Material & Methods
Animal model

The experimental animal model is Indian Goat, *Capra hircus*. Goats are seasonal and short day breeder. They are diurnal, semi domestic, grazing, herbivorous ruminants. They are having an average life span of 3-4 years. Morphologically males and females are easily identifiable. Monsoon is season of the reproductive preparatory phase of goats when green grasses are available. However mating and gestation occurs from middle of October to mid of February.

![Barbari Breed](image.png)

**Fig. 1 : Barbari Breed**

The gestational period varies between 150 ± 5 days. They generally give birth of 2 kids at a time. The goats are having a wide range geographical distribution (like the Himalayan range; chanthangi and chegu, North-Western arid and semi-arid region; sirohi, jhakrana, Southern region; malabari, and Eastern region; Black Bengal) in India. They are basically browser and browse if tree leaves are available but in northern India, due to plain topography, they are grazer. The vicinity of Varanasi (Lat. 25°18’N; Long. 83°01’E) is extremely rich of several breeds of goats such as Jamunapari, Jhakrana and most of them are either Barbari or their cross breeds like Anglo Nubian. The Indian domestic goats of local Barbari breed (Fig.1) are adapted for the tropical climatic condition. Goats are domesticated in several parts of the world (like arid and semi-arid regions, tropical and temperate climatic conditions) and its breeding and effective litter size is highest under captivity. The goats, having a high food value for common people are efficient meat machine that convert the residues of food crops into palatable meat. Very few experimental studies have been performed on physiology of the
Indian origin domestic goats. At first to establish the importance of tropical climatic condition on the goat physiology, we studied general status of immunity, metabolism and reproduction.

**Animal 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°18’ N, 83° 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 (*Pennisetum glaucum*), 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°F–103°F) 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 $43.87^\circ C \pm 1.02^\circ C$, percent relative humidity [%RH] $36.74 \pm 4.28\%$, day length, light–dark cycle-13.42 hours:10.18 hours), monsoon months (July–September, temperature $28.68^\circ C \pm 2.76^\circ C$, %RH 87.04 ± 3.50%, day length, light–dark cycle-12 hours:12 hours), and winter months (November–January, temperature $10.76^\circ C \pm 3.63^\circ C$, %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.

**Sample collections**

1. **Blood sampling**

   For the assessment of peripheral hormone, cytokine level and other plasma parameters, one night before the slaughtering, blood of male and female goats was collected from left jugular vein by venipuncture applying minimum stress (Kaushalendra and Haldar, 2012). 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$^\circ$C until the analysis of hormones (testosterone, estradiol, melatonin, cortisol and thyroxin), cytokines (IL-2, IL-6, TNF-α and IFN-γ) and other plasma parameters. Immediately after collection, fresh blood was used for haematological parameters (TLC, DLC, %LC, %Hb), free radical parameters and %SR of PBMCs.
2. **Sampling of pineal gland, spleen, thymus, liver and gonad:**

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. A small portion of that tissue was immediately fixed in neutral formalin for histological preparations and immunohistochemical studies. Remaining small portion was weighed and kept in a sterile vial containing chilled PBS for assessment of enzymatic parameters. Further, left tissues were kept in -20°C for different biochemical parameters. 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.

3. **General Histology of pineal gland, spleen, thymus and gonads**

**Preservation**

Pineal gland, thymus, spleen and gonads (testis and ovary) were collected from opened abdomen of the goats and cleaned with 0.9% NaCl (normal saline). The tissues were preserved in neutral formalin (37% formaldehyde, distilled water, Na$_2$HPO$_4$ and NaH$_2$PO$_4$) up to 48 hrs.

**Washing and dehydration**

The preserved tissues in neutral formalin were washed overnight under running tap water. The tissues were dehydrated through serial dilution of ethyl alcohol (30%, 50% and in 70% alcohol) by giving 2-3 changes at the interval of 30 minutes. Some times to remove the yellow colour of picric acid, the washing was initiated with Lithium carbonate. Small sized (0.125 cm$^3$) tissues were cut and then once more dehydrated in alcohol grade 30%, 50% and 70% by giving single change of 30 minutes and two changes in 90% and 100% alcohol at the interval of 1 hour. The dehydrated tissues were then ready for embedding.
**Embedding and block preparation**

The dehydrated tissues were cleared in absolute alcohol + xylene (1:1) and xylene (10 minutes) sequentially. The cleared tissues were kept in pre-warmed xylene + wax (1:1) solution for 30 minutes in an oven (maintained at 60-62 °C). These tissues were transferred to wax I and wax II for 30 minutes each and then, in wax III for 45 minutes. The blocks were prepared in pre-seasoned melted wax with the help of L-piece. Tissues in blocks were then ready for sectioning.

**Sectioning**

The block was fixed on the block-holder of the Leica semi-automated microtome (Leica Microsystems, RM2245) and was trimmed. The tissue was sectioned on a thickness of 6 µm. The sections were placed on gelatine pre-coated slide along with few drops of water. The slides were then placed on a mild hot plate and sections were allowed to spread. The slides were dried in air and allowed to attach firmly.

4. **Permanent slide preparation by Haematoxylene-Eosine (double-staining) method**

Slides with spread tissue sections were de-paraffinized in xylene for 30 minutes. The sections were rehydrated by putting them sequentially into a battery of downgraded alcohol (100%, 90%, 70%, 50% and 30%) and in water for 10 minutes in each. The slides were kept in the haematoxylin stain for 15-20 minutes and then washed in running tap water for 45 minutes for differentiation. The slides were transferred to diluted acid water (1 mL of acetic acid in 500 mL of distilled water) for a single dip and again washed in running tap water. The slides were dehydrated by putting them sequentially into 30%, 50%, and 70% alcohol each for 5 minutes and transferred to alcoholic (70%) eosine stain for 5 minutes. The sections were differentiated in acid alcohol (1 mL of acetic acid in 500 mL of 70% alcohol, if desired), and transferred sequentially in 90% alcohol for 2 minutes followed by two changes of 100% alcohol each for 5 minutes. The dehydrated sections were kept in 100% alcohol + xylene (1:1) for 5 minutes, and then transferred sequentially to xylene-I and xylene-II for 10 minutes. The permanent
slides were prepared by mounting with DPX (Distyrene Plasticizer Xylene, SRL, India), after 24 hrs were observed under microscope (Leitz MPV3 with photo-automat software) and were documented for general histology.

5. Morphometric analysis

The area of cortex and medulla (for thymus), size of red pulp and white pulp (for spleen), the size of normal pinealocyte along with the aged ones (for pineal), the area of seminiferous tubule (individually for testis), area of theca and granulose cells (for ovary) and endometrium and myometrium (for uterus) were measured with the help of ocular micrometer (Webcon, India). Ten sections of pineal gland, thymus, spleen, ovary, uterus and testis sample tissue were randomly selected for morphometric analysis.

6. Immunohistochemistry

For immunohistochemical localization of melatonin receptor types (MT1 and MT2), androgen receptor (AR), estrogen receptor (ERα) and glucocorticoid receptor (GR) spleen, thymus, testes, ovaries were fixed in neutral formalin and were processed (as described previously; see point 4). Endogenous peroxidase activity was blocked by H₂O₂ in 80% methanol for 20 min at room temperature. Sections were washed three times with phosphate-buffered saline (PBS) and pre-incubated with 3% blocking serum (Vectastain, USA) in PBS for 40 min. Sections were then incubated with primary antibody MT1; Mel1aR, ab 96502, at a dilution of 1:250 and MT2; Mel1bR, ab128469, at a dilution of 1:250 from Abcam, England, AR; anti-AR, N-20, sc-1004, at a dilution of 1:200, ERα; anti-ERα, HC-20, sc-543, at a dilution of 1:200 and GR; anti-GR, N-20, sc-2045 at a dilution of 1:250 all from Santa Cruz Biotechnology, USA for overnight at 4 °C in a humified chamber. Sections were washed three times in PBS and were incubated with biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, Vector laboratories, Burlinghame, CA, USA; dilution 1:10,000). Sections were washed with PBS and a pre-formed ABC reagent was conjugated to the free biotin of the secondary antibody. The antigens were visualized using the peroxidase substrate 3, 3-diaminobenzidine (DAB) (Savaskan et al., 2002). Further, the prepared slides
were observed and documented under microscope (Leitz MPV3 with photomat software) and were documented for immunohistochemical localization of different receptors.

7. **Assessment of hematological parameters:**

**Red Blood Cell Count**

The total blood (as collected from peripheral circulation) was taken in RBC counting tube with RBC fluid i.e. Ringer’s solution, mixed well and the entire mixture is placed on Neubaeur Chamber and RBCs were counted.

**%Hemoglobin (%Hb)**

% Hb in blood was estimated with the help of Sahli’s hemoglobin meter (Systonic Instruments, India). In principle, hemoglobin is converted to acid haematin by the action of HCL (0.1N). The acid haematin solution is further diluted with HCl until its color matches exactly with that of the permanent standard of the comparator block. The hemoglobin concentration is read directly from the calibration tube. The % hemoglobin was found to be calculated from the standard value as grafted on the standard tube.

**Estimation of Aspartate Transaminase (AST)**

Aspartate aminotransferase (AST) also known as glutamate oxaloacetic transaminase (GOT) is a transaminase. The principle of AST estimation is as follows:

Kinetic determination of the aspartate aminotransferase (AST) activity: L-Aspartate + α - Ketoglutarate \[\text{------}\rightarrow\] Oxaloacetate + L-Glutamate

Oxaloacetate + NADH +H\(^+\) \[\rightarrow\] L-Malate + NAD\(^+\). These levels may be estimated as plasma levels and can be regarded as an assay of hepatotoxicity upon hormonal/drug treatment.

**Estimation of Alanine Transaminase (ALT)**

Alanine aminotransferase (ALT) also known as glutamate pyruvate transaminase (GPT) is a transaminase. The principle behind the ALT test is as follows:
ALT
\[ \text{L-Alanine} + \alpha-\text{Ketoglutarate} \rightarrow \text{Pyruvate} + \text{L-Glutamate} \]

LDH
\[ \text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-Lactate} + \text{NAD}^+ . \]

8. **Hormonal preparation for *in vitro* supplementation**

All the drugs and hormones used were purchased from Sigma-Aldrich Chemicals, St. Louis, Missouri, USA. Concanavaline A (Con A), a T cell mitogen was dissolved and diluted to desired concentration (10µg/mL) in RPMI-1640 (incomplete medium). Melatonin, dexamethasone, estradiol and testosterone doses were prepared by dissolving it in few drops of DMSO (di methyl sulfoxide) and were further diluted in RPMI-1640 to get the desired concentrations. Both stock and working solutions of melatonin were kept at 4˚C in amber coloured glass bottle to avoid photo-degradation. Melatonin was added to cell culture plates in minimum visible red light diluting it with normal saline (0.85% NaCl) up to desired concentrations as per experimental requirement. Basal culture set was having cells only (without Con A). For each treatment, two sets of the culture were prepared. First set was with hormones only and another was hormones along with Con A to calculate the %SR of splenocytes and thymocytes.

9. **Immune parameters**

Peripheral hematological parameters:

Assessment of total leukocyte count (TLC), differential leukocyte count (DLC) and % lymphocyte count (%LC):

Blood was taken in a WBC pipette and diluted 20 times in Natt-Herrick diluents and white blood cells counted in Neubauer’s counting chamber (Spencer USA) under the microscope. For DLC, a thin blood film was prepared and stained with Leishman’s stain and leukocyte subpopulations were counted under oil immersion lens of Nikon microscope (Nikon, E200, Japan; Haldar et al., 2004). Lymphocyte counts (no./mm\(^3\)) was determined from total and differential leukocyte count by using the following formula:
Lymphocyte count \[= \frac{\text{TLC} \times \text{Lymphocyte percent}}{100}\]

10. Cell mediated immune parameters

**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% \(\text{NH}_4\text{Cl}\). 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 \(1 \times 10^6\) 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 \(1 \times 10^6\) 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).

**Isolation of Peripheral Blood Mononuclear Cells (PBMCs)**

Hisep LSM 1084 is based on the adapted method of isolating mononuclear cells using centrifugation techniques by Boyum (1968) in which defibrinated blood (heparinized) is layered on a solution of sodium diatrizoate and polysucrose and centrifuged at low speed for 30 minutes. Differential migration following centrifugation results in the formation of several cell layers. Mononuclear cells (lymphocyte and monocyte) are contained in the banded plasma-LSM interphase.
due to their density, and the pellet that is formed contains mostly erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube. Most extraneous platelets are removed by low speed centrifugation during the washing steps. Lymphocytes are recovered by aspirating the plasma layer and then removing the cells. Excess platelets, HiSep LSM, and plasma can then be removed by cell washing with isotonic PBS.

**Separation of Peripheral Blood Mononuclear Cells (PBMCs)**

Blood was diluted with PBS (RT) in 1:1 ratio. Three mL of Ficoll (HiSep, Cat. No. LSM 1084) was transferred to a 15 mL sterile centrifuge tube. Ficoll was carefully overlaid with 6 mL of diluted blood and mixing of both was avoided. The centrifuge tube was centrifuged (without brake) at 400 × g for 30 minutes at room temperature. Centrifugation at lower temperature was not performed to avoid the cell clumping and poor recovery. After centrifugation, the sedimentation of erythrocytes, polynuclear leukocytes and band mononuclear lymphocytes above was obtained. Supernatant containing plasma and most of the platelets were aspirated carefully without disturbing the interface band. The opaque interface containing the mononuclear cell band was aspirated with the help of a glass Pasteur pipette and transferred in a sterile 15 mL tube. Ten mL of PBS/appropriate cell culture medium was mixed gently with mononuclear cells. The tube was gently inverted several times only to ensure a proper mixing. The mixture was centrifuged for 10 minutes at 250 × g. The supernatant was discarded. This step was repeated for thrice and finally the cell number was counted and viability of the cells (≥95%) was determined with the help of trypan blue exclusion method.

**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°C with 5% CO₂ 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.

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

11. **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.

12. **Cytokine parameters**

*Estimation of plasma IL-2*

The concentration of IL-2 was measured in triplicate aliquots with the help of commercial kit (Koma Biotech, Seoul, Korea; Cat. No. K0331142). Sandwich ELISA was performed to measure the plasma level of IL-2 in all the groups according to manufacturer’s instruction. Lower and upper limits of analytic sensitivities were 32 pg/mL and 2000 pg/mL. All the assays were carried out in triplicate.

*Circulatory level of IL-6*

Sandwich ELISA was performed to quantify the level of IL-6 in plasma collected from the goats according to the manufacturer’s instruction (Koma
Biotech, Seoul, Korea; Cat. No. K0331230). Lower and upper limits of analytic sensitivities were 16 pg/mL and 1000 pg/mL. All the assays were carried out in triplicate.

**Circulatory level of TNF-α**

Sandwich ELISA was performed to quantify the level of TNF-α in plasma collected from the goats according to the manufacturer’s instruction (Koma Biotech; Cat. No. K0331186). Lower and upper limits of analytic sensitivities were 16 pg/mL and 2000 pg/mL. All the assays were carried out in triplicate.

**Circulatory level of IFN-γ**

Sandwich ELISA was performed to quantify the level of IFN-γ in plasma collected from the goats according to the manufacturer’s instruction (Koma Biotech; Cat. No. K0331209). Lower and upper limits of analytic sensitivities were 32 pg/mL and 4000 pg/mL. All the assays were carried out in triplicate.

13. **Estimation of circulatory hormone levels**

**Circulatory level of testosterone**

**Principle**

The ELISA of testosterone is a competitive solid-phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase- testosterone (HRP- testosterone) conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured developing coloured product from colourless substrate after incubation. Quantity of colour developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

**Procedure**

An ELISA kit for peripheral testosterone assay was purchased from Dia Metra (Lot No; DKO 002), Italy and was measured according to the manufacturer’s protocol. 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 minutes. Finally, 100 µL of stop solution (0.2 M H₂SO₄) was added and absorbance was recorded at 450 nm using a micro-plate 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.

