DISCUSSION

During the present study influence of immune cells and cytokines has been analysed in normal mature goat (Capra hircus) ovary. Changes in CD4$^+$, CD8$^+$, and IL-2$^+$ cell populations have been studied in different cellular components of normal growing follicles, atretic follicles, corpora lutea, interstitial cells and stroma. The quantities of cytokines such as IL-2 and GM-CSF have been determined in follicular fluid of normal and atretic follicles. The influence of cytokine (IL-2) on oocyte maturation and rescue of atresia in granulosa cells has been evaluated. Effect of IL-2 on progesterone and estrogen secretion and release in the presence and absence of FSH has also been analysed.

Immunohistochemical localization has revealed that the number of CD4$^+$ and CD8$^+$ cells and IL-2$^+$ cells was higher in the large-sized (≥ 8mm) atretic follicles as compared to the normal follicles of the same size. Effect of IL-2 on *in vitro* oocyte maturation revealed that the rate of oocyte maturation was not significantly different in control and that of IL-2 (25 and 50 ng/ml) treatment for 12 or 24 hrs of FSH supplemented cultures. The rate of oocyte maturation was significantly higher at dose level of 100 ng/ml of IL-2. TUNEL assay demonstrated the apoptosis of granulosa cells obtained from follicles having 4-6 mm diameter. The number of these cells was increased in 100 ng/ml IL-2 supplemented culture. Hormonal assay revealed that in the presence of IL-2, the production of progesterone increased with the increase in dose level of interleukin-2. The interleukin-2 only reduced the number of viable cells as compared to the control *in vitro*. As the number of passages was increased, the cell viability got reduced. The estrogen production decreased as the dose level of interleukin-2 was enhanced. The
quantification of GM-CSF and IL-2 in the follicular fluid of goat ovary revealed that the concentration of both cytokines were higher in atretic follicles as compared to that of normal follicles. The results of the present finding shall be compared with the recent findings in the subject and discussed under the following sections:

1. **Ovarian follicles**

   Results of present study indicate that the goat ovary contains immunologically dynamic cells which vary in nature and concentration depending upon the type of ovarian structure and the stage of development or atresia. The transverse sections of goat ovary were examined to study ovarian follicles and corpora lutea through many stages of development and regression. CD4\(^+\), CD8\(^+\) and IL-2\(^+\) cells were found in small numbers in developing follicles and newly formed corpora lutea, while atretic follicles and regressing corpora lutea contained these cells in abundance.

   The leukocyte populations have been reported in the ovaries of different mammalian species ranging from rodents to humans. The ovarian leukocytes are of two types i.e. residential and infiltrative. The best characterized residential leukocyte population in the ovary is macrophages, which are localized in the interstitium and corpus luteum (Cohen *et al*., 1999). In addition, a small number of lymphocytes, including CD4\(^+\) and CD8\(^+\) T cells, have been reported in the human ovary (Suzuki *et al*., 1998). It has been established that a large infiltration of leukocytes (macrophages and neutrophils) into the ovary occurs during the ovulatory process, probably in response to luteinizing hormone (LH) surge (Brannstrom *et al*., 1993b). Results of the present study strongly endorse the earlier finding and indicate that the presence of leucocytes is an ovarian characteristic of mammals in general.
Variable number of CD4\(^+\) cells was mapped in the different cellular components of the follicles. The reactivity of CD4\(^+\) cells was least in the membrana granulosa as well as in the oocytes of primordial follicles. In the primary follicles CD4\(^+\) cells were localized in the granulosa layer of the follicle where its intensity was weak in primordial and primary follicles but not in the oocyte. The reactivity of CD4\(^+\) cells were mapped both in the theca and granulosa layer of the developing follicle i.e. secondary and dominant follicles, their intensity increased from weak to moderate in granulosa and weak to mild in thecal cells but the percentage of CD4\(^+\) cells was greater in granulosa layer as compared to that of thecal layer of the follicle. The granulosa cells were more reactive to CD4\(^+\) antibodies than thecal cells of the primary, secondary and dominant follicles. In the oocytes of the large follicles reactivity of CD4\(^+\) cells was moderate to strong but it was very weak in the primordial and primary follicles. Cumulus cells around the antral follicle showed marked binding for CD4\(^+\) cells and their intensity was weak to moderate.

CD8\(^+\) cells were observed in both the granulosa and thecal compartments of developing follicles. In primordial and primary follicles reactivity for this antibody was not observed. The number of CD8\(^+\) cells was higher in theca cells as compared to granulosa cells in all stages of the developing follicles i.e. secondary and dominant follicles their intensity was weak to mild. Minimum reactivity for CD8\(^+\) in the granulosa of primordial and primary follicles was observed negative but while maximum binding was seen in the secondary and dominant follicles. Thecal cells were more reactive to CD8\(^+\) in secondary and dominant follicles. In the oocyte of the large follicles reactivity of CD8\(^+\) cells was observed but it was very weak in the primordial and primary follicles and mild to moderate in secondary and dominant follicles. Cumulus cells around the dominant follicle showed mild stain for CD8\(^+\) cells.
The present findings strongly support that the distribution and frequency of the leukocytes within the ovary vary with the stage of the developing follicle (Brannstrom et al., 1994a; Takaya et al., 1997). A variety of leukocyte subtypes has already been described in the ovary, including neutrophils, macrophage/monocytes, lymphocytes and mast cells (Brannstrom and Norman, 1993; Brannstrom et al., 1993a). A considerable number of leukocytes localized in the theca layer of the secondary follicles in the immature rat ovary (Katabuchi et al., 1989; Brannstrom et al., 1993a). Both the CD4\(^+\) and CD8\(^+\) cells were observed in the thecal compartment of primordial, primary, secondary and antral follicles of goat ovary. The reactivity of CD4\(^+\) and CD8\(^+\) cells were weak in the primordial follicles of goat ovary. The number of CD4\(^+\) cells was higher as compared to CD8\(^+\) cells in all stages of the developing follicles of goat ovary (Sharma and Gandhi, 2010). Brannstrom et al. (1994a) investigated localization of leukocyte subsets CD4\(^+\) and CD8\(^+\) in the thecal layer of the secondary follicle throughout the menstrual cycle in human ovary. Cavender and Murdoch (1988), Abisogun et al. (1988) observed small number of leukocytes in the thecal compartments of secondary follicle in human ovary and their number increased dramatically in antral follicles. In the antral follicle, the majority of leukocytes have been located in the theca, with a predominance of neutrophils and macrophages, as identified by immunostaining with specific antibodies having TNF-\(\alpha\) in their granules (Brannstrom et al., 1993a; Brannstrom and Norman, 1993).

The increase in the number of CD4\(^+\) and CD8\(^+\) cells in large follicles of goat demonstrate the similar pattern of changes as observed in rat where neutrophils were found to be located predominantly in the medullary region prior to ovulation, and as ovulation approached there was a 3-fold increase in the density of neutrophils in the medulla and an 8-fold increase in the theca layer of the secondary and antral follicles.
(Brannstrom et al., 1993a). Studies in the rabbit (Gerdes et al., 1992) and in the sheep (Cavender and Murdoch, 1988), using histological staining, has also shown a distinct increase in neutrophils in the thecal layer of the antral follicle.

Presence of weak to mild reactivity in small sized follicles for CD4+, CD8+ and IL-2+ cells in goat support the earlier finding wherein leukocytes were relatively scarce in the granulosa cells of primordial and primary follicles of human ovary, but may contribute to the regulation of follicular development by secretion of cytokines (Brannstrom and Norman, 1993). The granulosa cells were more reactive to CD4+ than CD8+. The minimum reactivity for CD4+ and CD8+ has already been reported in primordial follicles while maximum binding was seen in large sized follicles (Sharma and Gandhi, 2010).

Recent evidence has amassed indicating that factors produced by cells of the immune system, the leukocytes and cytokines, play potentially important roles in the regulation of granulosa cell functions. Gorospe et al. (1988) have all been demonstrated that leukocytes and cytokines suppress FSH-stimulated steroidogenesis as well as inhibit LH/hCG receptor induction in granulosa cells. Leukocytes are involved in the growth and differentiation of ovarian cells by secretion of a variety of cytokines (Adashi, 1992; Brannstrom et al., 1994a; Sunderkotter et al., 1994; Pate, 1995) such as interleukin-1 (Kokia and Adashi, 1993; Brannstrom et al., 1993c) and tumour necrosis factor-α (Terranova et al., 1993; Brannstrom et al., 1995). These leukocytes may act in a direct or indirect manner and influence the secretion of progesterone and prostaglandins (Fairchild et al., 1991; Townson and Pate, 1994). Interferons also alter bovine luteal cell prostaglandin production and progesterone synthesis (Fairchild and Pate, 1991), which indicate a role in luteolysis, and can also have direct cytotoxic effects on cells especially
when present in combination with some other cytokines (Campbell et al., 1988; Fairchild and Pate, 1991; 1992). T-cells up regulate the progesterone production by cumulus oophorus/oocyte complex which is mediated by various cytokines, (Fukuoka et al., 1992; Wang et al., 1992a). Yan et al. (1993) and Best et al. (1994) have reported that the leukocytes cytokine TNF-α inhibits granulosa cell oestradiol and progesterone biosynthesis in vitro. Since leukocytes observed in goat ovary were present in high numbers during follicular atresia, it is possible that leukocyte-secreted TNF-α may contribute to decreased steroid secretion during these periods. A number of studies have shown that unidentified lymphocyte derived products increase progesterone output of cultured granulosa cells of the rat (Hughes et al., 1991), pig (Hughes et al., 1990) and the human (Emi et al., 1991). While the stimulating agent was not identified, it appears to be a protein like substance with a mol. wt of <30 kDa (Emi et al., 1991).

