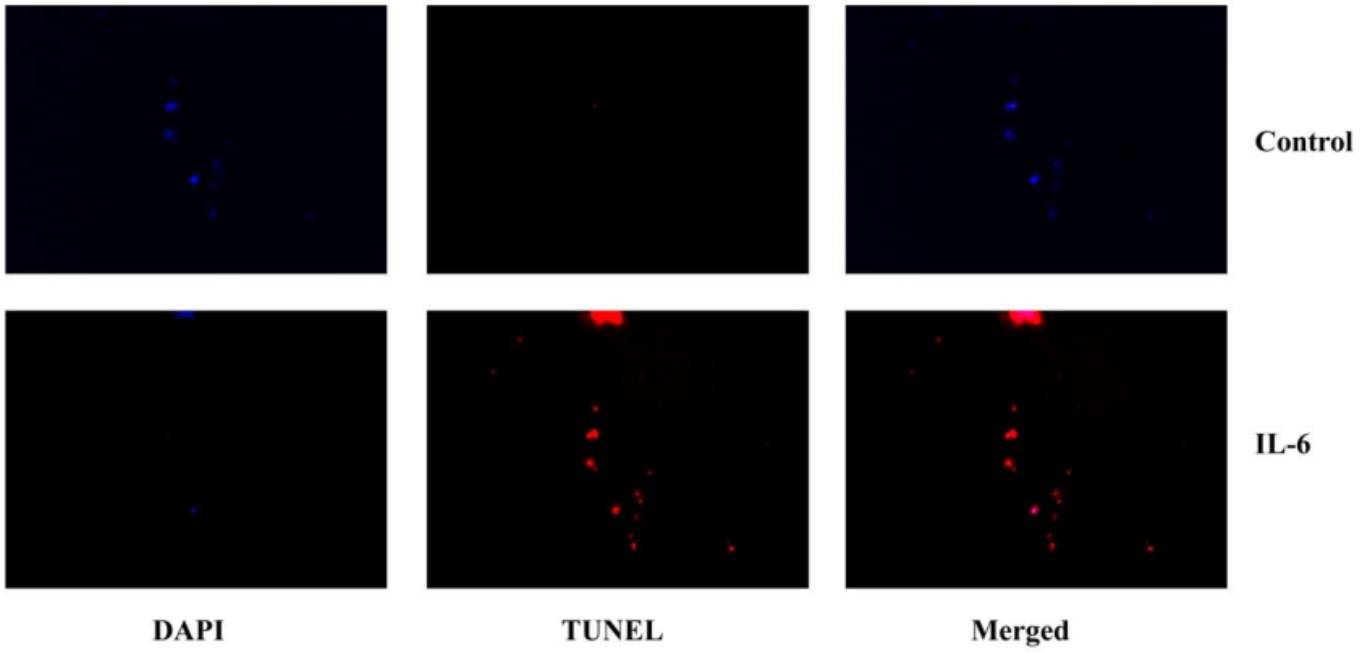


Fig 11. Tunnel analysis showing Il-6 induced oocyte maturation of highest dose (2 ng/ml). Follicular cells were isolated, mounted in the glass slides. Magnification was 440X and the bar is 20 μm.

Discussion:

Studies conducted in the past have shown that the ovary releases cytokines that have several functions (Zolti et al., 1990; Loret de Mola et al., 1998). (Terranova, 1997; Liu et al., 2009; Okuda and Sakumoto; Murdoch et al., 1997; Terranova, 1997). Cytokine release is regulated by gonadotropins (Gorospe and Spangelo, 1993; Breard et al., 1998). Iliev et al. (2007) provided the first account of a higher relative abundance of IL-6 mRNA transcript in the ovary around ovulation. Results from earlier mammalian studies suggest that cytokines, particularly TNF-α, play a significant role in the ovulation process (Wang et al., 1992; Loret de Mola et al., 1998; Stassi et al., 2017). The ovulatory process is brought on by altered enzymatic activity brought on by cytokines (Stassi et al., 2017). According to Liu et al. (2017), numerous conserved genes are thought to be involved in ovulatory processes. Present study explored the involvement of IL-6 and IL-10 during the time of oocyte maturation and ovulation by externally treating the isolated post-vitellogenic follicle by those recombinant proteins (rIL-6 and rIL-10). Our result showed that external IL-6 dosen't involve during the process of oocyte maturation rather it induce the rate of ovulation in dose dependent manner. Rate of ovulation is highest when treated with 2ng/ml dose. So, it was clear that during the time of ovulation IL-6 directly take part to conduct the process. The investigation also established when treated with hCG and TNF-α, but arrest the ovulation when treated with TAPI-1. The current study examined the relative abundance of matrix metalloproteinase activity to verify whether the IL-6 was a factor in ovulation induction. Our experiment showed that this cytokine is one of the primary cytokines influencing the breakdown of follicular walls and oocyte release by increasing the MMP activity during IL-6-induced in vitro ovulation. Furthermore, it increases the *pgf2,* which is one of the main physiological indicators of ovulation (Liu et al., 2017). The present study corroborates with earlier studies' findings (Crespo et al., 2015; Liu et al., 2017) which demonstrate that IL-6-induced ovulation in *A. testudineus* is accompanied by an increase in the expression of the gene encoding the primary enzyme involved in PGF2 synthesis, ptgs2. As in

