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Roles of Stress and Metabolic Hormones in Immunomodulation of Indian Goat *C. Hircus*.

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Abstract: *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 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) 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 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 role adrenal steroid in immunity was 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.*

Keywords: *Adrenal steroid, Dexamethasone, Goat, immunity, lymphoid organs, thyroxin.*

1. 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 [1]. Apart from the classical regulators of immunity, hormones are another important factor which can coordinate immune functions in different temporal and spatial manner [2]. 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; [3], [4], some are immune enhancer (e.g. melatonin, [5]) and some are playing both immune enhancing and immune suppressive roles hence, are regarded as immune neutral in nature (e.g. thyroxin; [6], [7], [8]) in nature. The immune suppressive activities of gonadal/adrenal steroids are well documented [9], [10]. along with immune enhancing property of melatonin [11] in different animals. However, role of thyroxin in immune modulation is not well established except for some partial reports [12], [13], [7]. 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 [14], [15]. 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; [16]) 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 [17]. Some partial reports [18], [19] 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; [20]). 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 [21]. 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 glucocorticoid, thyroxin and melatonin in goat immune modulation under *in vitro* conditions.

2. MATERIAL AND METHODS

2.1. 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), [22]. 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 (*Hordeumvulgare*, 1 part), crushed maize (*Zea mays*, 2 parts), linseed (*Linumusitatissimum*) 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 (*Pennisetumpurpureum), pearl millet (Pennisetumglaucum*), sorghum (*Sorghum* sp.) and oat (*Avena sativa*). The concentrate contained oilseed cakes and soaked gram (*Cicerarietinum*) 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), [23]. 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 $43.87^0 \pm 1.02^0$ 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 $28.68^0 \pm 2.76^0$ C, %RH 87.04 ± 3.50%, day length, light–dark cycle-12 hours:12 hours), and winter months (November–January, temperature $10.76^0 \pm 3.63^0$ 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.

2.2. 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).

2.3. Blood Sampling

For the assessment of peripheral hormone, one night before the slaughtering, blood of male and female goats was collected from left jugular vein by venipuncture applying minimum stress [20]. Blood samples were obtained during the night time (3 hours after sunset) in a 10 mL dispovan syringe coated with 10% EDTA (anticoagulant). All the goats were sampled within 40 minutes under dim red light (less than 1 lux at a distance of 20 cm) to avoid a direct illumination to the eyes of the goats. Blood was centrifuged (3000 × g) for collection of plasma and was immediately stored at -20^{0}C until the analysis of hormones (melatonin, cortisol and thyroxin).

2.4. Spleen and Thymus Sampling

The animals were electrically stunned and bled immediately till death after terminal cervical incision [20]. The desired tissues (spleen, thymus**)** were collected aseptically, weighed (Kern Instruments, Germany), and a small portion was cut, washed in PBS for three times then weighed. A small portion of that tissue was kept in a sterile vial containing chilled PBS. 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. The freshly prepared cell suspension was used for morphological analysis of apoptosis.

2.5. Circulatory Level of Cortisol

The ELISA kit of cortisol was generously gifted by Prof. T. G. Srivastava, National Institute of Health and Family Welfare (NIHFW), New Delhi, India. The estimation was carried out following manufacturer's protocol. According to the manufacturer's instruction, 25 µL of standard, control and samples were added in each well of ELISA plate followed by 100 µL of enzyme conjugate solution. The wells were then incubated with mild shaking at room temperature for two hours. The wells were then aspirated and washed three times with wash solution. Then, 100 µL of the TMB chromogenic solution (substrate) was added to each well and plate was incubated at room temperature for 30 minutes in dark. Finally, 100 µL of stop solution was added in each well and absorbance was recorded at 450 nm. The co-efficient of variation between intra and inter – assay variations ranged from 3.38% to 5.56% and 5.69% to 7.84% respectively. The recovery was 92% with an accuracy of 98.7%. The sensitivity or lower level of detection was 0.27µg/dL. The assay was carried out in triplicate.

