CHAPTER 1

INTRODUCTION

Goat, being the first ruminant to be domesticated, constitutes one of the important species of livestock industry and occupies a peculiar place in rural economy of India. In rural India, goat rearing is an enterprise which has been practiced from centuries. Goat is a hardy species and survives well on hillsides, deserts and in plain as well (Peters and Horst, 1981). This small ruminant has been used for meat, milk, hair and skin all over the world (Rout et al., 2002). Although, the goat attains puberty at the age of 8 months, has short post partum period and breeds throughout the year and is the prolific breeder having litter size 1 to 4 (Gall, 2001), yet no systematic study has been undertaken till date on its reproductive regulatory mechanisms. Infact, reproductive success of the species is determined by a number of extrinsic and intrinsic factors. To enhance the goat production (meat and milk yield) emphasis is being laid on (1) planned breeding programme, (2) nutrient rich feed of good quality, (3) proper management, and (4) health care.

Reproduction, the essence of survival, has remained of vital importance, largely because of intricate behavioral and evolutionary contributions. The physiology of reproduction from gametogenesis (spermatogenesis and oogenesis), fertilization, conception and maintenance of pregnancy to parturation is a complicated multifactor regulatory phenomenon. For sustainable economic benefits it is always in the interest of farmers to have healthy and productive animals in the flock. The basic understanding of its reproductive physiology the animal is thus of utmost importance (Guraya, 2000; Sharma, 2000; Sharma et al., 2000). With the advent of molecular and immunological techniques,
emphasis has remained to investigate the complex regulatory mechanisms of female reproduction. Moreover, various fertility and sterility problems confine themselves only to the ovary (Guraya, 1985). Therefore, a thorough understanding of the functional significance of various structural entities at cellular, subcellular and molecular level is an imperative need (Guraya, 1985).

Ovary, the primary female reproductive organ is comprised of five components (a) developing follicles (b) atretic follicles (c) corpus luteum (d) interstitial gland tissue and (e) stroma (Sharma and Batra, 2008). The developing follicles are committed for ensuring the development and maturation of normal growing oocytes (Guraya, 2000). The normal growing follicles act both as exocrine (ovulation) and endocrine (estrogen and progesterone) gland. Growth of the ovarian follicle is under the endocrine control of the pituitary gonadotropins. In the early stage of follicular development, granulosa cells undergo rapid proliferation with limited capacity for hormonal production. As the follicles grow and enlarge in size, granulosa cells show an increased ability to secrete hormones (Gougeon, 1993). Kotsuji et al. (1990; 1994); Kotsuji and Tominaga, (1994) have reported that granulosa cells are one of the important cell population for controlling the growth and differentiation of the steroidogenic cells. However, a growing body of evidence, indicating that steroidal and nonsteroidal factors produced by granulosa cells affect the function and proliferation of the theca cells (Hsueh et al., 1984; Roberts and Skinner, 1990; Ackland et al., 1992; Zachow et al., 1997; Parrott and Skinner, 1998a).

Granulosa cells in small antral follicles start to express FSH receptors and are selected to continue growth by increases in basal
concentration of gonadotropins. From this stage the follicles become dependent on FSH for survival (Gougeon, 1996). FSH-stimulation causes expression of aromatase in the granulosa cells, thus enabling the granulosa cells to convert androgens produced in the thecal cells to estrogens. Steroid hormones play a crucial role in gonadal development and function of these cells (Krege et al., 1998; Couse et al., 1999; Schomberg et al., 1999; Britt et al., 2001). Granulosa cells undergo a differentiation process during the reproductive cycle, where the immature form present in the preantral follicles transforms into a more mature counterpart present in preovulatory follicles (Richard, 1980). This process is characterized by the enhanced steroidogenic capacities and acquisition of LH/HCG receptors (Hsueh et al., 1984) and accompanied by cytomorphological changes. These changes are essential for both the development and maintenance of ovarian follicles.

In addition to the well established effects of gonadotropins, numerous growth factors can evoke significant modulation (Carson et al., 1989; Tonetta and Dizerega, 1989). The cytokines may also play potentially important role in the regulation of granulosa cell functions (Gottschall et al., 1987; Emoto and Baird, 1988; Gorospe et al., 1988 Roby and Terranova, 1988; Darbon et al., 1989; Kasson and Gorospe, 1989; Wood and Strauss, 2002). Over the last two decades, a wide range of regulators of gonadal function has been discovered to act largely in an autocrine or paracrine fashion. It has become apparent that in the ovary, the immune system contributes to the regulation of gonadal function (Mori, 1990; Tung et al., 2001). Leukocytes potentially act through local secretion of regulatory soluble factors present in the ovary hence constituting in-situ modulators of ovarian functions. These factors include numerous cytokines that largely originate by the action of immune cells.
within the ovary (Findlay et al., 1990; Adashi, 1992; Calkins et al., 2002).

Cytokines have been implicated as important regulators of steroidogenesis and gamete production (Adashi, 1992; Brannstrom and Norman, 1993). The evidence for this immune-endocrine interaction has been best developed in the ovary where the anatomy and vascularization permit migration of leukocytes in and out of the organ, as is evident by the histological presence of these cells and associated changes in the lymphatic flow (Olson and Ley, 2002). In addition, many other cell types in the ovary also produce cytokines independently of the presence of leukocytes.

