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Expression of genes associated with thermal stress in goats during different seasons

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ABSTRACT

Leptin, interleukin-2 (IL-2) and interleukin-6 (IL-6) have role in thermoregulation in animals. Therefore, the present study was undertaken to investigate the changes in expression profile of mRNA of leptin, interleukin-2 (IL-2) and interleukin-6 (IL-6) during peak winter, moderate cold and peak summer in PBMCs of goats (*Capra hircus*). Blood samples were taken from the goats of 2 different regions; tropical and temperate, each having 3 age groups (n=6 in each), during all 3 seasons. PBMCs were separated from blood samples and total RNA was isolated from which cDNA was synthesized using same amount of total RNA for each sample. Real time qPCR was applied to investigate mRNA expression of examined factors. Specificity of the desired products was documented using analysis of the melting temperature and high resolution gel electrophoresis to verify that the transcripts are of the exact molecular size and further confirmed by sequence analysis. Relative expression of each factor was calculated using respective efficiency by using Pfaffl equation. Our results indicated that during winter mRNA expressions of leptin, IL-2 and IL-6 were significantly higher than summer in all 3 age groups of both tropical and temperate goats. From the results, it is concluded that significant higher mRNA expressions of all these genes during cold stress suggest possible involvement of these genes in modulating cold stress by maintaining cellular homeostasis in goats.

Key words: Caprine, IL-2, IL-6, Leptin, PBMC, Thermal stress

In Indian conditions goats are prone to thermal stress because they are left outside for grazing for most of the day time where ambient temperature is high or low depending on the seasons. Genetic, physiological, immunological, biochemical and behavioral responses evolved for adaptation to different environmental conditions in different animal species (Mc Ewen 2000). The cellular response to various stress stimuli activates a specific set of genes which results in the selective synthesis of a group of proteins and they are thought to help the cell survive this stress by maintaining cellular homeostasis (Li and Werb 1982). Approximately 50 genes other than heat shock proteins (HSPs) changed expression during thermal stress (Sonna *et al*. 2002). Expression of leptin (Bernabucci *et al.* 2008, Lacetera *et al.* 2009) and interleukin-2 (Lan *et al.* 1995) and interleukin-6 (Parikh *et al.* 1998, Takii *et al.* 2010) changed markedly during thermal stress. Leptin, a peptide hormonal product of the *obese* (Ob) gene, acts through Janus kinase (JAK)-STAT-3 pathway, leading to transcriptional regulation of target genes (Zhang *et al.* 2005). It was reported that change in environmental temperature can affect leptin biology in dairy cows (Bernabucci *et al.* 2008) and mouse (Lacetera *et al.* 2009), mainly through an autocrine mechanism (Sanna *et al.* 2003).

Interleukins were first seen to be expressed by leukocytes (Brocker *et al.* 2010). IL-2 and IL-6 are thought to be responsible for immune system of body. IL-6 was reported to modulate thermal stress in some animal tissues (Parikh *et al.* 1998, Yildirim and Yurekli 2010). It is reported that stress induced activation of the hypothalamo-pituitary-adrenal (HPA) axis stimulates secretion of glucocorticoids and catecholamine, which are capable of modulating cytokine production and further modulating thermal stress (Han *et al.* 2010). However there is no report available on date regarding expression profile of leptin, IL-2 and IL-6 in goats during thermal stress but based on their roles and previous studies in other species, we hypothesized that these genes could play significant role in maintaining cellular homeostasis during thermal stress in goats, so the present study was undertaken.

MATERIALS AND METHODS

Animals and sampling: Healthy goats of 2 different regions, viz. tropical (Barberi breed) and temperate (Pashmina breed), maintained at experimental animal sheds

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of Division of Physiology and Climatology, Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh and Goat Farm IVRI, Mukteswar, Uttarakhand, were selected for the study. Goats were divided in 3 (n=6 in each group) age groups—group 1(up to 2 years), group 2 (2 to 5 years) and group 3 (Above 5 years).