2.6. Circulatory Level of Thyroxin

Peripheral thyroxin level was measured in the blood with the help of a commercial kit (Abcam, England, Cat. No.108686) according to the manufacturer's protocol. According to the manufacturer's instruction, 25 µL of standard, control and samples were added in each well of ELISA plate followed by 100 µL of enzyme conjugate solution. The wells were then incubated with mild shaking at room temperature for two hours. The wells were then aspirated and washed three times with wash solution. Then, 100 µL of the TMB chromogenic solution (substrate) was added to each well and plate was incubated at room temperature for 30 minutes in dark. Finally, 100 µL of stop solution was added in each well and absorbance was recorded at 450nm. The sensitivity of the assay is 0.5µg/dL with a recovery rate of 97.8%.

2.7. Circulatory Level of Melatonin

Peripheral melatonin level was measured in the blood collected at night with the help of a commercial kit (Biosource, Nivelles, Belgium; Cat. No. KIPL3300) according to the manufacturer's protocol.According to the manufacturer's instruction, 25 µL of standard, control and samples were added in each well of ELISA plate followed by 100 µL of enzyme conjugate solution. The wells were then incubated with mild shaking at room temperature for two hours. The wells were then aspirated and washed three times with wash solution. Then, 100 µL of the TMB chromogenic solution (substrate) was added to each well and plate was incubated at room temperature for 30 minutes in dark. Finally, 100 µL of stop solution was added in each well and absorbance was recorded at 450 nm. Analytic sensitivity (limit of detection) for melatonin serum was 2pg/mL. Inter and intra-assay variations were between 9.0% and 15%, respectively. The assay was carried out in triplicate.

2.8. Cell Mediated Immune Parameters with Hormonal Supplementation(s)

2.8.1. Isolation of thymocytes and splenocytes

The splenocytes and thymocytes were cultured following protocol of (KIaushalendra and Haldar, 2012 [20] with modifications as suggested by Ghosh et al., (2014) [14]. 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).

2.8.2. Cell harvesting and MTT assay

Cell harvesting and MTT assay was done following the protocol of Pauly et al., (1973) [24] with few modifications as suggested by Kaushalendra and Haldar (2012) [20]. 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.

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

2.8.3. Hormonal Supplementation in Vitro

Dexamethasone (a synthetic glucocorticoid), thyroxin and melatonin hormones were purchased from Sigma–Aldrich. Dexamethasone was dissolved in a few drops of DMSO (Super Religare Laboratories, Mumbai, India). Finally, desired concentrations of melatonin (500 pg/ mL), 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 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).

2.9. Morphological Analysis of Apoptotic Cells

Apoptotic cells were microscopically analyzed following Acridine Orange–Ethidium Bromide (AO–EB) double staining. AO–EB dye of volume 0.01 mL (1×) was admixed gently with 0.2 mL of the diluted sample (1×10^6 cells/mL in PBS). A drop of this mixture was placed underneath cover slip on a clean slide and cells were observed immediately under fluorescence microscope (Leitz MPV3, Wetzlar, Hesse, Germany) at 440–520 nm. A minimum of 200 cells was counted in every sample to observed cell death [25]. The colorization of the cells and the cellular outline is the marker for apoptosis detection. The cells with green colour and normal round shape were regarded as healthy and non apoptotic ones as the dye (acridine orange) binds to the double stranded DNA. The cells with yellow colour and blabbed out line were regarded as apoptotic cells (as acridin orange binds to the denatured single stranded DNA).

2.10. 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 (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) [26].

3. RESULTS

3.1. Circulatory Level of Cortisol

Circulatory level of cortisol was significantly high in both the sexes during monsoon ($p < 0.01$) and winter ($p < 0.05$; Fig. 1) There was no sex dependent variation or sex * season interactive effect ($p > 0.05$).

3.2. Circulatory Level of Melatonin

Circulatory level of melatonin was significantly high in both in cases of males and females during monsoon ($p < 0.05$) and winter ($p < 0.01$) in comparison to summer. Females always presented a significantly higher level of melatonin in comparison to males during summer ($p < 0.05$) and monsoon ($p < 0.01$) and summer and winter ($p < 0.01$; Fig. 2). Sex * season interactive effect was not significant ($p > 0.05$).

