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Original Article

FREE RADICAL LOAD IN LYMPHOID ORGANS (SPLEEN AND THYMUS) OF INDIAN GOAT *CAPRA HIRCUS*: ROLE OF SEX, SEASON AND MELATONIN

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ABSTRACT

Objective: Lymphoid organs (i.e. spleen and thymus) are important due to functional dynamicity. As a result, the generated free radicals may limit their function. Thus, present study was aimed to note seasonal and sex dependent variation in free radical status in Indian goat *Capra hircus* under the aegis of melatonin which is a well-known antioxidant.

Methods: Markers of oxidative stress (i.e. Super Oxide Dismutase; SOD, Catalase; CAT, Glutathione Peroxidases; GPx) were measured by standardized protocols. Total Antioxidant Status (TAS) was measured by 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) radical cation method and Lipid Per Oxidation (LPO) was measured by Thiobarbituric acid reactive substances (TBARS) level. Glucocorticoid Receptor (GR) expression in lymphoid organs was noted by Western Blot analysis. The circulatory level of cortisol and melatonin were estimated by commercial ELISA kits.

Results: We noted significantly high levels of SOD, Catalase, GPx activities and ABTS level in lymphoid organs during monsoon and low during winter. Malonaldehyde; MDA a marker for lipid peroxidation was significantly high during summer and was significantly low during monsoon and winter. Cortisol level was significantly high during monsoon whereas melatonin level was significantly high during winter. GR expression was significantly high in males during monsoon and winter, but the level was significantly high only during monsoon in females.

Conclusion: All the results suggest that monsoon and winter are the seasons of stress and to buffer the elevated stress level, melatonin coupled both the roles of free radical scavenger (as a free molecule) and elevation of antioxidant enzymes.

Keywords: Free Radical Load, Goat, Melatonin, Sex, Season, Spleen, Thymus.

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 [1]. During summer (April–June), they are under "heat stress" due to high temperature and scorching heat. Monsoon (July–September) is the favorable season for the growth of different pathogens due to high humidity levels [2]. Being the free grazing animals, the goats are easily infected by all of these pathogens (like bacteria, coccidia, nematodes, etc.) during monsoon. Further, the monsoon is the preparatory reproductive phase for goats and thus, during monsoon the circulatory levels of different gonadal steroids are also high in both the sexes [3]. 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, [4] and thus, can limit their mortality and productivity as well [5]. In the long day breeders (like squirrels and hamsters) and even in humans there are reports depicting the roles of free radicals in the modulation of different physiological processes like immunity [6], metabolism [7] etc. In the case of short day breeders the reports inadequate to depict the role of free radicals in the regulation of immunity.

Neurohormone melatonin is regarded as most important anti–stress hormone [8]. Melatonin itself or its metabolite 5-Sulfatoxy melatonin can directly scavenge free radicals [9] or it can up-regulate the expressions of a number of free radical scavenging enzymes [10]. In the case of goats, the circulatory level of melatonin is highest during winter and winter is the period of gestation for female goats [1]. 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 glucocorticoids [11] which is also anti-gonadotrophic in nature [12]. Till date only partial reports are available [13] demonstrating the levels of glucocorticoids and melatonin in circulation under thermal stress. But, detailed study considering the oxidative load in lymphoid organs of goats is totally lacking.

Therefore, the objective of the present study was to note the seasonal and sex dependent variations on oxidative load/status in 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 and Glucocorticoid receptor expression in lymphoid organs of goats and correlated it with circulatory levels of cortisol and melatonin.

MATERIALS AND METHODS

Animals and maintenance

Goats of approximately same age (~1 y) 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 [14]. 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 (*Linumusit atissimum*) 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 h outdoor for free grazing and 16 h indoor (during the night) conditions. The 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 [15]. 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 °±1.02 °C, percent relative humidity [%RH] 36.74±4.28%, day length, light–dark cycle-13.42 h: 10.18 h), monsoon months (July–September, temperature 28.68 °±2.76 °C, %RH 87.04±3.50%, day length, light–dark cycle-12 h: 12 h), and winter months (November–January, temperature 10.76 °±3.63 °C, %RH 64.12±3.05%, day length, light–dark cycle 10.35 h: 13.25 h). All of the results were validated with the samples collected from CIRG in a seasonal manner.

