Expression profile of cold shock protein genes in goats (*Capra hircus*) during different seasons

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Summary

The study was conducted to demonstrate expression profile of cold shock protein genes; cold inducible RNA binding protein (CIRP) and RNA binding motif protein 3 (RBM3) in goats (*Capra hircus*) during different seasons to check the gene expression in different thermal conditions. Blood samples were collected from healthy goats of tropical and temperate region during peak winter, moderate season and peak summer. Goats were divided in three (n=6 in each group) age groups- (I) up to 2 years, (II) 2 to 5 years and (III) above 5 years. RNA isolation was done from separated peripheral blood mononuclear cells (PBMCs) by Trizol reagent. Real-time PCR was applied to investigate mRNA expression of examined factors. The mRNA expression of CIRP and RBM3 was higher in all age groups during peak winter season as compared to peak summer season in both tropical and temperate region goats. To conclude, our results demonstrated expression of CIRP and RBM3 mRNAs in caprine PBMCs and increased expression of these genes during winter season could possibly play a significant role to ameliorate deleterious effects of cold stress to maintain cellular homeostasis in goats.

Key words: Goat, Cold stress, CIRP, RBM3, PBMCs

Introduction

Thermal stress stimulates complex responses which are fundamental in the preservation of cell survival (Sonna *et al*., 2002). Particularly in mammals, exposure to hypothermia or hyperthermia has been related to morphological and physiological modifications. At the molecular and cellular levels, cold stress reduces rates of enzymatic reactions, diffusion, and membrane transport whereas heat stress accelerates these processes (Sonna *et al*., 2002). Low temperature as well as high temperature induce the denaturation and misaggregation of proteins, slowing of progression through the cell cycle, inhibit transcription and translation, disrupt cellular cyto-skeleton elements and change membrane permeability (Fujita, 1999; Sonna *et al*., 2002). Thermoregulatory protective mechanisms in terms of heat shock proteins during heat stress, is well studied and explained in domestic animals including bovines (Collier *et al*., 2008), buffaloes (Patir and Upadhyay, 2007) and goats (Dangi *et al*., 2012) but very little information is available regarding such protective mechanism at molecular and cellular level during cold stress in domestic animals.

Mammalian cells are known to respond to cold stress via the induction of a number of cold shock proteins (CSPs) (Al-Fageeh and Smales, 2006; Roobol *et al*., 2009) like cold inducible RNA binding protein (CIRP), RNA binding motif protein 3 (RBM3) (Sonna *et al*., 2002). Among CSPs, CIRP and RBM3 have been well characterized in mammalian systems (Derry *et al*., 1995; Danno *et al*., 1997). These belong to a highly conserved glycine-rich RNA-binding protein family, and they modulate translation (Dresios *et al*., 2005; Smart *et al*., 2007) and function as RNA chaperones that facilitate translation upon the perception of cold stress (Fujita, 1999). CIRP and RBM3 proteins regulate gene expression at the level of translation by binding to different transcripts, thus allowing the cell to respond rapidly to environmental signals. Binding of these proteins to the 5′-untranslated region (UTR) or 3′-UTR of an mRNA stabilizes the transcript (Leonard, 2010).

The mRNA encoding CIRP is expressed constitutively in most tissues at relatively low levels but is strongly up regulated in the cells upon exposure to cold stress in various human cell lines (Nishiyama *et al*., 1997a), mouse fibroblast and (Nishiyama *et al*., 1997b; Al-Fageeh and Smales, 2009) rat neuronal cells (Liu *et al*., 2010). Similarly, hypothermic stress also up regulated RBM3 level in human (Derry *et al*., 1995; Danno *et al*., 1997) and mouse cells (Dresios *et al*., 2005). Both CIRP and RBM3 are expressed at high levels in testis and brain where the temperature is slightly less than the temperature of the other organs (Nishiyama *et al*., 1998; Danno *et al*., 2000; Dresios *et al*., 2005). The expression of CIRP and RBM3 decreases as the

temperature increases (Nishiyama *et al*., 1998), which indicates their possible protective functions during cold stress. It has been shown that these proteins have cytoprotective effects against UV rays (Sheikh *et al*., 1997) and hypoxia (Wellmann *et al*., 2004).