**Circulatory level of estradiol**

**Principle**

The ELISA of estradiol is a competitive solid-phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase-estradiol (HRP- estradiol) conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured developing coloured product from colourless substrate after incubation. Quantity of colour developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

**Procedure**

The ELISA kit for peripheral estradiol assay was purchased from Biotron Diagnostics Inc., Palm Ave Hemet, CA, USA and was measured 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
The ELISA of cortisol is a competitive solid-phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase-cortisol (HRP- cortisol) conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured developing coloured product from colourless substrate after incubation. Quantity of colour developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

**Procedure**

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.
Circulatory level of melatonin

Principle

The ELISA of melatonin is a competitive solid-phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase-melatonin (HRP- melatonin) conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured developing coloured product from colourless substrate after incubation. Quantity of colour developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

Procedure

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 2 pg/mL. Inter and intra-assay variations were between 9.0% and 15%, respectively. The assay was carried out in triplicate.

Tissue level of melatonin

For estimation of melatonin content in the lymphoid tissues commercial melatonin ELISA kit (RE54021, IBL, Hamburg, Germany) was used as per manufacturer’s instruction. The tissues were homogenised in PBS with 0.1% ethanol and centrifuged. The supernatants were used for determinations of
melatonin. Standards, controls and tissue homogenates were extracted using C18 reverses phase extraction columns according to the protocol of the manufacturer. The reaction was developed using p-nitrophenyl phosphate and optical densities were determined at 405 nm in an automatic microplate reader (Biotek, USA). The concentration of melatonin was expressed as pg/mg of protein measured in the tissue sample. The sensitivity of the melatonin assay was 1.6 pg/mL. Both the intra- and inter-assay coefficients of variation (CV) were 11.4% and 19.3% respectively.

**Circulatory level of thyroxin**

**Principle**

The ELISA of thyroxin is a competitive solid-phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase-thyroxin (HRP- thyroxin) conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured developing coloured product from colourless substrate after incubation. Quantity of colour developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

**Procedure**

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 450 nm. The sensitivity of the assay is 0.5µg/dL with a recovery rate of 97.8%.

14. 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 (1×10⁶ 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 (Sharma and Haldar, 2009). 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; Fig. 2).

![Fig. 2: AO-EtBr counter stain method for detection of apoptosis](image-url)
15. Free Radical Parameters

Estimation of Superoxide Dismutase (SOD) activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following the method of Das et al. (2000). 10% homogenates of tissues were prepared in 150 mM phosphate buffered saline (PBS, pH 7.4) and centrifuged for 30 min at 12,000 g at 4 °C. The supernatant was again centrifuged for 60 min at 12,000 × g at 4 °C and then processed for enzymatic activity based on a modified spectrophotometric method using nitrite formation by superoxide radicals. A 0.5 mL of homogenate was added to 1.4 mL of reaction mixture comprised of 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride, 50 mM ethylene diamine tetraacetic acid (EDTA) followed by a brief pre-incubation at 37 °C for 5 min. Next, 0.8 mL of riboflavin was added to all samples along with a control containing buffer instead of sample and then exposed to two 20W fluorescent lamps fitted parallel to each other in an aluminium foil coated wooden box. After 10 min of exposure, 1 mL of Greiss reagent was added and absorbance of the colour formed was measured at 543 nm on a spectrophotometer (ELx-800, Biotek Instruments, Winooski VT, USA). One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions. For the activity assay in plasma instead of tissue homogenate, equal volume of plasma was used.

Estimation of Catalase activity

Catalase (CAT; EC 1.11.1.6) activity was measured following the procedure of Sinha (1972). This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H₂O₂ with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured calorimetrically. The catalase preparation is allowed to split H₂O₂ for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H₂O₂ is determined by measuring chromic acetate calorimetrically after heating the reaction mixture. There is production of green color at the end of the process.
homogenate of tissues were prepared in PBS (10 mM; pH 7.0) and then centrifuged at 12,000 × g for 20 min at 4°C. Supernatant was taken for enzyme estimation. 5 mL of PBS was added to 4 mL of H₂O₂ (200 mM) and then 1 mL of enzyme extract was added. After 1 min 1 mL of this solution was taken in a tube and 2 mL of K₂Cr₂O₇ (5%) solution was added. Then it was boiled for 10 min and absorbance was measured at 570 nm (ELx-800, Biotek Instruments, Winooski VT, USA). The activity of CAT was expressed as amount of H₂O₂ degraded per minute. For the activity assay in plasma instead of tissue homogenate, equal volume of plasma was used.

**Estimation of Glutathione Peroxidase (GPx) activity**

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed as described by Mantha et al. (1993). The reaction mixture (1 mL) contained 50 μL sample (10% tissue homogenates prepared in chilled PBS and centrifuged at 12,000 × g), 398 μL of 50 mM phosphate buffer (pH 7.0), 2 μL of 1 mM EDTA, 10 μL of 1 mM sodium azide, 500 μL of 0.5 mM NADPH, 40 μL of 0.2 mM GSH and 1 U glutathione reductase. The reaction mixture was allowed to equilibrate for 1 min at room temperature. After this, the reaction was initiated by addition of 100 mM H₂O₂. The absorbance measured kinetically at 340 nm (ELx-800, Biotek Instruments, Winooski VT, USA) for 3 min. The GPx activity was expressed as nmol of NADPH oxidized to NADP⁺ per min per mg of protein using an extinction coefficient (6.22 mM/cm) for NADPH. For the activity assay in plasma instead of tissue homogenate, equal volume of plasma was used.

Estimation of lipid peroxidation (LPO) assay by thiobarbituric acid reactive substances (TBARS) level

Tissues of goats were weighed and homogenized in a tenfold excess of 20 mM Tris–HCl buffer (pH 7.4) and the 10% homogenates were centrifuged for 15 min at 3000 × g at 4 °C. The supernatant was subjected to thiobarbituric acid (TBA) assay by mixing with 8.1% sodium dodecyl sulphate (SDS), 20% acetic acid, 0.8% TBA and then digested it for 1 h at 95 °C (Sharma et al., 2008). The reaction mixture was immediately cooled in running water, vigorously shaken
with 2.5 mL of n-butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500 × g (Ohkawa et al., 1978). The absorbance of the upper phase was measured at 534 nm (ELx-800, Biotek Instruments, Winooski VT, USA). Total thiobarbituric acid reactive substances (TBARS) were expressed as malondialdehyde (MDA; nmol/g tissue weight) taking 1,1,1,1-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using different dilutions of 10 nM TEP. For the activity assay in plasma instead of tissue homogenate, equal volume of plasma was used.

**Estimation of Total Antioxidant Status (TAS)**

The free radical scavenging activity of antioxidants for 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cations was measured according to the method of Re et al. (1999). This method measures the antioxidant activity determined by decolorization assay of the ABTS radical cation, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. A stock solution of ABTS radical cations was prepared one day before the assay by mixing 5 mL of 7 mM ABTS with 1 mL of 14.7 mM potassium persulfate, followed by storage in the dark at room temperature. The stock solution of ABTS radical cations was diluted with water or ethanol. ABTS radical cation was generated by oxidation of ABTS with potassium persulfate. 2.95 mL of ABTS cation solution was mixed with 50 μL of 10% tissue homogenates and the decrease in absorbance was monitored for 10 min at particular interval of time at 734 nm (ELx-800, Biotek Instruments, Winooski VT, USA).

16. **Metabolic parameters**

**Glycogen estimation**

The estimation process was done by Anthrone-Sulphuric Acid method as suggested by Shavali and Haldar (1998). In brief a stock solution of glycogen was prepared (5mg/mL). It was then diluted in varying concentrations of glycogen (from 20μg/mL to 100μg/mL) for standard curve. A solution of anthrone (0.5%) in sulphuric acid was prepared. It was mixed with standard glycogen solution, placed
in boiling water bath till the characteristic colour (pinkish to red) was obtained and absorbance was measured in a spectrophotometer at 620 nm. For experimental estimation, the tissue glycogen was extracted in 50% KOH solution and 4 mL of Anthrone-Sulphuric Acid solution was added to each of samples. After mixing well, the samples were placed in boiling water bath till the characteristic colour of the solution were obtained and absorbance was measured in a spectrophotometer (ELx-800, Biotek Instruments, Winooski VT, USA) at 620 nm.

**Cholesterol assay**

The cholesterol estimation was done by method of Sackett, (1969). The stock solution of cholesterol was made of 1 mg/mL. Then serial dilutions were made from 0-200µg/mL in chloroform for standard curve. A mixture of acetic anhydride and sulphuric acid (20:1) was added to it and incubated in dark for 30 minutes and the O.D. was measured at 640 nm. For experimental samples the cholesterol was extracted in a mixture of ether: ethanol (3:1) following homogenization 10% (V/V). Then it was centrifuged at 3000 rpm for 10 minutes. The supernatant was taken out and evaporated to dryness in boiling water bath. Finally it was reconstituted in 5 mL of chloroform and 1 mL of Acetic Anhydride and sulphuric acid (20:1) mixture was added to it and incubated in dark for 30 minutes and the O.D. was measured at 640 nm (ELx-800, Biotek Instruments, Winooski VT, USA). For the cholesterol assay in plasma instead of tissue homogenate, equal volume of (0.5 mL) plasma was used.

**Glucose estimation**

The extraction of tissue glucose was done by the method of Moses et al. (2006). In brief the tissue was homogenized in 6 N Perchloric acid solutions (10% V/V) and then was spunned at 10,000 rpm for 10 minutes. Finally the supernatant was taken and glucose was estimated by commercially available glucose estimation kit (Beacon India Pvt. Ltd., Mumbai) following manufacturer’s protocol. The plasma was directly used for the assay.
% uptake of glucose by lymphoid organs and gonads

The circulatory level of glucose was considered to be 100%. The tissue level glucose (either of lymphoid organs or gonads) were measured as percentile values (in comparison to plasma level) and were represented as % up take values by the respective tissues.

% uptake of cholesterol by lymphoid organs and gonads

The circulatory level of cholesterol was considered to be 100%. The tissue level cholesterol (either of lymphoid organs or gonads) were measured as percentile values (in comparison to plasma level) and were represented as % up take values by the respective tissues.

17. Biochemical parameters

3β Hydroxysteroid dehydrogenase (3β HSD) estimations

The 3β HSD enzyme was assayed according to the protocol of Shivanandappa and Venkatesh (1997) using tissue homogenates. Ten percent tissue homogenate was prepared in 0.1 M Tris–Cl buffer (pH 7.8). The homogenate was centrifuged at 12,000 × g at 4°C and the supernatant was used as the source of enzyme. The enzyme was assayed in 0.1 M Tris–Cl buffer (pH 7.8) containing 500 mM of NAD, substrate (100 mM of dihydroxyepiandrosterone for males and pregnenolone for females), and enzyme (50 mL) in a total volume of 3.0 mL and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 2.0 mL of phthalate buffer (pH 3.0) and absorbance was noted at 490 nm. The enzyme activity was calculated from the standard curve of NADH (ranges from 0 to 150 nmol) and expressed as nanomoles of NADH formed per hour per milligram of protein.

Arylalkylamine N-acetyltransferase (AA-NAT) estimation

Arylalkylamine N-acetyltransferase activity was measured following the method of Chae et al (1999). In brief, the thymus was disrupted using a cell sonicator in 100 mL ice-cold 50mM phosphate buffer (16mM Na₂HPO₄, 40mM NaH₂PO₄, pH 6.8). Aliquots of 10 mL of the sonicate were added to reaction
mixtures containing 1.5mM tryptamine – HCl, 0.1mM acetyl coenzyme A, and 0.01 mM of $^3$H-acetyl coenzyme A (0.2 mCi per assay), and the total volume was adjusted to 20 mL with phosphate buffer. The reaction mixture was then incubated at 37°C for 20 minutes and the reaction was stopped by addition with 180 mL ice-cold phosphate buffer. Three millilitres of scintillation fluid (containing 0.4% 2, 5-diphenyl oxazole and 0.01% 1,4-bis [phenyloxazole-2-yl] benzene mixed with toluene) was rapidly added to the reaction mixture and the amount of radio labelled acetyl tryptamine was determined using a liquid scintillation counter (Beckman Instruments, USA). N acetyltransferase activity was expressed as nanomoles of N-acetyltryptamine produced per hour per milligram of protein.

**Glycogen phosphorylase estimation**

The glycogen phosphorylase (GP; EC 2.4.1.1) activity was measured following the protocol of Mason and Fasella (1971) with a few modifications as suggested by Zhang et al., (2012). The reaction mixture consisted of 50mM sodium glycerolphosphate (pH 7.1), 10mM potassium phosphate, 5mM MgCl$_2$, 0.5mM NAD$^+$, 1mM DTT, 1.6 unit phosphorglucomutase, 1.6 unit glucose-6-phosphate dehydrogenase, and 0.2% glycogen in a total volume of 0.3mL. Reaction was started by adding 200 µL of glycogen phosphorylase (for standard) and 200 µL of 10% tissue homogenate to make the volume of 500 µL and reaction was started at 25°C. The reaction was monitored by measuring the increase of absorbance (Ex. 350nm, Em.470nm, HITACHI F-4600 UV-Visual-Fluorescence spectrophotometer) for NADH generation and results were expressed as µmoles of NADH generated/min.

**Estimation of High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) in plasma**

Plasma level of HDL and LDL were measured using a commercial kit (Sigma Aldrich, USA, Cat. No. MAK045) following manufacturer’s protocol. The lower and upper limits of detections are 2mg/dL to 300mg/dL.
Estimation of protein

The tissues were lysed in lysate buffer and protein was estimated using commercially available Bradford’s reagent following the protocol of Bradford (1976). Plasma was directly used for the protein estimation.

18. Western blot analysis of melatonin receptor (MT1 and MT2), androgen receptor (AR), glucocorticoid receptor (GR) and estrogen receptor (ERα)

The Western blot analysis was performed according to the method published elsewhere (Ahmad and Haldar, 2010). Briefly, the thymus and spleen tissues were dissected in chilled PBS, homogenized, and lysed in lysate buffer. The protein content of the lysates was quantified using the Bradford method. The aliquots containing 100 µg protein of thymus were resolved on 12% (MT1/MT2) and 10% (GR, AR and ERα) of SDS-PAGE. Electrophoresis was followed by electrotransfer (Biometra, Goettingen, Germany) to nitrocellulose membranes (Bioscience, Keene, NH, USA) for 1 hour. The membranes were then blocked in Tris-buffered saline (TBS; Tris 50 mM, pH 7.5, NaCl 150 mM) solution containing 5% fat-free dry milk and 0.1% Tween-20 and were incubated with primary antibodies against MT1 (Mel1aR, ab96502, at a dilution of 1:250), MT2 (Mel1bR, ab128469, at a dilution of 1:250), AR (anti-AR, N-20, sc-1004, at a dilution of 1:200), GR (anti-GR, N-20, sc-2045, at a dilution of 1:250 and ERα (anti-ERα, HC-20, sc-543 at a dilution of 1:200). The membranes were washed thrice in TBS–Tween-20 and were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (donkey anti-rabbit HRP-IgG for MT1, MT2, AR, GR and ERα at a dilution of 1:500). Finally, the blots were washed thrice with TBS and developed with Super Signal West Pico Chemiluminescent substrate (#34080; Thermo Scientific, Rockford, USA). Further, the membranes were stripped with stripping buffer (10% sodium azide) and were immunostained with β-actin antibodies in 1:1000 dilutions (A-2228; Sigma–Aldrich) as internal loading control. Immune detection of β-actin was performed with donkey anti-mouse IgG-HRP (1:1000). Bands were quantified by the measurement of O.D. using Scion Image Analysis Software (Scion...
Corporation, MD, USA). Values were expressed as the ratio of the density of the specific signal to the β-actin signal. The ratio of density was calculated with respect to β-actin (house-keeping gene) and expressed as percent relative integrated density values of MT1, MT2, AR, GR and ERα. The values presented were as percent band intensity ± standard error of the mean.

