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Correlation between peripheral melatonin and general immune status of domestic goat, *Capra hircus*: A seasonal and sex dependent variation

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ABSTRACT

Caprine immune system has largely been neglected by ruminant researchers despite of its high economic importance in bioindustry. We made an attempt to note the annual/seasonal and gender dependent variation in immune status of the domestic goat, *Capra hircus*, Jamunapari breed and correlated with the concentration of peripheral gonadal hormones (testosterone, 17β-estradiol) and melatonin. Based on the data of general immune parameters such as total leukocyte count (TLC) and percent lymphocyte count (%LC) along with blastogenic response of splenocytes in terms of percent stimulation ratio (%SR), we found an annual variation in immune status of male, female and castrated male goats in a gender dependent manner. Female goats showed higher immune parameters than that of castrated as well as intact male goats. Testosterone in intact male goats was high throughout the year, which might be responsible for low immune status. However, in castrated male goats, plasma testosterone was undetectable and presented an immune status equivalent to that of female goats. We observed a high immune status during the winter season in all the groups of goats, which might be due to high circulatory concentration and longer duration of melatonin induced by short days. Being released by circulating lymphocytes, the concentration of interleukin-2 (IL-2) in blood plasma, showed an annual and seasonal variation being higher in female than castrated and intact male goats throughout the year, having a similar trend in plasma melatonin concentration. The higher immune status in female goats during the winter months might have helped them to overcome the winter bound stress, i.e. low temperature. Therefore, we may propose that testosterone acts as an immunosupressor while melatonin as an immunostimulator for goats as it showed a positive correlation with immune parameters noted.

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1. Introduction

Goat farming is a very popular animal husbandry in India, contributing to the majority of meat, milk and leather related income (MacHugh and Bradley, 2001). India is a vast subcontinent having 123 million goats with 20 identified breeds and several nondescript local breeds which together constitute approximately 20% of the world's goat population (http://fao.org). The grazing activity of domestic goats exposes them to a wide range of pathogenic organisms (cysts of helminthes and nematodes) and pesticides (used by local farmers) as a part of environmental challenges. Moreover, incidents of infectious diseases such as enterotoxaemia, diarrhea and various helminthic infections are more frequent during certain period of the year, i.e. rainy season. In tropical zone, rainy season provides high humidity and temperature for the growth of bacteria and several pathogens and facilitates their horizontal as well as vertical migration in domestic goat population. On the other hand, during rest of the year, the health of the

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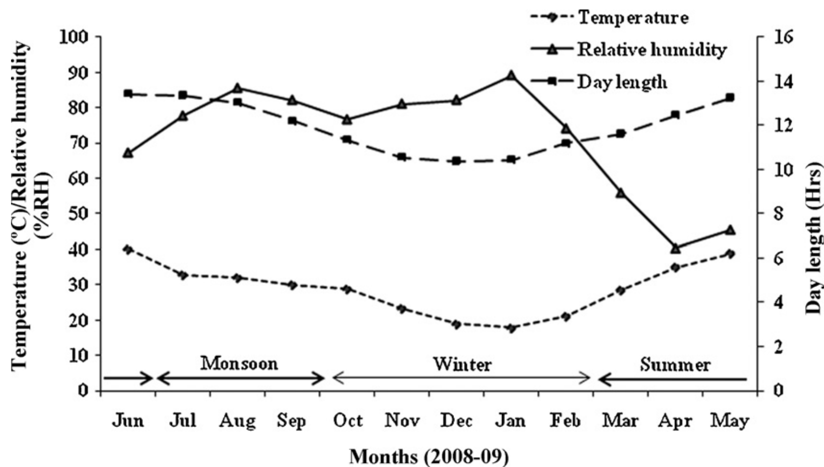

Fig. 1. Annual variation in the climatic factors – temperature (°C), day length (h) and relative humidity (%RH).

domestic goats is optimum and produce maximum amount of milk and meat. The breed "Jamunapari" is the most common domestic goat in Indian tropical zone. It received less attention correlating its immune physiology and reproductive pattern with climatic condition. The Jamunapari goats are non-seasonal breeder (Devendra, 1985; Hassan et al., 2010) and breed twice a year, i.e. late summer (May–June) and early winter (September–October). The length of estrous cycle in female goats ranges from 19 to 24 days. Female goats show estrous cycles periodically until they get pregnant. On the other hand, male goats are reproductively active year around (McDonald, 1963).

The role of melatonin, a neurohormone secreted mainly by pineal gland, has been less explored in goats except that it partially influences reproduction in seasonally breeding Payoya goats (Zarazaga et al., 2010). Further, a seasonal rhythm in melatonin concentration was noted in Finnish landrace goats suggesting that endogenous rhythm of melatonin concentration varies during the course of a year (Alila-Johansson et al., 2001). Study on partial cloning and polymorphism in Mel1a receptor gene of alpine breed goats suggest no relation between the expression of Mel1a and reproduction. Thus, the role of the peripheral melatonin in goat physiology is inconsistent. However, seasonality in reproduction seems to be influenced by the polymorphism of melatonin receptor gene, MTNR1A (Migaud et al., 2002). Despite of the fact that melatonin is a well-known immunostimulator (Ahmad and Haldar, 2010a,b); no report exists to suggest its role in caprine immunity. Further, the correlation of seasonal peripheral melatonin concentration and immunity has never been explored in any domestic goat. Taking this lacuna into consideration, we noted the seasonal immune status of a tropical ruminant *Capra hircus* of low latitude (Varanasi – Lat. 25° 18′N; Long. 83° 1′E) and correlated it with the concentration of melatonin, gonadal hormones (testosterone, 17β-estradiol) and climatic condition. To express the immune status, we used simple clinical parameters such as total leukocyte count (TLC), percent lymphocyte count (%LC) along with blastogenic response of splenocytes in terms of percent stimulation ratio (%SR) and estimation of a cytokine interleukin-2 (IL-2) concentration for the expression of seasonal variation in immunity in domestic goat, *C. hircus.*

2. Materials and methods

2.1. Climatic variation

Data of annual climatic variation, i.e. temperature (°C), day length (h) and relative humidity (%RH) for one year (June 2008–May 2009), recorded at Varanasi, was obtained from Indian Meteorological Department, Ozone Unit, Banaras Hindu University, Varanasi, India (Fig. 1).