Blood samples were taken from healthy goats using heparin (10 IU/ml) as anticoagulant during peak winter (temperature range –1 to 12°C and tropical 2 to 15°C), moderate cold (control) (temperature range 2 to 15°C and tropical 15 to 28°C) and peak summer (temperature range 15 to 25°C and tropical 28 to 44°C). To minimize the effect of ribonuclease activity during processing, all the plastic wares were treated with 0.1% DEPC prior to RNA isolation. Thereafter, PBMCs were isolated by density gradient centrifugation method using Histopaque 1077 and washed with PBS (pH 7.4) to get the cell pellet.

Total RNA extraction and quality determination: The PBMC pellet was re-suspended in 500 ml of DEPC-PBS and transfer to 2 ml nuclease free (DEPC treated) microcentifuge tube. Total RNA was isolated using trizol reagent by following standard protocol. The RNA was dissolved in nuclease free water and the purity of RNA was verified by optical density (OD) absorption ratio OD 260 nm/OD 280 nm between 1.8 and 2.0 using nanodrop spectrophotometer. The quality and integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Two intact bands of 28s, 18s with smearing indicated good quality and intactness of RNA.

RNA reverse transcription: Constant amount of 1µg of total RNA were reverse transcribed to cDNA using cDNA synthesis kit with following master mix: 11 µl RNA + nuclease free water, 1 µl random primer, 4 µl 5X reaction buffer, 2 µl dNTP mix (10 mM), 1 µl RNase inhibitor (20u/µl) and reverse transcriptase (200 u/µl) according to manufacturer's instruction.

Primers: Primers were designed for leptin, IL-2 and IL-6 by the integrated DNA technologies (IDT) using Beacon software. The sequences and expected PCR product length are shown in Table 1.

Quantitative real time PCR

Quantitative real-time PCR was performed with a commercial kit. A master mix of following components was prepared 8 µl nuclease free water, 0.5 µl forward primer (0.25µM), 0.5 µl reverse primer (0.25µM) and 10 µl SYBR mix. The master mix (19 µl) was added to the strip tubes and 1 µl of cDNA template was added. Following real time PCR protocol employed for all investigated factors: the PCR condition was as follows, initial denaturation at 95°C for 15 min, denaturation at 94°C for 30 sec, annealing at 58°C for all leptin, IL-2, IL-6 and beta actin for 25 sec, and extension at 72°C for 30 sec for 35 cycles and last cycle at 95°C for 30 sec, at 65°C for 30 sec and gradual increment from 65°C to 95°C @ 0.58°CDs and continuous fluorescence measurement, and a final cooling down to 40°C. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the SYBR green (with dissociation curve) method. Real-time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were obtained. The specificity of desired products was documented using analysis of melting temperature, which is product specific and a high resolution gel electrophoresis to verify that transcripts were of exact molecular size and further confirmed by sequence analysis. Relative expression of PCR product was determined by the equation suggested by Pfaffl (2001).

Gene expression analysis: The moderate season values were used as control for obtaining relative mRNA expression. Beta actin was used as housekeeping gene. Efficiency corrected relative quantification of mRNA was obtained by Pfaffl method (2001).

Statistical analysis: The statistical significance of differences in mRNA expressions of the examined factors was assessed by Paired t test using Prism 3.0 software. Differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

RNA integrity and purity: Total RNA was in good yield in all the samples. The bands of 28sRNA and 18sRNA reflected the high quality of extracted total RNA. Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 200–2000 ng/µl.

Primer efficiency: The efficiency for leptin was found 92.0%, for IL-2 105.9%, for IL-6 108.5% and for beta actin 107.4%.