These proteins have been studied in human, mouse and rat under cold shock conditions, but there is no such data available on domestic animals.

Goats are found in most countries but, in developing countries like India, they are reared mostly on open grazing system for long hours (10-12 h/day), grazing on the waste-land and therefore, during the peak of the season, they become vulnerable for exposure to thermal stress. It is known that a portion of the metabolizable energy used for production is diverted for thermal balance under uncomfortable environmental conditions, particularly beyond an animal's thermo-neutral zone. Therefore, under thermal stress, productivity like milk production, meat production and reproductive performance is affected. Heat and cold stress affects productivity of goats (Greenwood, 1992; De Souza *et al*., 2012), but very little information is available about how they respond to cold stress at a cellular level. A correlation exists between induction of CSPs and induction of tolerance to cold stress (Fujita, 1999; Leonard *et al*., 2010). Based on available literature, we hypothesized that expression of CSPs (CIRP, RBM3) may vary during different seasons to play a role in thermo tolerance in goats. The present study was therefore aimed at gaining insight into the mRNA expression profile of CSPs namely, CIRP and RBM3 during different seasons in goat peripheral blood mononuclear cells (PBMCs) from goat.

Materials and Methods

Animals and sampling

Healthy goats of two different regions viz. tropical (Barberi breed) and temperate (Pashmina breed), maintained at experimental animal sheds of the Division of Physiology and Climatology, Indian Veterinary Research Institute (IVRI), Izatnagar, U.P. and goat farm IVRI, Mukteswar; Uttarakhand were selected for the study. Semi intensive system of goat rearing was followed in which goats were allowed to graze during day hours and were kept in closed sheds during the night hours throughout the year. Goats were kept in a shed during the day hours and were divided in three (n=6 in each group) age groups- group I: up to 2 years, group II: 2 to 5 years and group III: above 5 years.

Blood samples were obtained from healthy goats using heparin (10 IU/ml) as anticoagulant by jugular vein puncture under sterile conditions during peak winter (temp. range -1 to 12°C and tropical 2 to 15°C), moderate cold (temp. range 2 to 15°C and tropical 15 to 28°C) and peak summer (temp. range 15 to 25°C and tropical 28 to 44°C). Precautions were taken to minimize the effect of ribonuclease activity while processing.

PBMCs isolation

PBMCs were isolated by density gradient centrifugation method using Histopaque 1077 (Sigma). The blood was layered carefully onto the Histopaque to produce a clean interface between the two layers. Further, it was centrifuged at 2000 rpm for 30 min at room temperature. The white opaque mononuclear fraction from the interface was collected between the plasma and the Histopaque. Further centrifugation was done for washing the cells with PBS (pH = 7.4). Finally, the cell pellet was obtained.

Total RNA extraction and quality determination

The PBMC pellet was re-suspended in 500 µl of DEPC-PBS and transferred to 2 ml nuclease free (DEPC treated) microcentifuge tube. Total RNA was isolated using Trizol reagent (Invitrogen, USA) 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 using nanodrop spectrophotometer. Samples having ratio more than 1.8 were considered as pure. 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 without smearing indicated good quality and intactness of RNA.

RNA reverse transcription

Constant amount of 1 µg of total RNA were reverse transcribed to cDNA using fermentas cDNA synthesis kit with the following master mix: 11 µl RNA + nuclease free water, 1 µl random primer (100 µM), 4 µl 5X reaction buffer, 2 µl dNTP mix (10 mM), 1 µl RNase inhibitor (20 u/µl) and reverse transcriptase (200 u/µl) according to manufacturer's instruction.

Primers

Primers were designed for CIRP and RBM3 by the Integrated DNA Technologies (IDT) using Beacon primer designer software (Version 7, USA). The sequences and expected PCR product length are shown in Table 1.