19. Statistical analyses

The data were presented as the mean ± standard error of the mean (SEM). All the data of annual variation in gonadal hormones (testosterone and estradiol) and expressions of AR and ERα were analyzed by a one-way ANOVA followed by post hoc test, i.e., the Dunnett test (two-sided; Bruning and Knitz, 1977). All the experimental data were analyzed by a two-way ANOVA. All the hematological, biochemical, hormonal, immunological and free radical data upon phyto-melatonin treatment were analyzed by one way ANOVA followed by Student’s unpaired t-test. In the Dunnett test, male and female goats of summer season were treated as control and compared with all other groups. 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 and Thy vs Thy + Mel in both the sexes), 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 (SPSS), IBM, software version 17.0.
**Fig. 1A.** Histology of young goat pineal gland (40X). **Fig.1B.** Histology of old goat pineal gland (40X). Con: Pineal concretions, DP: Dark Pinealocytes, LP: Light Pinealocytes, PGC: Pineal Glial Cells.

**Fig. 2A.** Histology of goat ovary (10X). **Fig. 2B.** Histology of goat ovary (40X). AF: Antral fluid, GC: Granulosa cell GF: Graafian follicle, TC: Theca cell.

**Fig. 3A.** Histology of goat testis (10X). **Fig. 3B.** Histology of goat testis (40X). GE- Germinal Epithelium, L- Lumen, Ldc- Leydig’s cells, ST- Seminiferous Tubule.
Fig. 4A. Histology of goat uterus (10X). Fig. 4B. Histology of goat uterus (40X).

Fig. 4C. Goat myometrium (40X). BL: Basal Lamina, E: Endometrium, L: Lumen, M: Myometrium, SC: Secretory Cells, UG: Uterine Gland.
**Fig. 5A.** Histology of goat thymus (40X).

**Fig. 5B.** Histology of goat thymus (100X). HC: Hassel’s Corpuscle.

**Fig. 6A.** Histology of goat spleen (40X).

**Fig. 6B.** Histology of goat spleen (100X). BC: Billroth’s Cord and GC: Germinal Centre.
Fig. 7A. & 7 B. Immunohistochemical localization of MT1 receptor in goat ovary (10X) and (40X). Arrows show immunoreactivity.

Fig. 7C. & 7 D. Immunohistochemical localization of MT1 receptor in goat ovary (10X) and (100X). Arrows show immunoreactivity.
Fig. 8A. & 8B. Immunohistochemical localization of MT1 receptor in goat uterus at (10X) and 40X. Arrows show immunoreactivity.

Fig. 8C. & 8D. Immunohistochemical localization of MT2 receptor in goat uterus at (40X) and 100X. Arrows show immunoreactivity.
Fig. 9A. & 9B. Immunohistochemical localization of MT1 receptor in goat thymus (10X) and (100X) magnifications. BV: Blood Vessel; HC: Hassel’s Corpuscle. Arrows show immunoreactivity.

Fig. 10. Immunohistochemical localization of MT1 receptor in goat spleen (100X) magnification. Arrows show immunoreactivity.

Fig. 11. Immunohistochemical localization of MT1 receptor in goat testes higher (40X) magnification. Arrows show immunoreactivity.
**Fig. 12.** Immunohistochemical localization of AR in male goat thymus (40X). Arrows show immunoreactivity.

**Fig. 13.** Immunohistochemical localization of ERα in female goat thymus (40X). Arrows show immunoreactivity.

**Fig. 14.** Immunohistochemical localization of GR in male goat thymus (40X). Arrows show immunoreactivity.
Chapter – 1

Histology and immunohistochemical localization of different receptors (MT1, MT2, AR, GR, ERα) in various organs (spleen, thymus, ovary, uterus and testes) of Indian goat C. hircus.
Histology and immunohistochemical localization of different receptors (MT1, MT2, AR, GR, ERα) in various organs (spleen, thymus, ovary, uterus and testes) of Indian goat C. hircus.

Introduction

Reproduction in both males and females in every animals, particularly in vertebrates is tightly regulated by different extrinsic factors like different environmental stressors i.e. scorching heat of summer, humidity of monsoon and sizzling cold of winter (Ungerfield and Bielli, 2012) and intrinsic factors (hormones, chemokines, lymphokines and cytokines (Wira et al., 2011) in a very timely and well coordinated manner.

As a part of intrinsic factor, hormones are the common factor which regulate both the important biological mega events i.e. immunity and reproduction. Among the hormones, the gonadal steroids (testosterone and estradiol) play an important role in regulation of immunity as immune suppressor in most of the animal models (Paavonen, 1994; Flatt et al., 2008; Wira et al., 2011). Apart from gonadal steroids, there are adrenal steroids (cortisone/corticosterone) expressed in a species specific manner which is important to ameliorate any type of stress. By the “Flight or Fight” mechanism the adrenal steroids play important role in maintaining body homeostasis under spontaneous or induced stress (Hadley and Levine, 2006).

Among the environmental cues, photoperiod is responsible for regulating different physiological functions in a season dependent manner and is chemically coded inside the body by a particular neurohormone, melatonin secreted from pineal gland (Haldar and Yadav, 2006). This hormone is regarded as both “Clock and Calendar” (Reiter, 1993) for the organisms. Melatonin is also regarded as an immune enhancer as documented in different animal models and systems (Miller et al., 2006; Haldar and Ghosh, 2014). This hormone acts as pro-gonadotrophic
(Evans et al., 1999; Pratalima et al., 2004) or anti-gonadotrophic (Benson et al., 1972) for reproductive management as per need of species in different ecological niches. Reports are available regarding the histoarchitecture of pineal (the main source of melatonin in circulation) and ovary in ruminants like sheep (Regodon et al., 1998; Redondo et al., 2003).

Goat is a ruminant short day breeder and female goats reproduce during winter under the positive influence of melatonin (du Preez et al., 2001) hence, melatonin acts as a pro-gonadotrophic hormone. Males are reproductively active throughout the year. Ovary and uterus are the most important and dynamic reproductive structures in females. The seasonality in reproduction and gestational efficacy in female goats are due to these two important reproductive structures and interestingly feature is that, in this animal maintenance of gestation and immunity is occurring side by side during winter.

The main immune organs of goats are the primary and secondary lymphoid organs i.e. thymus and spleen. However, being the most economically important free grazing animal, the health management of goat is neglected by the veterinarians and other basic researchers for a long period of time and till date no reports are available on basic histology of pineal, gonads (ovary, uterus and testes) and lymphoid organs (spleen and thymus) in Indian goat Capra hircus. In the present study we noted the general histology of pineal, ovary, uterus, spleen and thymus which were never reported in this ruminant short day breeder. Further, all the above mentioned organs are target for gonadal, adrenal steroids and melanin hormone hence, we also studied the immunohistochemical localization of different hormone receptors (membrane bound melatonin receptor MT1 and MT2, androgen receptor; AR, estrogen receptor α; ERα and glucocorticoid receptor; GR) to get a basic idea of areas of expression.

**Materials and methods**

**Animals and maintenance**

Details of animals and maintenance have been described in Materials and Methods section.
**Experimental design**

In order to study the histological architecture of pineal, ovary, uterus, spleen and thymus of goats 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. Samples of the desired tissues were collected from city abattoir in a seasonal manner as described in the Materials and Methods section and the results were validated with the samples collected from CIRG, Mathura, Uttar-Pradesh.

**General histology of tissues**

General histology of the target tissues were performed as described in the Materials and Methods section.

**Immunohistochemical localization of MT1, MT2, AR, GR and ERα in lymphoid organs (thymus and spleen) and gonads (testes, ovary and uterus) of goats**

The Immunohistochemical localization of MT1, MT2, AR, GR and ERα were performed following the protocol of Savaskan et al., 2002 with a few modifications as published elsewhere (Ahmad and Haldar, 2010) as described in the Materials and Methods section.

**Results**

**General histology of goat pineal**

The pineal glands of goats are having three different cell types (Fig. 1A). They are glial cells having a large and prominent nucleus but without dendritic protrusions, light pinealocytes having small nucleus and dark pinealocytes having
large nucleus almost covering the entire area of the pinealocyte. The entire histo-
architecture is totally filled with matrix and calcified concretions are scattered
throughout the structure if the goats are aged (Fig 1B).

**General histology of goat ovary**

The ovarian histology of goats shows a large Graafian follicle filled with
huge amount of antral fluid. They are surrounded by thick layer of thecal cells and
granulosa cells having secretory granules (Fig 2A and 2B).

**General histology of goat testes**

The general histology of goat testes shows the basic characteristics of a
mammalian testis. The histology shows a number of seminiferous tubules
surrounded by basal lamina. Just beneath this, there is the presence of germinal
epithelium having large and active germinal cells with a large nucleus. The testes
of goats are always in reproductively active phase; hence, the lumen of
seminiferous tubule is filled with sperms. Adjacent to the seminiferous tubule the
presence of large and triangular Leydig Cells are also evident (Fig. 3A and 3B).

**General histology of goat testes**

The uterine histology of goats shows the uterus surrounded by basal
lamina. Just beneath the basal lamina where the myometrium and endometrium
structures were clearly demarcated. The myometrium is having multiple layers of
non-voluntary muscle cells and the endometrium is having uterine gland cells
filled with secretory granules (Fig. 4A, 4B and 34). At higher magnification, the
secretory glands with empty lumen and secretory granules were visible (Fig. 34).

**General histology of goat thymus**

Histologically the thymus of goats can be generally divided into 2 parts the
cortex and the medulla. The cortex is the cell dense area of the tissue and medulla is
cell translucent in nature well vascularised and having the characteristic Hassel’s
corpuscles both in the cortex and medulla. The Hassel’s corpuscles are less in
number in aged goats. This structure is regarded as the “playing ground” of T-cells
and having a number of macrophages in them. The priming procedure of the T-cell is done here which is an important aspect of cell mediated immunity. Another important structure is PALS (Peri Arterial Lymphoid Sheath) which is regarded as the harbouring place for macrophages were also documented (Fig. 5A and 5B).

**General histology of goat spleen**

Histologically spleen is having the white pulp and red pulp areas (Fig. 6A and 6B). The red pulp is cell dense in nature and white pulp is cell translucent. The splenocytes which are immunologically important are located in the red pulp area.

**Immunohistochemical localization of MT1 and MT2 receptors**

**A. In goat ovary**

The goat ovary is having high expression for both MT1 and MT2 receptors. We found, high expression pattern for MT1 receptors in thecal and granulose cells (Fig. 7A and 7B) but the MT2 receptors were most abundantly present in thecal cells as well as on the cumulus oophorus layer of goat ovary surrounding the ovum (Fig. 7C and 7D).

**B. In goat uterus**

We noted high expression of both the membrane bound melatonin receptors (MT1 and MT2 receptor) in goat uterus. However, the MT1 was highly expressed only on the membranes of secretory cells of endometrium (Fig. 8A and 8B) but MT2 was highly expressed both on the non-voluntary muscle cells of myometrium and on the secretory cells of endometrium (Fig. 8C and 8D).

**C. In goat thymus**

The thymus was having a high expression of MT1 receptor and this receptor was cosmopolitan in distribution over the entire structure. The Hassel’s corpuscles and the PALS were having more distribution of this receptor. Even though, the macrophages which were present in the Hassel’s corpuscles were also having this receptor (Fig. 9A and 9B).
**D. In goat spleen**

The goat spleen is having higher expression of MT1 receptor on the splenocytes particularly in red pulp region. But, the white pulp region which is cell translucent is having less expression of MT1 or MT2 receptors (Fig. 10).

**E. In goat testes**

We noted high expression of MT1 receptor in the Leydig cells of testes of goats however; the MT2 expressions were less prominent in the Sertoli cells or in the Leydig cells of goat testes (Fig. 11).

**Immunohistochemical localization of AR in male goat thymus**

The male goat thymus is having high localization of androgen receptor (AR). Both the cortex and the medulla are having the distribution of AR but the cortex is having more expression in comparison to the medulla (Fig 12).

**Immunohistochemical localization of ERα in female goat thymus**

The female goat thymus is having localization of estrogen receptor (ERα). Both the cortex and the medulla are having the distribution of ERα. There was no zonal difference (cortex and medulla) in the estrogen receptor expression pattern (Fig 13) in the thymus of female goats.

**Immunohistochemical localization of GR in goat thymus**

Glucocorticoid receptor (GR) was localized in thymus of both the sexes however the result of male thymus is presented over here. We noted higher expression pattern of GR in the periphery of thymus (i. e. the basal lamina and peri lymphoid zones) in both the sexes. The Hassel’s corpuscle was having low expression of GR in both the sexes (Fig. 14).

**Discussion**

**The histomorphology of goat pineal**

Pineal gland is one of the most important structures in brain as it is the primary source of melatonin (Haldar and Yadav, 2006). In the present study, for the first time we have discussed about the basic histo architecture of this gland.
We noted high abundance of astrocytes, pineal glial cells, light and dark pinealocytes in young goats. But, the cells were degenerated with the advancement of age with huge pineal concretions laden with calcareous depositions. Our present result in goats is equivocal with the earlier report of Singh et al., (2014) in human pineal gland where the degenerative effect of age is very much evident.

**The histomorphology of gonads in goats**

**Testes**

Being reproductively active throughout the year, the histomorphological architecture of testes in goats is almost similar in a season round manner. However, depending upon the histology, the seminiferous tubules contain germ cells (embedded in Germinal epithelium), spermatogonia, primary spermatocyte, secondary spermatocytes and spermatids. In the germinal epithelium, the germ cells and particularly the primary spermatocytes were found in a mass of cells. This may be due to the fact that the germ cells and primary spermatocytes are connected with each other by inter cellular bridges (ICB). Thus, when they are under divisional stage, they are not separated from each other and finally form a horse shoe shaped cellular mass containing different types of germ cell layers in a linear manner but not in a tier. This interesting finding is in parallel with the previous report of Kojima (1992). Apart from this, the most interesting feature is in the Sertoli cells of goats, unlike other animals, they are having projections/processes. Morphologically, the Sertoli cell processes are classified into sheet-like and slender cord-like processes. The sheet-like process originated solely from the Sertoli cell column while the slender cord-like process projected either from the Sertoli cell column or the sheet-like process in a cumulative conjugation which is in equivocal with the previous report of Hess and Carnes, (2004).

**Ovary**

The basic ovarian structure is similar to the other mammals as suggested by other workers (Knobil and Neill, 1998). Outermost layer of ovary is germinal epithelium. In the internal structure the ovary is primarily demarked as cortex and
medulla. The cortical region contains ovarian follicles and stroma. The ovarian follicles are having cumulus oophorus, membrana granulosa (and the granulosa cells inside it), corona radiata, zona pellucida, and primary oocyte. The zona pellucida, theca of follicle, antrum and liquor folliculi is also contained in the follicle. Also in the cortex is the corpus luteum derived from the follicles. But, surprisingly we noted the presence of Graafian follicle for a prolonged time in histo architecture of ovary and our observation is similar to the previous reports of Sharma et al., (1996). Possible explanation may be that, also in goats, from the developing and to maturation of follicles there are seven different stages (from stage I to stage VII). This is the most important and remarkable feature of goat ovary which makes it different from others. The reason may be that in goats’ seasonality in reproduction is very tightly regulated and well managed by different gonadal steroids. Thus, during the reproductive inactive phase, the ovary is under quiescent phase with no regular cyclical activity. But, during reproductively active phase, the ovary switches from non-cyclical stage to cyclic ones and high level of circulatory estrogen facilitates this process (Thibault et al., 1987). Thus, due to prolonged estrous, Graafian follicle was found in dormant stage like delayed ovulation condition in bats (Srivastava and Krishna, 2010) which in turn may ovulate during reproductive active phase (i.e. winter; November to January) for a successful copulation.