CD8⁺ lymphocytes possess a receptor for the estradiol on its surface (Cohen et al., 1983) which is attracted by estrogen in the growing follicle and could stimulate their proliferation and differentiation. The activated T cells which arrive in the growing follicles secrete interferon 7 (Grasso et al., 1988; 1994) which caused a dramatic decrease of circulating oestradiol and progesterone without affecting the serum concentration of LH, FSH, and prolactin (Kauppila et al., 1982).

The secretion of cytokines by the granulosa cell appears to be a regulated event affected by the gonadotropin FSH and other cytokines. It is possibly because of enhanced demand of cytokines that are required in large follicle to execute variety of functions that number of CD4⁺ and CD8⁺ cells are higher.

The distribution of IL-2 cells was observed in the goat ovarian components. IL-2 was studied in the oocyte of primordial follicle but
granulosa cells remained unstained. In primary follicles, intensity of IL-2 in granulosa cells was weak to mild. IL-2 was localized in the oocyte and theca cells during secondary and dominant follicles their intensity was moderate in primordial and primary whereas strong to intense in dominant follicles. In the oocyte, cytoplasm and plasma membrane stained strongly, along with the germinal vesicle of the oocyte. IL-2 was not detected in cumulus cells of healthy follicles. It was detected in both granulosa and theca layer of dominant follicles and the percentage of IL-2+ granulosa cells were slightly higher than that of IL-2+ thecal cells.

Frequency of IL-2 was increased in atretic follicles both in granulosa cells and thecal cells in the dominant atretic follicles. In atretic follicles, number of IL-2+ cell type increased from primary to large sized follicles as compared to their counterpart normal developing follicles. The maximum reactivity was observed in atretic dominant follicles.

The present observation strongly endorse that the goat ovary is the site of production of ILs and their receptors as has already been demonstrated in both granulosa and theca cells, whilst maximal production occurs in preovulatory follicle after gonadotropin action (Brannstrom, 2004; Ingman and Jones, 2008). The ILs has several sites of synthesis in the ovary. The role of ILs in local processes is still poorly known, although there is evidence for its ability to stimulate ovarian cell proliferation, suppress apoptosis, and therefore to promote ovarian follicular growth. Cellular localization identifies IL-1 receptors in both granulosa and theca interstitial cells (Hurwitz et al., 1992) within the oocyte. Interleukin-1, a major cytokine, as well as receptors (IL-1R1 and IL-1R2) and antagonist (IL-1RA) are produced in the ovary of several species (Gerard et al., 2004).

IL-1 is an established immune mediator produced by resident macrophages, and has been suggested as the main source of this cytokine
in the ovary (Adashi, 1990; Hurwitz et al., 1991). During follicular
development, IL-1α, IL-1β and IL-1R type I staining were confined to the
theca-interstitial layer of growing follicles of the rat ovary (Hurwitz
et al., 1991) and the demonstrated effect of IL-1α on theca cells in antral
follicle of hamster ovary (Nakamura et al., 1990) contrary to IL-2. In this
regard IL-2 staining pattern in goat ovary is similar to IL-1 as recorded
earlier in mouse. IL-1α and IL-1β was only observed in some cells of
theca layer (Simon et al., 1994). IL-1Rt1 was localized in the theca layer
of secondary and antral follicle of mouse ovary (Simon et al., 1994). In
addition the absences of IL-1α and IL-1β staining in granulosa cells from
human preovulatory follicles have been reported by Polan et al. (1994).
IL-2 stimulates the thecal compartment without affecting the granulosa
cells and no correlation between follicular concentration of progesterone
and oestrogen was observed (Barak et al., 1992). Goat ovary resembles
with that of other mammals in this regard, the effect of IL-1 on the
follicular production of well-established ovulatory mediators have shown
that IL-1α increases the progesterone production from thecal tissue of
incubated pre-ovulatory follicles of the hamster (Nakamura et al., 1990).

The size of the follicle of goat selected for the estimation of IL-2
was in between 4-6 mm in diameter for both normal and atretic follicles.
The mean IL-2 concentration was 1.6 ± 0.09991 pg/ml (1.2 to 1.9 pg/ml)
in follicular fluids of normal follicle and 10.9 ± 1.2600 pg/ml (5.7 to 17.7
pg/ml) in follicular fluid of atretic follicle.

Similar studies have been carried in swine, IL-2-like activity is
present in ovarian follicular fluid and levels decrease as follicles enlarge
(Takakura et al., 1989). Although the intraovarian source of IL-2 has not
been identified, it is likely that some of the IL-2 in follicular fluid is
derived from lymphocytes including T-cells that infiltrate ovarian
follicles and corpora lutea (Standaert et al., 1991; Brannstrom et al.,
Concentrations of IL-2 in human preovulatory follicular fluid have been studied by Wang and Norman (1992) where a positive correlation between IL-2 concentration in follicular fluid and plasma was found. The titer of IL-2 does not show any relationship between progesterone and estrogen strongly supporting Barak et al. (1992) who investigated the relationship between IL-2 and oestradiol, progesterone and testosterone concentrations in periovulatory follicles of human ovary and observed no correlation between IL-2 concentration and oestradiol and progesterone concentrations in follicular fluid. In addition, they found no correlation between concentrations of IL-2 in follicular fluid and oestradiol in serum.

Increasing doses of IL-2 were tested on goat oocytes. The rate of oocyte maturation was not significant between control, the dose level of 25ng/ml of IL-2 and 50ng/ml of IL-2 in both 12 and 24hrs of culture which also contain FSH. But the rate of oocyte maturation was significantly higher at dose level of 100ng/ml of IL-2. The expansion of COCs was also observed and maximum expansion was seen at highest dose level of IL-2 i.e 100 ng/ml. The rate polar body extrusion was 45%, 48%, 46% and 68% in control, 25 ng/ml, 50 ng/ml and 100 ng/ml of IL-2, respectively.

The present observations on influence of IL-2 are similar to that of IL-1 observed in equine where IL-1 increases the germinal vesicle breakdown rate of oocytes in vitro in the rabbit (Takehara et al., 1994) and in vivo in the mare (Martoriati et al., 2003a, b). This demonstrates that IL-1 plays a role in the process of oocyte nuclear maturation, so IL-1 may be a paracrine factor involved either in follicular or oocyte-related aspects of ovulation. However, in the mare, IL-1 inhibits in vitro oocyte maturation (Martoriati et al., 2002; 2003a, b), leading to hypothesize that follicular factors produced by granulosa cells and secreted in the
follicular fluid could interact with IL-1, so that the culture medium used in vitro did not reflect in vivo maturation conditions.

Keskintepe et al. (1994), Izquierdo et al. (1998; 1999), reported that gonadotrophins (FSH and LH) and estradiol-17β improve maturation rates significantly when supplemented in IVM culture media. Efficacy of different growth promoting factors, vitamins or other substances alone or with supplementation in defined medium have been tested. Epidermal Growth Factor (EGF) influenced oocyte maturation and blastocyst production rates in a number of mammals. Goat cumulus cells express EGF receptors (Gall et al., 2004) and EGF triggers signaling through the MAPK pathway during IVM in goat cumulus cells (Gall et al., 2005). EGF involved in the regulation of follicular growth and oocyte maturation in goats. Recently, it is reported that EGF and its receptor are also expressed in goat ovarian follicles at all stages of follicle development, in corpus luteum and in ovarian surface epithelium (Silva et al., 2006). Goat oocytes matured in vitro in the presence of EGF had greater cumulus cell expansion, higher maturation and fertilization rates than the control oocytes (Nagar and Purohit, 2005). Insulin-like growth factor-I did not seem to affect oocyte IVM (Guler et al., 2000).