3.3. Circulatory Level of Thyroxin

Circulatory level of thyroxin was significantly in both the sexes during winter ($p < 0.01$). However, only during monsoon the level was significantly high in females ($p < 0.05$) than males. There was no sex* season interactive effect ($p > 0.05$; Fig. 3).

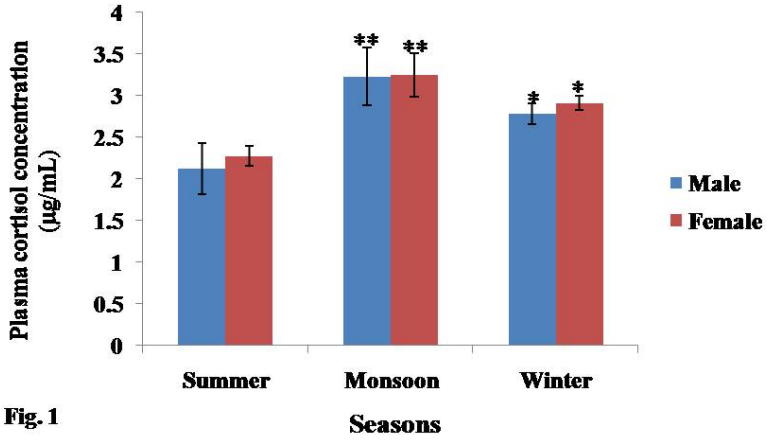

Fig. 1

Fig1. *Season and sex dependent variations in plasma cortisol level in male and female goats, C. hircus. Data represents mean ± SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *p< 0.05, ** p< 0.01; summer vs monsoon and winter.*

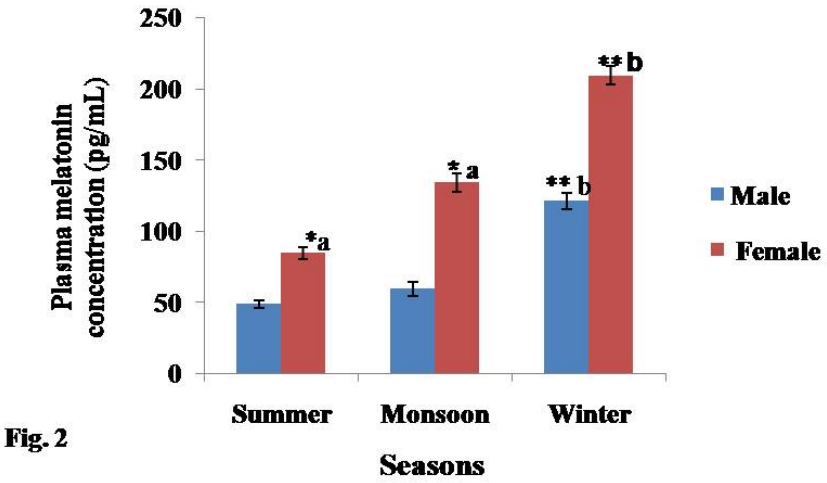

Fig. 2

Fig2. *Season and sex dependent variations in plasma melatonin level in male and female goats, C. hircus. Data represents mean ± SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *p< 0.05, ** p < 0.01; summer vs monsoon and winter. a p< 0.05, b p< 0.01; male vs female.*

