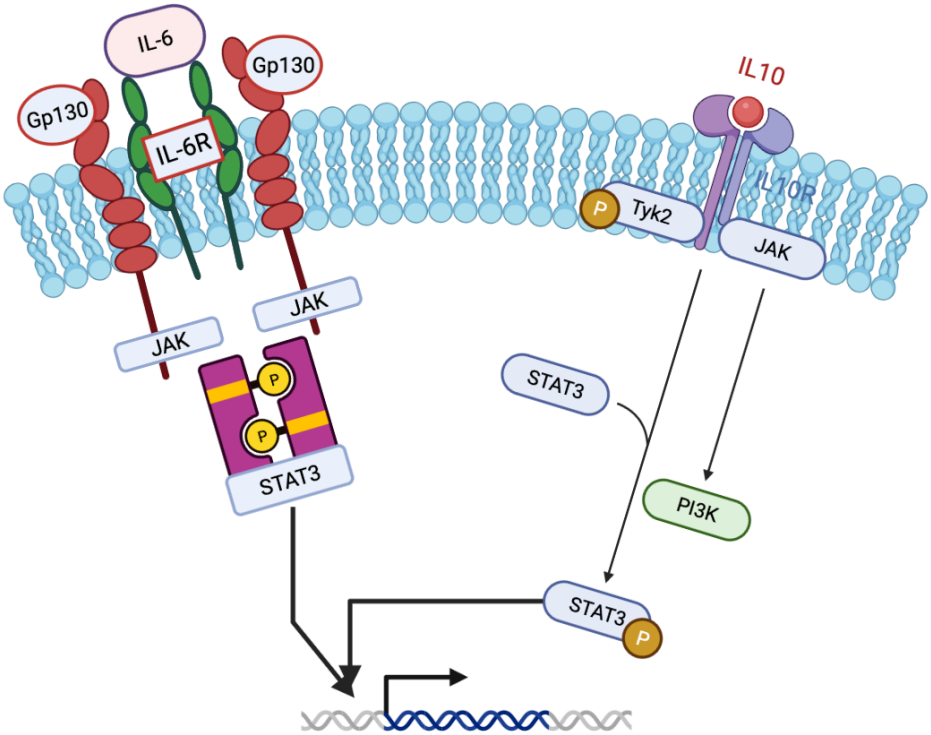
# Chapter IV

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

**Abstract:**

Signalling transduction pathway of these cytokines are studied variedly through out the species. In this study we elucidate the interaction of IL-6 and IL-10 with their receptors. IL-6-induced increase in abundance of receptors (IL-6 Rα and gp130) to which it binds indicating IL-6 autoregulates this population of receptors. The western blot analysis of stat-3 suggested that IL-6 further mediate the ovulation process via this pathway. IL-10 also induce the both IL-10 R1 and IL-10 R2 receptor and act via JAK/STAT pathway.




**Introduction**

The heterodimeric signalling complex composed of the IL-6 -receptor (IL-6R) and the signal-transducing subunit glycoprotein 130 (gp130), which has been shared by other cytokines, is actually what causes IL-6 to activate the cells (Garbers et al., 2012). Depending on whether the IL-6R is membrane-bound (IL-6 classic) or soluble (IL-6 trans-signalling), there is a clear distinction between the two types of IL-6 signalling. While gp130 is expressed in every tissue and cell type of the human body, IL-6R is primarily found in hepatocytes, megakaryocytes, and a few leukocyte subpopulations. According to studies available (Chalaris et al., 2011, Rose-Jhon 2012, Jones et al., 2011), the pro-inflammatory activity of IL-6 was accompanied by trans signalling or the soluble form of IL-6 R.

According to Kaneda et al., (2012), the IL-6R and gp130 two chains of the IL-6 receptor are expressed in fugu. Because IL-6 and its receptors have been found to have homologs in fish, this suggests that the IL-6 signaling pathway is conserved in fish. However, compared to mammals, fish appear to have a different arrangement of the IL-6 receptor complex. There is only one IL-6 receptor homolog in some fish species, including zebrafish and Atlantic salmon, and it combines the IL-6R and gp130 domains into a single protein. In contrast to mammals, where IL-6R and gp130 are two distinct proteins that work together to form a receptor complex. Two distinct IL-6 receptor homologs have been discovered in other fish species, including rainbow trout, one with a gp130 domain and the other with an IL-6R domain. These two receptors can combine to form a functional receptor complex that can bind to IL-6 and initiate the downstream signalling procedure.