In vitro results of present study endorse IL-2 blocks basal progesterone secretion in granulosa cells as well as that induced by LH (Barak et al., 1992). IL-1 blocks the apparition of granulosa cell receptors at LH (Gottschal et al., 1988). Both forms of interleukin-1 i.e IL-1α and IL-1β have been demonstrated to suppress FSH induced progesterone and estradiol synthesis in granulosa cells from immature rats (Kasson and Gorospoe, 1989). A number of studies have examined the effects of IL-1 on granulosa from immature follicles where they could potentially affect cell differentiation and follicular development by regulation of steroidogenesis, receptor formation, and eicosanoid as well as protein
secretion from undifferentiated cells. The effects of the multifunctional cytokine IL-1 on undifferentiated ovarian cells have been particularly well-studied in the rat. Initially it was found that recombinant human IL-1β inhibits FSH-induced development of luteinizing hormone receptors, and reduces progesterone as well as oestradiol secretion (Gottschall et al., 1988; 1989; Kasson and Gorospe, 1989) in undifferentiated granulosa cells. IL-1β induces prostaglandin synthesis in the granulosa cells (Kokia et al., 1992), and inhibits human chorionic gonadotrophin-stimulated androgen biosynthesis in granulosa-interstitial cells of immature rats (Hurwitz et al., 1991a). IL-1 also inhibits basal as well as HCG-stimulated progesterone production in cultures of porcine granulosa cells obtained from medium-sized follicles (Fukuoka et al., 1988), partly due to the inhibition of adenylate cyclase activity (Fukuoka et al., 1989a). A proliferative effect of IL-1 on undifferentiated pig granulosa cells (Fukuoka et al., 1989b) highlights the inverse relationship between effects on differentiation and growth.

The stimulatory effect of IL-2 on progesterone production in goat shows great similarity with IL-1β stimulates basal progesterone secretion by human granulosa cells (Chen et al., 2000) of small and large follicles (Baratta, 1996). When stimulated by gonadotropins, IL-1α inhibits both LH/hCG and FSH stimulated progesterone (Kasson and Gorospe, 1989; Dawood et al., 1997; Terranova and Rice, 1997; Breard et al., 1998; Donesky et al., 1998; Kohen et al., 1999; Chen et al., 2000) and estradiol (Kasson and Gorospe, 1989; Zhou and Galway, 1991; Chen et al., 2000) secretion by follicular granulosa cells, affecting cAMP production (Breard et al., 1998), suggesting an follicle-stage dependent regulatory role of IL-1 on ovarian follicles. Progesterone and estradiol are stimulated by IL-1 in small follicles while in antral gonadotropin-dependent follicles in the presence of cAMP analogs show an IL-1
induced decrease in estrogen production suggesting an interleukin steroidogenic downstream regulation (Zhou and Galway, 1991).

Presence of IL-2 in follicular fluid of goat strongly advocates the earlier findings wherein several cytokines were found to be produced in the ovary or contained in the follicular fluid and is pivotal in the generation and regulation of the immune response, and it has two major immunotherapeutic goals i.e. controlled, specific immunosuppression and immunoenhancement. The follicular aspirate has proved an invaluable source for the study of ovarian function. The primary cellular constituent of follicular aspirates is generally regarded as the granulosa cells; other cell types have also been identified. Most commonly reported amongst these non-granulosa cell populations are leukocytes, often accounting for more than half of all cells within follicular aspirates (Best et al., 1994; Evagelatou et al., 1996; Lachapelle et al., 1996; Smith et al., 2000). Follicular fluid provides microenvironment for the oocyte and contains likely key immunological factors for the regulation of its growth and development. There is growing evidence that interactions between the immune and endocrine systems can influence ovarian function. Consequently, the composition of regulatory factors in the follicular fluids is indirectly linked to fertilization and early embryonic development (Brannstrom and Norman, 1993; Brannstrom et al., 1994a; Gabrielsen et al., 1996; Hull et al., 1998). Follicular fluid and surrounding tissue contain various lymphocyte subsets which synthesize numerous cytokines. In addition to lymphocytes, cytokines can also be produced by ovarian somatic cells, including luteal, stromal, thecal and granulosa cells. It also contains a variety of factors responsible for the regulation of oocyte development, folliculogenesis and ovulation. The complex composition of the regulatory factors (hormones, cytokines and growth factors) and their concentration in the follicular fluid has an
influence, either direct or indirect, on oocyte viability and development potential.

Concomitant treatment with FSH and increasing doses of either rhIL-1 produced dose-dependent inhibition of the FSH-stimulated progesterone production. Significant suppression of progesterone production (P<0.01 vs. FSH alone) was obtained at doses greater than 0.8 ng/ml of rhIL-1α and at 0.4 ng/ml of rhIL-1β. Both forms of rhIL-1 suppressed progesterone production up to about 55%. In contrast to the inhibitory effects exerted by rhIL-1α and IL-1β on FSH-stimulated progesterone production, both forms augmented FSH-stimulated 20α-hydroxy-4-pregnen-3-one (20c~OH-P) production in a dose-dependent manner. The maximum augmentation was about 32% for rhIL-1α (EC=1.5 ng/ml) and 47% for rhIL-1β (EC=0.16 ng/ml) with significant increases (P<0.05 vs. FSH alone) obtained at doses greater than 8 ng/ml for rhIL-1α and 0.2 ng/ml for rhIL-1β. Neither rhIL-1 had any stimulatory effect in the absence of FSH, rhIL-1α or rhIL-1β alone had no stimulator effect. Treatment of the FSH-containing cultures with increasing doses of either rhIL-1α or rhIL-1β produced dose-dependent inhibition of the FSH-stimulated estrogen production. Significant suppression of estrogen production (P<0.01 vs. FSH alone) was obtained at doses greater than 0.8 ng/ml of rhIL-1α and at doses greater than 0.4 ng/ml of rhIL-1β (Kasson and Gorospe, 1989).

Interleuken-2 (IL-2) was one of the first hormones recognized in the immune system which is 15.5KDa glycoprotein released from activated T-lymphocytes. Its key role is to support clonal expansion of T-cells (Morgan et al., 1976; Ruscetti et al., 1977), including T-cell growth and in the function of other cell types like B-cells, monocytes, and lymphokine-activated killer cells (Harrison and Campbell, 1988; Minami
et al., 1993), which are important for the maintenance of normal protective immune function and auto reactive pathology.

IL-2 is generally required for expansion of populations of T lymphocytes *in vitro* (Smith, 1988). In many cases IL-2 proliferate Ag-specific CD8+ T cells *in vivo* (Kundig et al., 1993; Cousens et al., 1995; Wong et al., 2004; Williams et al., 2006). At low IL-2 concentrations 1) fewer cells are recruited and successfully complete their first division 2) the stochasticity of cell division is increased and 3) the rate, at which the death rate increases with the division number, increases (Vitaly et al., 2007). It is still a moot point whether these influences are because of direct effect of IL-2 or through altered endocrine profile. IL-2 production by ovarian follicular leukocytes is inhibited by granulosa cells, and interaction between granulosa cells and follicular leukocytes may provide important feedback machanisms (Maccio et al., 1993; 1994).

The presence of T-lymphocytes and IL-2 in theca and granulosa cells possibly reflect their physiological obligations in ovarian functions and thus support earlier findings. The most known cytokines related to reproduction are interleukins, colony stimulating factors and interferons. Interleukins produced by the granulosa cell can function in either an autocrine or paracrine fashion to play a significant modulatory role in the regulation of granulosa cell function and ovarian processes (Ingman and Jones, 2008). Among cytokine receptors involved in the control of ovarian functions the most known are tumor necrosis factors. Some cytokines of TGFβ family (activin, inhibin, anti-Mullerian hormone, AMH, bone morphogenetic protein, BMP 2, BMP4, and BMP6) are generated by ovarian follicles, whilst others (BMP6, BMP15 and growth and differentiation factors) are secreted by oocytes (Richards and Pangas, 2010). Furthermore, macrophages in the ovary also secrete cytokines,
including interferon gamma, interleukins, TNF-α and GM-CSF (Ingman and Jones, 2008; Spanel-Borowski, 2011).

The size of the follicle selected for the estimation of GM-CSF in goat ovary was in between 4-6 mm in diameter for both normal and atretic follicles. GM-CSF concentrations ranged from 19.6 to 21.3 pg/ml (mean 19.7 ± 2.9210 pg/ml) in follicular fluid of normal and from 68.4 to 69.1 pg/ml (mean 68.4 ± 0.3283 pg/ml) in follicular fluid of atretic follicle. GM-CSF concentrations were significantly higher in atretic follicle compared to normal follicle ($P < 0.001$). It is possibly because GM-CSF plays a role in inflammatory-like changes in ovary that induces production of TNFα and IL-1 (Hurwitz et al., 1991). This help to facilitate the recruitment of leukocytes to the thecal layer of the follicle through promoting leukocyte adhesion to endothelial cells and inducing somatic cells, including fibroblasts and endothelial cells, to release GM-CSF (Seelentag et al., 1987; Vogel et al., 1987; Wang et al., 1987).