2.2. Animals and management

The present study included 180 domestic goats (Jamunapari breed) of approximately same age (∼1 yr) and weight (∼20 ± 2 kg). In each month, 15 goats were randomly chosen and purchased from goat farm. In order to maintain a consistency in food and hygiene etc., the goats were acclimatized in the animal house under natural condition at least for 7 days. All the female goats used in the present study were neither pregnant nor lactating. The intact male goats were kept isolated from female goats in order to prevent mating. Castration (physical) of the young goats was performed by the goat farmers with the help of emasculators (before the development of secondary sexual characters). The goats were fed on green leaves and grasses as per seasonal availability along with usual roughages (hay, protein grains, mineral cake) and water ad libitum. Health of the goats was monitored by noting down the body temperature (normal rectal temperature: 102.5–103 F) and rumen movement by authorized veterinary doctors. Prophylactic measures were adopted in terms of vaccination against enterotoxaemia, foot and mouth diseases and peste des petits ruminant (PPR). The goats were treated with antihelmintics twice per year and 0.5% solution of malathion (acaricidal baths) was sprayed externally at the interval of 2 months as described by Chowdhury et al. (2002). No seasonality in any type of infection was observed during the study period.

2.3. Blood and spleen sampling

Fifteen goats (five males, five females and five castrated males) were selected from the flock and numbered. For the assessment of reproductive hormonal profile, blood of intact male, female and castrated male goats was collected from the jugular vein applying minimum stress when the female goats were in heat period. The detection of heat period was purely based on the visual observations, i.e. more vocalization, reddening of vulva and mucorrhea. Blood was heparinized and centrifuged at 3000 × *g*. The plasma was collected and stored at −20 °C until hormonal (testosterone and 17β-estradiol) analysis.

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One night prior to the slaughtering, blood samples were obtained during the night time (3 h after sunset) for the analysis of basic clinical parameters (TLC and %LC) along with estimation of melatonin and IL-2. Blood was collected by venipuncture (left jugular vein) in a 10 ml dispovan syringe coated with 10% EDTA (anticoagulant) and kept in ice box. All the goats were sampled within 40 min 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 processed for observation of basic clinical parameters, i.e. TLC and %LC and rest of it was centrifuged (3000 × g) for plasma collection. Plasma was immediately stored at −20 °C until the analysis of melatonin and IL-2. Next day, the animals were electrically stunned and bled immediately upto death after terminal cervical incision. The slaughtering of all the animals was performed according to the Slaughter of Animal Act under "Central Provinces Gazette" 1915 and modified in 2002. The spleens were collected aseptically. Testes and ovaries were collected and weighed. A small portion of the spleen was washed immediately in PBS three times and kept in a sterile vial containing chilled PBS on ice. Within 20 min of collection, spleen was processed for blastogenic response assay after challenging the splenocytes with a T cell mitogen, concanavalin A. This procedure of sampling was continued every month for one year (June 2008–May 2009).

2.4. Estimation of reproductive hormonal concentration (testosterone and 17β-estradiol)

2.4.1. Measurement of testosterone concentration

ELISA kit for testosterone assay was purchased from Dia Metra (Lot No. DKO 002). According to the manufacturer's instruction, 25 μl of standard, control and sample were added in each well of the ELISA plate followed by addition of 100 μl of the enzyme conjugate solution and 100 μl of the testosterone antiserum. The ELISA plate was incubated with mild shaking at room temperature for one hour. Wells were aspirated and washed thrice with double distilled water. Then, 100 μl of the TMB chromogenic solution was added to each well and the plate was further incubated at room temperature for 30 min. Finally, 100 μl of stop solution (0.2 M H_2SO_4) was added and absorbance was recorded at 450 nm using a microplate ELISA reader (BioTek). The coefficient of intra and inter assay variation was less than 9% and 15%, respectively. The assay was carried out in triplicate.

2.4.2. Measurement of 17β-estradiol concentration

ELISA kit for estradiol assay was purchased from diagnostics system laboratories (# DK0003; LOT 2130). 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 min in dark. Finally, 100 μl of stop solution was added in each well and absorbance was recorded at 450 nm. Intra and inter assay variation was less than 5% and 14%, respectively. The assay was carried out in triplicate.

2.5. Assessment of total leukocyte count (TLC) and percent lymphocyte count (%LC)

TLC and %LC were performed following the method of Haldar et al. (2004). WBCs were counted in Neubauer's counting chamber (Spencer USA). For %LC, a thin blood film was stained with Leishman's stain and lymphocyte subpopulation was counted under oil immersion lens of Nikon microscope (Nikon, Japan).