IL-10R1 and IL-10R2 are the two different types of IL-10 receptors found in mammals. The primary binding site for IL-10 is IL-10R1, and IL-10R2 serves as a co-receptor to increase IL-10's affinity for IL-10R1.The IL-10 R1 gene, which is found on chromosome 15, and its ligands are both highly conserved in zebrafish (Grayfer et al., 2012). The JAK/STAT pathway, MAPK pathway, and PI3K/AKT pathway are just a few of the downstream signalling pathways that are activated as a

result of IL-10 signalling through its receptors. Through the induction of SOCS-3, these pathways ultimately control gene expression and suppress inflammatory responses (Wei et al., 2014).

Recent studies showed that The FF-containing mature oocytes had significantly higher IL-6 and IL-6 sR concentrations than the fluid-containing immature oocytes (Wolf et al., 2014). The gp 130 protein was also present in the human oocytes (van Eijk et al. 1996). The evidence suggests that IL-6 and IL-6R mRNAs are present in the porcine CL throughout the estrous cycle (Sakumoto et al. 2006). Pitzel et al. (1993) discovered that the regressed CL had higher levels of IL-6R gene expression than the other stages. Telleria et al. (1998) study found that a decrease in P4, increased the expression of the IL-6R in rat CL. The expression of gp-130 in the adult female's primordial follicles is low, but it is upregulated in developing oocytes. Similar patterns can be seen in the male, where gp-130 expression is absent in spermatogonia but highly expressed in spermatocytes Manova et al (1990). Females homozygous for a mutated form of gp-130 that lacks the STAT-3 binding domain (gp130-STAT3) are not affected in ovulation, according to Ernst et al. (Ernst et al., 2001), but rather show an implantation defect akin to that seen in LIF-/- females (Stewart et al., 1992). The JAK family of proteins is activated upon binding of the IL6 ligands to their receptor complexes, which then causes the phosphorylation of STAT3 (signal transducers and activator of transcription). Alternately, the RAS/MAPK pathway can be used to transduce gp 130-mediated signals (Taga and Kishimoto, 1997).

In this chapter, we established the signalling pathway of both cytokines (IL-6 and IL-10) during the time of the ovulation, which can conclude the involvement of both cytokines in the reproductive process in *Anabas testudineus*.

**Materials and method:**

**Animals : As described in Chapter I**

**Chemicals : As described in chapter I**

**Methodology :**

**Regulation of IL-6 and IL-10 receptor mRNA transcript abundance :**

In order to isolate the RNA from 100 mg of post-GVBD follicular tissues and create cDNA to measure the relative abundances of mRNA transcripts for the IL-6 receptors (IL-6 R and gp 130) and the IL-10 receptors (IL-10 R1 and IL-10 R2; all samples were fixed in TRI reagent). hCG (100 ng/ml) was added to the tissues and other effectors like TNF-α and TAPI-1 were also added during the time of the incubation.

**Western blot analysis for stat-3 :**

To determine how IL-6 affects the amount of IL-6 protein that is stimulated by HCG, ovarian follicles were incubated with the hormone for 6 hours in the presence or absence of IL-6 and an inhibitor called TAPI-I. Following the incubation period, the follicles were homogenised in 1 ml of lysis buffer containing protease inhibitors (apotinin, leupeptin, PMSF, and trypsin: 1 mg/ml each), centrifuged at 8000 rpm for 5 minutes at 4°C , and the total protein concentration was determined using the Lowry et al. method (1951) from the supernatant. 30 mg of total protein were electrophoresed for immunoblotting using a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Fermentas, Life Sciences) before transferred it to a polyvinylidine difluoride membrane. Membranes were incubated for an hour in 5% blocking solution (Tris buffered saline with 0.1% Tween-20 and 5% nonfat milk), and then incubated overnight at 4°C with rabbit polyclonal anti-IL-6 antibody. The primary antibody that was bound was marked with alkaline phosphatase and then identified using the corresponding secondary antibody at a dilution of 1:5000. β-actin was used as a control.