Although the cellular sources of GM-CSF in the ovary is the epithelial-like granulosa cells (Robertson et al., 1992). Alternatively, GM-CSF can be produced by a variety of cell types, including fibroblasts, endothelial cells, T lymphocytes, macrophages and mast cells, which have been localized in the ovary (Gaytan et al., 1991; Brannstrom et al., 1993b). The result of present study support earlier finding using western blot and immunohistochemistry techniques where the GM-CSF protein and its receptor in the mouse ovary were mainly observed in the granulosa cells of the follicle (Salmassi et al., 2004). Cohen et al. (1997) and Tamura et al. (1998) observed a few signals of GM-CSF in the granulosa layer of primary follicles while significant number of GM-CSF was localized in secondary and antral follicle of rat ovary. In the human ovary, parallel and cell specific expression of GM-CSF and GM-CSFR were expressed in the granulosa cells of antral follicles during follicular
development and differentiation in human ovary (Zhao et al., 1998). GM-CSF could induce the cumulus granulosa cells to produce the cytokines or growth factors which are necessary for the development and implantation of the resulting embryo.

The intense reactivity for GM-CSF in large follicles of goat strongly advocates the observations of Ziltener et al. (1993) also reported intense staining mainly in granulosa cells before ovulation using anti-human GM-CSF monoclonal antibody indicating that GM-CSF protein is located mainly in granulosa cells of antral follicle in the human ovary (Brannstrom et al., 1994b). Oocytes appeared not to express any detectable GM-CSF mRNA they contained a weak immunoreactive GM-CSF that may possibly have originated from follicular fluid by perfusion (Zhao et al., 1998). In mouse, TNF was observed in the oocyte of secondary follicle (Chen et al., 1993). In rats, mouse and murine immunohistochemistry of adult ovaries revealed that the ooplasm of the oocyte was a primary site of TNF localization within the secondary follicle (Roby and Terranova, 1989; Marcinkiewiczj et al., 1994).

A potential role for GM-CSF in the ovulatory process was first suggested by the finding that bioactive GM-CSF is secreted from the rat ovary with maximal release occurring at the time of ovulation (Brannstrom et al., 1994a). GM-CSF was found to be present in follicular fluid of both naturally cycling and hyperstimulated women prior to ovulation at value ~2-fold less than that of plasma (Wang and Norman, 1992). The concentration of GM-CSF present in culture supernatants stimulates survival, proliferation and activation of myeloid cells in vitro (Lopez et al., 1992). GM-CSF helps in the regulation of myeloid cell recruitment and activation during inflammatory processes (Wang et al., 1987) and in the reproductive tract (Robertson et al., 1994), which helps to recruit leukocytes into the ovary at the time of ovulation and corpus
luteum development, and subsequently to regulate their behavioural and secretory profile. Indeed, GM-CSF may at least partially account for the chemotactic activity observed in follicular fluid (Herriot et al., 1986). GM-CSF may also target ovarian endothelium, inducing neutrophil adherence (Gamble et al., 1990) and the proliferation and migration of endothelial cells during angiogenesis (Bussolino et al., 1991).

GM-CSF output from the ovary was found to vary according to the stage of the follicle, with highest levels in large sized follicles corresponding with ovulation. Ovarian steroid hormones also involved in enhancing GM-CSF output, shown in murine (Robertson et al., 1994). The peak in GM-CSF secretion also coincides with a period in the cycle when the numbers of GM-CSF responsive leukocytes within the ovarian tissue are high (Brannstrom et al., 1993, Gaytan et al., 1991) suggesting that GM-CSF may have an active role in recruiting these cells.

Imunoactive GM-CSF was detected in human plasma with similar values throughout the menstrual cycle in both normal and ovariectomized women. However, gonadotrophin or steroid hormone-sensitive cells may contribute to the circulating concentrations of GM-CSF since plasma of hyperstimulated women contained significantly more GM-CSF than those undergoing a natural LH surge. Hyperstimulation also significantly increased the concentration of GM-CSF in follicular fluid. This increase suggests that the synthesis of GM-CSF by ovarian cells may be at least partially regulated by gonadotrophins or steroid hormones, as is the case for uterine epithelial cell GM-CSF synthesis (Robertson et al., 1996). Alternatively, the effect of gonadotrophins may be mediated through the enhanced recruitment of trafficking cells, such as macrophages, which can produce GM-CSF when activated.

The elevated level of GM-CSF in normal developing follicles of goat strongly endorse that plasma and follicular fluid concentrations of
GM-CSF are increased significantly in individuals undergoing ovarian hyperstimulation compared with naturally cyclic individuals. The association of GM-CSF levels in the follicular fluid and etiology of infertility was investigated by Calogero et al. (1998). Decrease in concentrations of GM-CSF in the follicular fluid of women with immunologically-based infertility compared to women with tubal factor. Hammadeh et al. (2002; 2003) studied on 160 patients showed no correlation between GM-CSF concentration in the follicular fluid and etiology of infertility or IVF-ET or ICSI outcome (1.45 ± 2.1 pg/ml versus 1.8 ± 3.3 pg/ml in low and high responders in ICSI procedure, respectively). The source of G-CSF synthesis into the follicular fluid is granulosa cells of the follicle and luteal cells (Yanagi et al., 2002). Salamassi et al. (2003) detected increased concentrations of G-CSF in the follicular fluid (median 117.98 pg/ml) compared to serum (median 67.5 pg/ml) in 82 patients undergoing IVF-ICSI program. Yanagi et al. (2002) also identified luteinized granulosa cells as the major ovarian source of G-CSF. The presence of M-CSF in the follicular fluid and serum of infertile patients undergoing IVF-ET procedure was shown by Nishimura et al. (n=90) and Kawano et al. (n=46) (Nishimura et al., 1998; Kawano et al., 2001). Both studies suggested an involvement of M-CSF in the follicular maturation. Follicular fluid from both spontaneous and stimulated cycles contains colony stimulating factor-1 (CSF-1) (Witt and Pollard, 1997; Shinetugs et al., 1999). CSF concentrations in the follicular fluid of 11 patients seem to be significantly higher in the ovulatory phase (median 2017 pg/ml, range 1131 ± 2236 pg/ml) compared with midfollicular phase (median 961 pg/ml, range 830 ± 1340 pg/ml) (Shinetugs et al., 1999), showed almost 7-fold higher concentrations of CSF-1 in the follicular fluid at oocyte retrieval (median 3116 pg/ml, range 1824 ± 5883 pg/ml) compared with blood (median 472 pg/ml, range 250 ± 105 pg/ml). Witt et al., (1997) reported significantly
higher levels of CSF-1 in the follicular fluid (10.0 ± 1.3 ng/ml) compared with serum (3.6 ± 0.3 ng/ml).

Presence of GM-CSF in higher concentration in the follicular fluid observed in normal follicles of goat support the findings of Witt and Pollard (1997) reported that immunoreactive CSF-1 concentrations in follicular fluid were 2.8-fold higher than those in serum, and cells isolated from follicular fluid express both CSF-1 and CSF-1R. GM-CSF concentrations in follicular fluid, obtained from the dominant follicle during the menstrual cycle and ovarian stimulation, were about 4-7-fold higher than those in plasma at the same time, respectively. The markedly elevated concentrations in follicular fluid of IVF patients and higher concentrations in the ovulatory phase compared to the follicular phase during the natural cycle implies that HCG/LH could be the natural trigger for increased follicular CSF-1 production. During ovarian stimulation, ovarian synthesis of CSF-1 may contribute to circulating concentrations of CSF-1, which may have systemic impact on immune cell populations. In addition, progesterone and oestrogen have been shown to induce CSF-1 mRNA expression by endometrium during mouse pre-implantation development (Pampher et al., 1991) and human endometrium (Azuma et al., 1990), which may indicate a steroid controlled expression of CSF-1. Increased concentrations of CSF-1 in follicular fluid may be important in macrophage chemoattraction and in supporting their activity within the follicle, and it may be linked to folliculogenesis, ovulation, corpus luteum function and regulation of progesterone synthesis via secretion of cytokines such as IL-1α or TNFα. In the mouse, the concentrations of CSF-1 bioactivity increased about 1000-fold at the time of pregnancy compared to the non-pregnant state, in contrast to only 1.4-fold increase in serum (Bartocci et al., 1986).
In atretic follicles, the number of CD4\(^+\) cells increased from primary to large-sized follicles as compared to their counterpart normal developing follicles. An influx of many CD4\(^+\) cells was observed not only in the theca and granulosa layer, but also in the follicular cavity of late atretic follicles in which loss of the granulosa layer and invasion of cells into the follicular cavity were observed. The weak reactivity for CD4\(^+\) was observed in primordial follicles. In thecal cells, the maximum binding was seen in secondary and dominant follicles and their intensity was increased from mild to moderate in atretic follicle. In oocyte the frequency of CD4\(^+\) cells was moderate to intense in atretic follicles. The percentage of CD4\(^+\) cells in granulosa, theca, cumulus cells and oocyte were increased from 41.22, 35.79, 57.39 and 70.83 to 78.51, 59.76, 78.27 and 90.01 respectively in normal large antral follicles and atretic antral follicles. The frequency of CD4\(^+\) cells was higher in the atretic follicles at all stages.