Uterus

Uterus is a dynamic structure in terms of reproduction and hormonal regulation. Like other mammals, the goat uterus is also having the basal lamina and two clearly distinguishable zones i.e. myometrium and endometrium. In the myometrium, non voluntary muscle layers are found. It is the uterus of reproductively inactive or post partum phase hence, the myometrial layer is thin. In the endometrium the secretory gland are found with huge amount of secretory granules present in the secretory cells and a large lumen inside it. We have also noted high involutions in goat uterus during this particular phase, which may be due to the post partum changes occurring in goat uterus. This observation is in

The histomorphology of lymphoid organs in goats

Spleen

Spleen is the most important secondary lymphoid organ in mammals. The spleen is located just beneath the peritoneum associated with the stomach and small intestine. Both in adults and in neonate pups they are having significant immunological importance and thus are not having age dependent degeneration. Like the basic splenic structure in other mammals, the goat spleen is also surrounded by outer capsule. In the internal structure it is mainly differentiated into white pulp and red pulp regions. Red pulp region contains sinusoids and splenic cords also called as Billroth’s cords. In our present histological observation we found the presence of three-dimensional network of fibroblastic reticular cells located among branched sinuses. This result is in parallel with the reports of Omotainse and Anosa (2009) in human spleen. The red pulp region is responsible for germination of red blood cells (at old age) and monocyte reserve. Further, the white pulp region is RBC translucent zone and contains Periarteriolar lymphoid sheaths (PALS; rich in T-lymphocytes) and lymphoid follicles. Thus, this region is most important for both cellular and humoral immunity.

Thymus

Thymus is the important primary lymphoid organs in all the mammals along with goats. The histomorphological observation of thymus in Indian goat Capra hircus is reported for the first time in our present study. The thymus being the most important lymphoid tissue for the neonates is highly developed in young ones but they gradually decrease in shape and size depending upon the age of the animal. The structure is distributed in a peculiar manner in goats. They are generally present just on the ventral side of the heart and two prolongations of the same are distributed on the two sides of trachea. In the histology, it is surrounded by parenchymatous capsule and having outer cortex and inner medullary regions. In the medulla, there is presence of Hassel’s corpuscles which is the playing
ground of T-lymphocyte for priming and maturation. Particularly in the medullary region, there is presence of Periarteriolar lymphoid sheaths and it is rich with T-lymphocytes.

**Immunohistochemical localization of MT1 and MT2 receptors in lymphoid organs and gonads of goats**

There is a long established hypothesis about the role of melatonin in immunomodulation and handful literatures are available in this respect. Melatonin receptors have been localized in the circulating lymphocyte and on lymphoid organs (Calvo et al., 1995), suggesting a direct effect of melatonin on the regulation of immune system (Guerrero and Reiter, 2002). High-affinity melatonin receptors have been localized on circulating lymphocytes from rodents, chickens, and humans (Calvo et al, 1995; Pang et al, 1996) and on thymocytes and splenocytes in humans (Carillo-Vico et al., 2013) and a number of rodent (Ahmad and Haldar, 2010). Thus it is quite evident that the melatonin is modulating the immune function and in this respect the immunocytochemical localization of MT1 and MT2 receptor in lymphoid organs (spleen and thymus) is having noteworthy significance. This study not only for the first time demonstrates about the localization of melatonin receptors in spleen and thymus of Indian goat but also pinpoints towards the immunomodulatory role of this hormone again in Indian goat. Most importantly we found MT1 and MT2 receptor expressions in PALS region of thymus and spleen. The PALS region is area abundant with T-lymphocytes. Thus, presence of MT1 and MT2 receptors suggest the direct role of melatonin in goat immune modulation.

Further, we noted high expression of MT1 and MT2 receptors in theca and granulosa cells of goat ovary. This result clearly suggests the role of melatonin in goat reproduction and is in equivocal with the reports of Tamura et al., (2012) in human. MT1 and MT2 receptors were also noted in the secretory glandular cells of goat uterus and particularly MT2 was abundantly present in the myometrium. Thus, we may conclude that melatonin might have some role in fetal development in goats (may be particularly by MT2) and the results are equivocal with the
reports of de Hafez et al., (2007) suggesting pro-gonadotrophic action of melatonin along with its role in maintenance of gestation.

We found high expression of MT1 receptor in goat testes (particularly in Leydig cells). Thus, it may be speculated that melatonin is not only having a positive role in maintenance of male goat reproduction but also might have role in steroidogenesis (via androgen receptor AR) present on Leydig cells.

**Immunohistochemical localization of gonadal steroid hormone receptors (AR and ERα) and glucocorticoid receptor (GR) in thymus of goats**

The immunosuppressive effects of different steroids (gonadal and adrenal steroids) are also evident and from time to time different authors have confirmed the same in different animal models (Ahmad and Haldar, 2010, Vishwas et al., 2013). For example glucocorticoid induces DNA fragmentation in lymphocytes and thereby maintains the morphological features of lymphoid organs (Reiter et al., 2000). However, melatonin can ameliorate the immunocompromising effect of glucocorticoids (Vishwas et al., 2013). There are reports demonstrating the inhibitory roles of pineal indoles in steroidogenesis (Ng and Lo, 1988) and inhibitory role of gonadal steroids in immunomodulation (Ahmad and Haldar, 2010). Thus, the presence of Androgen Receptor (AR) in the thymus of male goats and presence of Estrogen Receptor α (ERα) in thymus of female goats are having significant roles in describing the immunomodulatory action of these two gonadal steroids. The spleen is regarded as the negative control for the androgen receptor and estrogen receptor. Thus, no androgen receptor and estrogen receptor were localized in the spleen. In addition we have also found high expression of glucocorticoid receptor (GR) in thymus of goats. This result also suggests an immunomodulatory role of GR in goats.

Finally we may suggest that MT/MT2, AR, ERα and GR are expressed at high level at one phase has made this study more interesting and further *in vivo* and *in vitro* studies are needed to put forward the correlation of the hormones (melatonin, testosterone, estrogen and cortisol) in goat.
### Table 1: Total Leukocyte Count/mm³

<table>
<thead>
<tr>
<th>Season</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>5000</td>
<td>4000</td>
</tr>
<tr>
<td>Monsoon</td>
<td>6000</td>
<td>5000</td>
</tr>
<tr>
<td>Winter</td>
<td>7000</td>
<td>6000</td>
</tr>
</tbody>
</table>

### Figure 1: Seasonal Variation of Total Leukocyte Count

- Male: * (Significance level, not specified)
- Female: ** (Significance level, not specified)

### Figure 2: Seasonal Variation of Percentage of Eosinophil

- Male: ** (Significance level, not specified)
- Female: ** (Significance level, not specified)
Fig. 3

Percentage (%) of Basophil

- Summer
- Monsoon
- Winter

Male
Female

Fig. 4

Percentage (%) of Neutrophil

- Summer
- Monsoon
- Winter

Male
Female
Fig. 5

Fig. 6
Fig. 10
Plasma melatonin concentration (pg/mL)

Seasons

Male
Female

Fig. 11
Plasma thyroxin concentration (µg/dL)

Seasons

Male
Female

Fig. 11
Fig. 1 Season and sex dependent variations in Total Leukocyte Count (TLC) 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 Season and sex dependent variations in % Eosinophil 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.
Fig. 3 Season and sex dependent variations in % Baophil 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. 4 Season and sex dependent variations in % Neutrophil 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. 5 Season and sex dependent variations in % Lymphocyte 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.

Fig. 6 Season and sex dependent variations in % Monocyte 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. 7 Seasonal variations in plasma testosterone level in male 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 summer vs monsoon and winter.

Fig. 8 Seasonal variations in plasma estradiol level in 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. 9 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. 10 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. 11 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.
Chapter - 2A

Seasonal and sex dependent variations in haematological (TLC, DLC) parameters and hormonal (Melatonin, testosterone, estradiol, thyroxin and cortisol) levels of Indian goat *C. hircus*
Seasonal and sex dependent variations in haematological (TLC, DLC) parameters and hormonal (Melatonin, testosterone, estradiol, thyroxin and cortisol) levels of Indian goat *C. hircus*

Introduction

Goats are economically important short-day breeder ruminants (MacHugh and Bradley, 2001) which experience a variety of ecological challenges like wide variation in temperature, humidity and pathogenic invasions (Kaushalendra and Haldar, 2012), but never served as a favorable model and, hence, remained experimentally ignored. The tropical climate of Indian sub-continent provides various environmental signals to different animal species to develop adaptive strategies to cope up with ecological stress. Being short-day breeder, goats are mostly affected by temperature- and humidity-induced pathogenic infections during monsoon. Because of the seasonality in reproduction in female goats and their high tolerance level against stress i.e. high temperature of summer and low temperature of winter attracted our attention to study their adaptive strategies in a season- and sex-dependent manner.

Unlike the goats of temperate zone, those of tropical zone goats are capable enough to tolerate heat stresses, but during the months of monsoon and winter they get infected with several season-dependent diseases and may even succumb to death (Kumar et al., 2010). On the other hand, adaptation of animals for ecological stress makes them better survivors for which the metabolic strength of blood is highly important. Blood, being the most important specialized tissue of the body, creates an open channel system to provide the equal amount of nutrients, hormones and other important factors to different organs. Thus, blood biochemistry is not only the important marker for the general health and basic metabolic pattern of an animal but it may also throw light on the adaptive modifications to different geographical distributions/conditions. Recent literatures
on serum biochemistry of different species of goats under normal condition (Okonkwo et al., 2010), in relation to circulatory hormones (Nazifi et al., 2002) or under certain pathological conditions (Kiran et al., 2011) were reported. Serum level of glucose and lipid of Indian Osmanabadi (More et al., 2008) goats under normal and pathological conditions (Sharma et al., 2001) were also reported. But none of the reports suggest a sex- or season-dependent variation that might throw light on adaptive modifications. Therefore, we set as objectives to investigate the blood biochemistry of goat, *Capra hircus*, encompassing (i) immune (TLC, DLC), and hormonal (testosterone, estrogen, cortisol, melatonin and thyroxin), as features of immune adaptive strategy in season- and/or sex-dependent manner. We included the importance of sex in this study as the male goats are reproductively active throughout the year while females are cyclic in nature breeding mostly during short days (winter).

**Materials and Methods**

**Animals and maintenance**

Details of animals and maintenance have been described in Materials and Methods section.

**Experimental design**

In order to establish the effects of sex and season on peripheral immunological parameters and hormonal parameters of goats 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.
**Blood collection**

The blood was collected from the jugular vein of goats following the protocol of Kaushalendra and Haldar, (2012) as described in Materials and Methods section.

**Haematological parameters**

TLC was performed following the standardized method (Haldar et al., 2004) as described in Materials and Methods section.

**Hormonal parameters**

*Circulatory level of testosterone*

An ELISA kit for peripheral testosterone assay was purchased from Dia Metra (Lot No; DKO 002), Italy and was measured according to the manufacturer’s protocol as described in Materials and Methods section.

*Circulatory level of estradiol*

The ELISA kit for peripheral estradiol assay was purchased from Biotron Diagnostics Inc., Palm Ave Hemet, CA, USA and was measured according to the manufacturer’s protocol as described in Materials and Methods section.

*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 as described in Materials and Methods section.

*Circulatory level of thyroxin*

The ELISA kit for peripheral thyroxin was purchased from Arbor Assays, USA and was measured according to manufacturer’s protocol as described in Materials and Methods section.

*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 as described in Materials and Methods section.

**Statistical analysis**

The values for testosterone and estradiol were analyzed by one way ANOVA, followed by Dunnett Test (Post-Hoc Test). The remaining values were analyzed by two-way ANOVA. The significance was tested between sexes, between seasons and among the sexes and seasons at 0.05 (p < 0.05) and 0.01 (p < 0.01) levels. In the post hoc test the males and females of summer season were treated as control and were compared with all other groups. The p - values less than 0.05 (p < 0.05) and 0.01 (p < 0.01) were considered as significant and highly significant, respectively. Statistical analyses were done with Statistical Package of Social Sciences, IBM, software version 17.0 and in accordance with Bruning and Knitz (1977).

**Results**

**Hematological parameters**

**Total Leukocyte Count (TLC)**

Two way ANOVA revealed that the TLC had sex- and season-dependent variation. TLC increased significantly during monsoon (July to September; p < 0.05), reaching the peak in winter (November to January; p < 0.01) in female. Non significant variation was noted in males in season round manner (Fig. 1)

**Differential Leukocyte Count (DLC)**

In DLC, the % eosinophil was significantly high during winter months in both males and females. The variations were significantly high between seasons (p < 0.01). However, significant variations were absent between the sexes (p > 0.05) and sex * season interactions (p > 0.05; Fig. 2). % basophil was significantly high in males during monsoon (p < 0.01) and winter (p < 0.01) but high in females only during monsoon (p < 0.01). Significant variation was absent between sexes (p > 0.05), seasons (p > 0.05) and among sex * season interactions (p > 0.05; Fig. 3). The % neutrophil was highest during winter in males (p < 0.05) and females (p <
However, interactive effect of sexes and seasons was not significant (p > 0.05; Fig. 4). The % lymphocyte in males was significantly high during monsoon (p < 0.01) but in females it was significantly low during winter (p < 0.01). However, the sex * season interactive effect was not significant (p > 0.05; Fig. 5). During monsoon % monocyte level was significantly low in case of males (p < 0.01) and significantly high (p < 0.01) in case of females. But, both in cases of males and females % monocyte was significantly low during winter (p < 0.01 in case of males and p < 0.05 in case of females). However, sex dependent variation and sex * season interactive effect was not significant (p > 0.05; Fig. 6).

**Hormonal parameters**

**Circulatory level of testosterone**

One way ANOVA showed that the testosterone level was lowest during summer. The peak value of testosterone was obtained during monsoon and, hence, the effect of seasons was significantly high (p < 0.05; Fig. 7). Sex * season interactive effect was non-significant (p > 0.05).

**Circulatory level of estrogen**

The circulatory level of estrogen in female goats was significantly high (p < 0.05) during monsoon and the level was significantly low during winter (p < 0.01) when compared with summer season (Fig. 8). However, sex * season interactive effect was absent (p > 0.05).

**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. 9) There was no sex dependent variation or sex * season interactive effect (p > 0.05).

**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. 10). Sex * season interactive effect was not significant (p > 0.05).

*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).

**Discussions**

We recorded the season- and sex-dependent variations of hematological immune parameters of Indian goat *Capra hircus* to infer the immune adaptive significance. Earlier reports are available on the hematological profile of sheep (Abouzeid et al., 2010) and goat under clinical conditions like bacterial enteritis (Meshram et al., 2009), mycoplasma infection (DaMassa et al., 1992), diarrhea (Zaki et al., 2010) or in an age-dependent manner (Zumbo et al., 2011).

Some hormonal profiling in parts were recorded in respect of testosterone (Todini et al., 2007), progesterone (Estrada-Cortésa et al., 2009), estrogen (Paula et al., 2005), prolactin (Brackel-Bodenhause et al., 1994), and melatonin (Zarazaga et al., 2010) in goats. Some molecular approaches for separation of major and minor protein fractions in goat serum including IgG were also reported (Jain and Gupta, 2005). All these studies on goats were partial and till date no comprehensive report which deals with hormonal and haematological parameters of any tropical goat that can give an idea about adaptive significance for their survival under stressful seasonal or ecological conditions. Our study on the serum biochemistry (with a focus on hematological and hormonal parameters) of goats in season- and sex-dependent manner may provide some key feature for immunity and correlation of immunity with circulatory hormonal profiles in male and female goats under one roof of immune endocrine significance.