Cytokines are small proteins (15 ± 60KDa) that act locally through specific cell surface receptors to coordinate interactions of the immune system and surrounding tissues. Macrophages secrete a diverse repertoire of cytokines including IL-1, IL-2, IL-6, IL-10, IL-12, interferon-α, tumor necrosis factor-α and granulocyte macrophage colony stimulating factors. These cytokines have been identified in the ovaries of many species and are known to impact many aspects of ovarian function (Brannstrom and Norman, 1993; Terranova and Rice, 1997; Bukulmez and Arici, 2000) including follicle growth and differentiation, cell apoptosis, ovulation, and corpus luteum formation and regression (Saito, 2001; Paria et al., 2002; Schafer-Somi, 2003; Townson and Liptak, 2003). Therefore, it can be postulated that interaction between the endocrine and cytokine system play an important role in the regulation of follicular development and atresia (Moffett and Loke, 2006; Chaouat et al., 2007).
Interleukins are a group of cytokines (secreted proteins/signaling molecules) that were observed first to be expressed by white blood cells (leukocytes). The majority of interleukins are synthesized by helper CD4+ T lymphocytes, as well as by monocytes, macrophages, and endothelial cells. The interleukins promote the development and differentiation of T, B and hematopoietic cells. Interleukins are known best for their involvement in the immune system and their role during inflammation. Among all of the interleukins, interleukin-2 which is also known as T-cell growth factor was first described as a lymphokine capable of promoting the long term in vitro proliferation of activated T cell (Morgan et al., 1976). It has also been shown to modulate many other immunologic effects on cell including cytotoxic T-cells (Zarling and Bach, 1979; Gillis et al., 1980), natural killer cells (Henney et al., 1981; Ortaldo et al., 1984), activated B-cells (Mingari et al., 1984) and lymphokine activated killer cells (Grimm et al., 1982; Mazumder and Rosenberg, 1984). IL-2 has inhibitory or stimulatory effects on hormonal production by ovarian granulosa cells cultured in vitro (Grospe et al., 1988; Adashi et al., 1989; Kasson and Gorospe, 1989). The source of these cytokines within the ovary appears to be luteal and granulosa cells as well as immune cells such as macrophages, eosinophils, leukocytes and T-lymphocytes (Nakamura et al., 1987; Roby et al., 1990; Ji et al., 1991; Roby et al., 1999).

Increasing evidences suggest the existence of strong bidirectional and complex interactions between the ovary and the immune system. Animal experiments as well as certain human diseases demonstrate the influence of immune cells on ovarian physiology (Matasuyama et al. 1987; Turi et al., 1988; Kim et al., 1995; Marchetti et al., 1996; Pasoto et al. 1999; Bukulmez and Arici, 2000; Jasper et al., 2000; Kasteren
et al., 2000; Panda et al., 2001; Nelson, 2001). Furthermore, complex temporal changes of immune cell populations within the ovary and corpus luteum have been reported in cows, rats, rabbits, and humans (Bagavandoss et al., 1988; 1990; Petrovska et al., 1992; Brannstrom et al., 1994a; Brannstrom et al., 1994b; Spanel-Borowski et al., 1997; Gaytan et al., 1998; Penny et al., 1999; Pate and Keyes, 2001). The significant invasion of leukocytes into the corpus luteum of rabbits, rats, and cows at the end of luteal life demonstrates that leukocytes are involved in luteolysis (Bagavandoss et al., 1988; 1990; Brannstrom et al., 1994a; Spanel-Burowski et al., 1997; Penny et al., 1999). The mechanisms inducing the down regulation of luteal hormone production, luteal cell atrophy, and their selective clearance are not fully understood. In vitro co-culture experiments have shown that immune cells were able to modulate the function of the resident cells of the corpus luteum, namely granulosa-lutein cells, fibroblasts, and endothelial cells (Kirsch et al., 1981; Bagavandoss and Wilks, 1991; Yamanouchi et al., 1992; Fenyves et al., 1994; Evagelatou et al., 1997; Young et al., 1997; Castro et al., 1998; Wuttke et al., 1998; Kohen et al., 1999; Matsubara et al., 2000; Suter et al., 2001). Primarily proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and monocyte chemoattractant protein-1 (MCP-1), which are predominantly produced by immune cells, but in part also by resident luteal cells, are possibly involved in the regulation of luteolytic events (Wuttke et al., 1993; 1998; Castro et al., 1998; Zhao et al., 1998; Brannstrom et al., 1999; Roby et al., 1999; Penny et al., 1999; Senturk et al., 1999; Chen et al., 2000; Penny, 2000; Suter et al., 2001; Townson et al., 2002).
Numerous studies have investigated leukocyte populations in the ovaries of various species ranging from rodents to humans. The ovarian leukocytes can be classified into two categories: residential and infiltrative. In addition, a small number of lymphocytes, including CD4\(^+\) and CD8\(^+\) T cells, have been reported in the ovary (Suzuki et al., 1998). It has been shown that a large infiltration of leukocytes (macrophages and neutrophils) into the ovary occurs during the ovulatory process, probably in response to LH (luteinizing hormone) surge (Brannstrom and Norman, 1993). An equally important influx of monocytes into the corpus luteum characterizes the late post-ovulatory phase (Brannstrom et al., 1994a).