The maximum staining was observed in both normal and atretic dominant follicles, moderate in primary and least or absent in the primordial follicles. In atretic follicles, number of CD8\(^+\) cell type was not observed in primordial and primary follicles but in large sized follicles their number was increased as compared to their counterpart normal developing follicles and their intensity in granulosa and theca was mild to moderate and moderate to intense in secondary and dominant follicles, respectively. The number of percentage in granulosa, theca, cumulus cells and oocyte were increased from 37.10, 32.27, 52.11 and 67.73 to 68.65, 80.44, 73.03 and 94.81 respectively in normal large antral follicles and atretic antral follicles. The frequency of CD8\(^+\) cells was higher in the atretic follicles at all stages.
primordial follicles. The frequencies of CD4\(^+\) and CD8\(^+\) lymphocytes in the follicles changed during the process of atresia. The death of granulosa and theca cells by apoptosis is responsible for the regression of atretic follicles. Macrophages which are known to play a role in the removal of apoptotic cells and degraded tissue components increase in the follicles of the late atretic phase because CD4\(^+\) inducer T-cells in the late atretic follicles may play a role in stimulating macrophages to remove apoptotic cell bodies. CD8 molecules are expressed in the cytotoxic/suppressors T-cells, which are stimulated by intracellularly synthesized antigens in infected cells and then kill such cells by cytotoxic effects.

The results of present finding strongly support that the immune cells present in greater number both in theca and granulosa layer of atretic follicles (Pestka et al., 1987; Fukuoka et al., 1992; Wang et al., 1992a; Best et al., 1995; Sharma and Gandhi, 2010) and their number increased from the secondary follicle to dominant follicles which is responsible for macrophage activation and ovarian steroid inhibition through the secretion of IFN-\(\gamma\).

Neutrophils are the most prominent immune cells which are active during the initial stages of acute inflammation. They are specialized in phagocytosis and the destruction of bacteria, as well as having potent inflammation-promoting actions by the secretion of pre-formed toxic granule enzymes (cathepsins, collagenase, myeloperoxidase, lysozyme, elastase) and by the direct secretion of cytokines (TNF\(\alpha\), IL-1), eicosanoids, reactive oxygen metabolites, and platelet activating factor (Brannstrom and Norman, 1993). Neutrophils have been demonstrated in the ovary of several species by conventional histological techniques and by immunohistological methods using specific monoclonal antibodies. The presence and potential involvement of neutrophils in the human follicular development and atresia studied by Chang et al. (1998). Atretic
follicles contained relatively large numbers of neutrophils within the theca vasculature. In contrast, preantral follicles were found to be totally unassociated with the presence of surrounding neutrophils was 2-fold greater in atretic versus healthy follicles. The neutrophil index (neutrophils/granulosa cells X 1000) was inversely correlated with the number of granulosa cells per follicle.

Most authors have reported that macrophages are abundant in the ovarian medulla (Bulmer, 1964; Paavola and Boyd, 1979; Hume et al., 1984; Brannstrom et al., 1993b; 1994a; Simon et al., 1994; Bonello et al., 1996; Cohen et al., 1997; Hoek et al., 1997; Gaytan et al., 1997). Follicle-associated macrophages are located exclusively in the theca layer, related to perifollicular blood vessels but are absent from inside the follicle itself, except in the phase of atresia in which the presence of macrophages has been related to the phagocytosis of apoptotic granulosa cells (Brannstrom et al., 1993b; Petrovska et al., 1996; Hoek et al., 1997). Macrophages are found in the granulosa cell layer at only advanced stages of atresia (Petrovska et al., 1996) and because they are known to phagocytose apoptotic cells it is thought that they participate in the removal of cell debris created during granulosa cell apoptosis (Takaya et al., 1997; Gaytan et al., 1998a). However, they may also have an active role in the production of factors promoting follicular atresia.

In mouse ovary, TNF was observed in the granulosa cells in primary, secondary and antral follicles. In atretic follicles, granulosa cells exhibited TNF in large number (Chen et al., 1993). In cow, immunoreactivity of TNF was also observed in the granulosa layer of atretic antral follicles but not in the primordial, primary and secondary follicles (Roby and Terranova, 1989). In atretic antral follicles the number of TNF was increased in the granulosa layer (Roby et al., 1990). In atretic follicles of mouse ovary, IL-1Rt1 was observed in granulosa
layer (Simon et al., 1994). T-lymphocytes were found within the stroma of the ovary suggesting that these cells may be employed when needed for potential repair and clean-up function (Hill et al., 1987; Sharma and Esha, 2010). Other leukocytes include thymus derived T-lymphocytes, mostly of the cytotoxic/suppressor subtype, also present in the stroma. There is a fairly large population of macrophages close to capillaries in the stroma of the human ovary (Takaya et al., 1997). Katabuchi et al. (1989) identified a large population of macrophages close to the perifollicular capillary network of the stroma. Brannstrom et al. (1993b) demonstrated the presence of neutrophils in the stroma. During follicular development, localization of IL-1α and IL-1β was observed in the stroma (Simon et al., 1994).

For the experiment, 72hrs cultured granulosa cells were smeared on poly-L-lysine coated slide and TUNEL assay was carried out for the observation of apoptotic granulosa cells with increasing dose level of IL-2 (25 ng/ml, 50 ng/ml and 100 ng/ml). In 3’end labeling, single small densely staining nucleus having pyknotic appearance were frequently observed. A few cells with marginated chromatin material in the nucleus were also seen. The frequency of TUNEL positive apoptotic cells was sparse in granulosa cells without treatment. This number was highly increased in T3 (100 ng/ml) dose level of IL-2 thereby indicating the proapoptotic role of IL-2 in atresia. The molecular mechanism involved is similar to that of corpus luteum regression.

2. Corpus luteum

The changes in the distribution of CD4+, CD8+, and IL-2+ cell in the goat ovary during corpus luteum formation and regression were studied. Negligible reaction for these antibodies was seen in the healthy corpus luteum, but intense and pronounced reactivity in the corpus luteum at the regression stage was observed. The frequency of CD4+ and CD8+
cells were 93% and 89% respectively in regressing corpora lutea. Furthermore, it was examined that the changes in the localization of IL-2 in corpus luteum during regression. No obvious difference in the localization of IL-2 was seen. Scattered moderate positive staining for IL-2 was observed in all corpora lutea. Strong reactivity for IL-2 was also observed in stroma while negative in the blood vessels. IL-2 cells were also observed from early to late stages of corpus luteum development and regression with different intensity of staining.

In ruminants CD4+ and CD8+ cells were present in approximately equal numbers during dioestrus. The number of CD4+ cells increased significantly in the corpus luteum after luteolysis as compared with early and mid-luteal phases. As this increase occurred after functional luteolysis, the influx may not be relevant to the regression of the corpus luteum. However, CD8+ cytotoxic cells increased significantly before functional luteolysis, which indicates a role for these cells and their products in luteolysis (Lunn et al., 1991). Goat corpus luteum in this regard is similar to that of other domestic animals. In the horses, the ratio of CD4+: CD8+ cells is > 2:1 (Lunn et al., 1991), indicating that there is selective infiltration of CD8+ lymphocytes into the equine corpus luteum. In cows, CD8+ cells appear to be involved in functional luteolysis (Ndikum-Moffor et al., 1994). Thus, it seems that these cells may have a physiological role in control of ovarian function. Activated lymphocytes are a rich source of a variety of cytokines such as IL-2 and interferon (IFN), both of which affect steroidogenesis of human granulosa-lutein cells (Wang et al., 1991; 1992). IFN also alters bovine luteal cell prostaglandin production and progesterone synthesis (Fairchild and Pate, 1991), which indicates a role in luteolysis, and can also have direct cytotoxic effects on cells especially when present in combination with some other cytokines (Campbell et al., 1988; Fairchild and Pate, 1991; 1992).
Presence of CD4$^+$ and CD8$^+$ in corpus luteum of goat like other T-lymphocytes secrete most cytokines, of which some may influence the corpus luteum in a luteotrophic fashion, as demonstrated by the ability of lymphocytes to stimulate human luteinized granulosa cells to increase progesterone synthesis (Emi et al., 1991) yet in goat such studies are still lacking. The presence of neutrophils in the early and mid-cycle corpus luteum has not been particularly well investigated in any species. Relatively high density of neutrophils found in the corpus luteum during the early phases of pregnancy and pseudopregnancy in the rat (Brannstrom et al., 1993e). Using conventional histological techniques, Standaert et al. (1991) found extremely low numbers of neutrophils in the pig corpus luteum at these stages, while in the sheep a decrease in the density of neutrophils after ovulation was reported (Cavender and Murdoch, 1988). In the human corpus luteum the presence of high numbers of neutrophils (Brannstrom et al., 1993b), and there is a significantly greater density of neutrophils in the theca-lutein area as compared to the granulosa-lutein area observed (Brannstrom et al., 1993b). Neutrophilic granulocytes may have many functional roles in the early corpus luteum, such as regulating the secretion of plasminogen activator, collagenase and elastase in angiogenic processes.