The hematological parameters chosen for the present study were of clinical importance to provide first line information about immune status of goat, *Capra hircus*. During winter (November to January), summer (April to June) and
monsoon (July to September) seasons; the immune status (TLC & DLC) of females was higher than male and was in agreement with the report of others (Hotchkiss and Nelson, 2002) that experimental short day enhances lymphocyte proliferation in species ranging from mice to primates. Increased immune function under short days could be due to longer duration of melatonin secretion which acts as an immune stimulator has already been reported in rodents (Champney et al., 1998) including human beings (Carrillo-Vico et al., 2005). Melatonin is known to act directly or indirectly on target tissue within the immune system (Ahmad and Haldar, 2010). Recent studies (Auchtung et al., 2004) suggest that photoperiodic exposure can influence immune function in cattle due to increased circulatory level of melatonin. In goats, TLC has a positive correlation with the level of melatonin and gonadal steroids in both the sexes year round, suggesting that the immune regulation in goats is effected by both these hormones.

To delineate the role of melatonin in immune regulation of goats, we measured night time (22:00 h) melatonin and found it high in both sexes during short days of winter and low during long days of summer and monsoon. Our previous report (Kaushalendra and Haldar, 2012) suggests that high level of melatonin in winter was responsible for immune enhancement in goats. Male goats, being reproductively active throughout the year, showed least responses to melatonin and had little fluctuation in immune parameters even during the winter months when both melatonin and testosterone were high since C. hircus is a short-day breeder. Though having opposite physiological properties, melatonin and gonadal steroid (testosterone) maintained immunity and reproduction in a positive manner in male goats, suggesting an adaptive strategy. The melatonin level was high in males during winter season but might be lacking the threshold capacity to suppress testosterone and, hence, immunity was maintained by the basal level of melatonin. Hence, the interplay of both the hormones (melatonin and testosterone) is responsible for health and reproductive status of males.

A positive correlation was observed between the circulatory levels of estrogen and melatonin during winter months as female goats are cyclic and short-day breeder. Estrogen is reported to be a pro-inflammatory gonadal steroid
(Calippe et al., 2008) while melatonin is pro-gonadotrophic (Arendt, 1986). Simply, there may be a mutual synergism between the gonadal steroid and melatonin as an adaptive strategy in both the sexes to maintain the complex immune – reproductive crosstalk in goats. This suggestion gets further support from the results of metabolic parameters.

Further, we noted higher level of gonadal steroids (testosterone and estrogen) during monsoon suggesting that monsoon is the reproductive preparatory phase for both of the sexes. During monsoon and winter, stress hormone levels were also high (as suggested by higher level of circulatory level of cortisol). Thus, we may suggest that during monsoon goats are under inflammatory stress (due to high pathogenic invasion) and during winter both the males and females are under cold stress (due to low temperature and infection of guts) and gestational stress is prevalent particularly in female goats. Thus, our conclusion is that, to combat with inflammatory stress (during monsoon) and cold stress (during winter) the goats presented higher levels of peripheral immunological parameters. However, the correlation between cortisol and peripheral immunological parameters is negative; then, our results in goats are in parallel with the previous reports of Vishwas et al., (2013) in hamsters.

We observed higher level of thyroxin during winter in both of the sexes. This may be due to the fact that during winter goats are under cold stress and as of their adaptive strategy the Basal Metabolic Rate (BMR) was also high. During monsoon, females presented higher thyroxin level than males suggesting the fact that, during reproductive preparatory phase (i.e. monsoon) females are more metabolically active than males and hence, the requirement of energy was high. Our results are equivocal with the previous results of Rai et al., (2005) and Gupta and Thapliyal (1985). However, we are unable to draw any correlation between circulatory thyroxin level and peripheral immune parameters in goats and thus to delineate the exact roles of different hormones (gonadal/adrenal steroids, metabolic hormone and melatonin) some in depth in vivo and in vitro studies are needed which have been dealt in details in subsequent chapters.
Fig. 1

% Stimulation ratio of splenocyte culture

* * **

0 50 100 150 200
Summer Monsoon Winter

Seasons

Male
Female

Fig. 2

% Stimulation ratio of thymocyte culture

* * *

0 50 100 150 200 250
Summer Monsoon Winter

Seasons

Male
Female
Fig. 3A

Fig. 3B

Fig. 3C

Fig. 4

% apoptotic cells/100 cells

Seasons

Summer Monsoon Winter

Male
Female

Fig. 3B Fig. 3A

Fig. 3C

Fig. 4

% apoptotic cells/100 cells

Seasons

Summer Monsoon Winter

Male
Female

* * 
** ** 

Male
Female
**Fig. 5**

Circulatory concentration of IL-2 (pg/mL) across different seasons.

**Fig. 6**

Circulatory concentration of IL-6 (pg/mL) across different seasons.
Fig. 7

Circulatory concentration of IFN-γ (pg/mL)

Seasons

**

Fig. 8

Circulatory concentration of TNF-α (pg/mL)

* * **

Fig. 7

Male

Female

Summer Monsoon Winter

Fig. 8

Male

Female

Summer Monsoon Winter

Seasons
**Fig. 1** Season and sex dependent variations in % Stimulation Ratio (%SR) of splenocyte culture 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** Season and sex dependent variations in % Stimulation Ratio (%SR) of thymocyte culture 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. 3A** 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.

**Fig. 3B** 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. 3C** 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.

**Fig. 4** 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.
**Fig. 5** Season and sex dependent variations in circulatory concentration in IL-2 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. 6** Season and sex dependent variations in circulatory concentration in IL-6 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.
Fig. 7 Season and sex dependent variations in circulatory concentration in TNF-α 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. 8 Season and sex dependent variations in circulatory concentration in IFN-γ 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.
Chapter-2B

Seasonal and sex dependent variations in haematological (TLC, DLC) parameters and hormonal (Melatonin, testosterone, estradiol, thyroxin and cortisol) levels of Indian goat C. hircus
Seasonal and sex dependent variations in haematological (TLC, DLC) parameters and hormonal (Melatonin, testosterone, estradiol, thyroxin and cortisol) levels of Indian goat C. hircus

Introduction

Goats are economically important short-day breeder ruminants (MacHugh and Bradley, 2001) which experience a variety of ecological challenges like wide variation in temperature, humidity and pathogenic invasions (Kaushalendra and Haldar, 2012), but never served as a favorable model and, hence, remained experimentally ignored. The tropical climate of Indian sub-continent provides various environmental signals to different animal species to develop adaptive strategies to cope up with ecological stress. Being short-day breeder, goats are mostly affected by temperature- and humidity-induced pathogenic infections during monsoon. Because of the seasonality in reproduction in female goats and their high tolerance level against stress i.e. high temperature of summer and low temperature of winter attracted our attention to study their adaptive strategies in a season- and sex-dependent manner.

Unlike the goats of temperate zone, those of tropical zone goats are capable enough to tolerate heat stresses, but during the months of monsoon and winter they get infected with several season-dependent diseases and may even succumb to death (Kumar et al., 2010). On the other hand, adaptation of animals for ecological stress makes them better survivors for which the metabolic strength of blood is highly important. Blood, being the most important specialized tissue of the body, creates an open channel system to provide the equal amount of nutrients, hormones and other important factors to different organs. Thus, blood biochemistry is not only the important marker for the general health and basic metabolic pattern of an animal but it may also throw light on the adaptive modifications to different geographical distributions/conditions. Recent literatures on serum biochemistry of different species of goats under normal condition
(Okonkwo et al., 2010), in relation to circulatory hormones (Nazifi et al., 2002) or under certain pathological conditions (Kiran et al., 2011) were reported. Serum level of glucose and lipid of Indian Osmanabadi (More et al., 2008) goats under normal and pathological conditions (Sharma et al., 2001) were also reported. But none of the reports suggest a sex- or season-dependent variation that might throw light on adaptive modifications. Therefore, we set as objectives to investigate the blood biochemistry of goat, *Capra hircus*, encompassing (i) immune (TLC, DLC), and hormonal (testosterone, estrogen, cortisol, melatonin and thyroxin), as features of immune adaptive strategy in season- and/or sex-dependent manner. We included the importance of sex in this study as the male goats are reproductively active throughout the year while females are cyclic in nature breeding mostly during short days (winter).

**Materials and Methods**

**Animals and maintenance**

Details of animals and maintenance have been described in Materials and Methods section.

**Experimental design**

In order to establish the effects of sex and season on peripheral immunological parameters and hormonal parameters of goats 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.
**Blood collection**

The blood was collected from the jugular vein of goats following the protocol of Kaushalendra and Haldar, (2012) as described in Materials and Methods section.

**Haematological parameters**

TLC was performed following the standardized method (Haldar et al., 2004) as described in Materials and Methods section.

**Hormonal parameters**

**Circulatory level of testosterone**

An ELISA kit for peripheral testosterone assay was purchased from Dia Metra (Lot No; DKO 002), Italy and was measured according to the manufacturer’s protocol as described in Materials and Methods section.

**Circulatory level of estradiol**

The ELISA kit for peripheral estradiol assay was purchased from Biotron Diagnostics Inc., Palm Ave Hemet, CA, USA and was measured according to the manufacturer’s protocol as described in Materials and Methods section.

**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 as described in Materials and Methods section.

**Circulatory level of thyroxin**

The ELISA kit for peripheral thyroxin was purchased from Arbor Assays, USA and was measured according to manufacturer’s protocol as described in Materials and Methods section.

**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 as described in Materials and Methods section.

**Statistical analysis**

The values for testosterone and estradiol were analyzed by one way ANOVA, followed by Dunnett Test (Post-Hoc Test). The remaining values were analyzed by two-way ANOVA. The significance was tested between sexes, between seasons and among the sexes and seasons at 0.05 (p < 0.05) and 0.01 (p < 0.01) levels. In the post hoc test the males and females of summer season were treated as control and were compared with all other groups. The p - values less than 0.05 (p < 0.05) and 0.01 (p < 0.01) were considered as significant and highly significant, respectively. Statistical analyses were done with Statistical Package of Social Sciences, IBM, software version 17.0 and in accordance with Bruning and Knitz (1977).

**Results**

**Hematological parameters**

**Total Leukocyte Count (TLC)**

Two way ANOVA revealed that the TLC had sex- and season-dependent variation. TLC increased significantly during monsoon (July to September; p < 0.05), reaching the peak in winter (November to January; p < 0.01) in female. Non significant variation was noted in males in season round manner (Fig. 1)

**Differential Leukocyte Count (DLC)**

In DLC, the % eosinophil was significantly high during winter months in both males and females. The variations were significantly high between seasons (p < 0.01). However, significant variations were absent between the sexes (p > 0.05) and sex * season interactions (p > 0.05; Fig. 2). % basophil was significantly high in males during monsoon (p < 0.01) and winter (p < 0.01) but high in females only during monsoon (p < 0.01). Significant variation was absent between sexes (p > 0.05), seasons (p > 0.05) and among sex * season interactions (p > 0.05; Fig. 3). The % neutrophil was highest during winter in males (p < 0.05) and females (p <
0.01). However, interactive effect of sexes and seasons was not significant (p > 0.05; Fig. 4). The % lymphocyte in males was significantly high during monsoon (p < 0.01) but in females it was significantly low during winter (p < 0.01). However, the sex * season interactive effect was not significant (p > 0.05; Fig. 5). During monsoon % monocyte level was significantly low in case of males (p < 0.01) and significantly high (p < 0.01) in case of females. But, both in cases of males and females % monocyte was significantly low during winter (p < 0.01 in case of males and p < 0.05 in case of females). However, sex dependent variation and sex * season interactive effect was not significant (p > 0.05; Fig. 6).

Hormonal parameters

**Circulatory level of testosterone**

One way ANOVA showed that the testosterone level was lowest during summer. The peak value of testosterone was obtained during monsoon and, hence, the effect of seasons was significantly high (p < 0.05; Fig. 7). Sex * season interactive effect was non-significant (p > 0.05).

**Circulatory level of estrogen**

The circulatory level of estrogen in female goats was significantly high (p < 0.05) during monsoon and the level was significantly low during winter (p < 0.01) when compared with summer season (Fig. 8). However, sex * season interactive effect was absent (p > 0.05).

**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. 9) There was no sex dependent variation or sex * season interactive effect (p > 0.05).

**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. 10). Sex * season interactive effect was not significant (p > 0.05).

**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).

**Discussions**

We recorded the season- and sex-dependent variations of hematological immune parameters of Indian goat *Capra hircus* to infer the immune adaptive significance. Earlier reports are available on the hematological profile of sheep (Abouzeid et al., 2010) and goat under clinical conditions like bacterial enteritis (Meshram et al., 2009), mycoplasma infection (DaMassa et al., 1992), diarrhea (Zaki et al., 2010) or in an age-dependent manner (Zumbo et al., 2011).

Some hormonal profiling in parts were recorded in respect of testosterone (Todini et al., 2007), progesterone (Estrada-Cortésa et al., 2009), estrogen (Paula et al., 2005), prolactin (Brackel-Bodenhause et al., 1994), and melatonin (Zarazaga et al., 2010) in goats. Some molecular approaches for separation of major and minor protein fractions in goat serum including IgG were also reported (Jain and Gupta, 2005). All these studies on goats were partial and till date no comprehensive report which deals with hormonal and haematological parameters of any tropical goat that can give an idea about adaptive significance for their survival under stressful seasonal or ecological conditions. Our study on the serum biochemistry (with a focus on haematological and hormonal parameters) of goats in season- and sex-dependent manner may provide some key feature for immunity and correlation of immunity with circulatory hormonal profiles in male and female goats under one roof of immune endocrine significance.

The hematological parameters chosen for the present study were of clinical importance to provide first line information about immune status of goat, *Capra hircus*. During winter (November to January), summer (April to June) and
monsoon (July to September) seasons; the immune status (TLC & DLC) of females was higher than male and was in agreement with the report of others (Hotchkiss and Nelson, 2002) that experimental short day enhances lymphocyte proliferation in species ranging from mice to primates. Increased immune function under short days could be due to longer duration of melatonin secretion which acts as an immune stimulator has already been reported in rodents (Champney et al., 1998) including human beings (Carrillo-Vico et al., 2005). Melatonin is known to act directly or indirectly on target tissue within the immune system (Ahmad and Haldar, 2010). Recent studies (Auchtung et al., 2004) suggest that photoperiodic exposure can influence immune function in cattle due to increased circulatory level of melatonin. In goats, TLC has a positive correlation with the level of melatonin and gonadal steroids in both the sexes year round, suggesting that the immune regulation in goats is effected by both these hormones.

To delineate the role of melatonin in immune regulation of goats, we measured night time (22:00 h) melatonin and found it high in both sexes during short days of winter and low during long days of summer and monsoon. Our previous report (Kaushalendra and Haldar, 2012) suggests that high level of melatonin in winter was responsible for immune enhancement in goats. Male goats, being reproductively active throughout the year, showed least responses to melatonin and had little fluctuation in immune parameters even during the winter months when both melatonin and testosterone were high since C. hircus is a short-day breeder. Though having opposite physiological properties, melatonin and gonadal steroid (testosterone) maintained immunity and reproduction in a positive manner in male goats, suggesting an adaptive strategy. The melatonin level was high in males during winter season but might be lacking the threshold capacity to suppress testosterone and, hence, immunity was maintained by the basal level of melatonin. Hence, the interplay of both the hormones (melatonin and testosterone) is responsible for health and reproductive status of males.