2.6. Study of blastogenic response of splenocytes

2.6.1. Separation and culture of lymphocytes

Cell culture was performed following the method of Singh and Haldar (2005). A small piece of spleen was minced with the help of sterile syringe head in cold phosphate buffered saline (PBS) and passed through small sieve in order to prepare single cell suspension. For the lysis of red blood cells (RBCs), the total cell suspension was mixed with 0.84% cold ammonium chloride (1:10 ratio) for 20 min at 4 °C. The cell suspension was centrifuged (254 × g). The pellet was suspended in 2% complete medium and centrifuged (1000 × g). The procedure was repeated twice to exclude maximally RBCs and other cells. Finally, the cell pellet was suspended in 2 ml of 2% complete medium and filtered through 15 μm filters to get lymphocytes. The purity of the single cell suspension was monitored under inverted microscope and smear of cell suspension on glass slide was stained with Giemsa stain for more accuracy of purity. The appropriate cell viability (more than 95%) was checked with 1% trypan blue exclusion method. This single cell suspension of isolated spleen lymphocytes (∼95%) was adjusted to 1×10^6 cells/ml in 10% complete medium, containing antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml, gentamycin 100 μg/ml) and heat inactivated fetal calf serum (Sigma, USA). One ml of cell suspension (1×10^6 cells/ml) was plated in triplicates in 35 mm sterile culture plates. The control culture plates were incubated without T cell mitogen, concanavalin A (Con A; Sigma–Aldrich, USA) whereas test culture plates were incubated with 10 μg/ml of Con A. The optimal dose of Con A was standardized by performing a dose dependent assay. The culture plates were kept and maintained at 37 °C in a 5% CO_2 incubator (Heraeus) for 72 h. Eighteen hours before harvesting the cell culture, 1 μCi of tritiated thymidine (^3H) (BARC, India; specific activity 8.9 Ci/mM) was added to each culture plate.

2.6.2. Harvesting of the culture

Culture plates were harvested after 72 h of incubation following the modified method of Singh and Haldar (2005) earlier reported by Pauly and Sokal (1972). The cells were detached form the culture plate surface with flushing the medium gently with the help of a pasture pipette and were centrifuged to get the cell pellet. To wash the cells, the pellet was suspended in 2.0 ml of cold PBS and centrifuged (1000 × g). The procedure was repeated twice. The pellet was added with 2.0 ml of cold 5% trichloroacetic acid (TCA) and centrifuged (1000 × g) for 30 min at 4 °C. The precipitate was dissolved in 300 μl of 1 N NaOH and digested at 65 °C for 1 h. The cell suspension was filled with scintillation cocktail (naphthalene – 60 g/l, PPO – 4 g, dimethyl POPOP – 0.2 g, ethylene glycol – 20 ml, p-dioxane – 1 L) and counted in a liquid scintillation β-counter (BECKMAN, USA). The mean counts were expressed as counts per minute (CPM). The percent stimulation ratio (%SR) was calculated as follows:

$$\%\text{Stimulation ratio} = \frac{\text{CPM with mitogen}}{\text{CPM without mitogen}} \times 100$$

2.7. Measurement of melatonin concentration

Peripheral melatonin concentration in plasma was measured in triplicates from the blood collected during night time with the help of commercial kit (Biosource, NIVELLES, Belgium). The kit was of human origin and validated by using the goat plasma against the standard sample provided with it and no interference was observed. The lowest limit of detection (LLD) for plasma melatonin was 2 pg/ml. Inter and intra assay variations were between 9% and 15%, respectively.

2.8. Measurement of IL-2 concentration

The concentration of IL-2 was measured in triplicate aliquots with the help of commercial kit (Beckman Coulter, France). Sandwich ELISA was performed to measure the plasma concentration of IL-2 in all the groups according to manufacture's instruction. The ELISA kit used was of human origin and validation was carried out by using goat plasma against the standard samples provided. No interference was observed. Intra assay variation was between 3.3% and 7.2% and inter assay variation was between 6.2 and 8.2%. The LLD for plasma IL-2 was 5.0 pg/ml and recovery was between 80% and 132%.

2.9. Statistical analysis

The data were presented as the means ± SEM. The annual variation in gonadal weight (testis and ovary) and gonadal hormones (testosterone and 17β-estradiol) were analyzed by one-way ANOVA followed by post hoc test–Dunnett *t*-test (2-sided). Variation in TLC, %LC, %SR, plasma melatonin and IL-2 of male, female and castrated goats was analyzed by two-way ANOVA. When differences between months and groups of the goats were recorded, post hoc test–Dunnett *t*-test (2-sided) was applied. In Dunnett *t*-test, intact male goats were treated as control and compared with female as well as castrated male goats. The mean difference was statistically significant at the 0.05 level. Correlation analysis was performed to determine the possible linear relationship between melatonin and other immune parameters and expressed as Pearson coefficient (*r*).

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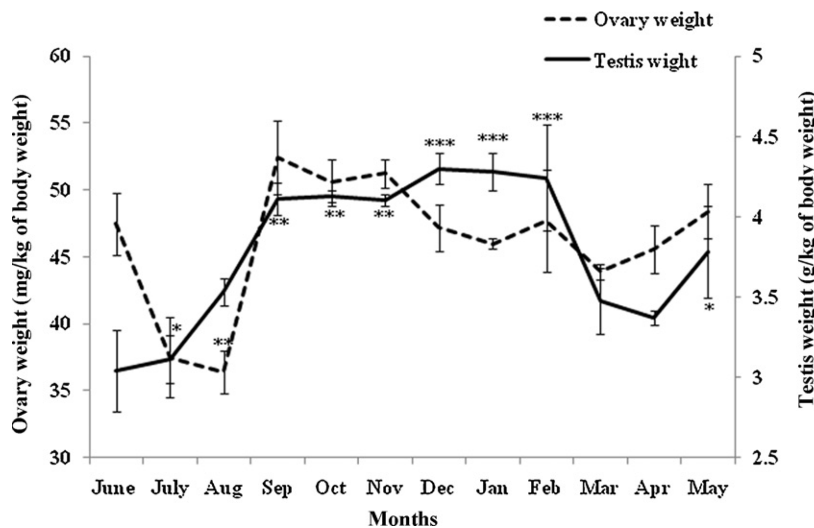

Fig. 2. Annual variation in testis and ovary weight in domestic goat, *Capra hircus*. Data of each point represent mean ± S.E.M; $N = 5$. Vertical bar on each point represents standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In Dunnett *t*-test, the data of June month were treated as control and compared with that of other months.