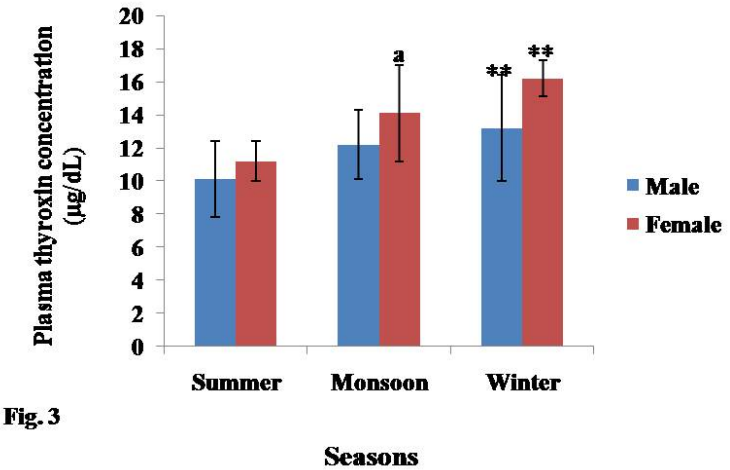

Fig. 3

Fig3. *Season and sex dependent variations in plasma thyroxin level in male and female goats, C. hircus. Data represents mean ± SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). ** p< 0.01; summer vs monsoon and winter. a p< 0.05, male vs female.*

3.4. 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. 4A and 4B).

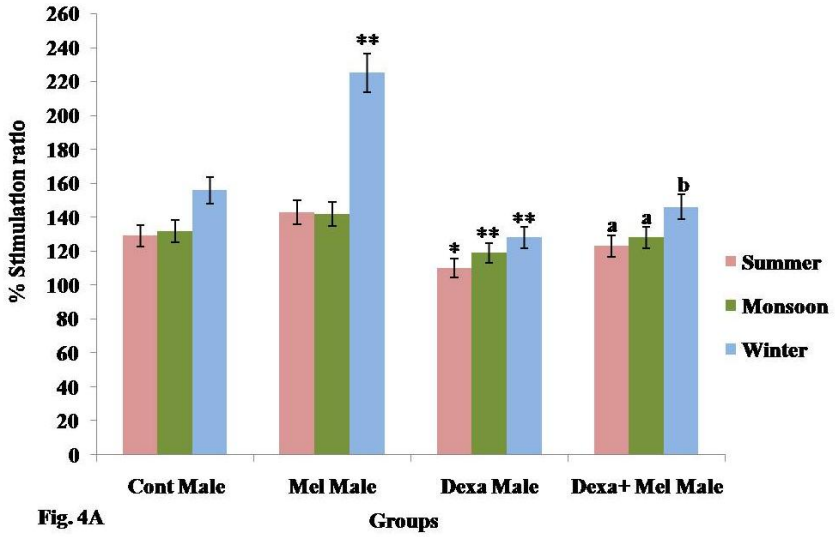

Fig. 4A

Fig4A. *Season and sex dependent variations in %SR of thymocytes culture 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.*

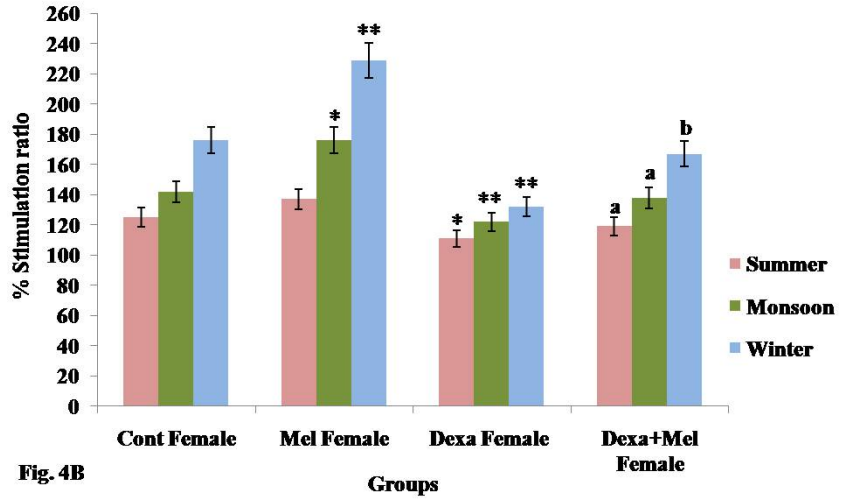

Fig. 4B

Fig4B. *Season and sex dependent variations in %SR of thymocytes culture 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 v sDexa+ Mel.*

3.5. 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. 4C and 4D).

