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Research Article
Influence of Different Environmental Stresses on Various Spleen Toll Like Receptor Genes Expression in Osmanabadi Goats

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Abstract

Background and Objective: Climate change related heat and nutritional stress weakens the animal's immune system and makes them more prone to diseases. Although this has been observed by various researchers, the impact of these stresses on immune gene expression and process of heat stress mediated immune suppression at molecular level has not been dealt in detail in goat. Hence, the study was conducted to establish the impact of heat stress, nutritional stress and combined stresses (heat and nutritional) on different spleen Toll Like Receptor (TLR) genes expression in Osmanabadi goats. **Materials and Methods:** Twenty four adult Osmanabadi male goats (average body weight 16.0 kg) were divided into four groups viz., C (n = 6, control), HS (n = 6, heat stress), NS (n = 6, nutritional stress) and CS (n = 6, combined stress). The study was conducted for a period of 45 days. The C and HS goats had *ad libitum* access to their feed while NS and CS goats were under restricted feed (30% intake of C bucks) to induce nutritional stress. The HS and CS goats were exposed to heat stress in outside environment for 6 h a day between 10:00-16:00 h to induce heat stress. The average minimum and maximum temperature and Relative Humidity (RH) during the study period were 27.23±3.46, 38.33±0.52 and 37.0±4.16, respectively. The animals were slaughtered and their spleen was collected for different TLR mRNA expression. The relative gene expression was calculated using the formula $2^{-\Delta\Delta CT}$. The results were expressed in fold change as compared to untreated control (control = 1 fold). **Results:** The fold expression level of TLR 1, 2, 3, 6, 7, 8, 9 and 10 mRNA in spleen followed the same trend in the current study where comparatively higher expression was noticed in CS group. These different TLR mRNA expressions in CS group were of higher magnitude as compared to both HS and NS group goats. This shows the severity of environmental stresses when occurring simultaneously and the consequences on immune response were much more severe than the individual stress. **Conclusion:** The activated splenic innate immune functions in terms of different increased TLR expression during combined stress indicate the Osmanabadi goat's adaptation and disease resistance mechanism under extreme environmental conditions.

Key words: Climate change, combined stress, diet restriction, goat, nutritional stress, PAMP, thermal stress, spleen, TLR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Climate change acts as a major threat to climate sensitive sectors such as agriculture and animal husbandry. With the changing climatic scenario, the frequency and duration of exposure of livestock to abiotic and biotic stressors increases. Abiotic stressors such as heat and nutritional stress has a major impact on livestock productivity[1]. In particular, heat stress is one of the crucial factors affecting livestock productivity[2]. Heat stress affects animal productive performances like milk yield, meat quality and reproductive performances like age at maturity, ovulation failure, embryo mortality etc.[3]. It also weakens the animal's immune system and makes them more prone to diseases. Although this has been observed by various researchers, the impact of heat stress on immune gene expression and process of heat stress mediated immune suppression at molecular level has not been dealt in detail in livestock.

Stressful stimuli are known to change the immune function. The immune system responds to stress by enhancement or suppression of immune functions[4]. Stress affects both innate and adaptive immune response in animals. The immune system does not respond directly to stress but act via neuroendocrine system. The stress related hormones act on the immune cell receptors to modulate the immune response. Innate immune response is one of the primary immune response which helps to tackle the pathogens that enter the host animals. Pathogen Recognition Receptors (PRR) play a crucial role in this process and they help to identify the conserved molecular signatures called Pathogen Associated Molecular Patterns (PAMPs) present in the pathogens[5] and help to kill the pathogen through phagocytosis[6]. Toll Like Receptors (TLRs) are the most studied among the PAMPs[7].

Animals reared in tropical environments are generally subjected to more than one stressor at a time. Multiple stressors greatly affect animal production, reproduction and immune status particularly in the extensive system of rearing. Most studies which have investigated the effects of environmental stress on livestock have generally studied one stressor at a time since comprehensive, balanced multi factorial experiments are technically difficult to manage, analyze and interpret[8]. Further, research efforts establishing the impact of heat and nutritional stress on immune response in livestock are very meager. Hence, considerable research efforts are needed to establish the cumulative impact of different environmental stresses in livestock. Only such attempt may yield fruitful results if one tries to establish the impact of climate change on livestock immune response. Since goat has been projected as animals to deal with in the climate change perspectives, research efforts establishing impact of heat and nutrition stress on immune response in goats will be of practical relevance. Hence, the present study was designed to establish the impact of heat and nutritional stress individually as well as cumulatively (both heat and nutritional stress) on TLR genes expression in spleen of Osmanabadi bucks.

