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Role of Different Hormones (Testosterone, Estrogen, Melatonin, Glucocorticoid, Thyroxin) in Immune Modulation of Thymocyte and Splenocyte Functions of Indian Goat *C. hircus*: An *in vitro* Study

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Abstract **Original Research Article**

The immune system is coordinated by a number of cytokines which are regarded as chemical messengers of immunity. Some of them are pro-inflammatory (e.g. IL-2), some are anti-inflammatory (e.g. IL-6), some can be regarded as a switch between pro and anti-inflammatory processes (e.g. TNF-α). Further, immune regulation in body is a proper balance between mitosis and apoptosis occurring simultaneously in the body. In the present chapter we explored the spleen and thymus functions by assessing %SR and % apoptotic rate of splenocytes and thymocytes being significantly high levels of cell proliferation (in terms of %SR) and apoptosis rate during monsoon and winter seasons. We noted IL-2 (a pro-inflammatory cytokine), IL-6 (an anti-inflammatory cykine), TNF-α (a switch between pro and anti-inflammatory cytokine) and IFN-γ (a marker of viral infection) in circulation of goats. We noted significantly high levels of IL-2 and TNF-α levels during monsoon and winter but IFN-γ and IL-6 levels were only high during monsoon. Hence, to ameliorate the elevated inflammatory stress level particularly during monsoon goats have evolved a number of adaptive strategies. But, simultaneously during monsoon the gonadal steroid levels are also high which are reported as immune suppressor. Thus, the basic query may arise how goats are proved to be a better survivor under the season of stress (particularly during monsoon) when the level of potent immune enhancer neurohormone melatonin level is also low.

Keywords: Estrogen, Goat, Glucocorticoid, Hormone, Immunity, Melatonin, Testosterone, Thyroxin.

INTRODUCTION

Immune system in the body is an "open circuit" system and is regulated by a number of factors. Among the factors cytokine, chemokine and lymphokines are most common which regulate immunity in autocrine/paracrine/juxtacrine manner (Kuby, 2006). Apart from the classical regulators of immunity, hormones are another important factor which can coordinate immune functions in different temporal and spatial manner (Flatt *et al*., 2008). The hormones can act as chemical messengers to regulate a number of biological processes like reproduction, metabolism etc. Thus, the hormonal regulation of immunity is most important and unique of its kind as not only the hormones but the other biological processes which hormones regulate can also modulate the immunity. In the neuroendocrine regulation of immunity some hormones are immune suppressor (e.g. gonadal and adrenal steroids; Haldar and Ahmad, 2010; Vishwas *et al*., 2013; Ghosh *et al*., 2014), some are immune enhancer (e.g. melatonin, Carrillo-Vico *et al*., 2005) and some are playing both immune enhancing and immune suppressive roles hence, are regarded as immune neutral in nature (e.g. thyroxin; Gupta *et al*., 1983; Singh *et al*., 2006; Haldar *et al*., 2006) in nature. The immune suppressive activities of gonadal/adrenal steroids are well documented (Dhabhar *et al*., 1996; Furman *et al*., 2014) along with immune enhancing property of melatonin (Guerro and Reiter, 2002) in different animals. However, role of thyroxin in immune modulation is not well established except for some partial reports (Hassman *et al*., 1985; Weetman *et al*., 1984; Singh *et al*., 2014). In the internal body milieu, cumulative effects of all of the hormones are finely orchestrated to modulate immunity and body homeostasis. Thus, supplementation of hormones *in*

vitro is one approach in measuring the effects of hormone on activities of immune cell proliferation and their roles in immune modulation.

Monsoon is stressful for the goats due to different kinds of pathogenic invasions (by helminths, cestodes and nematodes) during grazing. Reproductive preparatory phase of goats starts during monsoon season so that, successful conception and gestation may occur during winter (Ghosh *et al.*, 2014). Thus, during monsoon goats are not only under "inflammatory stress" but also they are under immune suppressive effect of gonadal steroids. Further, during winter cold stress and inflammatory stress (due to gastrointestinal pathogens; Scharko, 2008) is prevalent for both the sexes and gestational stress is particulate for females. Despite of higher adaptability of goats to different ranges of climatic conditions (in terms of temperature, percent humidity, etc.), their susceptibility to become diseased are more likely.

Melatonin is immune enhancer in nature as mostly reported (Conti *et al*., 2000; Maestroni, 2001). Some partial reports (Zarazaga *et al*., 2012) suggest the role of melatonin in regulation of reproduction in goats. But, the role of melatonin in goat immune modulation has never been studied in detail except for the report of Kaushalendra and Haldar (2012). Role of gonadal steroid (testosterone and estrogen) in immune modulation has never been studied in goats or sheep except for a single study of Kaushalendra and Haldar (2012) demonstrating the circulatory level of testosterone and seasonality in immune functions in Indian goats *C. hircus*. Particularly, in goats the circulatory level of corticosterone has been reported under normal as well as under thermal stress has been reported by correlating it with plasma melatonin level (Sejian *et al*., 2008). But, literature on the immune modulatory role of glucocorticoids in goats are completely lacking. In goats particularly, the role of thyroxin even including the circulatory level was not studied.

We identified the above lacuna and therefore, the aim of the present study was to note the role of gonadal steroids (testosterone and estrogen), glucocorticoid, thyroxin and melatonin in goat immune modulation under *in vitro* conditions.