Experimental design

In order to study the free radical parameters in 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 the 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.

Sample collections

Blood sampling

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

Sampling of spleen and thymus

The animals were electrically stunned and bled immediately till death after terminal cervical incision [1] 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 and was kept in a sterile vial containing chilled PBS for assessment of enzymatic parameters. The remaining tissue was lysed and kept in-20 °C for the assessment of Glucocorticoid Receptor (GR) expression by Western Blot.

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 [16]. 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 ethylenediaminetetraacetic 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 aluminum foil coated wooden box. After 10 min of exposure, 1 ml of Greiss reagent was added, and the absorbance of the color 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.

Estimation of catalase (CAT) activity

Catalase (CAT; EC 1.11.1.6) activity was measured following the procedure of Sinha, 1972 [17]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 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 H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate calorimetrically after heating the reaction mixture. There is the production of green color at the end of the process. 10% 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. The supernatant was taken for enzyme estimation. 5 ml of PBS was added to 4 ml of H_2O_2 (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 $K_2Cr_2O_7$ (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 the amount of H_2O_2 degraded per minute.

Estimation of glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed as described by Mantha *et al.*, 1993 [18]. The reaction mixture (1 mL) contained 50 µL sample (10% tissue homogenates prepared in chilled PBS and centrifuged at 12, 000 × g), 898 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 H_2O_2. 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.

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 mMTris–HCl buffers (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 [19]. 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 [20]. The absorbance of the upper phase was measured at 534 nm (ELx-800, Biotek Instruments, and 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.

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 [21]. 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 a particular interval of time at 734 nm (ELx-800, Biotek Instruments, Winooski VT, USA).

Hormonal parameters

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. The coefficient 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

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. 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.

Western blot analysis of glucocorticoid receptor (GR)

The Western blot analysis was performed according to the method published elsewhere [22]. 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 micrograms (µg) of the protein of thymus and spleen were resolved on 10% of SDS-PAGE. Electrophoresis was followed by electro transfer (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 GR (anti-GR, N-20, sc-2045, St Louis, USA at a dilution of 1:250). 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 GR 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 immuno stained with β-actin antibodies in 1:1000 dilutions (A-2228; Sigma–Aldrich, St Louis, USA) 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 (housekeeping gene) and expressed as percent relative integrated density values of GR. The values presented were as percent band intensity±standard error of the mean.

Statistical analysis

The data were presented as the means±Standard Error of Mean (SEM). Variation in lymphoid tissue level activities of SOD, CAT, GPx, TBARS, ABTS levels of male and female goats was analyzed by one-way ANOVA. To evaluate the interactive effect (male *vs* female), the Duncan multiple range t-test was used. 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 [23].

RESULTS

Circulatory level of cortisol

The circulatory level of cortisol was significantly high in both the sexes during monsoon (P<0.01) and winter (P<0.05; fig. 1A). However, sex-dependent variations were absent.

Circulatory level of melatonin

Circulatory level of melatonin was significantly high in both in cases of males and females during winter (P<0.01) and monsoon (P<0.05; only in case of females) in comparison to summer. Females always presented a significantly higher level of melatonin in comparison to males during summer and monsoon (P<0.05) and during winter (P<0.01; fig. 1B).

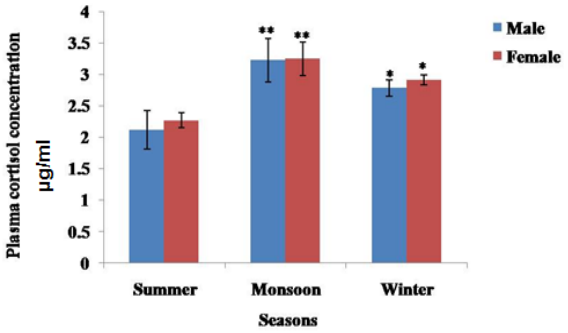

Fig. 1A: Season and sex dependent variations in plasma cortisol level in male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter

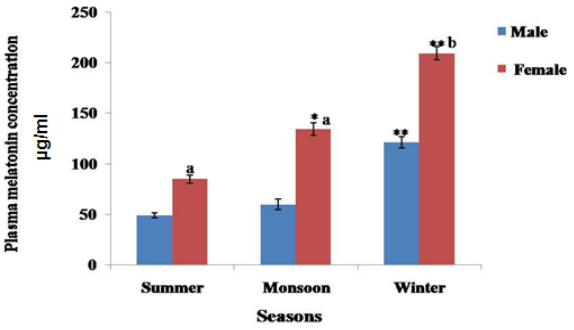

Fig. 1B: Season and sex dependent variations in plasma melatonin level in male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter. a P<0.05, b P<0.01; male *vs* female

SOD activity in lymphoid organs

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 the male thymus; fig. 2A and 2B). In the case of female spleen, the level of SOD was significantly low during monsoon and winter (P<0.05) and in case of female thymus the level was significantly low (P<0.01) during winter in comparison to males.

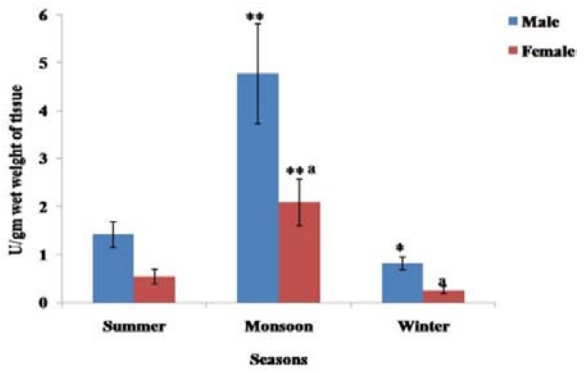

Fig. 2A: Season and sex dependent variations in Super Oxide Dismutase (SOD) activity in the spleen of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter. a P<0.05; male *vs* female

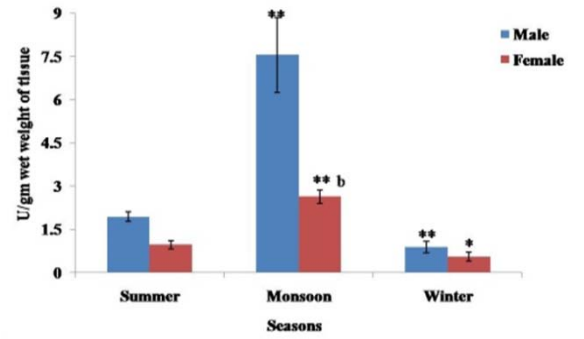

Fig. 2B: Season and sex dependent variations in Super Oxide Dismutase (SOD) activity in thymus of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter. b P<0.01; male *vs* female

Catalase activity in lymphoid organs

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. 3A and 3B). However, sex dependent variations were absent.

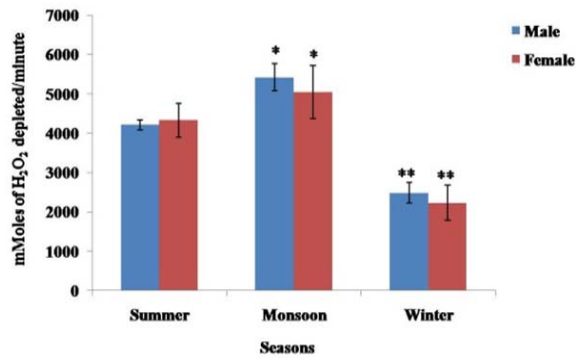

Fig. 3A: Season and sex dependent variations in Catalsase activity in spleen of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter

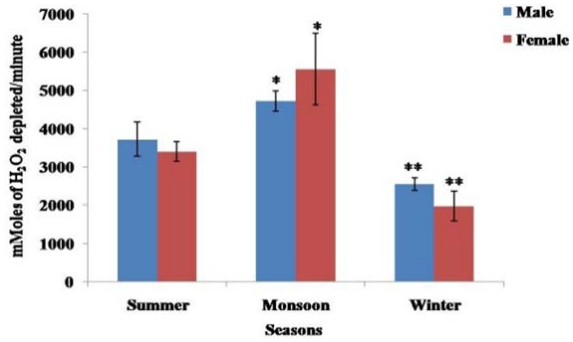

Fig. 3B: Season and sex dependent variations in Catalase activity in thymus of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter

Glutathione peroxidase (GPx) activity in lymphoid organs

Glutathione peroxidase (GPx) activity in spleen and thymus of both the sexes were significantly high during monsoon and winter (P<0.01; fig. 4A and 4B). However, sex-dependent variations were absent.