**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.

**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 |
|---|---|---|---|
| IL-6Rα(NM_001261449.1) | GTGTTTTGCGTGCTGTCAGT | CATCTGCCCAGCTGAGATGT | 234 |
| GAPDH (DQ107520.1) | CTGAGGCATCTCACAAACGA | TCACCCTCAACCTTGACCTC | 230 |
| Gp130 (AB018216.1) | ACAGTGTTTCCGACAGCGAT | TCTCGGCCCCGTCTAAAGTA | 216 |
| IL-10 R1 (NM_008348.3) | AAACCGAGGTGGAATCGGTC | CCACTGCTAAGGCTGTCCTC | 201 |
| IL10 R2 (NM_001405058.1) | GGTGTGTGAGGATGTGGAGG | CACTGGACGGAGGACCTAGA | 210 |

## Results

### Regulation of IL-6 receptor abundance

The data demonstrate that the relative abundance of IL-6 R and gp 130, both of which are IL-6 receptors, is unaffected by the presence of hCG, TNF-α, or hCG in combination with TAPI treatments (Fig. 17A). Evaluations of the relative abundances of IL-6-R and gp-130 mRNA transcripts after treatments with different doses of IL-6 (0, 0.1, 0.5, 1, 2, etc.) revealed that IL-6 autoregulates these mRNA transcript abundances at the doses of 1 and 2 ng/mL. (Fig.17 B).

### Regulation of IL-10 receptor abundance

In this set of experiment post vitellogenic follicles were isolated from *Anabas* and treated with the effector molecules where HCG act as an positive regulator in the process of ovulation. Our data suggested that the expression of IL-10 R1 and IL-10 R2 both are up regulated when treated with hCG and TNF-α respectively. In case of treated with hCG the expression is up regulated upto 4.87 and 2.39 for IL-10 R1 and IL-10 R2, respectively. Where as when the follicle treated with TAPI-1 the expression of both IL-10R1 and IL-10 R2 were down regulated. In case of TNF-α both the receptor of IL-10 is up regulated in post vitellogenic follicle of *Anabas* (Fig. 18)

### Stat-3 regulation :

Because IL-6 is an important regulator during the time of ovulation, now it is necessary to understand the transduction pathway of IL-6 which is specifically act through JAK/STAT pathway. In this study we conducted the experiment which reveal the pathway clearly and our result showed that STAT-3 was up regulated when treated with IL-6. When treated with TAPI-1 expression of STAT-3 was down regulated in post vitellogenic follicle (Fig. 19).