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

Target	Sequence of nucleotide	Fragment size (bp)	EMBL
CIRP	For: 5′AGACTGACTGGCTCATTAG3′ Rev: 5′CTCGGTGACAGGACTATC3′	178	NM_001034278.1
RBM3	For: 5′GGATACGGATATGGATATGGAAGG3′ Rev: 5′ATGGCAACACAGAAGTCTCAC3′	148	NM_001034363.1
Beta actin	For: 5′AGTTCGCCATGGATGATGA3′ Rev: 5′ TGCCGGAGCCGTTGT3′	54	NM_001009784.1

EMBL-accession number or reference of published sequence

Quantitative RT-PCR

Quantitative Real-time PCR was performed with DyNAmo^{TM} HS SYBR^{R} Green qPCR kit. A master mix of the following components was prepared: 8 µl nuclease free water, 0.5 µl forward primer (0.25 µM), 0.5 µl reverse primer (0.25 µM) and 10 µl SYBR mix (Finnzymes, Finland). The master mix (19 µl) was added to the strip tubes and 1 µl of cDNA template was added. The following real-time PCR protocol was employed for all investigated factors: initial denaturation at 95°C for 15 min, 35 cycles of amplification and quantification programme [denaturation at 94°C for 30 s, annealing at 56°C for CIRP, 58°C for RBM3 and Beta actin for 25 s, and extension at 72°C for 30 s] and last cycle at 95°C for 30 s, a melting step by slow heating from 65 to 95°C with a rate of 0.58°C/s and continuous fluorescence measurement, and a final cooling down to 4°C. After the run ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "SYBR green (with dissociation curve)" method of the MxPro3005 Stratagene real time (USA) machine. 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 this was further confirmed by sequence analysis. Relative expression of PCR product was determined by the equation suggested by Pfaffl (2001).

The moderate season values were used as calibrator for obtaining relative mRNA expression. Respective age groups were compared with same age groups of calibrator. Beta actin was used as housekeeping gene. Efficiency corrected relative quantification of mRNA was obtained by Pfaffl method (Pfaffl, 2001). For this, efficiencies of primer were determined by serial dilution of template cDNA sample and were run in triplicate. The efficiency for CIRP was found 101.1%, for RBM3 102%, and for beta actin 107.4%.

Statistical analysis

The statistical significance of differences in mRNA expressions of the examined factors was assessed by two way ANOVA followed by the Tukey's multiple comparison test (Hsu, 1996) using Prism 3.0 software. Differences were considered significant if $P<0.05$.

Results

The relative expressions of mRNA for CIRP in PBMCs of tropical and temperate goats during peak winter and peak summer are presented in Figs. 1 and 2, respectively. The moderate or thermo-neutral season values are used as calibrator.

In tropical goats, the mRNA expression was 1.42, 1.71 and 1.72 times higher as compared to calibrator during winter season in all three age groups respectively, whereas it was 0.86, 0.82 and 0.957 times as compared to calibrator during summer season.

The mRNA expression in each age group during winter is significantly higher than summer ($P<0.05$), however, mRNA expression among age groups was found statistically indifferent ($P>0.05$) in both winter and summer seasons.

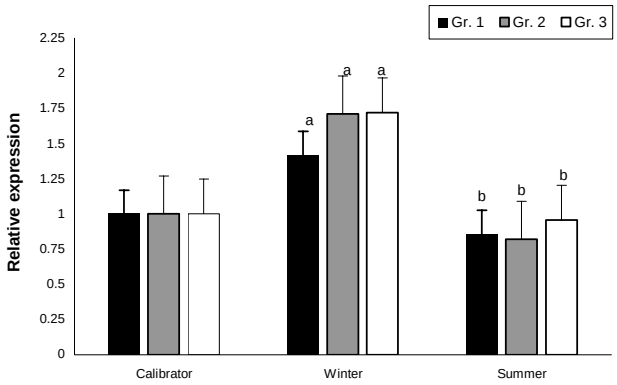

Fig. 1: Expression profile of CIRP mRNA during different seasons in tropical goats. Different superscripts denote statistically different values ($P<0.05$)

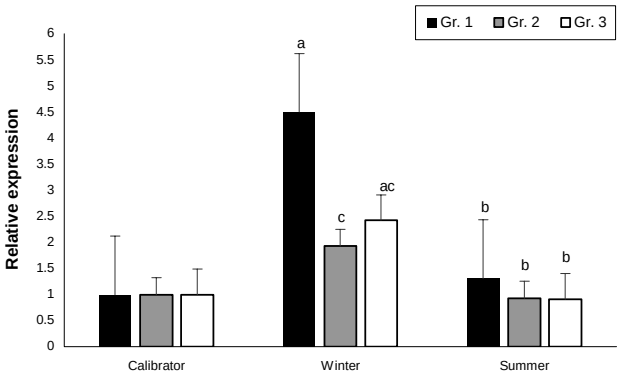

Fig. 2: Expression profile of CIRP mRNA during different seasons in temperate goats. Different superscripts denote statistically different values ($P<0.05$)