Table 1. Gene transcripts, primer sequences and resulting fragment size

Target	Sequence of nucleotide (52 -32)	Fragment size (bp)	EMBL
Leptin	For: 52 TAGCGGTTATGGGATATGC32 Rev: 32 GTGAGGTGTGCTGTAGAC52	128	GU944974.2
IL-2	For: 2 ACGGGGAACACAATGAAAGAAG32 Rev: 32 TGCATCCTGGAGAGCTTGAG52	101	AF535145.1
IL-6	For: 52 GCTGCTCCTGGTGATGAC32 Rev: 32 CGACGATGTGCTTAATGAGAG52	134	D86569.1
Beta actin	For: 52 AGTTCGCCATGGATGATGA32 Rev: 32 TGCCGGAGCCGTTGT52	54	NM_001009784.1

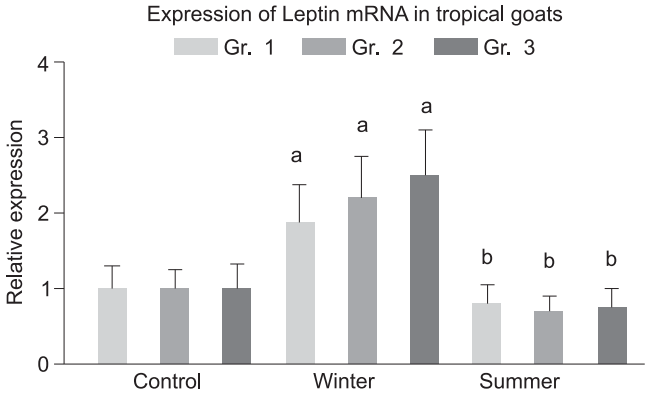

Fig. 1. Expression profile of leptin mRNA during different seasons in tropical goats. Different superscripts denote statistically different values (P<0.05).

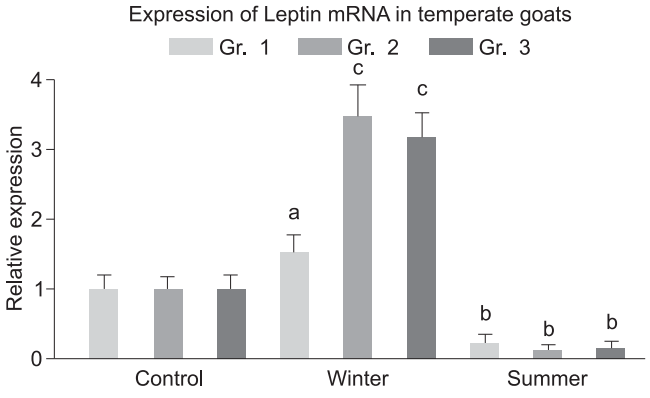

Fig. 2. Expression profile of leptin mRNA during different seasons in temperate goats. Different superscripts denote statistically different values (P<0.05).

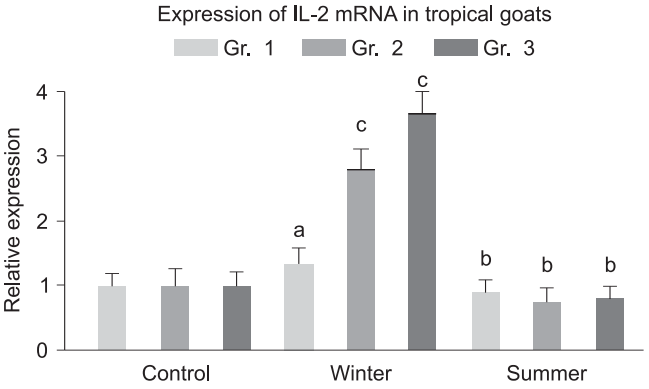

Fig. 3. Expression profile of IL-2 mRNA during different seasons in tropical goats. Different superscripts denote statistically different values (P<0.05).

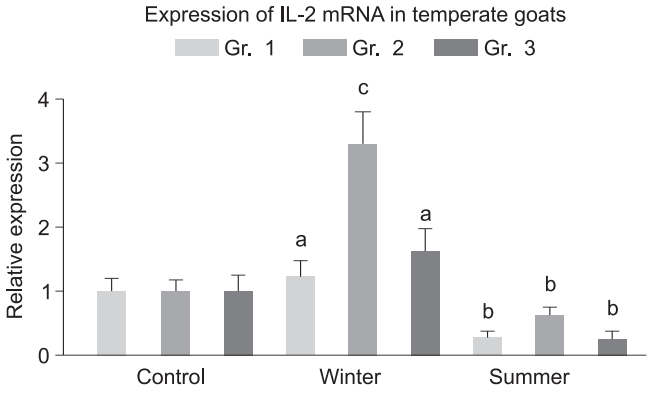

Fig. 4. Expression profile of IL-2 mRNA during different seasons in temperate goats. Different superscripts denote statistically different values (P<0.05).

mRNA Expression of leptin, IL-2 and IL-6: The relative expressions of mRNA for leptin, IL-2 and IL-6 in PBMCs of tropical and temperate goats during peak winter and peak summer are presented in Figs 1-6 and Table 2. The moderate or thermo neutral season values were used as control.