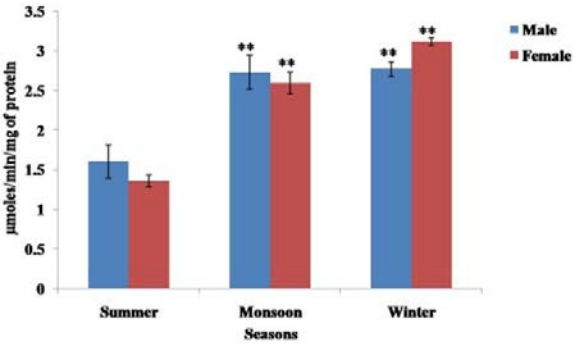

Fig. 4A: Season and sex dependent variations in glutathione peroxidase (GPx) activity in spleen of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). **P<0.01; summer *vs* monsoon and winter

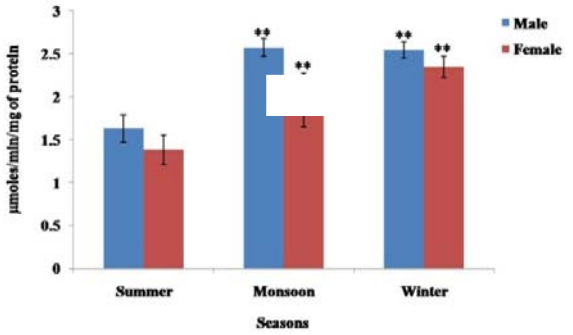

Fig. 4B: Season and sex dependent variations in glutathione peroxidase (GPx) activity in thymus of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). **P<0.01; summer *vs* monsoon and winter

ABTS level in lymphoid organs

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. 5A and 5B). However, sex dependent variations were absent

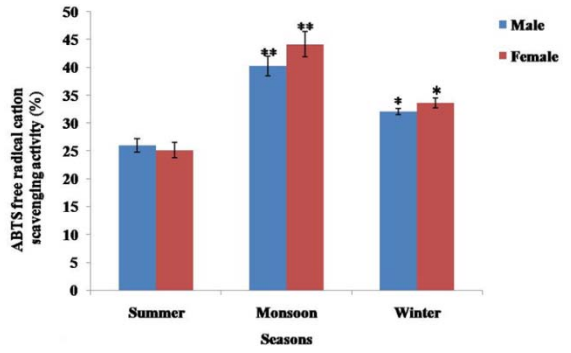

Fig. 5A: Season and sex dependent variations in 2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) activity in the spleen of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). *P<0.05, **P<0.01; summer *vs* monsoon and winter

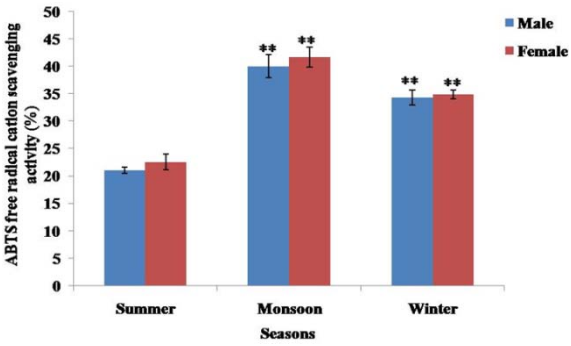

Fig. 5B: Season and sex dependent variations in 2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) activity in thymus of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). **P<0.01; summer *vs* monsoon and winter