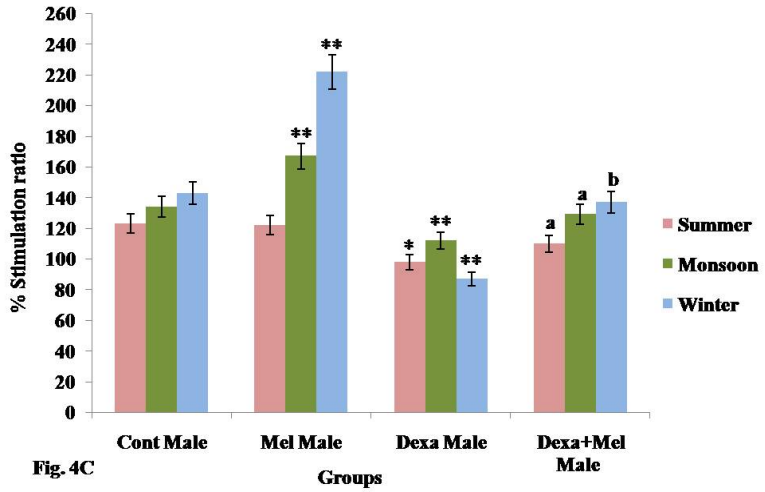

Fig4C. *Season and sex dependent variations in %SR of splenocytes culture upon dexamethasone supplementation in male goats, C. hircus. Data represents mean ± SEM, N =18 males/season. Vertical bar on each point represents standard error of mean (SEM).Dexamethasone; Dexa, Melatonin; Mel.*p < 0.05, **p < 0.01; control vs all other groups. a p <0.05, b p < 0.01; Dexa vs Dexa+ Mel.*

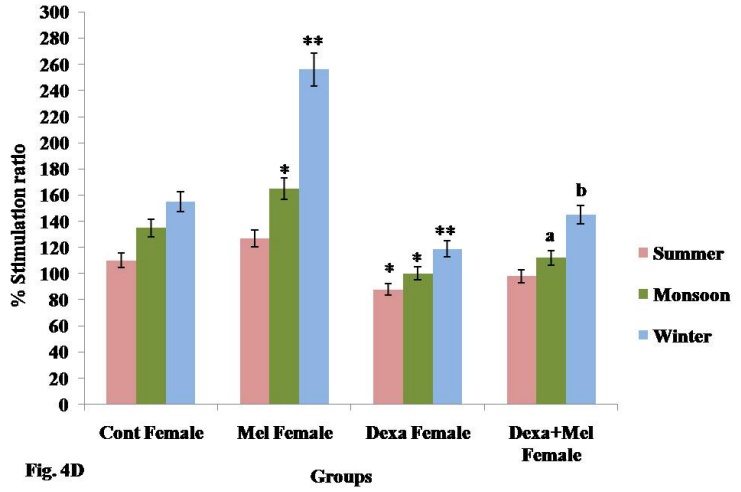

Fig4D. *Season and sex dependent variations in %SR of splenocytes culture upon dexamethasone supplementation in female goats, C. hircus. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Dexamethasone; Dexa, Melatonin; Mel. *p < 0.05, **p < 0.01; control vs all other groups. a p < 0.05, b p < 0.01; Dexa vs Dexa+ Mel.*

3.6. 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. 5A and 5B).

3.7. 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; 5C and 5D).