The analyses of data were computed with the help of SPSS Statistics 17.0 software programme.

3. Results

There are three major seasons in the northern part of India (Varanasi – Lat. 25° 18′N; Long. 83° 1′E), i.e. monsoon or rainy season occurs from July to September due to the seasonal cloud emerging from the Bay of Bengal. A short winter starts from October to February being extreme during December/January (0–1 °C). The summer is long from March–June being maximum temperature in May/June (40–47 °C). During 2008–09, Indian subtropical climatic presented the variation in temperature (highest during summer ~40 °C, lowest during winter ~17.78 °C) and humidity while variation of day length between summer and winter was only ~3 h (Fig. 1).

3.1. Annual variation in reproductive parameters

3.1.1. Annual variation in gonadal weight

There was a significant increase in testis weight during September to February ($P < 0.01$) and in May ($P < 0.05$) when compared with that of June. The testicular weight was maximum (4.3 ± 0.1 g/kg of body weight) in December and minimum (3.04 ± 0.251 g/kg of body weight) in June. On the other hand, a significant decrease ($P < 0.05$) in ovarian weight was noted in July and August when compared with that of June. A high ovarian weight was observed from September to February and May to June being maximum in

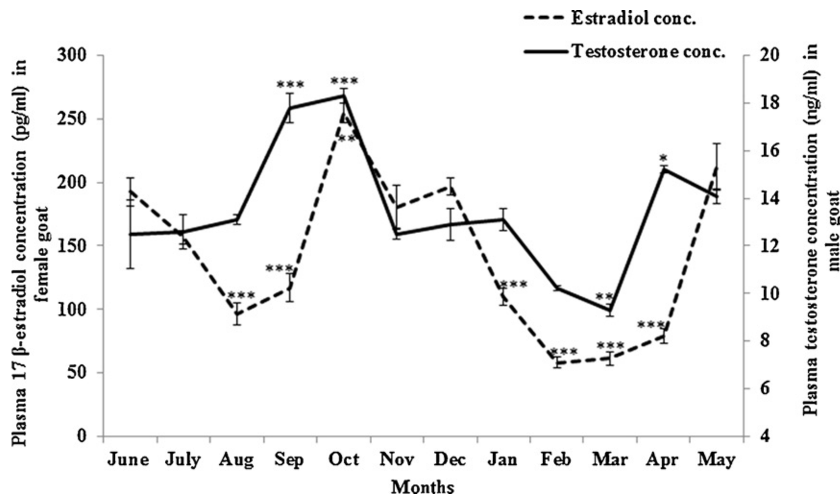

Fig. 3. Annual variation in plasma testosterone concentrations in intact male goats and 17β-estradiol in female goats, *C. hircus*. Data of each point represents mean ± S.E.M; $N = 5$. Vertical bar on each point represents standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In Dunnett *t*-test, the data of June month were treated as control and compared with that of other months.

September (52.42 ± 2.73 mg/kg of body weight) and minimum in August (36.4 ± 1.62 mg/kg of body weight) (Fig. 2).

3.1.2. Estimation of gonadal hormones

Testosterone concentration in intact male was maximum during the month of October (18.3 ± 3.4 ng/ml) and minimum during March (9.3 ± 4.9 ng/ml). Testosterone concentration was significantly high in September–October ($P < 0.001$) and May ($P < 0.05$) when compared with that of June while it was significantly ($P < 0.01$) low in March. The testosterone in the plasma of castrated male goats was undetectable and hence not presented. The peripheral plasma concentration of 17β-estradiol in female goats showed variation throughout the year presenting two peaks, first during the month of October (354.44 ± 40.4 pg/ml) and second during the month of May–June while the low concentration of the hormone was noted during February–April (48.27 ± 21.1). When compared with June, the concentration of 17β-estradiol was significantly high ($P < 0.01$) in October and low during August–September and January–April (Fig. 3).

3.2. Annual variation in immune status of male, female and castrated male goats

3.2.1. Peripheral total leukocyte count (TLC) and percent lymphocyte count (%LC)

An increased value of TLC during winter (November–February) had been noted in all the three groups of goats. The maximum TLC was observed in January in castrated male (5360 ± 292.57 cells/mm^3) and female goats (6700 ± 396.23 cells/mm^3) while intact male goats had moderately low value (3760 ± 324.96 cells/mm^3). February onwards, there was a sharp decline in TLC and the lowest count was observed in June for male (2100 ± 122.47 cells/mm^3) and castrated male goats (2800 ± 208.32 cells/mm^3) while for female goats, it was minimum (3700 ± 331.66 cells/mm^3) during July. A low value of TLC with minimum annual variation was noted in male goats (Fig. 4). A highly significant difference ($P \leq 0.001$) in TLC was noted for female and castrated male goats when compared with that of male goats. A significant variation in TLC was dependent on sexes ($P \leq 0.001$) as well as on months ($P \leq 0.001$) but, no significant interaction ($P = 0.102$) was observed between sexes and months. A similar trend was observed for the %LC being minimum in the month of July (male 21.4 ± 0.74, female 26.2 ± 1.82 and castrated male 23.4 ± 0.74 lymphocytes/100 cells) and maximum in the month of February for male (29.2 ± 0.73) and castrated male goats (33.2 ± 0.8) while in female goats, maximum %LC (37.8 ± 0.73) was noted in January. The variation in %LC among sexes ($P \leq 0.001$) and months was highly significant ($P \leq 0.001$) (Fig. 4).

3.2.2. Annual variation in percent stimulation ratio (%SR)

There was an increase in %SR from September to March, being maximum in the month of January (female 364.0 ± 12.75, male 219.4 ± 9.91 and castrated male 314.4 ± 31.13). It declined from March onwards with minimum value in the month of July (female 242.42 ± 14.89, male 154.4 ± 9.17 and castrated male 181.6 ± 24.32) (Fig. 4). A highly significant variation ($P \leq 0.001$) in blastogenic response of splenocytes was observed among sexes as well as months.