A positive correlation was observed between the circulatory levels of estrogen and melatonin during winter months as female goats are cyclic and short-day breeder. Estrogen is reported to be a pro-inflammatory gonadal steroid
(Calippe et al., 2008) while melatonin is pro-gonadotrophic (Arendt, 1986). Simply, there may be a mutual synergism between the gonadal steroid and melatonin as an adaptive strategy in both the sexes to maintain the complex immune – reproductive crosstalk in goats. This suggestion gets further support from the results of metabolic parameters.

Further, we noted higher level of gonadal steroids (testosterone and estrogen) during monsoon suggesting that monsoon is the reproductive preparatory phase for both of the sexes. During monsoon and winter, stress hormone levels were also high (as suggested by higher level of circulatory level of cortisol). Thus, we may suggest that during monsoon goats are under inflammatory stress (due to high pathogenic invasion) and during winter both the males and females are under cold stress (due to low temperature and infection of guts) and gestational stress is prevalent particularly in female goats. Thus, our conclusion is that, to combat with inflammatory stress (during monsoon) and cold stress (during winter) the goats presented higher levels of peripheral immunological parameters. However, the correlation between cortisol and peripheral immunological parameters is negative; then, our results in goats are in parallel with the previous reports of Vishwas et al., (2013) in hamsters.

We observed higher level of thyroxin during winter in both of the sexes. This may be due to the fact that during winter goats are under cold stress and as of their adaptive strategy the Basal Metabolic Rate (BMR) was also high. During monsoon, females presented higher thyroxin level than males suggesting the fact that, during reproductive preparatory phase (i.e. monsoon) females are more metabolically active than males and hence, the requirement of energy was high. Our results are equivocal with the previous results of Rai et al., (2005) and Gupta and Thapliyal (1985). However, we are unable to draw any correlation between circulatory thyroxin level and peripheral immune parameters in goats and thus to delineate the exact roles of different hormones (gonadal/adrenal steroids, metabolic hormone and melatonin) some in depth in vivo and in vitro studies are needed which have been dealt in details in subsequent chapters.
**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. a p < 0.01; Testo vs Testo + Mel.

**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** 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** 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 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. 3B** 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 vs Dexa + Mel.
**Fig. 3C** 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.

**Fig. 3D** 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.
**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** 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** 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.
Fig. 1A

Fig. 1B
Fig. 4A

Fig. 4B
Chapter-3

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
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

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), 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., 2005; 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., 2006). 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., 2005, 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**

Details of animals and maintenance have been described in Materials and Methods section.

**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. \( n = 6/\text{sex/every month of season} \)) 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**

Spleen and thymus were sampled from city abattoir following the protocol of Kaushalendra and Haldar (2012) as described in Materials and Methods section.

**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) as described in Materials and Methods section.

**Hormonal supplementation in vitro**

Doses of different hormones were prepared as described in Materials and Methods section.
**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).

**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 dexamethasone 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 be 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).

**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 be 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).

**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 neuroendocrine axis (Turnbull and Rivier, 1997; 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 extrathymic 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 lymphopoiesis in vitro. Estrogen also affects peripheral B cells and humoral 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 (Abou Akkada 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 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, 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. So, the effect of free radicals in modulation of immunity and reproduction will be discussed in the next chapter.
Fig. 1A

Seasons

Mean Testis
Mean Ovary

0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Fig. 1B

Seasons

Mean Testis
Mean Ovary

0
1.5
3
4.5
6
7.5
9

Fig. 1C

Seasons

Mean Testis
Mean Ovary

0
0.5
1
1.5
2
2.5
3
3.5
Fig. 4A

**ABTS free radical cation scavenging activity (%)**

- **Summer**: Male (25%) vs. Female (30%)
- **Monsoon**: Male (30%) vs. Female (40%)
- **Winter**: Male (25%) vs. Female (30%)

Fig. 4B

**ABTS free radical cation scavenging activity (%)**

- **Summer**: Male (20%) vs. Female (25%)
- **Monsoon**: Male (35%) vs. Female (40%)
- **Winter**: Male (25%) vs. Female (30%)

Fig. 4C

**ABTS free radical cation scavenging activity (%)**

- **Summer**: Male (30%) vs. Female (35%)
- **Monsoon**: Male (40%) vs. Female (45%)
- **Winter**: Male (30%) vs. Female (35%)

Key:
- **Male**
- **Female**
- **Testes**
- **Ovary**

Significance:
- *: P < 0.05
- **: P < 0.01
Fig. 5A

Fig. 5B

Fig. 5C
β Actin (~ 40 kDa)

GR (~ 95 kDa)

**Figure 6 A**

% band intensity of GR expression on male thymus

<table>
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<th>Monsoon</th>
<th>Winter</th>
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<td>60</td>
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**Figure 6 B**

% band intensity of GR expression on male spleen

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<th>Monsoon</th>
<th>Winter</th>
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<tbody>
<tr>
<td></td>
<td>60</td>
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</table>
**Figure 6 C**

Female Spleen

% band intensity of GR expression on female spleen

- Summer
- Monsoon
- Winter

**Figure 6 D**

Female Thymus

% band intensity of GR expression on female thymus

- Summer
- Monsoon
- Winter
Fig. 1A Season and sex dependent variations in Super Oxide Dismutase (SOD) activity in spleen of 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. 1B Season and sex dependent variations in Super Oxide Dismutase (SOD) activity in thymus of 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. 1C Season and sex dependent variations in Super Oxide Dismutase (SOD) activity in gonads of 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. 2A** Season and sex dependent variations in Catalsase activity in spleen of 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. 2B** Season and sex dependent variations in Catalsase activity in thymus of 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. 2C** Season and sex dependent variations in Catalsase activity in gonads of 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. 3A** Season and sex dependent variations in Glutathione Peroxidase (GPx) activity in spleen of 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.**

**Fig. 3B** Season and sex dependent variations in Glutathione Peroxidase (GPx) activity in thymus of 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.**

**Fig. 3C** Season and sex dependent variations in Glutathione Peroxidase (GPx) activity in gonads of 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. 4A** Season and sex dependent variations in 2, 2’- azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) activity in spleen of 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. 4B** Season and sex dependent variations in 2, 2’- azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) activity in thymus of 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.

**Fig. 4C** Season and sex dependent variations in 2, 2’- azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) activity in gonads of 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. 5A Season and sex dependent variations in Malonaldehyde (MDA) level in spleen of 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.

Fig. 5B Season and sex dependent variations in Malonaldehyde (MDA) level in thymus of 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; b p < 0.01, male vs female.

Fig. 5C Season and sex dependent variations in Malonaldehyde (MDA) level in gonads of 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. 6A Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in thymus of male goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 males/season. Vertical bar on each point represents SEM. *p < 0.05; summer vs monsoon and winter.

Fig. 6B Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in spleen of male goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 males/season. Vertical bar on each point represents SEM. *p < 0.05; summer vs monsoon and winter.
**Fig. 6C** Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in spleen of female goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 females/season. Vertical bar on each point represents SEM. *p < 0.05; summer vs monsoon and winter.

**Fig. 6D** Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in thymus of female goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 females/season. Vertical bar on each point represents SEM. *p < 0.05; summer vs monsoon and winter.
Chapter-4

Seasonal and sex dependent variations in free radical load of reproductive and lymphoid organs of Indian goat *C. hircus.*
Seasonal and sex dependent variations in free radical load of reproductive and lymphoid organs of Indian goat *C. hircus.*

**Introduction**

The caprine species (particularly the goats) have to survive in different adverse climatic conditions in different parts of the world. In tropical environments (like India) they are under the threat of huge changes in the environmental temperature and humidity levels during different seasons (Kaushalendra and Haldar, 2012). During summer (April – June) they are under “heat stress” due to high temperature and scorching heat. Monsoon (July – September) is favourable season for growth of different pathogens due to high humidity levels (Ghosh et al., 2013). Being the free grazing animals the goats are easily infected by all of these pathogens (like bacteria, coccidian, nematodes etc.) during monsoon. Further, monsoon is the reproductive preparatory phase for goats and thus, during monsoon the circulatory levels of different gonadal steroids are also high in both the sexes (Ghosh et al., 2014). Being the ruminant short day breeder, winter season (November – January) is stressful particularly for female goats due to “gestational” as well as “cold” stress. Simultaneously, in the internal body milieu a number of physiological processes are going on in a regular manner to maintain the body homeostasis. Further, also to cope up with the environmental stress some important physiological metabolic functions were also elevated. All the physiological responses of elevated environmental stress and routine metabolic processes of the body can give rise to a number of free radicals which responsible for immune compromised condition for the animal (may be due to elevated level of apoptosis, Kalyanaraman and Sohnle, 1985) thus can limit their mortality and productivity as well (MacHugh and Bradley, 2001). In the body physiology, gonads (i.e. testes and ovaries) are most important tissues because of their spontaneous involvement in gametogenesis. As spermatogenesis and oogenesis
both are multistep and energy consuming processes (Jauoux et al., 2013) they are always performing a number of biochemical reactions. As a causative effect of the same generation of free radicals are quite obvious. In the seasonal breeders, the gametogenesis process is dependent upon the status of reproductive physiology like active and inactive phases of reproduction (Chemineau et al., 2008; Ungerfield and Bielli, 2012). In the long day breeders (like squirrels and hamsters) and even in humans there are reports depicting the roles of free radicals in modulation of different physiological processes like immunity (Knight, 2000), metabolism (Droge, 2002) etc. In case of short day breeders the reports inadequate to depict the role of free radicals in regulation of reproduction.

Neurohormone melatonin is regarded as most important anti – stress hormone (Vishwas et al., 2013). Melatonin itself or its metabolite 5-Sulfatoxy melatonin can directly scavenge free radicals (Tan et al., 2000) or it can up regulate the expressions of a number of free radical scavenging enzymes (Toma’s-Zapico and Coto-Montes, 2005) In case of goats, the circulatory level of melatonin is highest during winter and winter is the period of gestation for female goats (Kaushalendra and Haldar, 2012). Thus, the role of melatonin as a pro-gonadotrophic and anti-stress hormone is mostly prevalent in goats. Further, it is also well reported that physiological manifestation of elevated stress is high circulatory level of glucocorticods (Gupta and Haldar 2013) which is also antigonadotrophic (vanDiepen, 2012) in nature. Till date only partial reports are available (Sejian, 2008) demonstrating the levels of glucocorticoids and melatonin in circulation under thermal stress. But, detailed study considering the oxidative load in gonads and lymphoid organs of goats is totally lacking.

Therefore, objective of the present study was to note the seasonal and sex dependent variations on oxidative load/status in gonads and lymphoid organs of Indian goat Capra hircus. To establish the above objective we noted Total Antioxidant Status (TAS), levels of lipid peroxidation (by estimation of TBARS), different free radical scavenging enzyme (SOD, CAT, GPx) activities in lymphoid organs and gonads of goats.
Materials and methods

Animals and maintenance

Animals and maintenance is described in Materials and Methods section.

Experimental design

In order to study the free radical parameters in gonads and lymphoid organs of goats 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.

Gonads, spleen and thymus sampling

Samples of desired tissues were collected following the method of Kaushalendra and Haldar (2012) as described in Materials and Methods section.

Estimation of Superoxide Dismutase (SOD) activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following the method of Das et al. (2000) as described in the Materials and Methods section.

Estimation of Catalase activity

Catalase (CAT; EC 1.11.1.6) activity was measured following the procedure of Sinha (1972) as described in the Materials and Methods section.

Estimation of Glutathione Peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was assayed as described by Mantha et al. (1993) as described in the Materials and Methods section.
**Estimation of lipid peroxidation (LPO) assay by thiobarbituric acid reactive substances (TBARS) level**

Lipid peroxidation was measured by estimating thiobarbituric acid reactive substances (TBARS) level as described by Ohkawa et al., (1978) as described in the Materials and Methods section.

**Estimation of Total Antioxidant Status (TAS)**

The free radical scavenging activity of antioxidants for 2,2’- azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) radical cation was measured according to the method of Re et al. (1999) as described in the Materials and Methods section.

**Western blot analysis of glucocorticoid receptor expression pattern in lymphoid organs of goats**

The western blot analysis was performed according to method as published elsewhere (Ahmad and Haldar, 2010) with only a modification that the membranes were incubated with primary antibody against GR (anti-GR, N-20, sc-2045, Santa Cruz Biotechnology, USA, at a dilution of 1:250) as described in the Materials and Methods section.

**Statistical Analysis**

The data were presented as the means ± Standard Error of Mean (SEM). Variation in tissue (gonads and lymphoid organs) level activities of SOD, CAT, GPx, TBARS, ABTS levels of male and female goats were analyzed by two-way ANOVA. The expressions of GR was analyzed by one-way ANOVA followed by post hoc test i.e. Dunnett test (2-sided). In Dunnett t-test, male and female goats of summer season were treated as control and compared with all other groups. 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 (SPSS) software version 17.0 and in accordance with Bruning and Knitz (1977).
Results

SOD activity in lymphoid organs and gonads

SOD activity in spleen and thymus were significantly high in both the sexes during monsoon (p < 0.01) and significantly low during winter (p < 0.05 in male spleen and female thymus and p < 0.01 in male thymus; Fig. 1A and 1B). Further, SOD activity in gonads of both the sexes was significantly high during monsoon (p < 0.01) and winter (p < 0.05; Fig. 1C). However there was no sex dependent variation.

Catalase activity in lymphoid organs and gonads

Catalase activity in spleen and thymus of both the sexes were significantly high during monsoon (p < 0.05) and significantly low during winter (p < 0.01; Fig. 2A and 2B). Further, Catalase activity in testes and ovaries of both the sexes was significantly high (p < 0.01) during monsoon and significantly low (p < 0.05) winter in comparison to summer (Fig. 2C). Sex dependent variations were not observed.

Glutathione peroxidase (GPx) activity in lymphoid organs and gonads

Glutathione peroxidase (GPx) activity in spleen and thymus of both the sexes were significantly high during monsoon and winter (p < 0.01; Fig. 3A and 3B). Glutathione peroxidase (GPx) activity in testes and ovaries of both the sexes was significantly high during monsoon (p < 0.05) and winter (p < 0.01; Fig. 3C). However there was no sex dependent variation.

ABTS level in lymphoid organs and gonads

During monsoon the Total Antioxidant Status (TAS) of lymphoid organs of both the sexes were significantly high (p < 0.01). During wither also the similar trend was observed. The TAS level was significantly high in spleen (p < 0.05) and thymus (p < 0.01) of both the sexes (fig. 3A and 3B). During monsoon and winter the Total Antioxidant Status (TAS) of gonads of both the sexes were significantly high (p < 0.01 during monsoon and p < 0.05 during winter; Fig. 4C). However, sex dependent variations were not observed.
Estimation of lipid peroxidation (LPO) assay by thiobarbituric acid reactive substances (TBARS) level

In case of both thymus and spleen during summer the level was highest. But, the level was significantly low monsoon (p < 0.01 in female spleen and male thymus). In case of female thymus, the level was significantly low (p < 0.01) during summer in comparison to males and the level was significantly high (p < 0.01) during monsoon in comparison to males as well as in comparison to summer. (Fig.5A and 5B). In case of both testes and ovaries during summer the level was lowest in both the sexes. But, the level was significantly high in gonads of both the sexes during monsoon (p < 0.05) and winter (p < 0.01; Fig. 5C).

Western Blot analysis of expression of Glucocorticoid Receptor (GR)

The glucocorticoid receptor (GR) expression pattern in males was significantly high in both thymus and spleen during monsoon and winter (p < 0.05, Fig. 6A and 6B). But in case of female spleen and thymus the level was significantly high only during monsoon (p < 0.05; Fig. 6C and 6D).