In temperate goats, the mRNA expression was 3.50, 1.93 and 2.42 times more as compared to calibrator during winter season in the three age groups respectively, whereas it was 1.31, 0.93 and 0.91 times as compared to calibrator during summer season. The mRNA expression during winter season in age group I was significantly ($P<0.05$) higher than age group II and age group III. Expression of mRNA in each age group during winter is significantly higher than summer ($P<0.05$).

The relative expressions of mRNA for RBM3 in PBMCs of tropical and temperate goats during peak winter and peak summer has been shown in Figs. 3 and 4, respectively.

The mRNA expression was 1.56, 2.12 and 2.39 times higher as compared to calibrator during winter season in all three age groups respectively, whereas it was 0.55, 0.53 and 0.46 times as compared to calibrator during summer season in tropical goats. In temperate goats, the mRNA expression was 1.18, 1.37 and 1.25 times higher as compared to calibrator during winter season in the

three age groups respectively, whereas it was 0.53, 0.20 and 0.33 times as compared to calibrator during summer season. Expression of mRNA was statistically non significant (P>0.05) among age groups during winter and summer season in both tropical and temperate region goats.

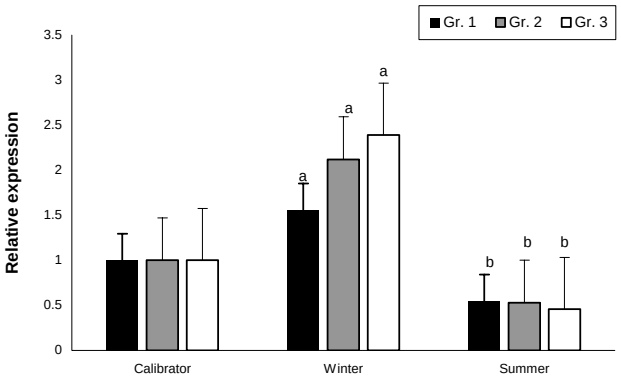

Fig. 3: Expression profile of RBM3 mRNA during different seasons in tropical goats. Different superscripts denote statistically different values (P<0.05)

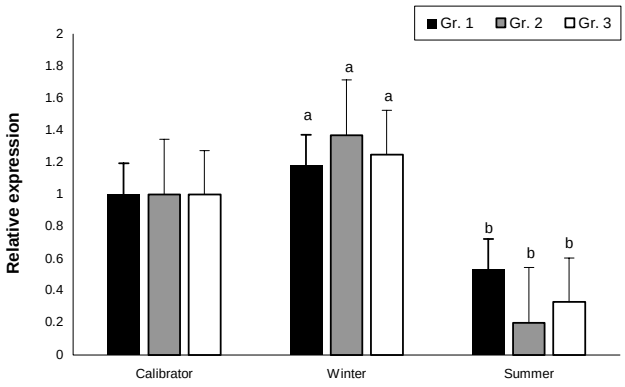

Fig. 4: Expression profile of RBM3 mRNA during different seasons in temperate goats. Different superscripts denote statistically different values (P<0.05)

Discussion

To the best of our knowledge, no studies have been designed so far to gain insight into the seasonal variation effects on CSPs gene expression profile in goats. In the present investigation, the effects of thermal stress on the mRNA expression profiles of CIRP and RBM3 in PBMCs of tropical and temperate region goats were studied *in vivo* during winter, moderate, and summer seasons. As blood cells play a major role in thermal regulation and homeostasis, blood cells are used to study gene expression changes. Since mononuclear cells are in maximum number in ruminants among leukocytes and they express almost all stress proteins, PBMCs were used to check gene expression level. The environmental temperature range existing during the study in summer was between 28 and 44°C in tropical region and 20 to 29°C in temperate region; in winter the range was -2 to 10°C in temperate region and 2 to 13°C in tropical region. Comfort zone is a range of environmental temperature within which body temperature is maintained constant with a minimal effort from thermoregulatory mechanism and within which the sensation of cold or heat is absent. Although the lower critical temperature for goat is not specified, the limits of thermo-neutrality for goats may be taken as a climatic environment having an air temperature of 13-27°C, relative humidity of 60-70% and wind velocity of 5-8 km/h, and a medium level of solar radiation (Misra and Puneet, 2009). During summer and winter, animals were exposed to ambient temperature beyond the comfort zone, so they were under thermal stress in both seasons.