MATERIALS AND METHODS

Animals and maintenance

Goats of approximately same age (~1 year) and weight (~20 ± 2 kg) were procured from commercial goat raiser and then were housed in goat shelter under natural conditions of Varanasi ($25^0$18' N, 83^0 01' E, India) in order to maintain a consistency in food and hygiene throughout the year. At the time of procurement, the goats were weighed (Calf Weighing Sling, Munk's Livestock, Kansas, USA) and the age was determined by dentition as described by Fandos *et al*., (1993). The male and female goats were kept separately to avoid mating or pheromonal effects. The detection of heat period was purely based on the visual observations i.e. more vocalization, reddening of vulva and mucorrhea. Goats were fed with usual ration of roughages (dry and green) and concentrate as suggested by Central Institute for Research on Goats, (CIRG), Mathura, Uttar-Pradesh, India. Single goat generally requires 4-5 kg of fodder/day and was fed with usual ration made up of roughages (dry and green) and concentrate. Dry roughages contained crushed barley (*Hordeum vulgare*, 1 part), crushed maize (*Zea mays*, 2 parts), linseed (*Linum usitatissimum*) or mustard seed cake (*Brassica juncea*, 2.25 parts), rice bran (*Oryza sativa*, 2 parts) along with small amount of molasses or a pinch of salt when required. Green roughages contained maize (*Zea mays*), elephant grass (*Pennisetum purpureum)*, *pearl millet (Pennisetumglaucum*), sorghum (*Sorghum* sp.) and oat (*Avena sativa*). The concentrate contained oilseed cakes and soaked gram (*Cicer arietinum*) and water *ad libitum*. They were exposed to 8 hours outdoor for free grazing and 16 hours indoor (during night) conditions. Health of the goats was monitored by noting down the body temperature (normal rectal temperature, 102.5^0F–103^0F) and rumen movement by authorized veterinary doctors. Goats were treated with helminthicide twice per year and 0.5% solution of malathion (acaricidal baths) as described by Chowdhury *et al.*, (2002). The slaughtering of the goats was performed according in the city abattoir to the Slaughter of Animal Act under "Central Provinces Gazette" 1915 and modified in 2002. All the experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and Institutional practice within the framework of revised Animal (Specific Procedure) Act of 2007 of Government of India on animal welfare. The study was carried out during three major seasons of a year i. e. summer, monsoon and winter. Thus, the climatic condition during summer months was (April–June, temperature 43.87^0 ± 1.02^0 C, percent relative humidity [%RH] 36.74 ± 4.28%, day length, light–dark cycle-13.42 hours:10.18 hours), monsoon months (July–September, temperature 28.68^0 ± 2.76^0 C, %RH 87.04 ± 3.50%, day length, light–dark cycle-12 hours:12 hours), and winter months (November–January, temperature 10.76^0 ± 3.63^0 C, %RH 64.12 ± 3.05%, day length, light–dark cycle 10.35 hours: 13.25 hours). All of the results were validated with the samples collected from CIRG in a seasonal manner.

Experimental Design

In order to study the role of different hormones (testosterone, estadiol, melatonin, thyroxin and glucocorticoid) on cell mediated immunological parameters in sex and season dependent manner throughout the year, a total number of 108 male and female goats were included for the study. The study was conducted during three seasons, i.e., summer (April–

June), monsoon (July–September) and winter (November–January). A total number of 12 goats (six males and six females) were selected from the flock for every month of a season (i.e. n = 6/sex/every month of season) and were numbered on ears. Thus, for summer, the total numbers of male goats were 18 and the total numbers of female goats were also 18. Hence, for summer the total number of males and females were 36 (18 males + 18 females). The same numbers of goats were used for monsoon and winter months. The results were validated with the samples collected from CIRG, Mathura, Uttar-Pradesh.

Spleen and thymus sampling

The animals were electrically stunned and bled immediately till death after terminal cervical incision (Kaushalendra and Haldar, 2012) in the city abattoir. The desired tissues (pineal, spleen, thymus, liver and gonads) were collected aseptically, weighed (Kern Instruments, Germany), and a small portion was cut, washed in PBS for three times then weighed. Within 20 minutes of collection, spleen and thymus were processed for blastogenic response assay (%SR) after challenging the splenocytes and thymocytes with a T-cell mitogen, Concanavalin A (Con A) with or without hormonal supplementations.

Cell mediated immune parameters with hormonal supplementation(s)

Isolation of thymocytes and splenocytes

The splenocytes and thymocytes were cultured following protocol of Kaushalendra and Haldar (2012) with modifications as suggested by Ghosh et al., (2014). In brief, pieces of thymus and spleen were minced between glass slides in cold PBS. 2 mL of minced spleen tissues were treated with equal volume of 0.84% NH_4Cl. Then, the splenocytes and thymocytes were passed through sieve to prepare single cell suspension. The cell suspension was centrifuged (254 × g) and the pellet was suspended in 2% complete medium and filtered through 15 µm filters to get lymphocytes. The appropriate cell viability (> 95%) was checked with 1% trypan blue exclusion method and then was adjusted to 1×10^6 cells/mL in 10% complete medium (RPMI-1640), containing antibiotics (1% penicillin 100 IU/mL, streptomycin 100 µg/mL, gentamycin 100 µg/mL), 1% L-glutamine 2mM/mL, 0.1% 2-mercaptoethanol (5×10^{-2}M/ mL) and heat inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, USA). Viable cell number was adjusted in cell suspension to 1×10^6 cells/ mL and was plated in triplicates in sterile 96 well-culture plates. The basal culture plates were incubated without T cell mitogen, Concanavalin-A (Con-A) whereas challenged culture plates were incubated with 10µg/ mL concentration of Con-A (with or without hormonal supplementations).

Hormonal supplementation *in vitro*

Testosterone, estradiol, dexamethasone (a synthetic glucocorticoid), thyroxin and melatonin hormones were purchased from Sigma–Aldrich. Testosterone, estradiol and dexamethasone were dissolved in a few drops of DMSO (Super Religare Laboratories, Mumbai, India). Finally, desired concentrations of melatonin (500 pg/ mL), testosterone (10 ng/mL), estrogen (10 nM), dexamethasone (10 nM) and thyroxin (100 nM) were freshly prepared in complete media and were used for hormonal supplementation analysis *in vitro* .

Experimental protocol in vitro (for testosterone)

Group-I had male splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II had male splenocytes and thymocytes supplemented with testosterone (Testo; 10 ng/ml).

Group-III had male splenocytes and thymocytes supplemented with melatonin (Mel; 500 pg/mL)

Group-IV had male splenocytes and thymocytes supplemented with testosterone and melatonin (Testo; 10 ng/ml + Mel; 500pg/ mL).

Experimental protocol in vitro (for estrogen)

Group-I: had female splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II: had female splenocytes and thymocytes supplemented with estrogen (Estro; 10 nM).

Group-III: had female splenocytes and thymocytes supplemented with melatonin (Mel; 500pg/mL).