The possible involvement of T lymphocytes in corpus luteum regression has been reported (Aust et al., 2000). Several physiological processes in the ovaries, especially ovulation/luteinization, show similarities to the inflammatory process. A growing body of evidence, including discovery of a leukocyte influx into the ovary during ovulation, suggests that the ovulation/post-ovulatory process may indeed constitute a local inflammatory reaction (Espey, 1980; Brannstrom et al., 1994b; 1993a; Standaert et al., 1991; Adashi, 1990). However, controversy remains as to which populations participate in the processes. Neutrophils and macrophages have been the most common candidates. However, a recent study showed that depletion of peripheral blood leukocytes, mainly neutrophils, did not affect ovulation (Chun et al., 1993). Although it remains unclear, a few studies do suggest that cytokines released by those leukocytes might be directly involved in the ovulatory or post-ovulatory functions (Ujioka et al., 1998; Garcia-Velasco and Arici, 1999).

In the ovary, leukocytes have been found during the different phases of the menstrual cycle (Kasuya and Kawabuchi, 1998) localized primarily in the vascular connective tissue and theca-lutein areas of the
corpus luteum, while some are found in the granulosa-lutein cell layer (Wu et al., 2004; Balasch et al., 2004). Leukocytes are important regulator of angiogenesis. Cytokines like IL-1, IL-6, IL-10, IL-12, IFNα, TNFα etc., secreted by macrophages, are known to have impact on many important aspects of the ovarian function (Brannstrom et al., 1993b; Vinatier et al., 1995; Terranova and Rice, 1997; Chung et al., 2000), including follicle growth and differentiation, ovulation and corpus luteum formation and regression. The ovarian leukocytes remove apoptotic granulosa cells and apoptotic luteal cells through phagocytosis (Gaytan et al., 1998; Kasuya, 2002), thereby contributing to the processes of follicular atresia and luteolysis.

Most of cells have the ability to self-destruct by activation of an intrinsic cellular suicide program referred to as programmed cell death or apoptosis. Apoptosis plays an important role in regulation of ovarian function in mammals (Kaipia and Hsueh, 1997; Martimbeau and Tilly, 1997). Over the course of reproductive lifespan, only a few follicles mature and ovulate, while majority of follicles i.e., up to 99%, do not grow to ovulate but degenerate during various stages of follicular development due to the process of initiation of apoptosis in granulosa cells (Hughes and Gorospe, 1991; Tilly et al., 1995; Tajima et al., 2007). Apoptosis is physiologically controlled form of cell death that occurs in the ovarian follicles where lymphocytes and cytokines play an important role in its regulation (Kaipia and Hsueh, 1979).

Ovulation is the final stage in the growth and differentiation of the follicle where leukocytes play an important role (Espey, 1980; 1994). The infiltration of macrophages and T-cells into the ovarian follicles is a cytological marker of the ovulatory process (Norman and Brannstrom, 1994). Just before ovulation, leukocytes penetrate into the area
surrounding the preovulatory follicle (Brannstrom and Norman, 1993; Brannstrom et al., 1994a; Bukovsky et al., 1995). Immediately after ovulation there is a rapid influx of macrophages and lymphocytes into the follicle. In preovulatory follicles, these cells comprise of 5-20% of follicular tissue cells (Castilla et al., 1990; Loukides et al., 1990). They may play an active role in ovulatory process (Hellberg et al., 1991; Machelon and Emilie, 1997; Simon et al., 1998). Although IL-2 has been recognized as a well known cytokine but very little is known about its physiological significance in the ovary. As a cytokine, it has the ability to communicate with the neighbouring cells, but no information is available on impact of cytokine (IL-2) on rescue of granulosa cells and its localization in the ovary. It is still not clear whether or not passages induce any alterations in the hormonal production of progesterone and estrogen in vitro. Keeping in view the lacunae the present study was planned with the following main objectives:

2. Estimation of interleukin-II (IL-II) in follicular fluid of normal and atretic follicles.
3. Immunohistochemical localization of IL-2, CD4+, CD8+ T-lymphocytes within ovarian compartments of normal and atretic follicles.
4. Impact of cytokines and IL-II on
   (i) Oocyte maturation
   (ii) Rescue of atresia in granulosa cells.
5. Ultrastructural analysis of normal and atretic granulosa cells.
6. Hormonal assays of the follicles at different phases of development and atresia.
The results of the present findings would be of great help in understanding basic aspects of cellular interaction in small and large follicles and will explain the regulation of local immunological events, including infiltration of immune cells and cytokines in various biological activities in a stage specific manner and will elaborate functional significance of cytokines and immune cells in the process of follicular atresia. The results will help in developing information and experimental data to establish a close relationship between immune cells and reproductive functions.