The pattern of changes in CD4$^+$ and CD8$^+$ cells observed within the corpus luteum of different categories in goat is similar to the changes observed in other mammals. Temporal changes of immune cell populations within the ovary and within the corpus luteum are reported in cows, rats, rabbits, and humans (Bagavandoss et al., 1988; 1990; Petrovska et al., 1992; Brannstrom et al., 1994a, b; Spanel-Borowski et al., 1997; Gaytan et al., 1998; Penny et al., 1999; Pate and Keyes, 2001). Especially the significant invasion of macrophages into the corpus luteum of rabbits, rats, and cows at the end of luteal life demonstrates that macrophages are involved in luteolysis (Bagavandoss et al., 1988; 1990;
Immune cells are 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).

The preponderance of lymphocytes in goat corpus luteum is primarily proinflammatory source of 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; Senturk et al., 1999; Penny et al., 1999; Chen et al., 2000; Penny, 2000; Suter et al., 2001; Townson et al., 2002). The leukocyte subtype, which has been most-well studied in relation to the luteolytic process, is the macrophage. Class II MHC positive macrophages are the most prominent immune cells within the human corpus luteum throughout its lifespan (Petrovska et al., 1992). The variation recorded during corpus luteum life span of goat depicts similar pattern there by indicating an identical physiological regulatory mechanism.

T-lymphocytes and macrophages were commonly observed in the regressing corpus luteum of goat. Several studies also have analysed macrophages during corpus luteum lifespan. Macrophages have been described predominantly in the theca-lutein layer (Lei et al., 1991; Wang et al., 1992) at a maximum in the late luteal phase (Duncan et al., 1998). Another study found that in the human ovary the number of CD68
positive macrophages increased up to the end of the early luteal phase, remained relatively unchanged during the mid luteal phase, and decreased at the late luteal phase (Gaytan et al., 1998b), paralleling the functional activity of the corpus luteum. Another study, however, found that the proportion of macrophages in the human corpora lutea did not differ between different stages of the luteal phase (Castro et al., 1998). The number of macrophages in the corpus luteum is highest during regression, implicating these cells in luteolysis (Paavola and Boyd, 1979; Lei et al., 1991; Brannstrom et al., 1994b; Hameed et al., 1995; Best et al., 1996; Senturk et al., 1999). Inflammatory cell infiltration begins in the theca lutein and gradually invades the granulosa lutein, but this infiltration can be prevented by inhibition of luteolysis with HCG to mimic pregnancy (Duncan et al., 1998). In the human, the progressive infiltration of lymphocytes and macrophages during luteal regression may occur in response to MCP-1 expression in blood vessels within the corpus luteum (Hameed et al., 1995). In the rat, increased endothelial cell expression of the intercellular adhesion molecule (ICAM)-1 occurs concurrently with macrophage infiltration, suggesting that ICAM-1 mediates macrophage migration into the regressing corpus luteum (Olson et al., 2001). The influx of macrophages also coincides with increased MMP activity in luteal cells (Duncan, 2000) and increased macrophage phagocytosis (Takaya et al., 1997), both of which are thought to be important for ingestion of cellular remnants that result from luteal cell apoptosis (Pate and Keyes, 2001). Macrophages comprised at least 10% of the total cells in the corpus luteum, distributed equally between the theca-luteal and granulosa-luteal areas. A significant number of macrophages could also be detected in the central loose connective tissue area of the corpus luteum. Concentration of macrophages in the boundary area between the granulosa-luteal and theca-luteal cells, indicating that there may be production of a chemoattractant by cells in that area.
Zhao et al. (1995), suggests that GM-CSF is not synthesized by granulosa cells but rather than cells of the theca may produce GM-CSF which targets granulosa cells, while luteal cells appear to be both a source and a target for GM-CSF. Leukocytes, which comprise up to 20% of granulosa-lutein cell preparations (Wang et al., 1995), and connective tissue and endothelial cells as well as leukocytes, which comprise up to 80% of the cells in the corpus luteum (Lei et al., 1991), are also potential sources of GM-CSF in the culture preparations.

Variations in the frequency of different types of lymphocytes clearly indicate that immune cells are the mobile cells that can affect both endocrine function and the structure of the corpus luteum during luteinization and maintenance of luteal function. No macrophages or T lymphocytes were seen in healthy corpus luteum, but abundant macrophages and increasing T lymphocytes were seen in corpus luteum at the functional regression stage (early stage of regression). At the structural regression stage (late stage of regression), abundant T-lymphocytes but no macrophages were demonstrated in the corpus luteum.

The high frequency of CD4+ and CD8+ cells in regressing corpus luteum of goat may be because of the cellular interaction between leukocytes and luteal endothelial cells probably determines the recruitment of leukocyte populations, or may induce proliferation of resident leukocytes within a corpus luteum (Schlatt et al., 1999). The major proportion of proliferating cells in the corpora lutea of rats, cows and primates are leukocytes (Jablonska-Shariff et al., 1993; Zheng et al., 1994; Christenson and Stouffer, 1996). These cells represent approximately 50% of the total number of cells in a fully developed corpus luteum (Farin et al., 1986; Lei et al., 1991; Reynolds and Redmer, 1999). First time T lymphocytes were observed in the rabbit corpus
luteum where its density was about 10-20% during early and mid-luteal phases of cycle (Bagavandoss et al., 1990). In cow and pig abundance of leukocytes was observed during regressing corpus luteum (Jablonka-Shariff et al., 1993; Zheng et al., 1994; Spanel-Borrowski et al., 1997; Reynolds and Redmer, 1999). Thus, leukocytes that are proliferating may occur in the regressing bovine corpus luteum throughout the estrous cycle. The changes observed in regressing corpus luteum in terms of enhanced frequency of lymphocytes strongly endorse the earlier findings on mammalian corpus luteum.

The majority of the leukocytes were localized in the theca-luteal area. There were relatively few leukocytes in the granulosa-luteal area (Wang et al., 1992). T lymphocytes in the human corpus luteum detected by using different antibodies to show that the ratio of T4 to T8 cells was about 2:1 in the theca-luteal area and 1:1 in the granulosa-luteal area (Hill et al., 1987).

Although during present study no significant change in IL-2 reactivity was observed in corpora lutea of different categories in goat. However in humans a significant number of T cells in the corpus luteum where activated T cells were detected by an anti-IL-2 receptor antibody and these cells corresponded with T4 cells in that area. This indicated that there are functional T cells in the human corpus luteum with IL-2 receptors permitting the possible effect of IL-2 on cells of the corpus luteum. Small groups of T cells were frequently observed near the capillaries of the theca-luteal area, indicating that these cells may migrate from the blood vessels into the substance of the corpus luteum. One of the most important functions of the T4 cells is to help the B cell respond to protein antigen (Wang et al., 1992).

CD4+ cells were also observed from early to late stages of corpus luteum development and regression with different intensity of stain. A
small number of CD4\(^+\) cells were also found in the blood capillaries around the follicles. A few of the CD4\(^+\) cells were also observed in the ovarian interstitial gland tissue and stroma surrounding follicles. Macrophages also have the capacity to secrete factors that may stimulate apoptosis in the corpus luteum, such as reactive oxygen intermediates, and particularly TNF-\(\alpha\). TNF-\(\alpha\) mRNA has been observed in the corpus luteum of the mouse (Chen et al., 1993), human (Kondo et al., 1995) and rat (Marcinkiewicz et al., 1994), and in the rabbit, where it was associated with macrophage numbers (Bagavandoss et al., 1988; 1990). In the bovine corpus luteum, macrophages induces apoptosis, via TNF-\(\alpha\) receptor type I as macrophages are primary source of TNF-\(\alpha\) (Zhao et al., 1998; Friedman et al., 2000).

The peak density of macrophages within the corpus luteum occurs during luteal regression (Bagavandoss et al., 1990; Lei et al., 1991; Brannstrom et al., 1994a, b). A large portion of the macrophages expresses class II MHC antigen (Brannstrom et al., 1994a). Phagocytic activity of the macrophages within the corpus luteum was demonstrated in guinea pig (Paavola, 1979). The human corpus luteum during the menstrual cycle can be rescued temporarily from luteolysis by exogenous LH and HCG (Duncan et al., 1998). T-helper lymphocytes are present within the regressing corpus luteum (Bagavandoss et al., 1990; Brannstrom et al., 1994b), with a modest increase in numbers just before luteolysis (Penny et al., 1999).