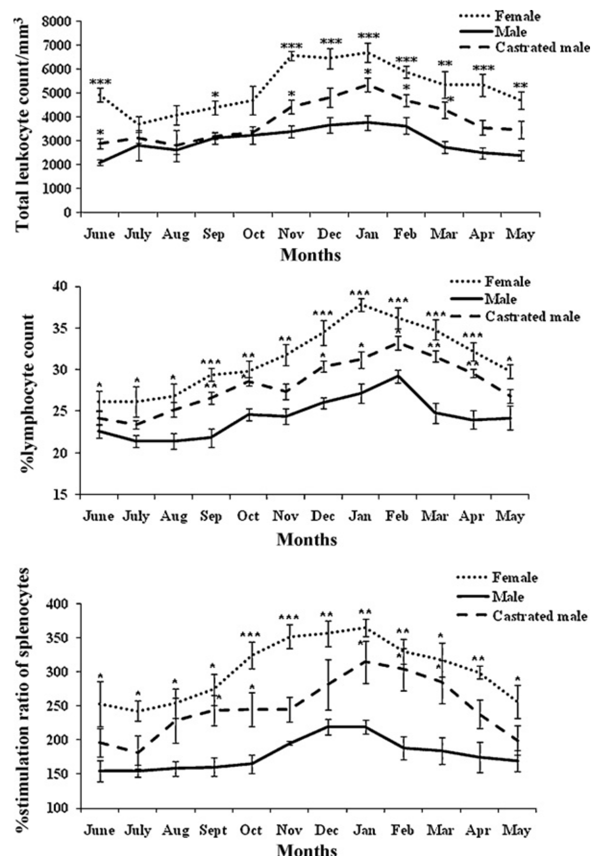

Fig. 4. Annual variation in total leukocyte count (upper panel), %lymphocyte count (middle panel) and %stimulation ratio of splenocytes (lower panel) in domestic goats, *C. hircus*. Data of each point represent mean ± S.E.M; $N = 5$. Vertical bar on each point represent standard error. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$. In Dunnett *t*-test, intact male goats were treated as control and compared with all other groups (female and castrated male goats).

3.3. Annual variation in melatonin concentration

In female and castrated male goats, night time (22:00 h) melatonin concentration was higher than that of intact male goats. In all the three groups of goats, the annual melatonin concentration started increasing from August onwards till November being the highest in February for female (295.7 ± 1.05 pg/ml) while in castrated and intact male goats, the highest concentration of melatonin was observed in Jan (castrated male 197.58 ± 1.56 and intact male 151.88 ± 1.30 pg/ml). Low concentration of melatonin was recorded during March to July, i.e. in summer months. The variation in plasma melatonin was highly significant ($P \leq 0.001$) among the three groups of goats as well as the months of the year. The interaction between genders and months was highly significant ($P \leq 0.001$) (Fig. 5).

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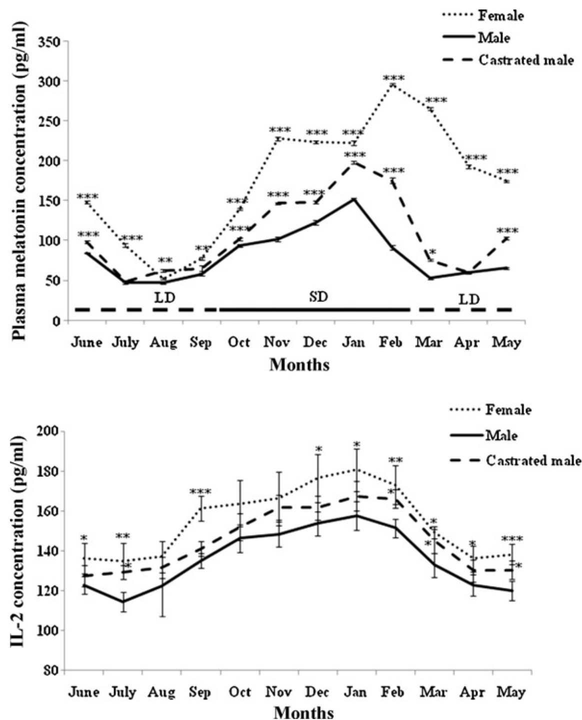

Fig. 5. Annual profile of melatonin (upper panel) and IL-2 (lower panel) in goat plasma. LD = long day length (broken line) and SD = short day length (solid line). Data represent mean ± S.E.M; $N = 5$. Vertical bar on each point represents standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In Dunnett *t*-test, intact male goats were treated as control and compared with all other groups (female and castrated male goats).

3.4. *Annual variation in interleukin-2 concentration*

IL-2 concentration in plasma of female, male and castrated male goats presented an increasing trend from August and had peak value in January (female 180.714 ± 5.62, male 157.518 ± 3.71 and castrated male 167.454 ± 3.71 pg/ml). It then sharply declined reaching to the lowest concentration in July for female (134.812 ± 5.16 pg/ml) and male (114.498 ± 3.05 pg/ml), while IL-2 concentration in castrated goats (127.242 ± 3.37) was minimum in June. There was a highly significant variation ($P \leq 0.001$) of peripheral IL-2 concentration among the three groups of goats and months (Fig. 5).

3.5. *Seasonal variation in immune status of male, female and castrated male goats*

The seasonal variation in immune status of goats was judged by general immune parameters of blood, i.e. TLC, %LC and %SR of splenocytes during winter (October–February), summer (March–June) and monsoon (July–September). TLC was significantly high in female ($P < 0.001$) as well as in castrated male goats ($P < 0.01$) during winter and summer. TLC in male goats was significantly high ($P < 0.05$) only during winter and presented minimum variation. Percent lymphocyte count presented a significant variation in all the groups of goats during winter ($P < 0.01$) and summer ($P < 0.05$) when compared with monsoon. The goats also presented a significant variation in %SR of splenocytes during winter ($P < 0.001$) and summer ($P < 0.01$) when compared with that of monsoon (Fig. 6).