At the molecular and cellular levels, thermal stress strongly affects many biochemical processes which include denaturation and misaggregation of proteins, a slowing of progression through the cell cycle, inhibit transcription and translation (Fujita, 1999). However, under hypothermia, transcription and translation of certain proteins such as CIRP and RBM3 are reported to be increased. CIRP and RBM3 protein have a possible role in protection of mRNA of other proteins from degradation (Sonna *et al*., 2002; Leonard, 2010). Although mammalians are an endothermic animal and they maintain core body temperature within a narrow range, extreme low or high environmental temperature causes stress to animals and changes in gene expression of stress related genes might be observed.

The results obtained using qPCR indicate that CIRP expression was significantly higher (P<0.05) in all three age groups during peak winter season as compared to summer season in both tropical and temperate region goats. We also found that a basal level of CIRP is present in the cells at thermo-neutral temperature. Our findings are consistent with *in vitro* study in rat fibroblast and neuronal cells (Nishiyama *et al*., 1997a, b) which shows that CIRP expression increases when cells are exposed to mild hypothermia (32-33°C) as compared to 37°C. The present findings are also in agreement with the reported increased CIRP expression during winter season as compared to summer in Bullfrog (Saito *et al*., 2000). Our results also follow an earlier study in which CIRP expression was found to be decreased under heat stress conditions (Nishiyama *et al*., 1998). Possible reason of increased CIRP at low temperature might be transcription by alternative splicing and internal initiation of translation IRES (Al-Fageeh and Smales, 2009). As an RNA binding protein CIRP binds to 5′ or 3′ end of un-translated region (UTR) mRNA of various important proteins and stabilizes them to prevent degradation during stress.

The mRNA expression of RBM3, another cold shock protein, maintained a higher (P<0.05) level during winter season as compared to summer season in all three age groups of both tropical and temperate goats. It was observed that during summer a basal level of RBM3 is also maintained as in case with CIRP. Our results are in agreement with the previous work done by Danno *et al*. (2000) and Chip *et al*. (2011) in which they found increased expression of RBM3 upon mild hypothermic treatment to various cell lines in human. Similarly,

Stephen *et al*. (2001) also found increased expression on RBM3 mRNA under mild hypothermia in c-*myc* cell lines. It has been suggested that they have either IRES or another unknown mechanism that promotes translation of these proteins in hypothermic condition (Leonard, 2010), however, they protect mRNA of various proteins from degradation by binding to 5' or 3' UTR.

We hypothesized that thermoregulatory protective mechanism develops with age of animal, so differential expression of CSPs was expected among different age groups, with the exception of CIRP in temperate region, we did not find any significant difference regarding expression of CSPs in both regions goats. It is possible that some other mechanism may exist among age groups to combat thermal stress and it may vary with breed. While stress-induced CSPs expression represents a generalized molecular mechanism displayed by almost all cells, individual age groups differ in their capacity to manage with stress.

The present investigation on the CIRP and RBM3 expression during different seasons showed significantly higher levels of their induction, particularly in winter, in comparison to summer, showed the important role of these CSPs in the thermoregulation at cellular level and overall functioning of the body in the tropical and temperate agro-ecological zones of the world. The CSPs help in conferring the thermo adaptability and high level of thermo-tolerance. The reports about the CSPs responses to thermal stress at cellular levels in the livestock species are scanty, and future research is required to find out the exact role of CSPs in conferring protection under stressful conditions in goats.

In conclusion, our results are the first to demonstrate that (1) CSP genes are expressed in caprine PBMCs, and (2) higher expression of CSPs during cold stress suggest their possible involvement in ameliorating the deleterious effect of thermal stress to maintain cellular integrity and homeostasis in goats.

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