Group-IV: had female splenocytes and thymocytes supplemented with melatonin and estrogen (Estro, 10 nM + Mel, 500 pg/mL).

Experimental protocol in vitro (for glucocorticoid)

Group-I had male splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II had male splenocytes and thymocytes supplemented with dexamethasone (Dexa; 10 nM).

Group-III had male splenocytes and thymocytes supplemented with melatonin (Mel; 500pg/mL)

Group-IV had male splenocytes and thymocytes supplemented with dexamethasone and melatonin (Dexa; 10 nM + Mel; 500pg/mL).

Similarly the basal and challenged culture plates of female splenocytes and thymocytes were also grouped into four sets.

Group-I had female splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II had female splenocytes and thymocytes supplemented with dexamethasone (Dexa; 10 nM).

Group-III had female splenocytes and thymocytes supplemented with melatonin (Mel; 500pg/mL)

Group-IV had female splenocytes and thymocytes supplemented with dexamethasone and melatonin (Dexa; 10 nM + Mel; 500pg/mL).

Experimental protocol in vitro (for thyroxin)

Group-I had male splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II had male splenocytes and thymocytes supplemented with thyroxin (Thy; 100 nM).

Group-III had male splenocytes and thymocytes supplemented with melatonin (Mel; 500pg/mL)

Group-IV had male splenocytes and thymocytes supplemented with thyroxin and melatonin (Thy; 100 nM + Mel; 500pg/mL).

Similarly the basal and challenged culture plates of female splenocytes and thymocytes were also grouped into four sets.

Group-I had female splenocytes and thymocytes without any hormonal supplementation but only with DMSO (Con).

Group-II had female splenocytes and thymocytes supplemented with thyroxin (Thy; 100 nM).

Group-III had female splenocytes and thymocytes supplemented with melatonin (Mel; 500pg/mL)

Group-IV had male splenocytes and thymocytes supplemented with thyroxin and melatonin (Thy; 100 nM + Mel; 500pg/mL).

Cell harvesting and MTT assay

Cell harvesting and MTT assay was done following the protocol of Pauly *et al*., (1973) with few modifications as suggested by Kaushalendra and Haldar (2012). Plates were incubated at 37^0C with 5% CO_2 in incubator (Heracell, Germany) for 48 h and blastogenic response of thymocytes and splenocytes were measured by using a colorimetric assay based on the reduction of tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, SRL, Mumbai, India) following the protocol of Mosmann, (1983). At 48 h, 200 µL of acidified propanol (0.04M HCl in isopropanol) was added to each well and the optical density (OD) of each well was determined with a micro-plate reader (ELx-800, Biotek Instruments, Winooski VT, USA) equipped with a 570 nm wavelength filter. Mean OD values for each set of triplicate were used in subsequent statistical analysis. Response was calculated as percent stimulation ratio (%SR) representing the ratio of absorbance of mitogen stimulated (challenged with Con A) cultures to basal cultures (without Con-A) for each groups.

$$\% \text{ Stimulation ratio (\%SR)} = \frac{\text{Optical density of Challenged (Con A)} \times 100}{\text{Optical density of Basal}}$$

STATISTICAL ANALYSIS

The data were presented as the mean ± standard error of the mean. For the *in vitro* hormonal supplementation experiments the data were analyzed by a one-way ANOVA. To evaluate the interactive effect (Testo*vs*. Testo ± Mel in males; Estro*vs*. Estro ± Mel in females; Dexa*vs*. Dexa ± Mel both in males and females; Thy *vs*. Thy ± Mel both in males and females), the Duncan multiple range t test was used. The mean difference was considered to be statistically significant at the 0.05 level ($p < 0.05$). Statistical analyses were done with Statistical Package of Social Sciences, IBM, software version 17.0 and in accordance with Bruning and Knitz (1977).

RESULTS

Effect of testosterone and melatonin co-supplementation on thymocytes

Testosterone supplementation presented immune suppression by decreasing %SR than control ($p < 0.01$, during monsoon; $p < 0.05$, during winter). Melatonin supplementation alone presented immune suppression both during monsoon ($p < 0.01$) and winter ($p < 0.01$). But, Co-supplementation of melatonin and testosterone significantly ($p < 0.01$ during monsoon and winter) increased the thymocyte proliferation when compared with testosterone supplementation alone (Fig 1A).

Effect of testosterone and melatonin co-supplementation on splenocytes

Testosterone supplementation presented immune suppression by decreasing %SR than control ($p < 0.05$ during summer and monsoon and $p < 0.01$ during winter). However, co-supplementation of testosterone and melatonin recovered back the immune cell proliferation to the normal level (Fig. 1B). The %SR upon co-supplementation of melatonin and testosterone significantly ($p < 0.01$ during summer and winter and $p < 0.05$ during monsoon) increased the splenocyte proliferation when compared with testosterone supplementation alone (Fig. 1B).

Effect of estrogen and melatonin co-supplementation on Thymocytes

Estrogen supplementation decreased significantly ($p < 0.01$; during monsoon and winter) the cell mediated immune parameters in terms of %SR of thymocytes when compared with control. But, co-supplementation of melatonin and estrogen improved immunity to control level and the level was significantly high ($p < 0.05$ during summer and $p < 0.01$ during monsoon and winter) when compared with estrogen supplementation alone (Fig. 2A).

Effect of estrogen and melatonin co-supplementation on splenocytes

Estrogen supplementation only significantly ($p < 0.05$ during summer and winter; $p < 0.01$ during monsoon) decreased cell mediated immune parameters in terms of %SR of splenocytes than control. However, co-supplementation with estrogen and melatonin improved immunity to the control level along with significantly higher level when compared to estrogen supplementation alone ($p < 0.05$ during summer and $p < 0.01$ during monsoon; Fig. 2B).

Effect of glucocorticoid and melatonin co-supplementation on thymocytes

In case of both male and female thymocyte culture dexamethasone significantly suppressed immunity during summer ($p < 0.05$), monsoon ($p < 0.01$) and winter ($p < 0.01$) in terms of %SR. Melatonin supplementation significantly improved immunity during monsoon ($p < 0.05$ in case of females) and winter ($p < 0.01$) in case of both the sexes. Co-supplementation of melatonin and dexamethasone significantly improved immunity during in both the sexes; summer ($p < 0.05$), monsoon ($p < 0.05$) and winter ($p< 0.01$; Fig. 3A and 3B).