Table 1
Correlation between annual profile of melatonin with immune parameters, i.e. TLC, %LC and %SR of splenocytes in female, male and castrated male goats. Correlation was expressed in terms of Pearson coefficient (*r*).

Immune parameters	Groups of goats	Correlation with melatonin (*r*)
TLC	Female	0.798, ($P < 0.001$)
	Male	0.702, ($P < 0.001$)
	Castrated male	0.838, ($P < 0.001$)
%LC	Female	0.837, ($P < 0.001$)
	Male	0.675, ($P < 0.001$)
	Castrated male	0.607, ($P < 0.001$)
%SR	Female	0.720, ($P < 0.001$)
	Male	0.814, ($P < 0.001$)
	Castrated male	0.691, ($P < 0.001$)

3.6. *Gender dependent variation in immune status of male, female and castrated male goats*

Data of TLC, %LC and %SR of splenocytes of male, female and castrated male goats reflected that the immune status of female goats were significantly higher ($P \leq 0.001$) than that of intact male goats.

3.7. *Correlation of plasma melatonin concentration with gonadal and immune parameters*

Table 1 showed the correlation coefficient (*r*) of melatonin with TLC, %LC and %SR in female, male and castrated male goats. Correlation analysis clearly suggested that annual melatonin profile in female goats had no correlation with annual ovarian weight ($r = 0.331$) as well as 17β-estradiol concentration in plasma. However, plasma melatonin in intact male goats had a weak correlation ($r = 0.562$) with testicular weight but not with plasma testosterone concentration ($r = -0.257$). Further, in female goats, there was a strong positive correlation of melatonin with TLC ($r = 0.798$), %LC ($r = 0.837$) and %SR of splenocytes ($r = 0.720$) while IL-2 concentration ($r = 0.516$) had a weak correlation with plasma melatonin concentration (Fig. 7). In male goats, melatonin had a strong positive correlation with TLC ($r = 0.702$), %SR of goat splenocytes ($r = 0.0814$) and IL-2 concentration ($r = 0.841$) but moderately correlated with %LC ($r = 0.675$) (Fig. 7). In castrated male goats, TLC ($r = 0.843$) and IL-2 concentration ($r = 0.865$) had a strong positive correlation with melatonin while a moderate positive co-relationship of %LC ($r = 0.607$) and %SR of splenocytes ($r = 0.691$) was noted with plasma melatonin (Fig. 7).

4. Discussion

In general, goats are short day breeder (Zarazaga et al., 2009), being reproductively active during the winter months (September–October). Kidding occurs in spring season (February–March) which provides abundant food and favorable environment for the survival of the

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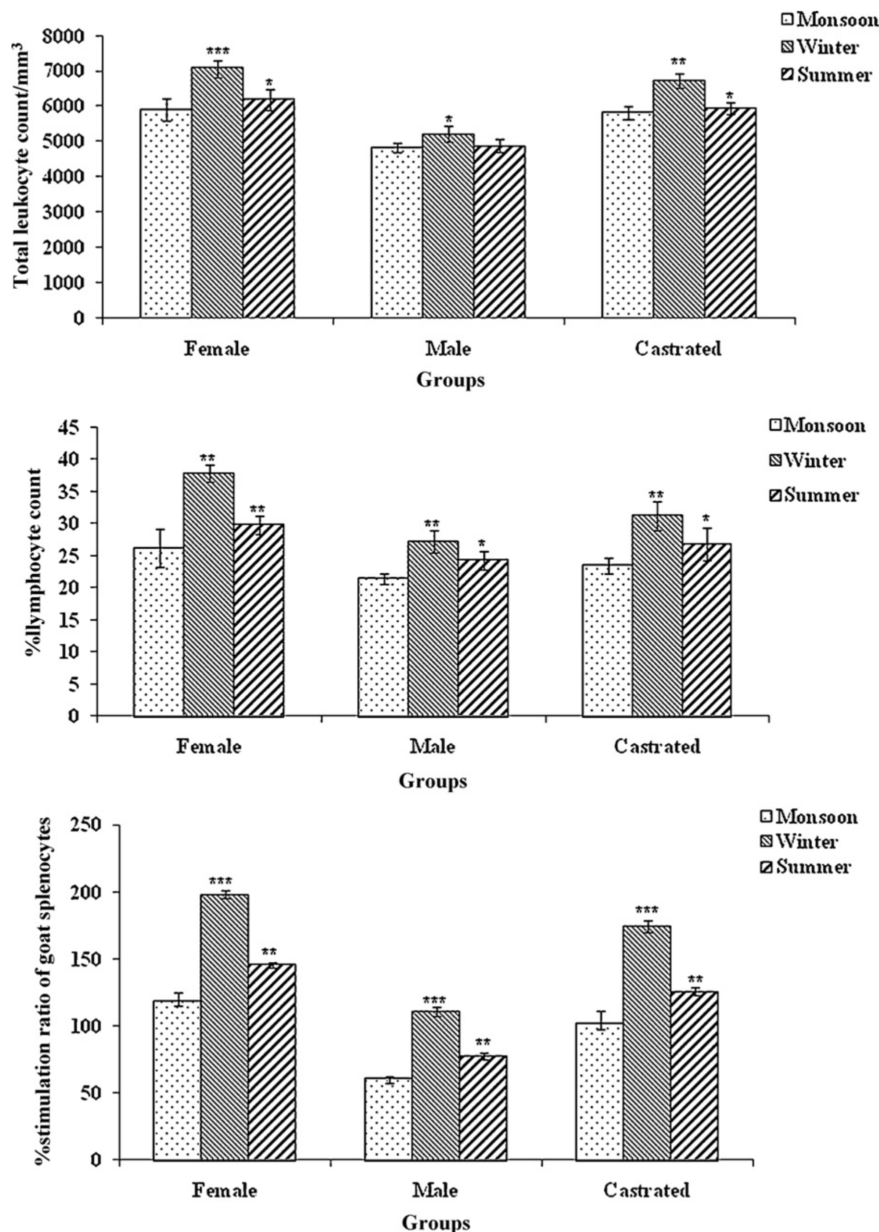