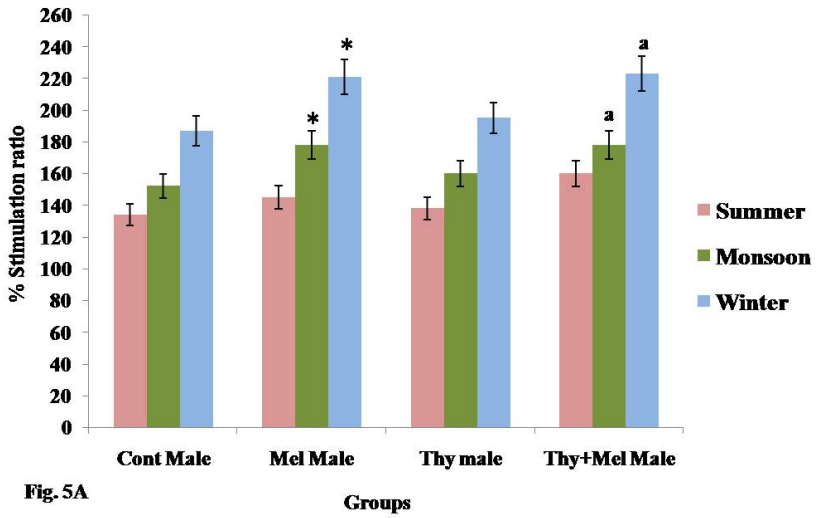

Fig5A. *Season and sex dependent variations in %SR of thymocytes culture upon L-thyroxin supplementation in male goats, C. hircus. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM).Thyroxin; Thy, Melatonin; Mel. *p < 0.05; control vs all other groups. a p < 0.05, Thy vs Thy+ Mel.*

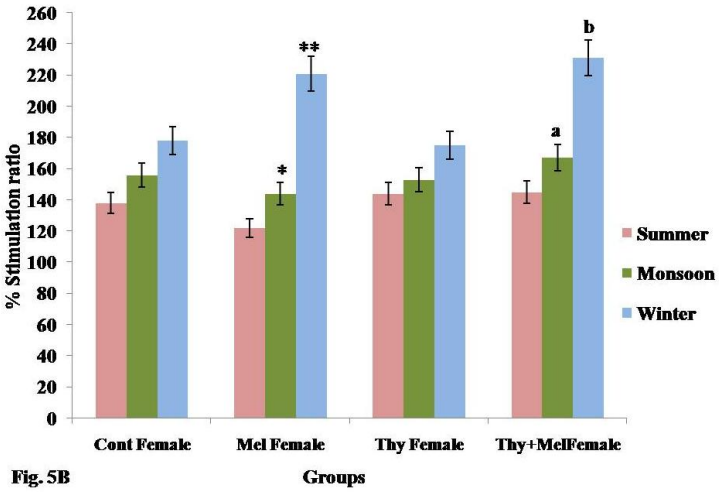

Fig5B. *Season and sex dependent variations in %SR of thymocytes culture upon L-thyroxin supplementation in female goats, C. hircus. Data represents mean ± SEM, N=18 females/season. Vertical bar on each point represents standard error of mean (SEM). Thyroxin; Thy, Melatonin; Mel. *p < 0.05, **p < 0.01; control vs all other groups. a p < 0.05, b p < 0.01; Thy vs Thy+ Mel.*

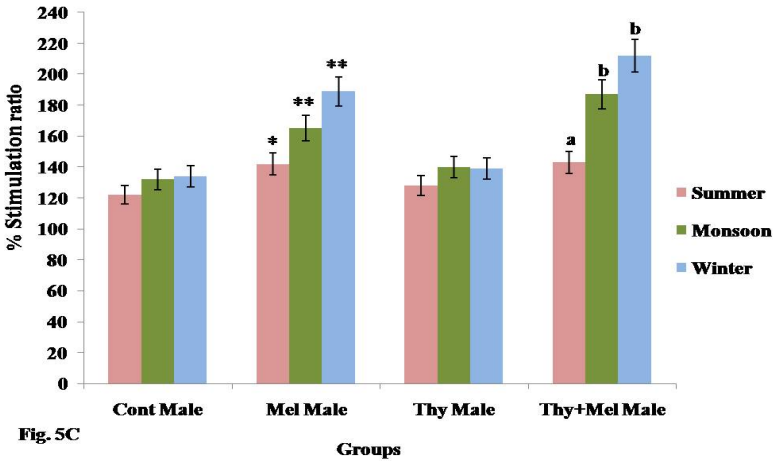

Fig5C. *Season and sex dependent variations in %SR of splenocytes culture upon L-thyroxin supplementation in male goats, C. hircus. Data represents mean ± SEM, N=18 males/season. Vertical bar on each point represents standard error of mean (SEM). Thyroxin; Thy, Melatonin; Mel. *p < 0.05, **p < 0.01; control vs all other groups. a p < 0.05, b p < 0.01; Thy vs Thy+ Mel.*