The result showed that mRNA expression of leptin was significantly higher during winter in comparison to summer in both, tropical and temperate region goats. It was observed that during winter the leptin mRNA expression of all 3 age groups was significantly (P <0.05) higher in comparison to summer in tropical goats. Among the age groups, mRNA expression in groups 2 and 3 was found significantly (P<0.05) higher than group 1 during winter in temperate goats.

Our results are consistent with qPCR studies in the bovine PBMC (Lacetera *et al.* 2009) which showed that heat shock impairs DNA synthesis and down-regulates gene expression

Table 2. Relative expression values of mRNA for different genes

Gene	Winter season			Summer season		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Leptin (Trop.)	1.87	2.21	2.51	0.82	0.698	0.756
Leptin (Temp.)	1.54	3.47	3.19	0.243	0.125	0.153
IL-2 (Trop.)	1.33	2.80	3.65	0.896	0.735	0.787
IL-2 (Temp.)	1.23	3.30	1.62	0.293	0.632	0.252
IL-6 (Trop.)	1.16	2.47	2.33	0.427	0.981	0.608
IL-6 (Temp.)	2.31	4.59	1.58	0.176	0.457	0.124

Values are given in comparison to calibrator. Trop.- tropical, Temp.- temperate

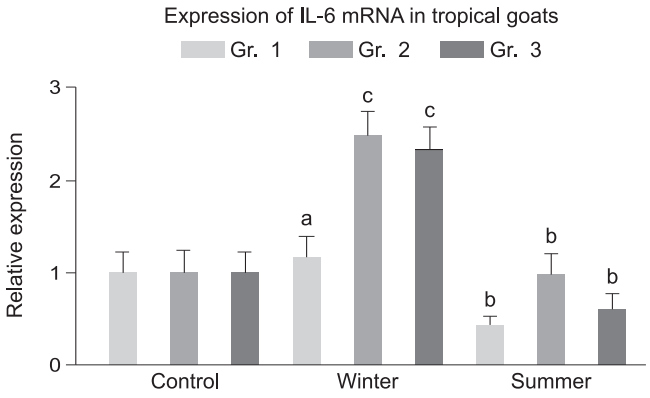

Fig. 5. Expression profile of IL-6 mRNA during different seasons in tropical goats. Different superscripts denote statistically different values (P<0.05).

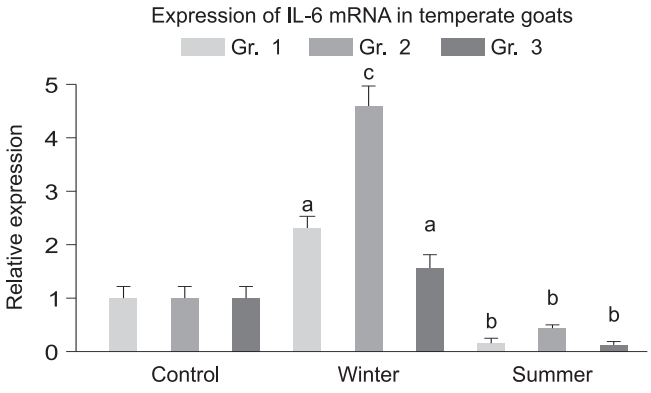

Fig. 6. Expression profile of IL-6 mRNA during different seasons in temperate goats. Different superscripts denote statistically different values (P<0.05).