Fig. 6. Seasonal variation in total leukocyte count, %lymphocyte count in blood as well as %stimulation ratio of goat splenocytes. Data represent mean ± S.E.M. Vertical bar on each point represents standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In Dunnett *t*-test, intact male goats were considered control and compared with all other groups (female and castrated male goats). Data of each season is the mean values of respective months.

offsprings. However, Jamunapari female goats present estrous throughout the year (Devendra, 1985, Hassan et al., 2010), mating occurs twice in a year, i.e. May–June and September–October. The annual ovarian weight and estrogen profiles of the female goats noted in the present study, showed a well-defined two peaks during the above-mentioned period. McDonald (1963) reported that intact male goats were capable of producing fertile ejaculates year around, we found a high testicular weight from September to February and in May, with a peak in peripheral testosterone concentration. The high testosterone concentration might be due to the rut activity during which the intact male goats displayed mounting behavior.

Melatonin physiology in relation with reproduction has less been discussed. In most of the temperate goat breeds, melatonin is progonadotropic in nature as its administration during the last month of spring in autumn born female goats, advanced the breeding season (Papachristoforou et al., 2007). Melatonin also mimics the stimulatory effect of short days on reproduction in goats (Haresign et al., 1990; Croker et al., 1992; Williams et al., 1992; Belibasaki et al., 1993), but no report on annual profile of melatonin in goats in relation with other hormone exist. We correlated the annual profile of plasma melatonin of intact male and female goats with their respective reproductive hormones and parameters. The annual variation in

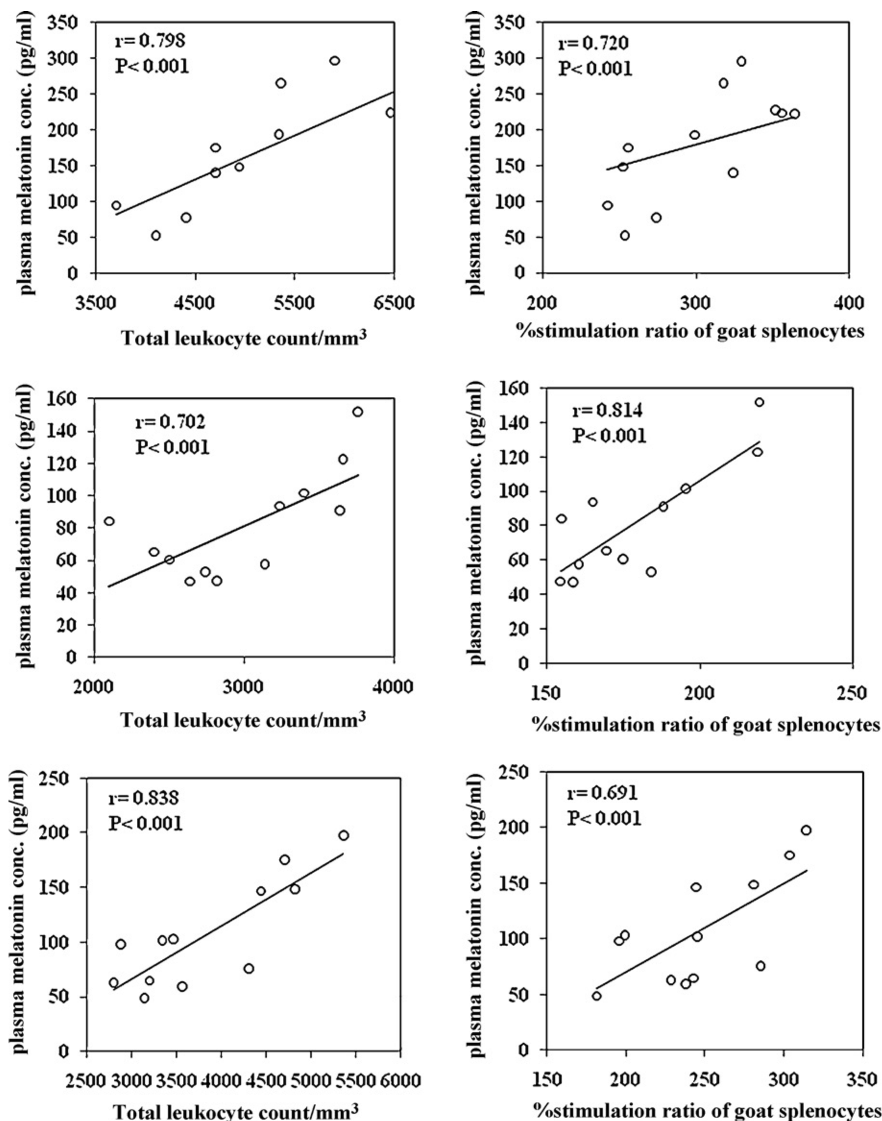

Fig. 7. Scatter plot for the correlation between plasma melatonin concentration and TLC, %SR of splenocytes in female (upper panel), male (middle panel) and castrated male goat (lower panel).