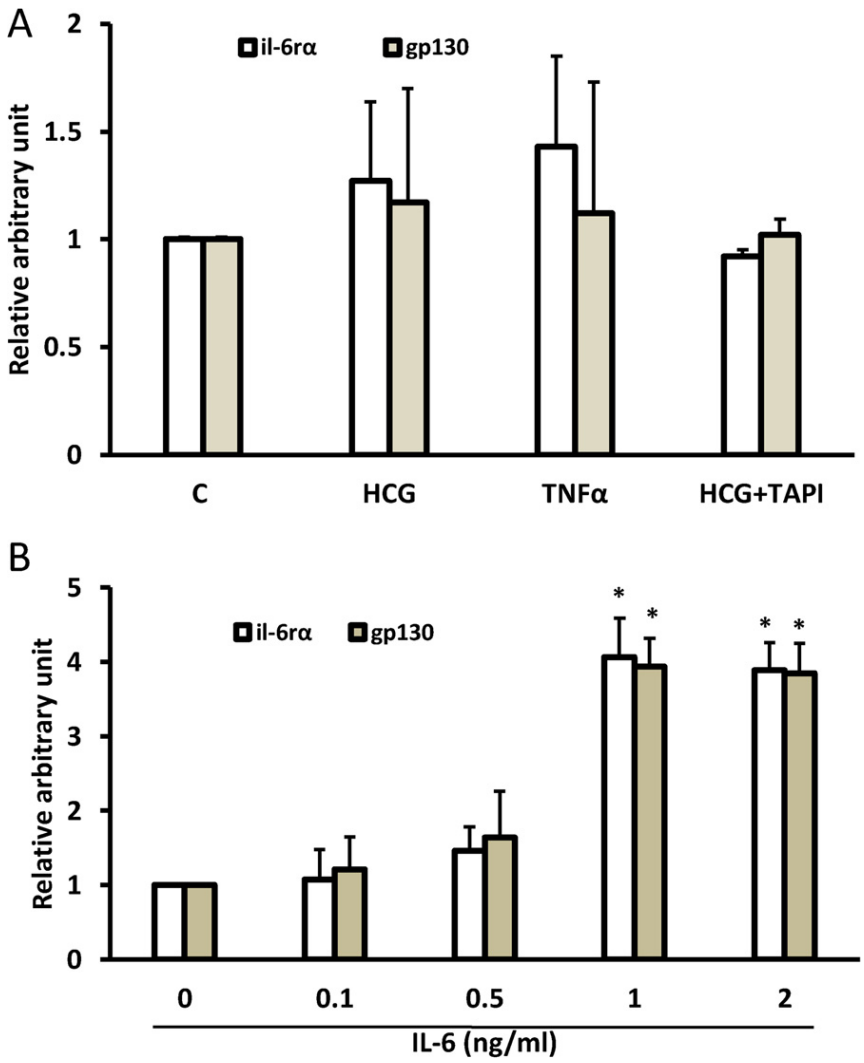



*Fig 17. Pattern of IL-6 receptors in A. testudineus in post-GVBD follicles; (A) Relative abundances of IL-6 rα and gp130 mRNA transcriptswere similar when there were treatments of follicles with hCG, TNFα and hCG combined with TAPI-I in comparison to control follicles; (B) Relative abundance of IL-6rα and gp130 mRNA abundances were greater when there were treatments with IL-6 at 1 and 2 ng/mL doses; Experiments were conducted in five fish with triplicate incubations for each developmental stage; Standard bar is the mean ± SEM; *denotes the differences in values compared values in the control group ($P \leq 0.05$).*

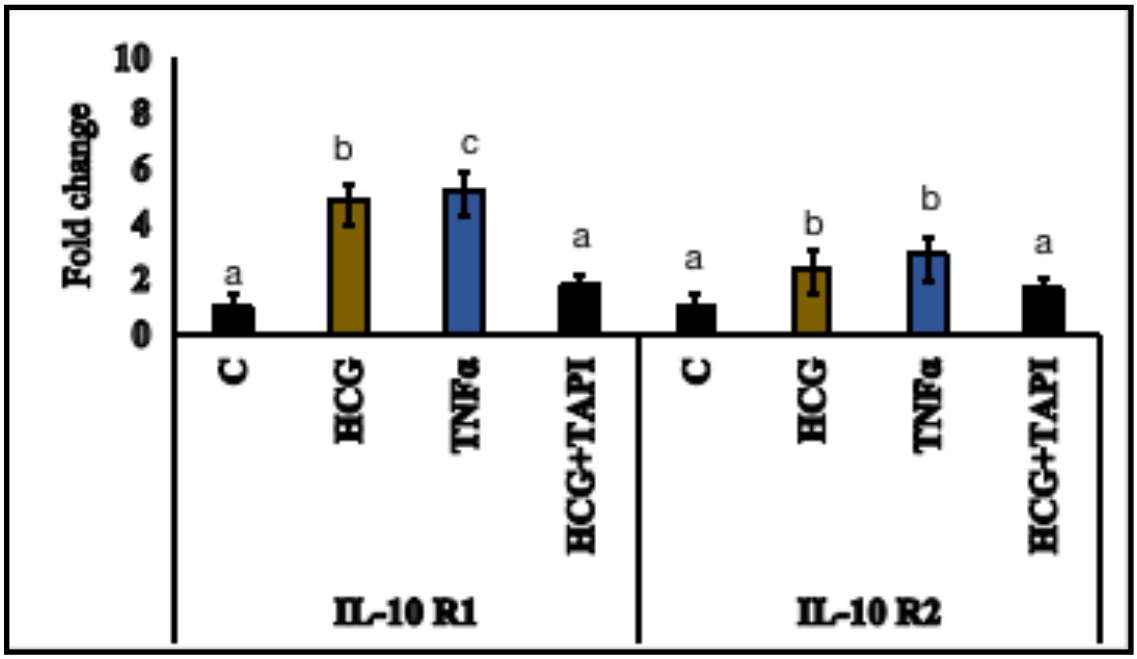



*Fig 18. Pattern of IL-10 receptors in A. testudinieus post vitellogenic follicle when treated with hCG in compared to control follicles. Experiments were conducted in five fish with triplicate incubations for each developmental stage; Standard bar is the mean ± SEM; *denotes the differences in values compared values in the control group ($P \leq 0.05$).*