Effect of glucocorticoid and melatonin co-supplementation on splenocytes

In case of both male and female splenocyte culture dexamethasone significantly suppressed immunity during summer ($p < 0.05$ in both the sexes), monsoon ($p < 0.05$ in case of females and $p < 0.01$ in case of males) and winter ($p < 0.01$ in both the sexes). Supplementation with melatonin alone increased immune cell proliferation during monsoon ($p < 0.01$ in case of males; $p < 0.05$ in case of females) and winter ($p < 0.01$ in both the sexes). But, co-supplementation with dexametahsone with melatonin increased immunity (in terms of immune cell proliferation) during summer ($p < 0.05$ in case of males), monsoon ($p < 0.05$) and winter ($p < 0.01$) in cases of both the sexes (Fig. 3C and 3D).

Effect of thyroxin and melatonin co-supplementation on thymocytes

In cases of both male and female thymocyte culture, thyroxine supplementation was found to immune neutral in terms of % SR. Melatonin supplementation significantly increased %SR of thymocytes ($p < 0.05$) in both the sexes during monsoon and ($p < 0.05$ in males; $p < 0.01$ in females) during winter. However, result with co-supplementation with melatonin and thyroxin is of most importance. We noted significant increase of %SR of thymocytes upon melatonin and thyroxin co-supplementation during monsoon ($p < 0.05$, in both the sexes) and winter ($p < 0.05$ in males and $p < 0.01$ in females) (Fig. 4A and 4B).

Effect of thyroxin and melatonin co-supplementation on splenocytes

Like the thymocyte culture, in cases of both male and female splenocyte culture, thyroxine supplementation was found to immune neutral in terms of % SR. Melatonin supplementation significantly increased %SR of splenocytes during summer ($p < 0.05$; in both the sexes), monsoon ($p < 0.01$; in males) and winter ($p < 0.01$ in males and $p < 0.05$ in females). Co supplementation with melatonin and thyroxin significantly increased %SR of splenocytes during summer ($p < 0.05$; in both the sexes), monsoon ($p < 0.05$ in males and $p < 0.01$ in females) and winter ($p < 0.01$ in both the sexes) (Fig. 4C and 4D).

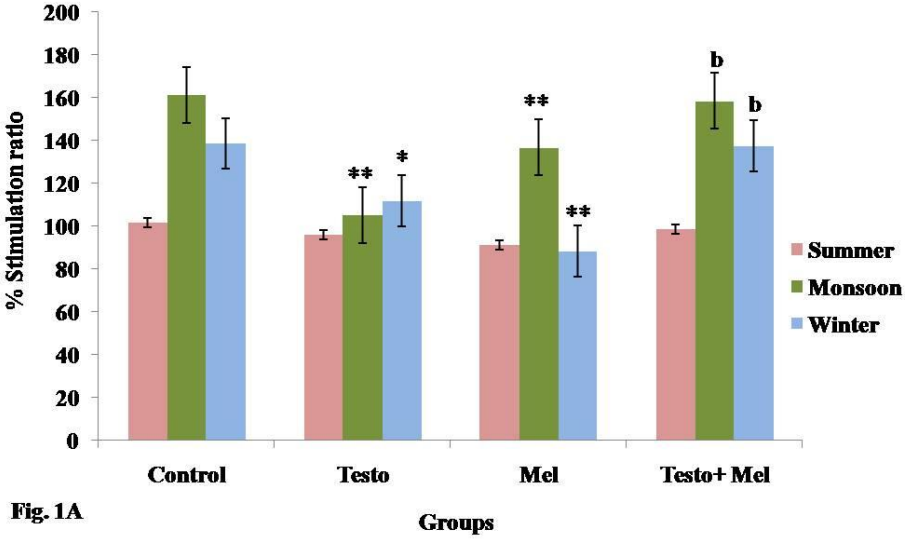

Fig. 1A

Fig-1A: Season and sex dependent variations in %SR of thymocytes culture upon testosterone supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N =18 males/season. Vertical bar on each point represents standard error of mean (SEM). Testosterone; Testo, Melatonin; Mel. *p < 0.05, **p < 0.01; control *vs* all other groups. b p < 0.01; Testo *vs* Testo+ Mel

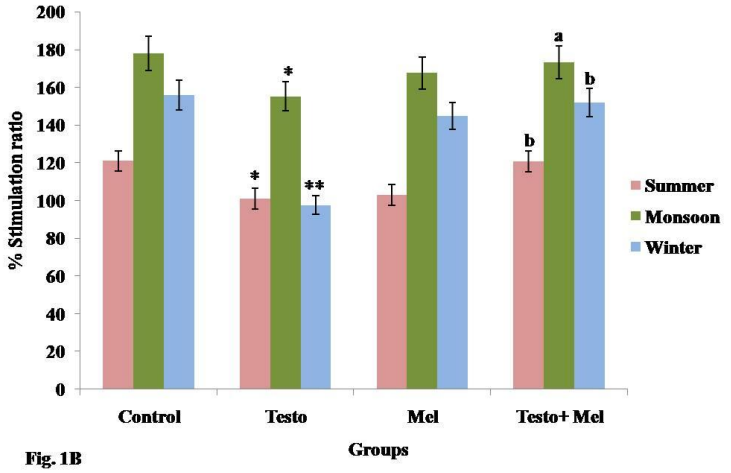

Fig. 1B

Fig-1B: Season and sex dependent variations in %SR of splenocytes culture upon testosterone supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM). Testosterone; Testo, Melatonin; Mel.*p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Testo*vs*Testo+ Mel