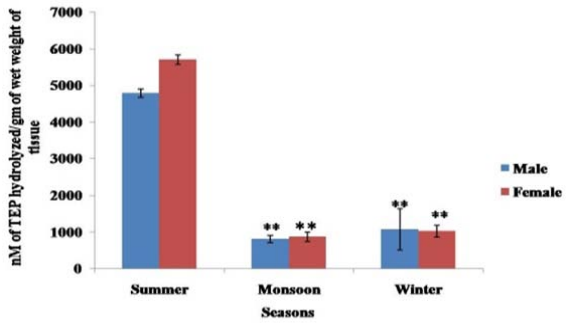

Fig. 6A: Season and sex dependent variations in malonaldehyde (MDA) level in spleen of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). **P<0.01; summer *vs* monsoon and winter

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

In the case of the spleen of both the sexes and thymus of males during summer the level was highest. But, the level was significantly low during monsoon (P<0.01 in the spleen of both the sexes and thymus of males only). In the case of the 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. During winter, the MDA level was significantly low in spleen of both the sexes and in male thymus only (P<0.01) in comparison to summer (fig.6A and 6B).

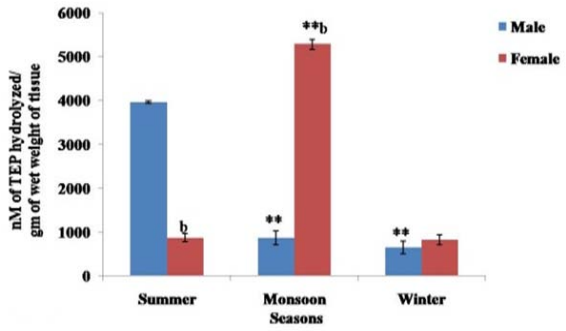

Fig. 6B: Season and sex dependent variations in Malonaldehyde (MDA) level in thymus of male and female goats, *C. hircus*. Data represents mean±SEM, N=18/sex/season. Vertical bar on each point represents standard error of mean (SEM). ** P<0.01; summer *vs* monsoon and winter; b P<0.01, male *vs* female

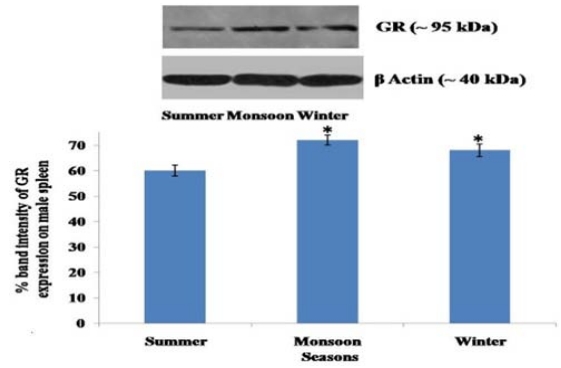

Fig. 7A: Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in the spleen of male goats. The data are expressed as percent band intensity of receptor expression in spleen. β-Actin expression was used as loading control. Data are expressed as the mean±SEM, N=18 males/season. Vertical bar on each point represents SEM. *P<0.05; summer *vs* monsoon and winter

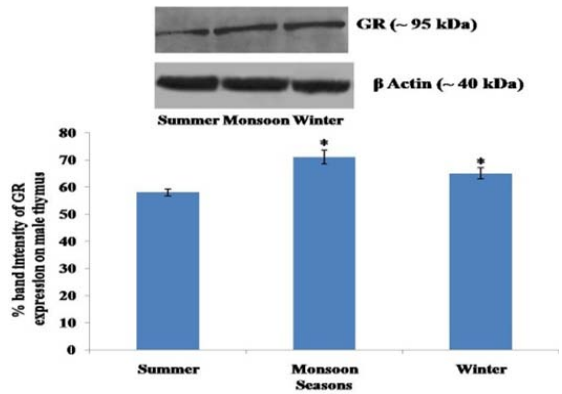

Fig. 7B: Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in thymus of male goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean±SEM, N=18 males/season. Vertical bar on each point represents SEM. *P<0.05; summer *vs* monsoon and winter

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. 7A and 7B). But in the case of the female spleen and thymus the level was significantly high only during monsoon (P<0.05; fig. 7C and 7D).