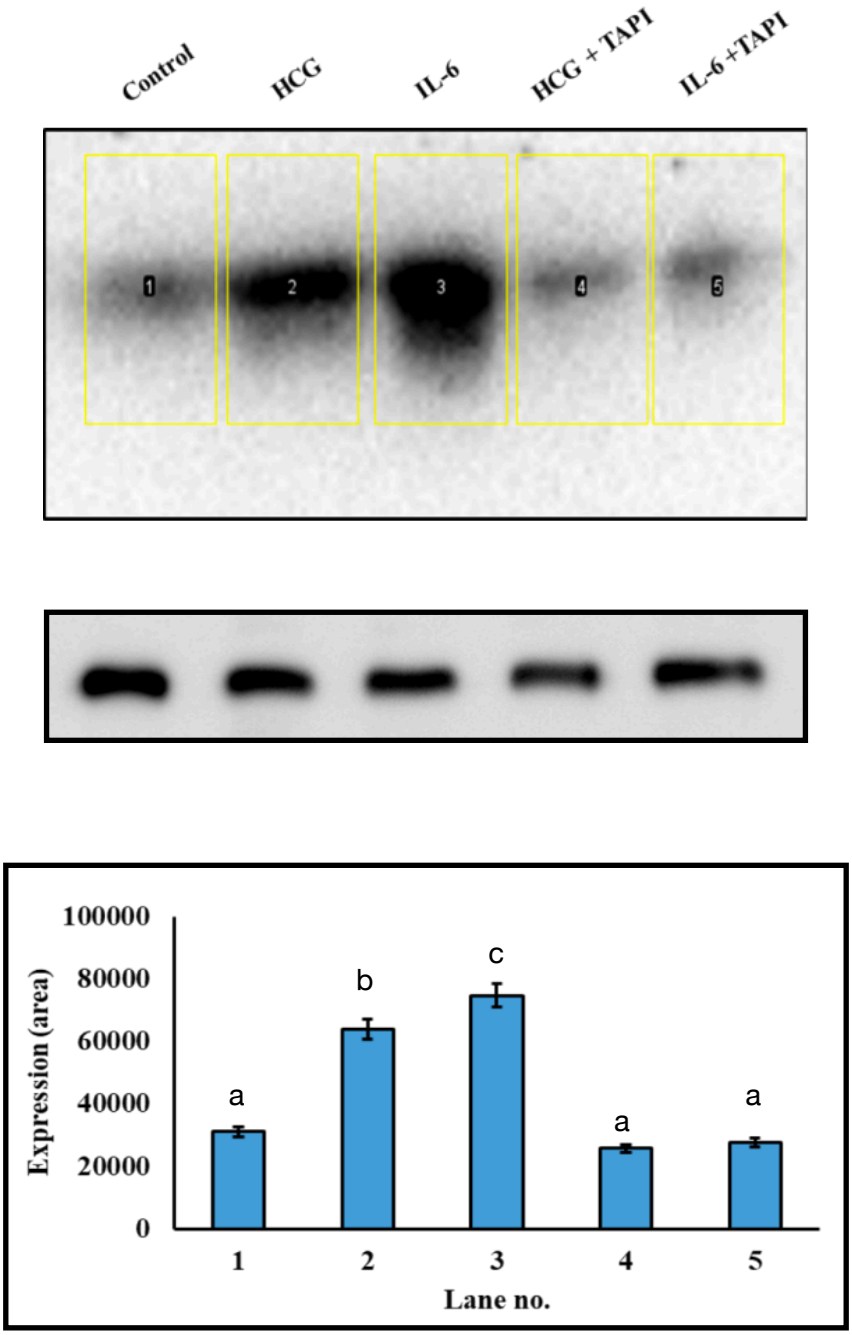



*Fig 19. Western blot analysis of STAT3. Data validated in respect to the expression of β-actin.*