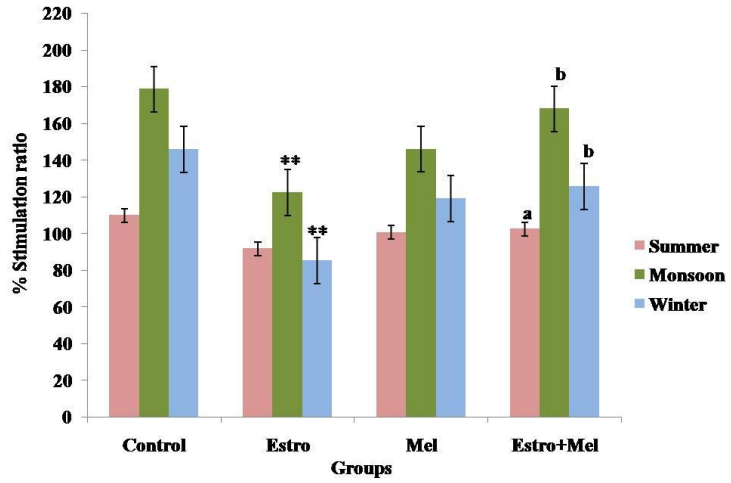

Fig. 2A

Fig-2A: Season and sex dependent variations in %SR of splenocytes culture upon estrogen supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM).Estrogen; Estro, Melatonin; Mel.p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Estro *vs* Estro+ Mel**

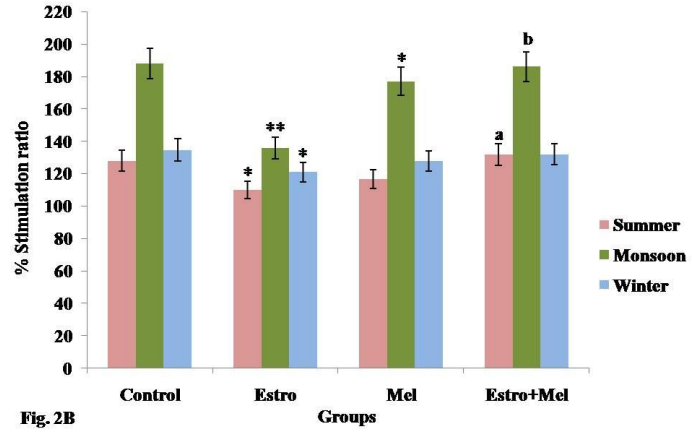

Fig. 2B

Fig-2B: Season and sex dependent variations in %SR of thymocytes culture upon estrogen supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM).Estrogen; Estro, Melatonin; Mel.*p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Estro *vs* Estro+ Mel

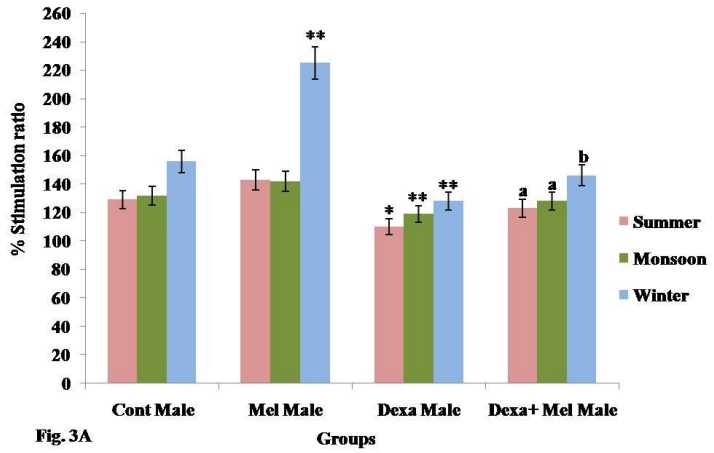

Fig-3A: Season and sex dependent variations in %SR ofthymocytesculture upon dexamethasone supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM).Dexamethasone; Dexa, Melatonin; Mel.*p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Dexa*vs*Dexa+ Mel

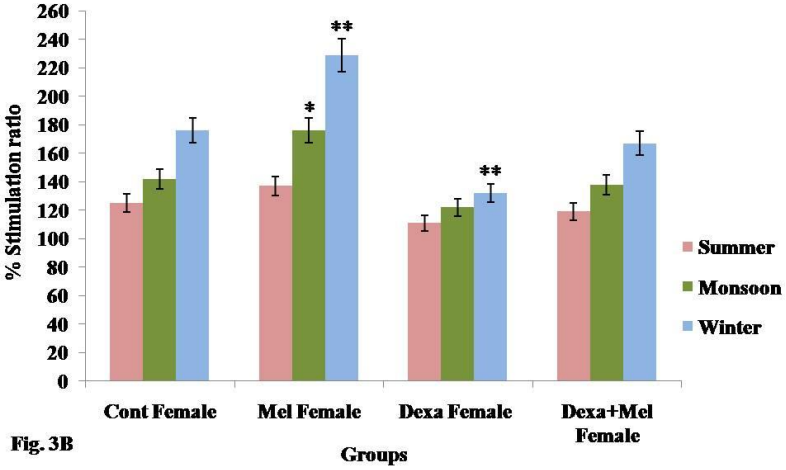

Fig-3B: Season and sex dependent variations in %SR ofthymocytesculture upon dexamethasone supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Dexamethasone; Dexa, Melatonin; Mel. *p < 0.05, **p < 0.01; control *vs* all other groups. ap < 0.05, bp < 0.01; Dexa *vs* Dexa+ Mel

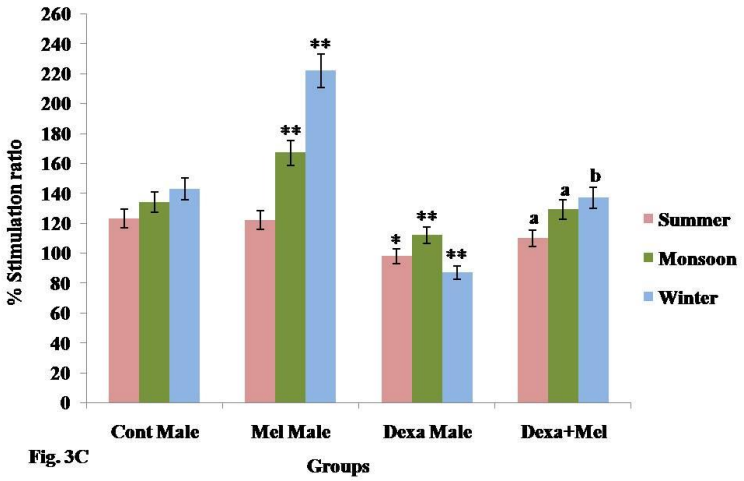

Fig-3C: Season and sex dependent variations in %SR ofsplenocytesculture upon dexamethasone supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N =18 males/season. Vertical bar on each point represents standard error of mean (SEM).Dexamethasone; Dexa, Melatonin; Mel.*p < 0.05, **p < 0.01; control *vs* all other groups. a p <0.05, b p < 0.01; Dexa *vs* Dexa+ Mel