MATERIALS AND METHODS

Location: The experiment was carried out at the National Institute of Animal Nutrition and Physiology Experimental Livestock Farm, Bengaluru, India which is located in Southern Deccan plateau of the country at longitude 77°38′E and the latitude of 12°58′N and at altitude of 920 m above mean sea level. The average annual maximum and minimum ambient temperature ranges between 15-36°C. The mean annual RH ranges between 20 and 85%. The annual rainfall in this area ranges from 200-970 mm with an erratic distribution throughout the year. The average annual minimum and maximum temperature ranges between 15-22 and 27-34°C, respectively. The average annual RH ranges between 40-85%. The experiment was carried out during April-May. The average meteorological data for the entire study period both inside the shed as well as outside are given in Table 1 and 2, respectively.

Animals: Osmanabadi is a dual purpose (meat and milk) hardy goat breed, which originated in the semi-arid areas of central tropical India. The study was conducted in 24 (1 year old) Osmanabadi bucks weighing between 15-18 kg. The animals were housed in well-ventilated sheds made up of asbestos roofing at the height 2.4 m and open from side and maintained under proper hygienic conditions. Prophylactic measures against goat diseases like goat pox, peste des petits ruminants, enterotoxaemia, endo and ectoparasitic infestations were carried out as prescribed by the health calendar of the institute to ensure that the animals were in healthy condition throughout the study.

Technical program: The study was conducted for a period of 45 days. Twenty four adult Osmanabadi bucks were used in the study. The bucks were randomly allocated into four groups of 6 animals each viz., C (n = 6, control), HS (n = 6, heat stress), NS (n = 6, nutritional) and CS (n = 6, combined stress). The animals were stall fed with a diet consisting of 60% roughage (hybrid napier) and 40% concentrate (maize 36 kg, wheat bran 37 kg, soybean meal 25 kg, mineral mixture 1.5 kg and common salt 0.5/100 kg of feed). The C and NS bucks were

Table 1: Mean and SEM of climatological data during the experimental period inside the shed

	Temperature (°C)					
Time of recording	Minimum	Maximum	Dry bulb	Wet bulb	RH (%)	THI
Morning (8:00 h)	21.50±0.60	32.37±0.20	23.80±0.21	20.10±0.17	78.67±4.67	72.21±0.25
Afternoon (14:00 h)	24.4±0.56	35.60±0.72	26.47±0.28	20.93±0.09	45.67±7.21	74.73±0.22

SEM: Standard error mean, RH: Relative humidity, THI: Temperature humidity index

Table 2: Mean and SEM of climatological data during the experimental period in the outside environment

	Temperature (°C)					
Time of recording	Minimum	Maximum	Dry bulb	Wet bulb	RH (%)	THI
Morning (8:00 h)	26.93±2.87	34.03±0.52	23.13±0.20	22.97±0.66	61.00±7.77	73.92±0.57
Afternoon (14:00 h)	27.23±3.46	38.33±0.52	29.57±0.38	26.53±0.71	37.00±4.16	80.99±0.25

SEM: Standard error mean, RH: Relative humidity, THI: Temperature humidity index

Table 3: Primers used for gene expression studies

Gene ID	Primers	Primer sequence	Annealing temperature	Product size	Accession No.
TLR1	F	5-ACTTGGAATTCCTTCATTACGA-3	60	176	HQ263209.1
	R	5-GGAAGACTGAACACATCATGGA-3			
TLR2	F	5-TTCCGTCTCTTTGATGAG-3	55	114	JQ911706.1
	R	5-CTTGGTGTTCATGATCTTC-3			
TLR3	F	5-GATGTATCGCCGTGCAAAGACA-3	55	195	HQ263210.1
	R	5-TGCATATTCAAACTGCTCTGCT-3			
TLR4	F	5-CTTGCGTCCAGGTTGTTCCTAA-3	60	153	JF825527.1
	R	5-CTGGGAACCTGGAGAAGTTATG-3			
TLR5	F	5-CCTCCTGCTCAGCTTCAACTAT-3	60	172	FJ659852.1
	R	5-TATCTGACTTCCACCCAGGTC-3			
TLR6	F	5-CCTTGTCTTTCACCCAAATAGC-3	61	150	HQ263211.1
	R	5-GTTGGTCTTCCAGTGAGT-3			
TLR7	F	5-TCTTGAAGGAAAGGACTGGTTA-3	60	205	HQ263216.1
	R	5-AAGGGGCTTCTCAAGGAATATC-3			
TLR8	F	5-CGCACCGTCTAGGATTTATT-3	55	209	JF825528.1
	R	5-AAGCCGGGTCAGATTGGT-3			
TLR9	F	5-CTGACACCTTCAGCCACCTGAG-3	61	156	HQ263217.1
	R	5-TGGTGGTCTTGGTGATGTAGTC-3			
TLR10	F	5-ATGGTGCCATTATGAACCCTAC-3	60	248	HQ263213.1
	R	5-CACATGTCCCTGTGGTGTCTAA-3			
GAPDH	F	5-GGTGATGCTGGTGCTGAGTA-3	55-60	265	AF030943
	R	5-TCATAAGTCCCTCCACGATG-3			