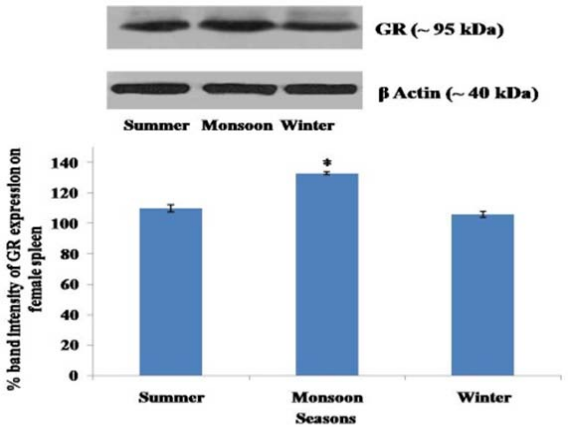

Fig. 7C: Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in the spleen of female goats. The data are expressed as percent band intensity of receptor expression in spleen. β-Actin expression was used as loading control. Data are expressed as the mean±SEM, N= 18 females/season. The vertical bar on each point represents SEM. *P<0.05; summer *vs* monsoon and winter

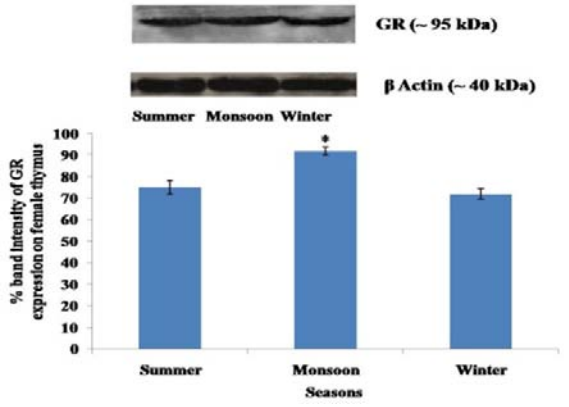

Fig. 7D: Western blot analysis for seasonal variations in receptor expression of glucocorticoid receptor (GR) in thymus of female goats. The data are expressed as percent band intensity of receptor expression in thymus. β-Actin expression was used as loading control. Data are expressed as the mean±SEM, N = 18 females/season. The vertical bar on each point represents SEM. *P<0.05; summer *vs* monsoon and winter

DISCUSSION

Pesticides are well known for increasing the free radical loads of different metabolically active organs such as kidney [24], liver [25], brain [26] and immune system [27] of animals including human being [28]. In the present agro ecosystem, the use of pesticides and chemical fertilizers is increasing continuously to fulfil the requirement of food for ever expanding the 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 in different seasonal [29] and spontaneous breeders [28]. 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 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 [29]. Endogenously produced melatonin may have a significant role in deferring a number of free radical-related disease and some patho physiological changes [30]. 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. Our data gets support from the studies of small mammals suggesting that changes in oxidative load are dependent on the circadian melatonin rhythm [31]. 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. This season is also the preparatory reproductive phase of goats with higher levels of gonadal steroids [3]. Due to a high level of temperature and humidity, the 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 the circulation of a seasonal breeder is highly relevant in terms of the total antioxidant capacity of lymphoid organs. Effects of lipid peroxidation, 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 peroxidation is considered as the biomarker of free radical load [32]. As oxidative stress is an 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 [33]. 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 an 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 (during monsoon) and high (during winter) but the cortisol level was high 9during both monsoon and winter). This suppressed the general immunity and increased the free radical load. Our data gets further support from the result of up-regulated GR expression in lymphoid organs (i. e spleen and thymus) 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, 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.

CONCLUSION

In conclusion, we may suggest that monsoon and winter are the most important seasons in terms of stress to both the sexes of goats as suggested by an elevated level of free radical scavenging enzymes, stress hormone glucocorticoid and its receptor. In this context, the role of neurohormone melatonin is noteworthy. Melatonin acted as a "coupler" which not only increased the free radical scavenging enzymes but also scavenged free radicals as an amphipatic free molecule, particularly during winter.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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