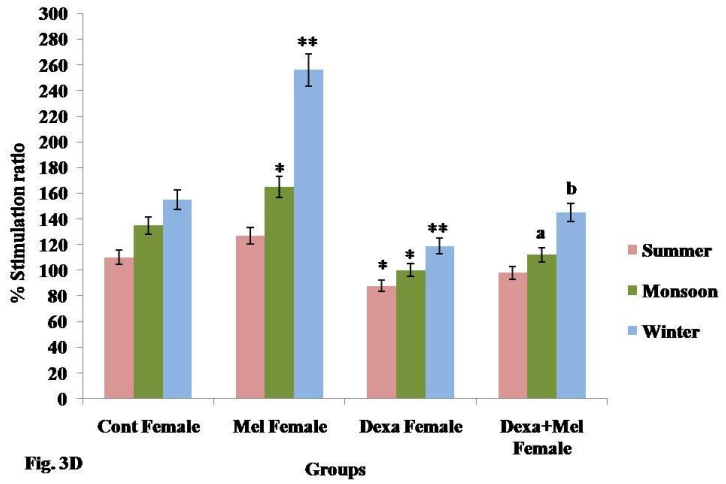

Fig-3D: Season and sex dependent variations in %SR ofsplenocytesculture upon dexamethasone supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Dexamethasone; Dexa, Melatonin; Mel. *p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Dexa *vs* Dexa+ Mel

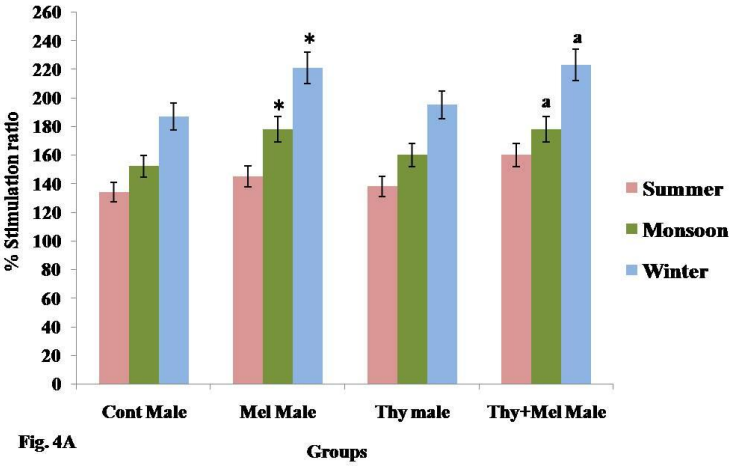

Fig-4A: Season and sex dependent variations in %SR of thymocytes culture upon L-thyroxin supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM).Thyroxin; Thy, Melatonin; Mel. *p < 0.05; control *vs* all other groups. a p < 0.05, Thy *vs* Thy+ Mel

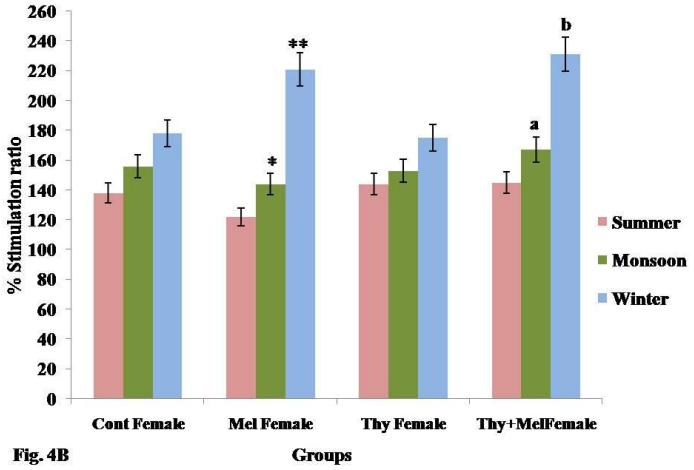

Fig-4B: Season and sex dependent variations in %SR of thymocytes culture upon L-thyroxin supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Thyroxin; Thy, Melatonin; Mel. *p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Thy *vs* Thy+ Mel

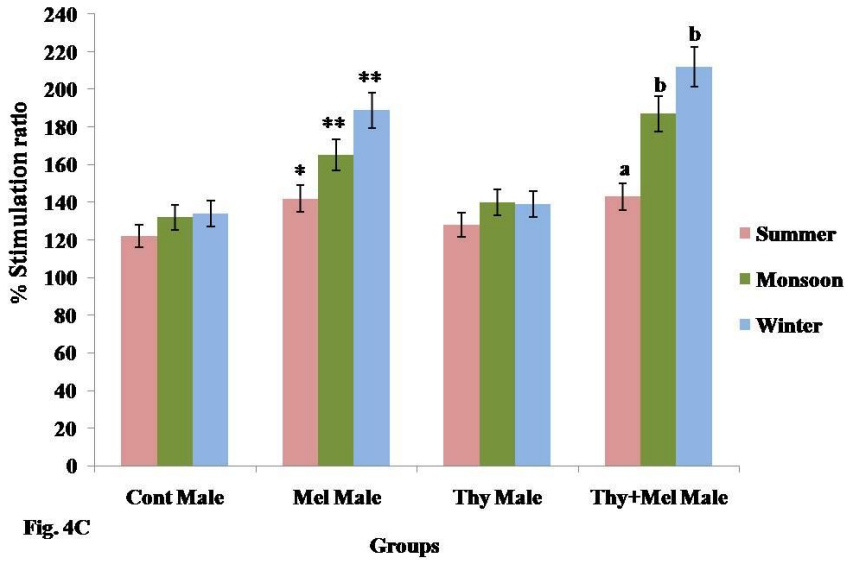

Fig. 4C

Fig-4C: Season and sex dependent variations in %SR of splenocytes culture upon L-thyroxin supplementation in male goats, *C. hircus*. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM). Thyroxin; Thy, Melatonin; Mel. *p < 0.05, **p < 0.01; control *vs* all other groups. a p < 0.05, b p < 0.01; Thy *vs* Thy+ Mel