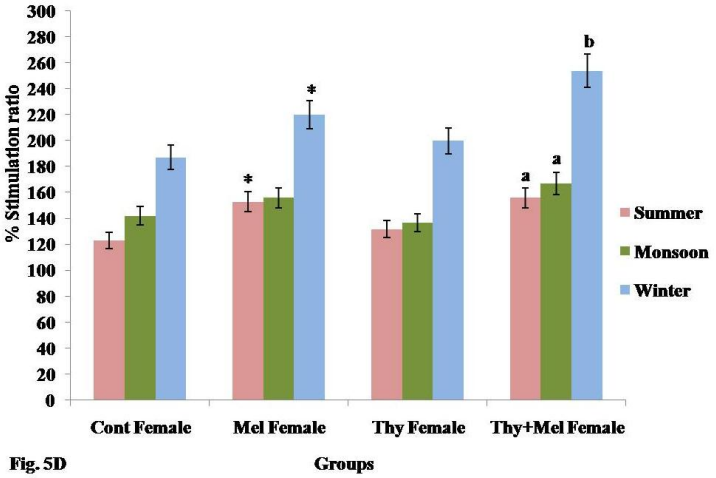

Fig. 5D

Fig5D. *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.*

3.8. % of Apoptosis

Apoptosis was detected by AO-Et-Br double staining method (Fig. 6A and 6B). We noted significant increase in % of apoptosis during monsoon ($p < 0.01$) and significant decrease in apoptosis during winter ($p < 0.05$) in both spleen and thymus in both the sexes (Fig. 6C and 6D).

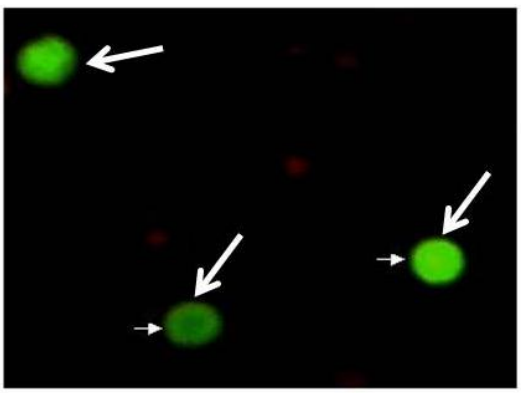

Fig. 6A

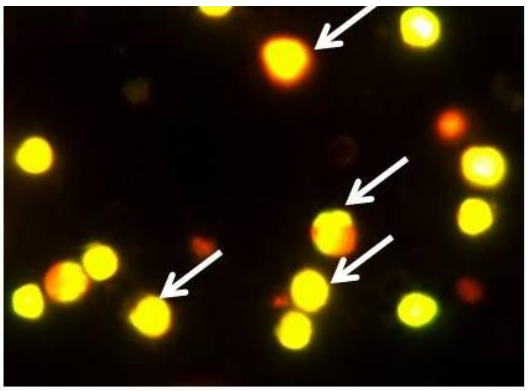

Fig. 6B

Fig6A. *Detection of apoptosis by Acrydin Orange Ethidium Bromide (AO-EtBr) counter stain method. The white arrows show non apoptotic living cells with bright green colour.*
Fig6B. *Detection of apoptosis by Acrydin Orange Ethidium Bromide (AO-EtBr) counter stain method. The white arrows show apoptotic cells with blabbing and orangish yellow colour…*