for leptin and Ob-Rb receptor in concanavalin A-stimulated bovine peripheral blood mononuclear cells. The probable mechanism behind that may be through an autocrine mechanism in which leptin secretion by activated T cells reduced during heat stress (Lacetera *et al.* 2009). In the present study mRNA of leptin increased (P<0.05) during cold stress in both tropical and temperate goats. It can be explained by probable mechanism that high altitude and cold stress stimulates leptin synthesis as hypoxia-inducible factor-1 (HIF-1) trans activates the leptin gene promoter (Rissanes *et al.* 2006). HIF-1 is stimulated not only by hypoxia (Grosfeld *et al.* 2002) but also by low temperature (Rissanes *et al.* 2006), which probably explains that during cold temperature HIF-1 was stimulated and it further increased leptin expression. Our study provided new evidence, which encourage considering leptin as a pleiotropic hormone in caprine species. Finally, present findings also suggest that changed leptin expression in PBMCs *in vivo* may represent an adaptive mechanism to environmental temperatures in goats suffering from severe thermal stress.

Expression profile of IL-2 mRNA during different seasons in temperate goats. Different superscripts denote statistically different values (P<0.05).

The relative mRNA expression if IL-2 and IL-6 was found significantly higher in winter as compared to summer in tropical as well as temperate region goats. For IL-2, It was observed that during winter the mRNA expression in groups 2 and 3 was significantly (P<0.05) higher than group 1 in tropical goats, whereas in temperate goats the mRNA expression in winter, in group 2 was significantly (P<0.05) higher than groups 1 and 3 while during summer it was nonsignificant (P>0.05) among all the age groups.

It was observed that, in tropical goats, the IL-6 mRNA expression during winter, was significantly (P<0.05) higher in groups 2 and 3 than group 1 whereas during summer IL-6 mRNA expression of group 2 was significantly (P<0.05) higher than groups 1 and 3. In temperate region goats, during winter the mRNA expression in group 2 was significantly (P<0.05) higher than groups 1 and 3 and during summer it was nonsignificant (P>0.05) among all the age groups.

Our results obtained using qPCR indicated that in winter the mRNA expressions of IL-2 and IL-6 were significantly (P<0.05) higher than summer in all 3 age groups of both tropical and temperate goats. Our results are consistent with studies of Yildirim and Yurekli (2010) and Guo *et al.* (2012) on rats, which explained that under stress conditions, the HPA axis gets stimulated, which stimulated secretion of catecholamine and glucocorticoides, which modulate immune cells and thus cytokine production and modulation of cold stress occurs due to higher expression of pro-inflammatory cytokines like IL-2 and IL-6.

Han *et al.* (2010) observed that acute heat stress enhanced the secretion of IL-2 by splenic lymphocytes significantly in broiler chickens. Many studies indicated that acute or chronic stress induces immunomodulatory effects in animal models. It was found that cold stress enhanced IL-6, IL-2 and tumour necrosis factor (TNF)-a cytokine levels. Many studies have also demonstrated that restraint stress could induce elevation of the plasma IL-6 level. Immobilization stress may increase plasma IL-6 via central and peripheral catecholamines (Takaki *et al.* 1994, Hideyuki *et al.* 2001). The decrease in IL-6 expression during summer in present study may be due to inhibition of expression of IL-6, mediated through activating transcription factor 3 by heat shock factor 1 as observed in murine cells (Takii *et al.* 2010).

Thermoregulatory protective mechanism develops with age of animal and is well developed in adults as compared to infants and aged ones (Kolesar 1996). The higher expression of leptin, IL-2 and IL-6 in adults as compared to young ones suggests that these genes helps in thermal protection in better way in adults as compared to young ones.

The results of our experiment suggested that cytokines as leptin, IL-2 and IL-6 expressions are observed normally during all the seasons due to having role in cell metabolism

and immunity. From our experiment it is concluded that significant higher mRNA expressions of leptin, IL-2 and IL-6 genes during cold stress suggest possible involvement of these genes in modulating cold stress by maintaining cellular homeostasis in goats.

Further investigations are required in this regard to elucidate the specific roles and mechanisms of regulation of leptin, IL-2 and IL-6 in response to cold and heat exposure in goats. A better understanding would allow for development of new strategies for improving goat's production efficiency in the tropical and temperate regions.

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