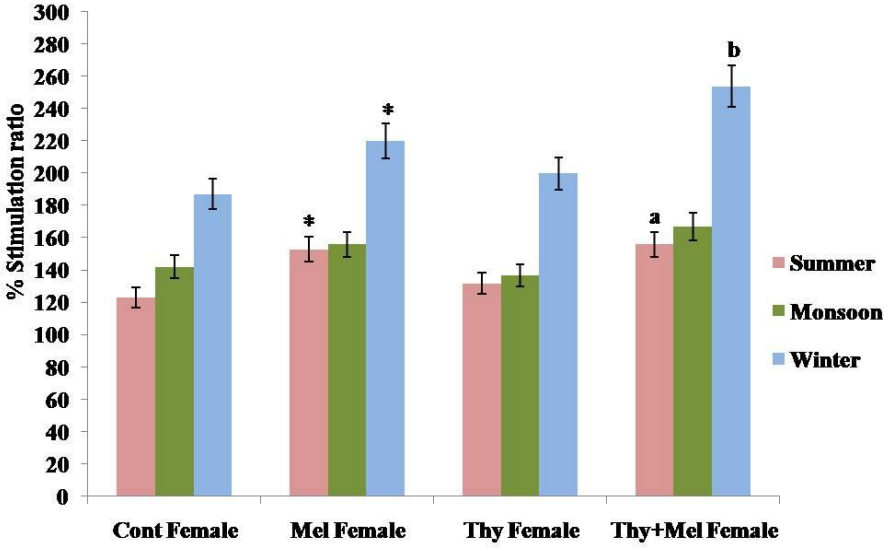

Fig. 4D

Fig-4D: Season and sex dependent variations in %SR of splenocytes culture upon L-thyroxin supplementation in female goats, *C. hircus*. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Thyroxin; Thy, Melatonin; Mel. *p < 0.05; control *vs* all other groups. a p < 0.05, b p < 0.01; Thy *vs* Thy+ Mel

DISCUSSION

The role of different hormones in immune modulation in ruminants general and goats in particular is less explored area. Thus, in the present study we wish focus on the role of different hormones (gonadal/adrenal steroids, thyroxin and melatonin) in immune modulation of goats. Among different hormones the gonadal/adrenal steroids are regarded as immune suppressive as suggested by others (Vishwas *et al*., 2013). A unique aspect of our study is that, the results were discussed under the special focus of melatonin which is a known immune enhancer (Currier *et al.*, 2000).

Sex steroids act as negative regulators in both the thymus and bone marrow, but androgens and estrogen tend to affect different subsets of immune cells. In general, androgens seem to inhibit immune activity, while estrogen seems to have a more powerful effect on immune cells and to stimulate immune activity. It is apparent that the immune and reproductive systems are intimately interconnected and that androgens are important components of these interactions. Indeed, the immune system can be

modulated by androgens in some cases; conversely, activation of the immune system, particularly the innate arm, is associated with suppression of the reproductive neuro endocrine axis (Ahmad and Haldar, 2010). Several studies in both animals and humans have been performed to understand the influence of sex steroids on the immune system. Androgen receptors have been identified in thymic tissues, particularly in the epithelial, lymphatic portion of the thymus (McCruden and Stimson, 1984). Androgenic effects on lymphocytes may be in direct or through aromatization of androgens to estrogens, because no androgen receptors have been found on circulating lymphocytes (McCruden and Stimson, 1991). But, role of testosterone on goat/sheep immune modulation is totally lacking. In the present studies on goats in our lab, we found interesting results on splenocyte and thymocyte cultures to note cell mediated immunity upon testosterone supplementation *in vitro* in a season and sex dependent manner. An immune suppressive role of testosterone was noted *in vitro* which reduced cell proliferation in terms of % Stimulation Ratio (%SR) in a season dependent manner.

Androgens and estrogen tend to affect different subsets of immune cells. In general, androgens seem to inhibit immune activity, while estrogen seems to have a more powerful effect on immune cells and to stimulate immune activity (Calippe *et al*., 2008). Estrogen receptors have been localized in the cytosol of circulating lymphocytes Estrogen stimulates CD4+CD8- cells and can activate an extra-thymic pathway of auto-reactive T cell differentiation in the liver (Muller et al., 1995). Several studies have established that estrogen is a potent inhibitor of stromal cell-dependent B cell lympho poesies *in vitro* . Estrogen also affects peripheral B cells and hum oral immunity. Manipulation of female reproduction by exogenous estrogen treatment is a very common and ancient practice in milk cattle breeds. In the study of sheep particularly the effect of estrogen is very much prevalent in female reproduction **(**AbouAkkada and El-Shazly, 1976) but not in immunity. Studies were performed in goats with circulatory levels of estrogen (Paula *et al*., 2005) but the immunomodulatory role or estrogen has never been tested in sheep or goats. In the present study we noted immune suppressive role of estrogen in cell mediated immunity (in terms of %SR of thymocytes and splenocytes) under *in vitro* conditions. Our present result on hormonal supplementation *in vitro* is in parallel with the previous report of Kaushalendra and Haldar (2012) suggesting that cell mediated immune parameters (i.e. %SR of splenocytes) are in opposite correlation with circulatory level of gonadal steroids (both testosterone and estrogen).