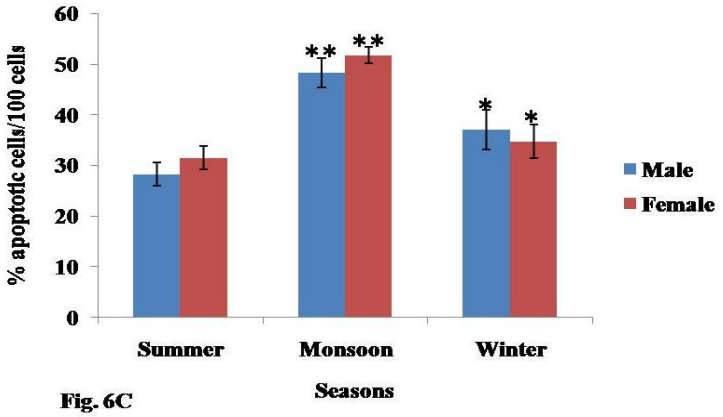

Fig. 6C

Fig6C. *Season and sex dependent variations in % apoptotic rate of splenocytes in male and female goats, C. hircus. Data represents mean ± SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *p< 0.05, ** p< 0.01; summer vs monsoon and winter*

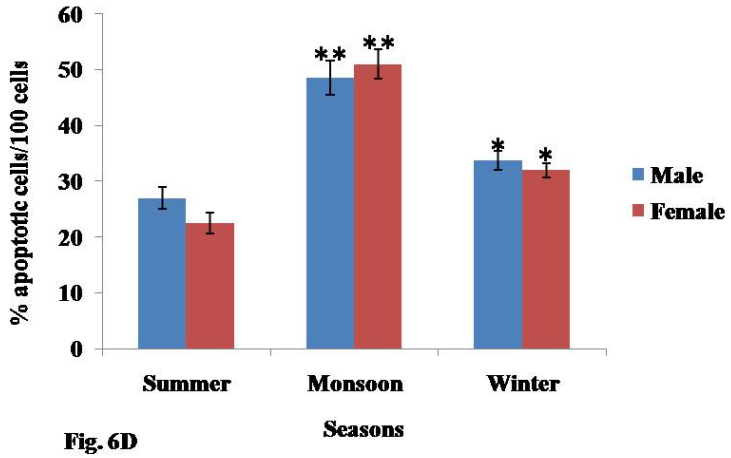

Fig6D. *Season and sex dependent variations in % apoptotic rate of thymocytes in male and female goats, C. hircus. Data represents mean ± SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *p< 0.05, ** p< 0.01; summer vs monsoon and winter.*

4. DISCUSSION

The role of different hormones in immune modulation in ruminants general and goats in particular is less explored area. Further, goats are susceptible to different immune compromised conditions particularly during monsoon. Because during monsoon they are under the threats of pathogenic invasions [27] and it is also well documented by elevated level of apoptosis in both thymus and spleen of both the sexes. Thus, in the present study we wish focus on the role of different hormones (adrenal steroids, thyroxin and melatonin) in immune modulation of goats. Among different hormones the adrenal steroids are regarded as immune suppressive as suggested by others [4]. A unique aspect of our study is that, the results were discussed under the special focus of melatonin which is a known immune enhancer [28].

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 [29]. Males and females often differ in the types of stressors they encounter, especially during the breeding season [30]. Thus, exposure to stressors may influence sex differences in immune function and subsequent resistance to infection [31]. 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 [32]. 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 [33]. A wealth of information demonstrates how glucocorticoids suppress immune function [32], which led to the conjecture that glucocorticoids are largely responsible for decrements in immune activity in free-living animals in winter [34]. Now there is compelling evidence that in certain contexts glucocorticoids can enhance aspects of immune function which may be immune redistribution in disguise [35]. 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 [21]. 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 [12]. However, thyroidectomy resulted in hypoplasia of lymphoid organs [36] 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 [37]. Some of the reports are contradictory to the previous citations some scientist [13] reported that under *in vivo* and *in vitro* conditions thyroxin has no role in immune modulation. Some other report [38] 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 [11]. 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 [39]. 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 [17]. According to some reports [40], 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 [16]. The role of melatonin in modulation of goat reproduction and maintenance of seasonality is well documented [19] particularly focussing 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, [14]) 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).

5. CONCLUSION

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. So, the effect of free radicals in modulation of immunity may be another important aspect of experimentation.

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