mammals, PGs are also involved in the ovulatory process in teleost fish (Stacey and Pandey, 1975; Jalabert and Szollosi, 1975; Fujimoria et al., 2011; 2012). The granulosa and cumulus cells activate their receptors in response to the increased cytokine concentrations, which induces the genes necessary for COC enlargement or PGF2 synthesis in the period prior to ovulation from follicles (Richards et al., 2008; Liu et al., 2009; Smolikova et al., 2012). Current study found that preovulatory teleost follicles synthesise PGF2 in LH-dependent manner, which is consistent with findings from numerous earlier studies (Lister and Van Der Kraak, 2008; 2009; Crespo et al., 2011; Hagiwara et al., 2014; Crespo et al., 2015; Zhu et al., 2015; Tang et al., 2017; Takahashi et al., 2018). According to Crespo et al. (2015), LH-induced ovulation in trout is thought to be influenced by the production of PGF2 by the pro-inflammatory cytokine TNF-α. The results of the present study supported the hypothesis that in *Anabas*, IL-6 mediates gonadotropin-induced ovulation. The results of the present study also demonstrate that IL-6 regulates ovulatory functions by altering PGF2 synthesis and matrix metalloproteinase activation. The transcription level expression analysis of the ovulatory genes (eg. *ptgs2, cpla2*, and *mif* in this study) also supports the involvement of IL-6 in ovulation; all of these genes are markedly up-regulated in response to IL-6 treatment but down-regulated in response to TAPI-1. Furthermore, the fact that both *mmp-2/9* were up-regulated in response to IL-6 demonstrated that IL-6 functions as a crucial regulator during the ovulation process. Another study that shows *il-1beta* is upregulated in response to IL-6 and is supported by the Brannstrom, 1993 study, which explains why the ovulation rate was increased in cases where IL-6 was used, as this causes IL-1β to be induced and ultimately upfolds LH-induced ovulation.

In contrary, IL-10 mostly considered as an immunosuppressive cytokine which plays a crucial part in regulating immune responses (Moore, 2001). By activating macrophages IL-10 suppresses the inflammatory response. Proinflammatory cytokines like IL-1β, TNF-α, IL-12, IL-6 and COX-2 are inhibited by IL-10 at both transcriptional and post translational level and also they play a role in inhibition of nitric oxide production. An interesting study in Fugu shows that TNF-α can upregulate

il-10 expression via NFAT pathway (Secombes, 2003). In our current investigation we found that rIL-10 neither involved in the process of ovulation nor it triggered the maturation of oocyte single handedly. The relative abundance of other pro inflammatory cytokines like *nfκβ*, *tnf*-α and *il-6* were all down regulated in post-vitellogenic follicle when treated with rIL-10 as compare to that of hCG which is generally a positive regulator of those cytokines. Most specific findings in this investigation was its interaction with IL-6. When post-vitellogenic follicle was treated with rIL-10 it down regulate the expression of *il-6* while those oocyte treated with rIL-6 the expression of *il-10*.

Furthermore, to understand the role of IL-10 in oocyte we investigated its role when the follicle was subjected to stress. When treated with polystyrene, reactive oxygen species upregulated the expression of TNF-α and IL-10 in post-vitellogenic oocyte follicle and the percentage of GVBD was high which indicates the early maturation of follicles tells a story where we can say that the expression TNF-α and IL-10 in early stage of oocyte is related and play a crucial role to maturing an oocyte, where as an anti inflammatory cytokine, IL-10 may play a vital role to prevent early oocyte maturation or maintain the homeostasis of pro inflammatory response through out the processes.