Discussion

Pesticides are well known for increasing the free radical loads of different metabolically active organs such as kidney (Ozbek, 2012), liver (Muriel, 2009), brain (Poon et al., 2004) and immune system (Ahmad et al., 2012) of animals including human being (Fantone and Ward, 1985). In the present agro-ecosystem, the use of pesticides and chemical fertilizers is increasing continuously to fulfil the requirement of food for ever expanding population. But, pesticides pose a large threat to primary consumers. Hence, like other herbivores, goats being free grazing animals are directly exposed to pesticides and other environmental stresses during different seasons of the year. All these factors cumulatively have weakened their immune system. Reports are available regarding oxidative and nitrosative stress in lymphoid organs and gonads in different seasonal (Ahmad et al., 2013) and spontaneous breeders (Fantone and Ward, 1985). But, reports on the free radical load in goats are totally lacking. In this context our results are significant and first
of its kind depicting the free radical status in gonads and lymphoid organs of goats.

The generation of reactive oxygen species by aerobic organisms comes with a high physiological price, which can be lowered by antioxidants such as melatonin (Reiter, 2000). Endogenously produced melatonin may have a significant role in deferring a number of free radical-related disease and some patho-physiological changes (Siu et al., 2006). Being amphipathic molecule this indoleamine is acting as free radical scavenger because it has the capability of penetrating all physiological barriers and can enter all sub-cellular compartments. Thus, high level of circulatory melatonin during winter season might be responsible for lowered lipid peroxidation and increased antioxidant enzyme level in lymphoid organs of goats. The antioxidant enzymes (SOD, CAT, GPx) showed a clear-cut variation in a season dependent manner. Maximum levels of antioxidant enzymes (SOD, CAT and GPx) were observed in all the groups of goats during monsoon and winter season when the peripheral melatonin level was high (Chapter 2B). Our data gets support from the studies of small mammals suggesting that changes in oxidative load are dependent on the circadian melatonin rhythm (Haldar et al., 2006b). Therefore, a physiological level of melatonin appears to be adequate to alter the antioxidant defence system as reflected by the level of activities of antioxidant enzymes in goats. During monsoon the circulatory level of melatonin is moderately high but cortisol level is highest (Chapter 2B). This season is also the reproductive preparatory phase of goats with higher levels of gonadal steroids (Ghosh et al., 2014). Due to high level of temperature and humidity, monsoon is the most important season for parasitic growth and infections. Being free grazing animals goats are under inflammatory stress and can generate high level of free radicals. To scavenge them the free radical scavenging enzyme level and TAS levels were also high. During winter, both the male and female goats are under cold stress and particularly females are under gestational stress. Melatonin might be stimulating the protective activity of antioxidant enzymes as designated by ABTS radical cation reduction. The more free radicals, the less ABTS percentage inhibition occurs and vice-versa. The present results
also suggested that physiological level of melatonin in circulation of a seasonal breeder is highly relevant in terms of total antioxidant capacity of lymphoid organs and gonads. Effects of lipid per-oxidation in particular are under intense investigation because of their involvement in several pathological conditions. The unsaturated lipids are more prone to free radicals damage and hence, lipid per-oxidation is considered as the biomarker of free radical load (Wagner et al., 1994). As oxidative stress is indicative imbalance between oxidants and antioxidants, methods for quantifying oxidative stress mostly include direct or indirect estimation of oxidants and antioxidants. Malonaldehyde (MDA) is a low molecular weight end product which is generated as the free radical damages the lipids (Wong-ekkabut, 2007). Further, melatonin reduced lipid peroxidation during monsoon and winter. This supports our observation of seasonal variation in enzyme activity, TAS levels as recorded in lymphoid organs and gonads of goats.

In general, the level of melatonin has inverse correlation with the cortisol level. Whenever the goats were under ecological stress during monsoon (seasonal infection etc.) and winter (cold stress and gestational stress) the level of melatonin was moderate and high but the cortisol level was high. This suppressed the general immunity and increased the free radical load. Our data gets further support from the result of up regulated GR expression during monsoon and winter in male and female goats. During monsoon, the levels of cortisol and its receptor (GR) in lymphoid organs of both the sexes were significantly high. This may be due to elevated inflammatory stress and moderately high level of melatonin. During winter, moderately high level of cortisol and GR expression suggest that due to high level of melatonin the level of free radical was decreased. This is an adaptive modification particularly suggested for female goats for maintenance of pregnancy and perfect gestation.

We noted higher level of free radical scavenging enzyme parameters (SOD, CAT and GPx) and TAS level in gonads during monsoon. This may be due to higher metabolic activity of gonads of both the sexes in terms of steroidogenesis and gametogenesis. The sophisticated machinery of steroidogenesis and gametogenesis might have involved a number of local biochemical activities which in turn have
generated huge amount of free radicals which is equivocal with the reports of other workers (Jana et al., 2010; Das et al., 2002). Thus, to cope up with the elevated level of free radicals, the free radical scavenging enzyme activities and TAS levels were high. Another reason may be that, during monsoon melatonin level was moderately high and the free radical scavenging activity of melatonin as a free molecule was low. So, the gonadal level of stress management was solemnly performed by gonads it selves. This point was further supported by moderately high level of free radical scavenging enzymes and TAS level during winter when plasma level of melatonin attended its yearly peak. Being an amphipathic molecule, melatonin might have crossed the Blood Testes Barrier (BTB) in males and scavenged free radicals. Further, it is also well reported that both testes and ovaries are well equipped with melatonin synthesizing machinery and locally can synthesize melatonin at tissue level (Ghosh et al., 2014). Thus, this local melatonin and one of its metabolite (5-sulfatoxymelatonin) can scavenge free radicals as 5-sulfatoxymelatonin is more potent free radical scavenger than melatonin itself (Rosen et al., 2009). Another possibility is that, tissue melatonin might have elevated some of the key enzyme for free radical scavenging (like GPx) during winter which either alone was sufficient enough to scavenge free radicals or have elevated the levels of other free radical scavenging enzymes (like SOD and CAT; Liu and Ng, 2000).

We noted higher level of MDA (a marker of lipid peroxidation) in gonads during monsoon and winter in both the sexes. The reason behind is that, high cholesterol was taken up by both testes and ovaries during monsoon and winter. But the paths of utilization in both the sexes were different. During monsoon it was utilized by both the sexes for steroidogenesis and during winter it was utilized by females for maintenance of gestation and by males for spontaneous gametogenesis.

Being ruminant short day breeder maintenance of immunity and reproduction is a simultaneous process in goats. But, both the processes are highly energy demanding. Thus, our next aim was to note the path of energy allocation in goats, Capra hircus to modulate reproduction and immunity particularly during
monsoon (which is the reproductive preparatory phase and season of inflammatory stress) and winter which is the season for cold stress for both the sexes and gestational stress particularly for females.
Fig. 1A

**b**
Conc. of Glucose in blood (mg/dl)

Fig. 1B
Conc. of Glucose in Spleen (μg/dl/mg of wet weight of tissue)

Seasons
Fig. 2A

Cholesterol Concentration in Blood (mg/ml)

- **a**
- **b**

Fig. 2B

Cholesterol Concentration in Spleen (μg/ml/gm wet weight of tissue)

- **a**
- **b**
Cholesterol Concentration in Thymus

- **b**
- **a**

**Fig. 2C**

Cholesterol Concentration in Gonads

- Testis
- Ovary

**Fig. 2D**

Seasons

- Summer
- Monsoon
- Winter
Fig. 3A

Seasons

Conc. of Glycogen in Liver (mg/100mg of tissue weight)

- Summer
- Monsoon
- Winter

** Male
* Female

Fig. 3B

Seasons

Conc. of Glycogen in Spleen (mg/100mg of tissue weight)

- Summer
- Monsoon
- Winter

** Male
** Female
Fig. 4A

% Uptake of Glucose by Spleen from blood

Seasons

Summer Monsoon Winter
Mean Male Mean Female

Fig. 4B

% Uptake of Glucose by Thymus from blood

Seasons

Summer Monsoon Winter
Mean Male Mean Female

** **
Fig. 5A

% uptake of cholesterol by spleen from blood

<table>
<thead>
<tr>
<th>Season</th>
<th>Mean Male</th>
<th>Mean Female</th>
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<tr>
<td>Monsoon</td>
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<tr>
<td>Winter</td>
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Fig. 5B

% uptake of cholesterol by thymus from blood

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<th>Season</th>
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<th>Mean Female</th>
</tr>
</thead>
<tbody>
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<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Winter</td>
<td>120</td>
<td>120</td>
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</tbody>
</table>

* Significant difference
** Highly significant difference
**Fig. 6A**
Activity of phosphorylase in spleen (μ moles/min)

- **Summer**: Male 1.5, Female 1.2
- **Monsoon**: Male 2.5, Female 2.3
- **Winter**: Male 1.7, Female 1.5

**Fig. 6B**
Activity of phosphorylase in thymus (μ moles/min)

- **Summer**: Male 2.0, Female 1.5
- **Monsoon**: Male 2.8, Female 2.3
- **Winter**: Male 1.9, Female 1.6

**Fig. 6C**
Activity of phosphorylase in gonads (μ moles/min)

- **Summer**: Testes 1.5, Ovary 1.2
- **Monsoon**: Testes 3.0, Ovary 2.5
- **Winter**: Testes 1.5, Ovary 1.4
Fig. 10A

MT1 (~43 kDa)
MT2 (~39 kDa)
β-Actin (~40 kDa)

% band intensity of MT1 and MT2 expression in female thymus

Seasons

Summer
Monsoon
Winter

Fig. 10B

MT1 (~43 kDa)
MT2 (~39 kDa)
β-Actin (~40 kDa)

% band intensity of MT1 and MT2 expression in male thymus

Seasons

Summer
Monsoon
Winter

** Significant difference

* Significant difference

Fig. 10A

Fig. 10B
Fig. 11 A

% band intensity of AR expression in male thymus

Seasons

Summer Monsoon Winter

Fig. 11 B

% band intensity of ERα expression in female thymus

Seasons

Summer Monsoon Winter

*
Fig. 1A Season and sex dependent variations in glucose concentration in blood of 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, b p < 0.01; male vs female.

Fig. 1B Season and sex dependent variations in glucose concentration in spleen of 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. 1C** Season and sex dependent variations in glucose concentration in thymus of 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, b p < 0.01; male vs female.

**Fig. 1D** Season and sex dependent variations in glucose concentration in gonads of 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, b p < 0.01; male vs female.
**Fig. 2A** Season and sex dependent variations in cholesterol concentration in blood of 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, b p < 0.01; male vs female.

**Fig. 2B** Season and sex dependent variations in cholesterol concentration in spleen of 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; male vs female.
**Fig. 2C** Season and sex dependent variations in cholesterol concentration in thymus of 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. 2D** Season and sex dependent variations in cholesterol concentration in gonads of 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. 3A** Season and sex dependent variations in glycogen concentration in liver of 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. 3B** Season and sex dependent variations in glycogen concentration in spleen of 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.
Fig. 3C Season and sex dependent variations in glycogen concentration in thymus of 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; male vs female.

Fig. 3D Season and sex dependent variations in glycogen concentration in gonads of 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.
**Fig. 4A** Season and sex dependent variations in % up take of glucose from blood by spleen of 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.

**Fig. 4B** Season and sex dependent variations in % up take of glucose from blood by thymus of 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.
**Fig. 5A** Season and sex dependent variations in % up take of cholesterol from blood by spleen of 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. 5B** Season and sex dependent variations in % up take of cholesterol from blood by thymus of 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. 6A** Season and sex dependent variations in phosphorylase activity in spleen of 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. 6B** Season and sex dependent variations in phosphorylase activity in thymus of 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. 6C** Season and sex dependent variations in phosphorylase activity in gonads of 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. 7** Season and sex dependent variations in AA-NAT activity in thymus of 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. 8** Season and sex dependent variations in 3βHSD activity in thymus of 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; *p < 0.05, male vs female.

**Fig. 9** Season and sex dependent variations in tissue level of melatonin concentration in thymus of 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.
**Fig. 10A** Western blot analysis for seasonal variations in expression of membrane bound melatonin receptors (MT1 and MT2) in thymus of female goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 females/season. Vertical bar on each point represents standard error of mean (SEM). **p < 0.01; summer vs monsoon and winter.

**Fig. 10B** Western blot analysis for seasonal variations in expression of membrane bound melatonin receptors (MT1 and MT2) in thymus of male goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 males/season. Vertical bar on each point represents standard error of mean (SEM). *p < 0.05, **p < 0.01; summer vs monsoon and winter.
**Fig. 11A** Western blot analysis for seasonal variations in expression of androgen receptor (AR) in thymus of male goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 males/season. Vertical bar on each point represents standard error of mean (SEM).

**Fig. 11B** Western blot analysis for seasonal variations in expression of estrogen receptor (ERα) in thymus of female goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean ± SEM, N = 18 females/season. Vertical bar on each point represents standard error of mean (SEM). *p < 0.05; summer vs monsoon and winter.
Chapter-5A

Seasonal and sex dependent variations in energy allocation for reproduction and immunity of Indian goat *C. hircus*: Existence of hormonal micro-circuit in lymphoid tissues.
Seasonal and sex dependent variations in energy allocation for reproduction and immunity of Indian goat *C. hircus*: Existence of hormonal micro-circuit in lymphoid tissues.

**Introduction**

Reproduction and immune modulation both are extremely dynamic biological mega events. Modulation in immunity is in need of a balance of mitosis and apoptosis. Reproduction in both males and females involves gametogenesis and steroidogenesis process. Hence, both reproduction and immune modulations are high energy demanding processes. In most of the mammals including human the energy balance between reproduction and immune modulations are well managed due to their opportunistic behaviour in reproduction (García-Garcia, 2012). But, it is a matter of concern in seasonal breeders (Nelson and Demas, 1997). However, in the long day breeders (like squirrel, hamsters) the energy allocation pattern is partially documented. Their reproductive phase is divided into two different phases Reproductive Active Phase (RAP) and Reproductive Inactive Phase (RIP). During, RAP the circulatory level of melatonin is low with basal level of immune parameters and high reproductive behaviour and performances. But during RIP; reproductive performances are low while immunological parameters are high (Haldar and Ahmad, 2010; Demas and Nelson, 2003). The main problem is with the short day breeders (sheep and goats) where reproduction and immune modulations are occurring simultaneously. Thus, it is a matter of investigation that how both the high energetically important mega events are occurring simultaneously in short day breeders (Demas and Nelson, 1996; Ruckstuhl et al., 2003). In sheep, the reproductive energy demand is partially documented (Sayed, 2009), but till date no report is available regarding the energy balance between reproduction and immunity in goats.
Further, in some animals (like mice and rats) it is evident that there may be a local hormonal circuit in peripheral endocrine organs (like ovary for GnIH and GnRH; Singh et al., 2010), salivary gland (Harris and Kaufman, 1985), GI tract (Ahlman and Nilsson, 2001; Clarke et al., 2014) etc. But, no report is available regarding the primary and secondary lymphoid organ can be regarded as endocrine organ and also a site for local micro circuit of hormones. This kind of results or even speculations are totally lacking in any seasonal (including long day or short day) breeders.

We identified the lacunae of previous studies and therefore, aim of the present study was to note the seasonal and sex dependent variations in energy allocation pattern in reproduction and immune modulation in goats with a special focus on hormonal microcircuit in lymphoid organs.

**Materials and methods**

**Animals and maintenance**

Details of animals and their maintenance have been described in Materials and Methods section.

**Experimental design**

In order to study the energy allocation pattern in regulation of reproduction and immunity in goats 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.
**Blood collection**

The blood was collected from the jugular vein of goats following the protocol of Kaushalendra and Haldar, 2012 as described in Materials and Methods section.