Glucocorticoids are the principal negative regulators of an important neuroendocrine axis (Hypothalamus–Pituitary–Adrenal (HPA) axis. Glucocorticoids are now recognized as powerful mediators of many physiological processes including reproduction and immune activity (Khansari et al., 1990). Males and females often differ in the types of stressors they encounter, especially during the breeding season (Klein and Nelson, 1999). Thus, exposure to stressors may influence sex differences in immune function and subsequent resistance to infection (Zuk and McKean, 1996). Interaction between glucocorticoids and the immune system is complex and bidirectional. Stressor- induced elevated glucocorticoid concentrations can modulate immune activity; however, activation of the immune system can also drive the production of glucocorticoids (McEwen *et al*., 1997). Because glucocorticoids tend to suppress inflammation but be induced by pro-inflammatory stimuli, they have been conceptualized as 'brakes' on the immune system, having evolved to prevent runaway inflammation and promote fine-tuning of the immune response (Sapolsky *et al*., 2000). A wealth of information demonstrates how glucocorticoids suppress immune function (McEwen *et al*., 1997), which led to the conjecture that glucocorticoids are largely responsible for decrements in immune activity in free-living animals in winter (Nelson *et al*., 2002). Now there is compelling evidence that in certain contexts glucocorticoids can enhance aspects of immune function which may be immune redistribution in disguise (Braude *et al*., 1999). Particularly, in goats the circulatory level of corticosterone has been reported under normal as well as under thermal stress has been reported by correlating it with plasma melatonin level (Sejian *et al*., 2008). But, literature on the immunomodulatory role of glucocorticoids in goats/sheep are completely lacking. Our data on the *in vitro* supplementation of dexamethasone (a synthetic glucocorticoid) to delineate its role goat immune modulation in a season and sex dependent manner suggest that in both males and females it is immune suppressive in terms of %SR. However, effect of dexamethasone supplementation in females is more prominent during winter under *in vitro* proliferation assay as during winter females are under gestational as well as cold stress.

Thyroid hormones are basically known to regulate Basal Metabolic Rate (BMR) of the body. But the immunomodulatory role of this hormone is least known and in need to be elucidated. Some previous reports suggest that thyroxin (T4) caused thymus enlargement and increase in number of peripheral lymphocyte (Hassman *et al*., 1985). However, thyroidectomy resulted in hypoplasia of lymphoid organs (Rai *et al*., 2005) as thyroid hormones are reported to increase the nucleated cells in spleen and thus improving the immune status of an immune compromised animal to the threshold level (Baroni *et al*., 1969). Some of the reports are contradictory to the previous citations some scientist (Weetman *et al.,* 1984) reported that under *in vivo* and *in vitro* conditions thyroxin has no role in immune modulation. Some other report (Gupta and Thapliyal, 1984) suggests that thyroxin in immune inhibitor in nature. Most of these

reports are mainly from birds but not from mammals. Our *in vitro* results of goat thymocyte and splenocyte culture in sex and season dependent manner is first of its kind suggesting that there is lack of immune enhancing or immune suppressive role of thyroxin alone in goat immune modulation, however, in combination with melatonin it acts as immune stimulatory.

In recent years much attention has been devoted to the possible interaction between melatonin and the immune system (Guerrero and Reiter, 2002). Melatonin has significant immune modulatory roles in immune compromised states. Late afternoon injection of melatonin increases both the primary and secondary antibody responses to SRBC (Maestroni *et al*., 1987). Melatonin enhances both cell-mediated and humoral immunity. The immune enhancing effect of melatonin involves opioid peptides; melatonin stimulates cells to secrete opioid peptides that have up-regulatory effects on a variety of immune cells (Maestroni, 2001). According to some reports (Nelson and Drazen, 2000), melatonin is a part of a complex physiological system that coordinates reproductive, immunological and other physiological processes to cope up with energetic stressors during winter. There is a possibility that melatonin could act as an autocue in bone marrow as shown by the demonstration of melatonin synthesis in bone marrow cells of mice and humans (Conti *et al*., 2000). The role of melatonin in modulation of goat reproduction and maintenance of seasonality is well documented (Zarazaga *et al*., 2012) particularly focusing on its regulatory role in reproductive seasonality. In our *in vitro* study of thymocyte and splenocyte culture melatonin supplementation not only improves immunity but also ameliorates gonadal steroid (testosterone/estrogen, Ghosh *et al*., 2014) and dexamethasone induced immune compromised condition up to the control level. Thus, melatonin acts as a buffer-hormone to regulate immunity even under stressful conditions and under immune-suppressed condition caused due to gonadal and steroid. The role of melatonin supplementation with thyroxin was quite interesting. In our study, thyroxin played non-significant role in improvement of immunity. But, co-supplementation with melatonin; significantly improved immune status; particularly in females during winter. This may be due to the fact that winter is stressful for both the sexes due to "cold stress" and particularly for the females due to gestational stress. At that time circulatory level of thyroxin was also high in females due to high level of metabolism to maintain both the high energy demanding biological processes (i.e. maintenance of gestation and immunity).

Thus, the roles of different hormones were evident as one of the important factors in regulation of immunity. But, there are so many other factors which can limit the immune modulation and reproduction. Being the most metabolically active tissue (lymphoid organs and gonads) are highly prone to generate huge amount of free radicals.

CONCLUSION

Apart from the classical regulators of immunity, hormones are another important factor which can coordinate immune functions in different temporal and spatial manner. The aim of the present study was to note the role of gonadal steroids (testosterone and estrogen), glucocorticoid, thyroxin and melatonin in goat immune modulation under *in vitro* conditions. We noted significant decrease in spleen and thymus functions (in terms of %SR of thymocytes and splenocytes) upon gonadal (testosterone and estrogen) and adrenal (dexamethasone, a synthetic glucocorticoid) steroid supplementation alone in both the sexes in a season dependent manner. Thyroxin supplementation alone played immune neutral role as it presented non-significant influence on %SR of thymocytes and splenocytes in both the sexes in a season dependent manner. Melatonin supplementation alone significantly increased %SR of thymocytes and splenocytes in both the sexes during three seasons. However, co-supplementation of melatonin along with gonadal and adrenal steroids improved immune suppressed condition to the control level. But, co-supplementation of melatonin along with thyroxin significantly increased %SR of thymocytes and splenocytes than control as well as thyroxin and melatonin supplementation alone. Thus, from the results of the present chapter we may conclude that the immune suppressive roles of gonadal and adrenal steroid in immunity were ameliorated by melatonin to improve the immune status and to act as a "buffer hormone" or as "opportunistic hormone" to influence the functioning of immune neutral hormone to immune enhancing one.

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