Macrophages may be one source of PGF2\(\alpha\) or, via TNF-\(\alpha\) secretion, may stimulate PGF2\(\alpha\) production by luteal cells (Fairchid and Pate, 1992; Wang et al., 1992). Macrophages may also inhibit progesterone production directly, since removal of leukocytes from luteal cell cultures has been shown to increase progesterone levels (Kohen et al., 1999). TNF-\(\alpha\) has also been shown to inhibit progesterone secretion by mouse
luteal cells (Adashi et al., 1990) and TNF-α and IL-1β both decrease progesterone production and inhibit survival of bovine luteal cells (Fairchild and Pate, 1992). A major involvement of macrophage-derived products in enhancing preovulatory progesterone production is also possible. In co-incubation studies it was demonstrated that peritoneal macrophages stimulated both human granulosa-lutein cells (Halme et al., 1985) and mouse granulosa cells (Kirsch et al., 1981), to increase progesterone production.

IL-2 has been proved to influence steroidogenesis in goat but IL-1 has no effect on hormone secretion of differentiated rat luteal cells (Fukuoka et al., 1992). This cytokine is able to block the synthesis of androgens induced by gonadotrophins. The shortfall in the provision of androgens could explain the negative effects of IL-1 on estrogen synthesis (Hurwitz et al., 1991). Some researchers have reported a positive regulation of IL-1 on progesterone production released from human granulosa-luteal cells (Sjogren et al., 1991; Fukuoka et al., 1992) and rat granulosa cells (Brannstrom et al., 1993a). However, Gottschall et al. (1988) shown that IL-1α inhibits LH-induced luteinization and progesterone production of rat granulosa cells and porcine granulosa cells (Fukuoka et al., 1988; 1989).

The result of present in vitro findings are contrary to human granulosa-lutein cell culture, wherein (3H) thymidine incorporation into cultured cells is increased by IL-2, while basal progesterone levels are not changed by this cytokines (Wang et al., 1991; Norman and Wang, 1991). IL-2 at a concentration 19.4 fmol/l suppresses HCG-stimulated progesterone secretion from luteinized granulosa cells grown in vivo (Wang et al., 1991). In the presence of FSH; rhIL-2, at doses of 28 and 56 ng/ml, significantly (P < 0.01 vs. FSH) augmented FSH-stimulated progesterone production by up to 50%. IL-2 stimulates cell proliferation
and inhibits hCG-induced progesterone production by luteinized human granulosa cells (Wang et al., 1991).

Many studies indicate luteal cells and macrophages as the main sources of TNF-α in the human (Roby et al., 1990), bovine (Roby and Terranova, 1989; Zolti et al., 1990) and rabbit corpus luteum (Bagavandoss et al., 1990). Changes in TNF-α immunostaining were observed throughout the luteal phase. This may suggest the involvement of TNF-α in corpus luteum development and maintenance. Moreover, the presence of TNF-α in luteal cell possibly indicates a paracrine and autocrine luteotrophic role for TNF-α (Adashi et al., 1990). Kirsch et al. (1983) and Terranova et al. (1991) assumed that TNF-α is one of the factors mediating luteotrophic function and stimulating luteal progesterone secretion. Increase in granulosa proliferating pig cells treated with TNF-α was noticed in vitro (Prange-Kiel et al., 2001). Omar et al. (2008) observed the intense immunostaining during late regression phase suggests a possible role for TNF-α in luteal regression.

A small number of CD8+ cells were also found in the blood capillaries around the follicles. CD8+ cells were also observed from early to late stages of corpus luteum development and regression with different intensity of stain. A few of the CD8+ cells were also observed in the interstitial gland tissue and ovarian stroma surrounding follicles. Lymphocytes and macrophages observed during luteolysis in goat are indicative of their involvement in the regression of corpus luteum. Bioactivity of TNF-α was found in the sheep corpus luteum after pharmacologic induction of luteolysis (Ji et al., 1991), and in the bovine corpus luteum, elevated TNF-α mRNA levels were seen at luteolysis (Petroff et al., 1999). TNF-α seems to have direct luteolytic effects, because exogenous TNF-α decreased progesterone secretion from bovine luteal cells (Fairchild and Pate, 1992). A potent cytotoxic function of
TNF-α on luteal cells was demonstrated when the cytokine was combined with IFN-γ. A functionally different luteolytic effect by TNF-α may be stimulation of apoptosis, as demonstrated in human luteinized granulosa cells (Matsubara et al., 2000) and also on the endothelial cells of the bovine corpus luteum (Friedman et al., 2000).

As TNF-α can induce necrosis of endothelial cells of tumors, it was proposed that similar mechanisms operate in luteal regression. Immunoactivity against TNF-α is present in the bovine, human and rat corpus luteum (Roby and Terranova, 1989) and incubated regressing corpus luteal tissue from the rabbit secretes bioactive TNF-α when stimulated by lipopolysaccharide IFN-γ in vitro (Bagavandoss et al., 1990). The effect of TNF-α on corpus luteum cells in vitro has been particularly well-studied in the cow, where it was found that recombinant bovine TNF-α inhibits basal progesterone synthesis, while stimulating prostaglandin secretion (Fairchild and Pate, 1992). In the sheep corpus luteum TNF-α bioactivity could be extracted at luteal regression, but the drop in luteal progesterone concentrations occurred before any detected rise in TNF-α bioactivity (Ji et al., 1991). This suggests that TNF-α is not involved in the immediate onset of luteal regression, but may well be operative in later phases. In the pig, TNF-α administration by microdialysis induces functional luteolysis (Wuttke, 1992). TNF-α by itself does not alter progesterone output of cultured human luteal cells, but significantly potentiates the IFN-γ-induced progesterone inhibition (Wang et al., 1992c).

TNF-α, has also been implicated in angiogenic processes (Vassalli, 1992), and it augments progesterone production from human luteinized granulosa cells in culture (Yan et al., 1989). TNF-α links to the functional and structural regression process of corpus luteum (Bagavandoss et al., 1987; Quirk et al., 2000) but, Komatsu et al. (2003) found that no
obvious changes in TNF-α expression in corpus luteum during the regressing process.

Ultrastructure of membrana granulosa in caprine ovary was studied by Sharma and Sawhney (1999). The caprine granulosa cells showed resemblance in their fine structure with that of rat, mouse, sheep, bovine and human granulosa cells (Zamboni, 1974; 1980; Dvoark and Tesarik, 1980; Espey and Lipner, 1994; Greenwald and Roy, 1984). The ultrastructure of normal granulosa cells revealed the same observations. The ultrastructural details of granulosa cells studies are indicative of protein and mucopolysaccharide synthesizing nature as reported in cattle (Guraya, 1997).

The fine structure of mural and cumulus granulosa cells of antral follicles shows similar ultrastructural organization. However, these cells differ from the preantral granulosa in terms of smooth endoplasmic reticulum and lipid droplets thereby suggesting their changed metabolic obligations as suggested by Ax et al. (1984) in bovine. These workers attributed these changes to alterations in estrogen content of developing bovine granulosa cells.

The cytoplasmic extensions include cell organelles like mitochondria, lipid droplets and vacuoles morphologically resembling the reminiscent lysosomes. A morphologically similar process was reported in a number of animals (Merk et al., 1973; McGaughey et al., 1990; Philips and Dekel, 1991; Sharma and Shawney, 1999).

The ultrastructural studies of cumulus oophorus complexes have demonstrated the presence of structural interactions between oocyte and granulosa cells. Present electron microscopic studies revealed that gap junctions, structures implicated in cell to cell communication and metabolic contact both of the cumulus cells and oocyte, and cumulus cells
themselves. Furthermore, these studies have also revealed an elaborate gap junctional network throughout follicular membrana granulosa. These observations are in agreement with reports of Sotelo and Porter (1959), Bjorkman (1962), Larsen et al. (1986), Wert and Larsen (1989), McGughey et al. (1990), Guraya (1997). The caprine ovarian follicular fine structural details suggested follicle as a syncytium involving membrane granulosa and the oocyte that provides the structural basis for the transfer of nutritional and regulatory molecules from somatic cells into oocyte.

Membrana granulosa of goat showed a great degree of pleiomorphism than cumulus junctions and may be very extensive in the mature antral follicle. Larsen et al. (1981, 1987) and Racowksy et al. (1989) have demonstrated similar gap junctional variations in rat, hamster and rabbit, respectively.

In granulosa cell apoptosis the primary event is the nuclear compaction: the chromatin collapses into large irregular masses surrounded by nuclear envelope (Kerr et al., 1972) and the plasma membrane interflexes forming deep incisions that confer to the cell a very irregular appearance which supports the present studies. Swollen mitochondria, irregular nuclear membrane, large vacuoles were observed during electron microscopy which is in accordance with the studies of Sharma and Shawney (1999); Sharma and Bhardwaj (2009).

3. **Hormonal changes**

Estradiol concentration in follicular fluid increased significantly (0.05) on the 3rd day of the culture in the large non-atretic follicles and afterwards it declined. The same pattern was observed in the large atretic follicles also but the concentration of estrogen was higher in the large non-atretic follicles. Progesterone concentration in the follicular fluid of large non-atretic follicle was increased up to the 5th day of the
culture, in contrast, in large atretic follicle, the concentration of progesterone was increased up to the 3\textsuperscript{rd} day of the culture and afterwards the level of progesterone was decreased. The total amount of the two principal steroids (estrogen and progesterone) secreted into culture medium by large atretic and non-atretic follicles on 1\textsuperscript{st}, 3\textsuperscript{rd} and 5\textsuperscript{th} days of culture. During the first 24 hrs in culture large non-atretic follicle secreted significantly more steroid (P<0.05) than large atretic one. By day 3 steroid output by both groups had fallen, but atretic follicles were still secreting twice as much steroid as they had been on day 1\textsuperscript{st}.