**Discussion:**

IL-6 activates the JAK-STAT, MAPK, and PI3 kinase pathways when it binds to the appropriate membrane receptors with two subunits, IL-6 R and gp130 (Rose-John and Heinrich, 1994; Garbers et al., 2012). The current study's preliminary experiment measured IL-6R and gp130 in follicles to ascertain whether gonadotropins regulate the receptors or whether these receptor populations are autoregulated by IL-6. The results demonstrate that gonadotropins release TNF-α and IL-6, but that hCG and TNF-α have no effect on the expression of IL-6R and gp130. A significant finding in the current study is that IL-6 is released by granulosa cells, increasing the number of IL-6 receptors and consequently starting the signalling cascades for ovulation. Some of the earlier study established that 17β-estradiol and dihydrotestosterone decreased IL-6R expression, and thus ovariectomy in mice caused an increase in IL-6R mRNA (Lin et al., 1997). 17β-estradiol down-regulated cilia-localized IL-6R in human and murine fallopian tubes (Shao et al., 2009). Our result from this experiment in accordance with the discussion of the previous chapter we can say that IL-6 is a key regulator during the time of the ovulation which acts through its receptor IL-6R and gp-130 and auto regulates its own receptor.

IL-10-IL-10RI interaction is thought to occur within two hydrophobic patches, where the IL-10 ligand is thought to bind the receptor (Grayfer et al., 2012), it is likely that these hydrophobic residues facilitate the formation of those patches. A specific cell surface receptor complex made up of two distinct chains, IL-10 RI and IL-10 RII, both from the cytokine class II family receptor (CRF2), mediates the pleiotropic expression of IL-10. The majority of cells express IL-10RII, also known as cytokine receptor family member b4 (CRFB4/CRF2-4), widely and at relatively high levels. In this present study we found out that IL-10 RI and IL10-RII both were present in the ovary but the relative abundance of IL-10 RI was much higher that of IL10RII. The striking fact is that, when treated with TNF-α the expression of IL-10 RI and IL-10 RII were unregulated and the expression were going down when treated with TAPI-1. This evidence proved the fact that IL-10

expression in oocyte is regulated by TNF-α in post vitellogenic follicle which corroborated the study of Kube et al., (1995). STAT-3 homodimer is released from the receptor and translocated into nucleus, where it binds to STAT binding elements in the promoter of various gene including IL-10 itself. Among IL-10 induced genes, SOCS-3 is well known for its function in controlling STAT-3 activation and negatively regulating the expression of some cytokine genes viz, IL-6.

Previous study reports that when IL-6 binds with its receptor (IL-6R and gp-130) eventually it activate the JAK/STAT pathway. Previous reports also suggested that stat-3 can activate MMP (Hung et al., 2016). IL-6 acts via stat-3 in the ovulation process, according to the most recent stat-3 protein expression data, which supports earlier research (Rose-John and Heinrich, 1994; Garbers et al., 2012). Western blot data analysis reveals that during the ovulation period, IL-6 upregulates the stat-3 receptor, through which they act. This is confirmed when TAPI-I subsequently blocks the expression of stat-3. From this study, we can also infer that stat-3 may function as a key regulator for activating MMP2 and facilitating ovulation.

The up regulation of IL-6 receptors in post vitellogenic follicle by IL-6 itself but not by the gonadotropin explain the pivotal role of IL-6 during the time of the ovulation in *Anabas*. The eventual mechanism then controlled by the MMPs which may be activated by the stat-3 and ptgs2 tells us the fact that the ovulation process in fish is all interdependent between the gonadotropin-steroids-paracrine factors in that particular microenvironment of that oocytes. The up regulation of IL-10 receptors when treated with gonadotropin and TNF-α establish an argument whether IL-10 is important for the reproductive processes or not. From our investigation it was clear that the dependency of Il-10 release with TNF-α and its constant presence in vitellogenic and post vitellogenic processes establish the scenario that IL-10 may take part to maintain the homeostasis of other cytokines to ensure proper environment for oocyte to grow and eventually matured.