melatonin had no correlation with ovarian weight ($r = 0.331$, $P \leq 0.001$), estrogen concentration ($r = -0.257$, $P \leq 0.001$) as well as testosterone concentration ($r = -0.034$, $P \leq 0.001$). However, testicular weight ($r = 0.562$, $P \leq 0.001$) showed a mild correlation with plasma melatonin concentration in intact male goats. These data suggested that melatonin might have less or no role in the regulation of reproductive functions of domestic Jamunapari goats. Our result is in consistent with the report of Zarazaga et al. (2003) who observed that plasma melatonin concentration had little role in controlling the reproduction in sheep and Mediterranean goats. Migaud et al. (2002) also did not observe any correlation between Mel1a receptor expression and reproduction. Further, polymorphism of melatonin receptor 1A (MTNR1A) was found only in some goat breeds like Sarda goats involved in seasonality of reproduction (Carcangiu et al., 2009). Chu et al. (2007) showed an association between MTNR1A gene and seasonality in estrous of goats. Taking circulatory concentration of melatonin into account, we observed a distinct seasonal variation, i.e. being high during short days of winter (October–February) in all the three groups of goats. Similarly, a six-month rhythmicity in melatonin concentration and rectal temperature was reported in Maltese female goat kids (Piccione et al., 2006) being high during short days. Support to our data comes from the result of the photoperiodic experiment done earlier in Finnish landrace goats by Alila-Johansson et al. (2001) to note the possible relation between the overt and endogenous melatonin rhythms.

Our next step was to observe the annual variation in immune function and to correlate it with peripheral gonadal steroids, melatonin and the climatic condition. Interestingly, intact male goats having reproductive

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activity throughout the year with an equally high concentration of testosterone had little difference in the immune parameters during winter, summer or monsoon. It might be due to high concentration of testosterone which had an immunosuppressive effect (Ahmad and Haldar, 2010a,b). On the other hand, castration before puberty, inhibited testosterone surge and provided a higher immune status almost equivalent to female goats when compared with those of intact male goats. Our results are in consistent with the report of Schuurs and Verheul (1990) in which they reported that castration of adult rodents resulted in an increased immunoglobulin level, humoral and cell mediated immunity but were not equivalent to those of female goats.

Sex differences in immune function are well established in vertebrates (Alexander and Stimson, 1988; Billingham, 1986; Schuurs and Verheul, 1990). Males generally exhibit lower immune response than female conspecific and under pathogenic condition (Billingham, 1986; Schuurs and Verheul, 1990; Zuk and McKean, 1996). We also found a gender dependent variation in annual/seasonal immune status in domestic goats, *C. hircus*. Since, estrogen enhances both cell and humoral mediated immune responses (Olsen and Kovacs, 1996, 2002), the female goats presented a higher immune status than that of intact male as well as castrated male goats.

Our data suggested that during winter, the immune parameters (TLC, %LC and %SR of splenocytes) were high in all the three groups of the goats, i.e. intact male, castrated and female goats. Our results are in agreement with the report of Hotchkiss and Nelson (2002) that experimental short day enhanced lymphocyte proliferation in species ranging from mice to primates. Their explanation is quite applicable for goats as far as the winter months/short days are concerned. It has already been hypothesized that enhanced immune function during short days is due to the increase in the duration of melatonin secretion (Brainard et al., 1988; Champney and Matthews, 1991; Champney et al., 1998). In *C. hircus*, we may suggest that short days of winter months increased the peripheral melatonin concentration that might had stimulated the splenocytes for proliferation in vivo. When these prestimulated cells were treated with mitogen concanavalin A, more induced mitogenic activity of splenocytes was noted. On the other hand, moderately low concentration of peripheral melatonin during summer and monsoon season could not induce cell proliferation of splenocytes in vivo to the extent observed in winter.

Recently in seasonal breeder ewe, the ability of leptin to modulate melatonin release was observed (Zieba et al., 2007). Leptin, secreted by adipose tissues suppressed melatonin secretion during long days and stimulated the same during short days when tested in vitro on ovine pineal gland. Melatonin influences the release of cytokine (IL-2) from circulatory lymphocytes (Carrillo-Vico et al., 2005). We measured circulatory concentration of IL-2 and found no seasonal variation in male and castrated goats. However, female goats showed a peak value during winter (January–February) when compared with summer (May–June) and monsoon months (July–August). Interestingly, the IL-2 and melatonin concentration in plasma showed a parallel trend along with TLC, %LC and %SR of splenocytes in all the genders of *C. hircus*, suggesting a seasonal variation in immunity. Studies suggest that exposure of cattle to different photoperiod can influence immune function (Auchtung et al., 2004) which could be due to an increase in circulatory concentration of melatonin. We therefore, measured at least one time point, i.e. night time (22:00 h) melatonin throughout the year and recorded a high concentration of melatonin in all the three groups of the goats during short days of winter months (from October to February), being the lowest from March to September when long days prevails. Therefore, high concentration of circulatory melatonin in winter months (due to short days) can be correlated and might be responsible for immune enhancement as noted in domestic goats, *C. hircus*. This further supported our earlier finding in the present study that melatonin had no effect on reproduction of Jamunapari domestic goats breeding in winter. High melatonin concentration during the winter months might have boosted the immunity in goats and thus a successful survival during the cold stress of winter months was noted. This advantage is extremely important for female goats, (where the maximum immunity was noted) as they breed and undergo the gestation during winter months.

5. Conclusion

A gender dependent annual/seasonal variation in immune status existed in domestic goats, *C. hircus*, as reflected by the variation in TLC, %LC, %SR of splenocytes along with circulatory concentration of IL-2. Though, no direct correlation exist between gonadal steroids and melatonin, seasonal variation in gonadal steroids (testosterone and estradiol) and melatonin might be responsible for regulation of immunity in domestic goats for a successful survival during the cold stress of winter months. Thus, melatonin as an immunostimulator, is extremely useful for female goats, (maximum immunity) as they breed during the winter months. Our results of seasonal variation in gonadal steroids and melatonin concentration led us to propose that immune regulation in goats is a complex phenomenon and more experiments are required to unravel it.

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