**Gonads, spleen and thymus sampling**

Samples of desired tissues (gonads, spleen and thymus) were collected from city abattoir following the procedure as suggested by Kaushalendra and Haldar (2012) which has been described in details in Materials and Methods section.

**Estimation of glucose**

Tissue level glucose was extracted following the protocol of Moses (2006). Blood and tissue level (spleen, thymus and gonad) glucose was estimated with a commercial kit according to manufacturer’s protocol as described in details in the Materials and Methods section.

**Estimation of cholesterol**

Blood and tissue level (spleen, thymus and gonad) cholesterol was estimated following the protocol of Sackett, (1969) as described in details in the Materials and Methods section.

**Estimation of glycogen**

Tissue level (liver, spleen, thymus and gonad) glycogen was estimated following the protocol of Shavali and Haldar (1998) as described in details in the Materials and Methods section.

**Estimation of AA-NAT enzyme activity**

Tissue level (spleen and thymus) AA-NAT activity was estimated following the protocol of Chae et al., (1999) as described in details in the Materials and Methods section.
Estimation of 3β HSD enzyme activity

3β HSD enzyme activity was assayed following the protocol of Shivanandappa and Venkatesh, (1997) as described in details in the Materials and Methods section.

Estimation of Glycogen phosphorylase enzyme activity

Glycogen phosphorylase enzyme activity was assayed following the protocol of Mason (1971) as described in details in the Materials and Methods section.

Western Blot analysis of expression of membrane bound melatonin receptor MT1 and MT2, androgen receptor (AR) and estrogen receptor (ERα)

The western blot analysis was performed according to method as published elsewhere (Ghosh et al., 2014) with only a modification that the membranes were incubated with primary antibody against MT1 (anti-Mel1aR, ab 96502, Abcam, England at a dilution of 1:250), MT2 (Mel1bR, ab128469, Abcam, England at a dilution of 1:250), AR (anti-AR, N-20, sc-1004, Santa Cruz Biotechnology, USA, at a dilution of 1:200) and ERα (anti-ERα, HC-20, sc-543, Santa Cruz Biotechnology, USA at a dilution of 1:200) as described in the Materials and Methods section.

Statistical analyses

The data were presented as the mean ± standard error of the mean (SEM). The results of plasma and tissue level of glycogen, glucose and cholesterol concentrations, tissue level enzyme activities and MT1 and MT2 receptor expression patterns were analyzed by two way ANOVA. The data of AR and ERα expression pattern was analyzed by one way ANOVA followed by post hoc Dunnett test (2-sided). In Dunnett t-test, male and female goats of summer season were treated as control and compared with all other groups. 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 (SPSS) software version 17.0 and in accordance with Bruning and Knitz (1977).
Results

Glucose concentration in blood, lymphoid organs and gonads

We noted significantly high level of plasma glucose in females than males and other seasons (p < 0.01; Fig. 1A). In spleen the tissue level glucose was significantly high in both the sexes during monsoon (p < 0.01 in males and p < 0.05 in females) and winter (p < 0.01 in males and p < 0.05 in females; Fig. 1B). In thymus, the glucose level was significantly high during monsoon (p < 0.01 in males and p < 0.05 in females) and winter (p < 0.01) only in females than males and other seasons (Fig. 1C). In the gonads the glucose level is significantly high in females during monsoon (p < 0.01) than other seasons and (p < 0.05) than males. During monsoon the level is significantly low in males (p < 0.01) than other seasons and significantly high in females (p < 0.01) when compared with other seasons and males (Fig. 1D).

Cholesterol concentration in blood, lymphoid organs and gonads

We noted significantly high level of plasma cholesterol during monsoon and winter in females (p < 0.01) in comparison to males (p < 0.05 during monsoon and p < 0.01 during winter; Fig.2A). Cholesterol concentration in female spleen was significantly high during monsoon and winter (p < 0.01) than summer and also in comparison to males (p < 0.05) particularly during monsoon. In male spleen the level was significantly high only during winter (p < 0.05; Fig.2B). Cholesterol concentration in male thymus was significantly low during monsoon (p < 0.01) and was significantly high during winter (p < 0.01). However, in females cholesterol level was significantly high during monsoon (p < 0.01) and winter (p < 0.05). Females showed higher cholesterol level than males in monsoon (p < 0.05) and males showed higher cholesterol level than females (p < 0.01) during winter (Fig. 2C). Gonad level of cholesterol was significantly high in both the sexes during monsoon (p < 0.05 in males and p < 0.01 in females) and winter (p < 0.05 in males and p < 0.01 in females; Fig. 2D).
Glycogen concentration in liver, lymphoid organs and gonads

We noted a decreasing pattern in glycogen level in liver. The level was significantly low during monsoon (p < 0.01 in males and p < 0.05 in females) and winter (p < 0.01 in both the sexes). However, gender dependent variation in liver glycogen level is also prominent being significantly low in females than males during monsoon (p < 0.05) and winter (p < 0.01; Fig. 3A). Stored glycogen level in spleen was significantly low during monsoon and winter in females (p < 0.01) and in males it is significantly low (p < 0.01) only during winter (Fig. 3B). Stored glycogen level was significantly low in thymus of both the sexes (p < 0.01 in males and p < 0.05 in females) during monsoon and winter (p < 0.01 in both the sexes). However, during monsoon females showed higher level of glycogen (p < 0.05; Fig. 3C). In gonads, the stored glycogen was significantly low (p < 0.05) in testes only during monsoon. Gender dependent variation in gonadal glycogen storage was not significant (Fig. 3D).

% uptake of glucose by lymphoid organs from circulation

% uptake of glucose was significantly low in female spleen during monsoon (p < 0.01) but the level is significantly low in male spleen (p < 0.01) during winter (Fig. 4A). In case of thymus, the % uptake of glucose is significantly high in both the sexes during monsoon (p < 0.01) and winter (p < 0.01; Fig. 4B).

% uptake of cholesterol by lymphoid organs from circulation

We noted significantly low level of % uptake of cholesterol by male spleen during monsoon and winter (p < 0.05) but the level was significantly high in female spleen during monsoon (p < 0.05) and winter (p < 0.01). The female goats always showed significantly higher level of % uptake of cholesterol than males during monsoon (p < 0.05) and winter (p < 0.01; Fig. 5A). In case of male thymus, the % uptake of cholesterol was significantly low during monsoon (p < 0.05) and significantly high (p < 0.01) during winter. But, the female thymus showed significantly increased % uptake of cholesterol during monsoon (p < 0.05) and winter (p < 0.01; Fig. 5B).
Glycogen phosphorylase activity in lymphoid organs and gonads

The glycogen phosphorylase activity in spleen of both the sexes was significantly high during monsoon (p < 0.05) and winter (p < 0.01; Fig. 6A). In thymus of both the sexes the level was significantly high during monsoon (p < 0.01) and winter (p < 0.05; Fig. 6B). In gonads the level was significantly high in testes during monsoon (p < 0.01) only and in case of ovaries the level was significantly high during monsoon (p < 0.01) and winter (p < 0.05; Fig. 6C).

Estimation of AA-NAT activity in thymus

AA-NAT activity was found to be significantly high in thymus of both the sexes during monsoon (p < 0.05) and winter (p < 0.01; Fig. 7).

Estimation of 3β-HSD activity in thymus

3β-HSD enzyme activity was significantly high in thymus of both the sexes during monsoon (p < 0.01) and only in females during winter (p < 0.05; Fig. 8).

Estimation of tissue level melatonin in thymus

Tissue level melatonin was significantly high in thymus of both the sexes during winter (p < 0.01; Fig. 9).

Expression of MT1, MT2, AR, and ERα in thymus of goats

In males, MT1 and MT2 were significantly high during winter (p < 0.01); however, the level of MT2 was significantly low during monsoon (p < 0.05; Fig. 10 B). In females both MT1 and MT2 were significantly high (p < 0.01; Fig. 10 A) during winter. AR expression pattern was found to be static in male thymus during three seasons (Fig. 11A), whereas the ERα showed only a significant increase (p < 0.05 Fig. 11 B) during monsoon in female thymus.

Discussion

The main energy distribution of the body is being channelized to modulate the two mega physiological events they are the modulation of immunity and maintenance of reproduction. However, some literature suggesting the energy allocation pattern and seasonal modulation of reproductive energetic has been
reported by some authors (Nelson and Demas, 1997; Viney et al., 2005; Martin and Festa-Bianchet, 2010) in sheep, seal and other animals residing in polar regions (e.g. polar bear). But, those reports are partial and never included patterns of immunity. Goats being the short day breeder provide an opportunity to study the path of energy allocation in both immunity and reproduction simultaneously. But, there is no literature available in this regard and our study provides first report to describe the energy allocation pattern primarily in immune organs as well as in gonads during reproductively active (i.e. winter) and inactive (i.e. summer and monsoon) phases of goats. To delineate the energy allocation pattern, we studied the glycogen, glucose and cholesterol content of both the tissue (lymphoid organs and gonads) as well as at circulatory level.

We noted significantly higher level of glucose (the primary source of energy) in circulation of goats during monsoon only. But, in lymphoid organs of both the sexes of goats and in their gonads the tissue level of glucose was significantly high both during monsoon and winter. Both the gonads and lymphoid organs of females presented a high level of tissue glucose than males in year round manner, suggesting that females are in need of high energy to maintain immunity during monsoon and both immunity and gestation during winter.

We considered the circulatory level of glucose as the primary source of energy and tried to draw a relation between circulatory glucose level with that of tissue level i.e. in gonads and lymphoid organs. We noted that the thymus of both male and female goats had a higher level of % uptake of glucose which was even high in circulation. Thus, we looked for an alternate source of energy and our primary focus on glycogen level.

For glycogen we took liver of both males and females as internal positive control and we found that stored glycogen level was significantly high during summer. But, the stored glycogen level significantly goes down during monsoon and winter when compared with summer as the utilization rate (in the form of glucose by glycogenolysis) in those two season (summer and monsoon) might had increased. Further we noted tissue level glycogen storage in lymphoid organs and gonads as well. Our study clearly showed that, gonads, thymus and spleen were
capable enough to store the glycogen as a source of energy and the level was substantial when compared to the liver (as an internal positive control). This result is in parallel with the previous report of Taira et al., (1982). But, the level of stored glycogen was significantly low particularly in lymphoid organs during monsoon and winter. This may be due to the fact that physiologically elevated energy demand was not sufficiently quenched by circulatory level of glucose coming either from dietary sources or from glycogenolysis in liver. Thus, we may suggest that during the months of extreme stress i.e. in terms of immunity and reproduction the elevated energy consumption was counter balanced by the tissue level glycogenolysis. Hence, to check the level of glycogenolysis in both the lymphoid organs and gonads we studied the tissue level glycogen phosphorylase activity as a marker of glycogenolysis.

We noted significantly high level of glycogen phosphorylase activity in gonads, spleen and thymus of both the sexes of goats during monsoon and winter. Our result clearly suggests that, the lymphoid organs and gonads are not only acting as the major action sites of immune modulation and reproduction respectively, but also, are the “local power house” of the body. When there is a need of high energy (for reproduction and immunity maintenance) then these power houses may act promptly by utilizing their own energy resources. This exceptional mechanism of energy allotment and utilization is a typical and particular phenomenon occurring in goats.

Cholesterol, either being in the circulation or in the tissue, may be used as secondary source of energy. We noted significantly high level of cholesterol in circulation, spleen and in gonads both during monsoon and winter months in both the sexes of goats. But, the level was significantly low during monsoon and winter in thymus of male and female goats. Further, % uptake level of cholesterol was significantly high in lymphoid organs of both the sexes during monsoon and winter months. In most of the cases females presented a higher level of cholesterol than males, may be due to the fact that monsoon being the reproductive preparatory phase for females (with high level of estrogen) while males are reproductively active throughout the year. Thus, during monsoon and winter the
gonads needed higher cholesterol level for high level of steroidogenesis. Contrary to this, lymphoid organs (particularly the thymus) had low level of tissue cholesterol. This might be due to tissue level of steroidogenesis (Vacchio et al., 1994), a process which is less common phenomenon but were reported particularly for some seasonal breeders squirrel (Ghosh et al., 2014). To, check the similar phenomena we noted tissue level of expression of AR, ERα and activity of 3βHSD particularly in thymus as because spleen is regarded as the negative control for gonadal steroid receptors (e.g. AR and ERα, Ahmad and Haldar, 2010).

We recorded significantly high level of 3βHSD activity during monsoon in thymus of both the sexes of goats with significantly high level of 3βHSD activity in females only during winter months. Further, ERα expression in thymus of females was significantly high during monsoon while AR expression pattern in male goat thymus had no seasonal variation. With the help of the present data we may suggest that, the thymus of both the sexes has utilized tissue level cholesterol for balancing steroidogenesis. This unusual utilization of cholesterol is due to the fact that sometimes gonadal steroids may work as immune stimulator (particularly Estrogen) by increasing different pro-inflammatory cytokine particularly by PI3K/Akt pathway (Calippe et al., 2008). Thus, it may be possible that during monsoon when plasma level of melatonin is moderately high, the gonadal steroids might have played some balancing role in goat immune modulation via the local inflammatory process. Further, to delineate the local inflammatory process under influence of melatonin (a known immune stimulator) we noted AA-NAT activity, tissue level melatonin and MT1 and MT2 receptor expression pattern in thymus of both the sexes. We found AA-NAT activity is significantly high in both the sexes during monsoon and winter. Tissue level melatonin was significantly high only during winter with significantly high level of melatonin receptor MT1 and MT2 expression. Cumulatively, all these results may suggest that the thymus is acting as most important organ in goat immune modulation either via gonadal steroids or via melatonin pathways.

Thus, in conclusion we may suggest that, to adjust with seasonal stress, the tissue level of melatonin in goats is not only acting as a buffer hormone to
maintain the threshold level of immune status but also playing an important role in regulating local “microcircuit” of melatonin and gonadal steroid hormones in lymphoid organs to modulate immunity. In this regard, the role of thymus is of highest importance (in terms of energetic), to maintain two energetically most important events i.e. immunity and reproduction simultaneously. It might also develop a functional synergism between the immune suppressive role of gonadal steroids and immune enhancing role of melatonin. Thus, it could be a special physiological adaptation of goats that made this ruminant short day breeder, a better survivor under different environmental odds as well as physiological stress.
Fig. 2B ALT Activity I.U./L

Fig. 2C RBC Count ($\times 10^7$ cells/µL)
Fig. 3C

% Lymphocyte Count

Groups

*...

Fig. 4A

Glucose conc. in plasma (mg/dL)

Groups

Fig. 4B

Cholesterol conc. in plasma (μg/mL of plasma)

Groups
Fig. 4C

Protein conc. in plasma (mg/ml)

<table>
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<th>Control male</th>
<th>Control Female</th>
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Fig. 4D

HDL conc. in plasma mg/dl)

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Fig. 4E

LDL conc. in plasma mg/dl)

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Fig. 5C

Fig. 5D

[Bar graph showing MDA level in plasma (μmoles of TEP hydrolyzed/min) for different groups: Control Male, Control Female, Exp. Male, Exp. Female. Significant differences are indicated with **]
Fig. 6A

Fig. 6B

Fig. 6C
Fig. 7A: Circulatory concentration of IL-6 (pg/mL)

- Control Male: 20 pg/mL
- Control Female: 40 pg/mL
- Exp. Male: 50 pg/mL
- Exp Female: 60 pg/mL

Fig. 7B: Circulatory concentration of TNF-α (pg/mL)

- Control Male: 70 pg/mL
- Control Female: 30 pg/mL
- Exp. Male: 80 pg/mL
- Exp Female: 90 pg/mL

Significance:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001

Note: The figure shows the circulatory concentration of IL-6 and TNF-α in different groups, with significance levels indicated for each group.