Granulosa cells were obtained from medium sized follicles (6 to 8 mm) and treated with increasing doses of IL-2 in the presence or absence of FSH (100 ng/ml) for culture at 37\degree C and supernatant for hormonal assay was collected at every 3\textsuperscript{rd} day of culture. It was found that IL-2 evoked a dose-dependent effect on estrogen and progesterone production. The level of estrogen was decreased and progesterone was increased as the dose of IL-2 was increased. The effect of interleukin-2 on FSH-induced progesterone production by caprine granulosa cells, cells were obtained from ovarian follicles cultured with FSH (10 µg/ml) and testosterone (1 µg/ml) to maximize aromatase activity. After 24 hr of culture, interleukin-2 (25, 50 and 100 ng/ml) was also added to the culture medium. In culture of cells, interleukin-2 had stimulatory effect on FSH-induced progesterone production. If we compare the control value with the treatment at the dose level of 25 ng/ml interleukin-2, progesterone production was not so effected in passage 1. However as the dose level was increased by 50 and 100 ng/ml the production of progesterone was also increased up to 20.83%. With every passage, the production of progesterone was increased as the dose level of interleukin-2 was increased. In culture of cells, interleukin-2 only reduces the number of viable cells but had no effect on control. As the passages were increased cell viability also reduced.
In the presence of IL-2, the production of progesterone increased as the dose level of interleukin-2 was increased. In culture of granulosa cells of goat ovary, interleukin-2 only reduces the number of viable cells but had no effect on control. As the passages were increased cell viability also reduced (Sharma and Gandhi, 2011). Whereas, the estrogen production decreased as the dose level of interleukin-2 increased. In culture of granulosa cells, interleukin-2 only reduced the number of viable cells but had no effect on control. As the passages were increased cell viability also reduced (Sharma and Gandhi, 2011).

The direct in vitro effects of rhIL-2 on progesterone production by granulosa cells are depicted by Kasson and Gorospe (1989), in the absence of FSH; rhIL-2 not induced production of progesterone. IL-2 has no effect on LH/hCG induced steroid secretion, while it increases FSH induced progesterone synthesis and has no effect on estradiol (Mikuni, 1995). In granulosa cells culture, it provokes an increase in progesterone production by stimulating the 3β-HSD activity (Gorospe and Kasson, 1988). Lymphomonocytes have been found in preovulatory follicles and their specific products, cytokines (IL-2), were demonstrated to inhibit steroidogenesis. Ovarian steroids, in turn, reduce the cytokine production from immune-competent cells. However, unfavorable effects of IL-2, cytotoxic and antisteroidogenetic activities, are counteracted by the products of granulosa cells.

The demonstration of IL-8 as a proliferative agent on endometrial stromal cells (Arici et al., 1998), and the evidence that FSH induces follicular growth through an indirect regulation of growth factor expression in granulosa cells (Parrott and Skinner, 1998), supports the hypothesis that the FSH-induced IL-8 production from granulosa cells contribute to the proliferative events occurring in the developing follicle.

The effect of interleukin-2 on FSH-induced estradiol production by caprine granulosa cells, cells were obtained from ovarian follicles,
cultured with FSH (10 µg/ml) and testosterone (1 µg/ml) to maximize aromatase activity. After 24 hr of culture, interleukin-2 (25, 50 and 100 ng/ml) was also added to the culture medium. In culture of cells, interleukin-2 had inhibitory effect on FSH-induced estradiol production. If we compare the control value with the treatment at the dose level of 25 ng/ml, 50 ng/ml and 100 ng/ml the maximum inhibition was observed in the highest dosage of interleukin-2 i.e 100 ng/ml about 20.081%. With every passage, the production of estrogen was decreased as the dose level of interleukin-2 was increased. In culture of cells, interleukin-2 only reduces the number of viable cells but had no effect on control. As the passages were increased cell viability also reduced.

Progesterone production by caprine granulosa cells in the absence of FSH, cells were cultured without FSH and testosterone. After 24 hr of culture, interleukin-2 (25, 50 and 100 ng/ml) was added to the culture medium. In culture of cells, interleukin-2 had not detectable effect on progesterone production in the absence of FSH. If we compare the control value with the treatment at the dose level of 25 ng/ml, 50 ng/ml and 100 ng/ml interleukin-2 on the progesterone production, there was not so change observed in all dose levels. In culture of cells, interleukin-2 only reduces the number of viable cells from in control as well as in treatment also. As the passages were increased cell viability also reduced.

In culture of cells, interleukin-2 had not detectable effect on estradiol production in the absence of FSH. If we compare the control value with the treatment at the dose level of 25 ng/ml, 50 ng/ml and 100 ng/ml interleukin-2 on the estradiol production, there was not so change observed in all dose levels. In culture of cells, interleukin-2 only reduces the number of viable cells from in control as well as in treatment also. As the passages were increased cell viability also reduced. FSH is the most important factor for follicle growth in the preovulatory phase and a lack of FSH could lead to follicular atresia through apoptosis. Our results
suggest that FSH may exert a promoting effect on granulosa cells. Correlation coefficient was computed between control and treated variables. Correlation coefficient was obtained in order to study the degree of relationship between control and treated variables. Two tailed Pearson correlations ($r = 0.998$) was significant at 0.01 level.

In the absence of interleukins, FSH increased (125) iodo-hCG binding capacity and when cells were incubated with FSH and increasing amounts of rhIL-2 it inhibit the induction of LH/hCG receptors by FSH. Fukuoka *et al.* (1987) and Gottschall *et al.* (1988), reported that IL-2 had no effect on progesterone production by granulosa cells, FSH-stimulated progesterone was significantly affect at a relatively high dose (28ng/ml) of rhIL-2. In addition, the progesterone metabolite, 20c~OH-P, and total progestins were also increased at this dose. In contrast, IL-2 did not affect estrogen production or LH/hCG receptor induction.

Effect of IL-2 on viability (by hemocytometer) and size of granulose cells was analysed in the presence and absence of FSH at 12 and 24hrs of culture. In the presence of FSH, the viability decreased from passage I to passage VI but it increased as the dose level of IL-2 was increased from 25 to 100 ng/ml. In the absence of FSH, the viability decreased from passage I to passage IV and also decreased as the dose level of IL-2 was increased from 25 to 100 ng/ml. On the size of granulosa effect of IL-2 was not significantly observed in any of the respective doses i.e. 25, 50 and 100 ng/ml.

Salmassi *et al.* (2001) observed that relatively low concentrations of IL-6 are capable of exerting a potent inhibitory effect on the estradiol production of human granulosa cells. The inhibitory effect of IL-6 on the FSH driven estradiol and progesterone production may suggest that gonadotropin regulation of granulosa cells steroidogenesis can be modulated by non-steroidal factors, including cytokines. This track seems to be one of the mechanisms of the ovarian autoregulation. IL-6
production by granulosa cells may well mean that the steroidogenesis of granulosa cells is coupled to IL-6 production. The inhibitory effect of IL-6 could then readily be regarded as part of a rebound mechanism to down regulate hormone production. This effect is concentration-dependent. It has also been observed that locally available IL-6 possibly originated from resident ovarian macrophages or ovarian stromal cells. The contribution of these cells to local IL-6 concentration has been shown to represent the centerpiece of an intra-ovarian regulatory loop promoting granulosa cells differentiation into lutein cells (Hurwitz et al., 1991, Schoester et al., 1994; Breard et al., 1998). The results of the influence of IL-2 on granulosa viability and regulation of steroidogenic activity potential observed in goat ovary show a close similarity to that of IL-6 observed in human granulosa cells.

IL-6 added to granulosa cells cultures inhibits differentiated functions induced by FSH such as aromatase activity, LH receptor expression and progesterone production. IL-6 has no effect on stimulatory human chorionc hormone effects on progesterone release. IL-6 might act likely by reducing FSH binding capacity (Machelon et al., 1994). In bovines, rabbits and rats, IL-6 has been found to be produced by cultured granulosa cells, influencing folliculogenesis and granulosa cells steroidogenesis (Adashi, 1990; Gorospe et al., 1992; Gorospe and Spangelo, 1993; Alpizar and Spicer, 1994; Breard et al., 1998). There is a communication between endocrine and immune systems and this is of particular relevance to the ovary, where the role of IL-2 in ovarian endocrinology studied in some animal models in vitro, based on the concept of a putative bi-directional network between the immune and reproductive endocrine systems in that organ (Wang and Norman, 1992).