GAPDH used as reference gene to normalize the gene expression of target genes

maintained in the shed while HS and CS bucks were exposed to summer heat stress in the outside environment between 10:00-16:00 h to expose them to heat stress. The C and HS bucks were provided with *ad libitum* feeding, while NS and CS bucks were provided with restricted feed (30% of intake of GI and GII bucks) to induce nutritional stress. All four group animals were fed and watered individually throughout the study period. At the end of the study period the animals were slaughtered and their spleen samples were collected in aseptic condition. The study was conducted after obtaining approval from the institute ethical committee for subjecting the animal to both heat and nutritional stresses and to slaughter the animals for collecting the spleen for TLR gene expression.

Slaughter and tissue collection: The animals were slaughtered on the 46th day of the experiment and spleen was collected in sterile containers containing RNA shield (Zymo Research, California, USA) and stored at -80°C until further processing.

RNA extraction and cDNA synthesis: Total RNA was extracted from the spleen using RNAsol kit (Chromous Biotech Pvt., Ltd., Bangalore, India) as per the manufacturer's instructions. The cDNA synthesis was performed using thermo scientific maxima first strand cDNA synthesis kit (Lithuania, EU) using oligo dTs and random hexamer primers. The reaction was performed as per the manufacturer's instructions. About 2 µL of cDNA (50 ng reaction^{-1}) was used for real-time PCR.

TLR1-10 primers: The following TLR1-10 primers were used in the present study were taken from already published sequences[9]. The primer sequences were described in Table 3.

Real-time qPCR: Real-time PCR was performed using SYBR green chemistry with 20 µL reaction using maxima SYBR green/ROX qPCR master mix (Lithuania, EU). The reaction mixture consists of maxima SYBR green/ROX qPCR master mix (2X)-10 µL, forward primer 5 µM, reverse primer 5 µM, template DNA 50 ng, nuclease-free water to make up 20 µL. The reaction conditions were as follows: Enzyme activation at 95°C for 10 min and 40 amplification cycles consisting of initial denaturation at 95°C for 15 sec, annealing at 61°C for 30 sec and extension at 72°C for 30 sec. Melt curve analysis was performed to check the non-specific amplification. The GAPDH gene (Glyceraldehyde 3 phosphate dehydrogenase) was used as an internal control.

Statistical analysis: The relative gene expression was calculated using[10] the formula $2^{-\Delta\Delta CT}$. The results were expressed in fold change as compared to untreated control (control = 1 fold).

RESULTS

Spleen TLR1 mRNA expression: The results revealed that spleen TLR1 mRNA expression was evident in C (1 fold), HS (3.25 fold), NS (8.21 fold) and CS (11.43 fold) groups (Fig. 1). Within the stress groups, the highest spleen TLR1 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 1).

Spleen TLR2 mRNA expression: The results revealed that spleen TLR2 mRNA expression was evident in C (1 fold), HS (2.07 fold), NS (2.50 fold) and CS (5.24 fold) groups (Fig. 2). The highest spleen TLR2 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 2).

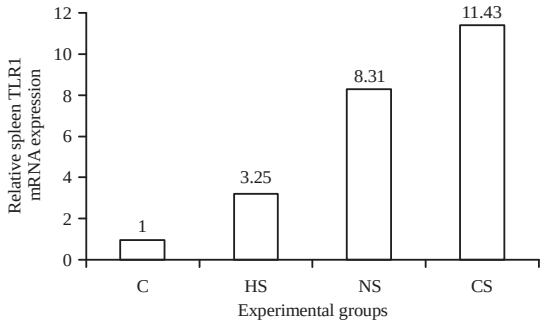

Fig. 1: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR1 mRNA expression in Osmanabadi goats

Spleen TLR3 mRNA expression: The results revealed that spleen TLR3 mRNA expression was evident in C (1 fold), HS (2.86 fold), NS (20.87 fold) and CS (24.19 fold) groups (Fig. 3). Within the stress groups, the highest spleen TLR3 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 3).

Spleen TLR4 mRNA expression: Spleen TLR4 mRNA transcript expression was down regulated in all experimental groups (HS, NS, CS) as compared to C group. The fold expression difference in spleen TLR4 mRNA transcript expression in C, HS, NS and CS groups are 1, 0.54, 0.44 and 0.45, respectively (Fig. 4). Within the stress groups, the highest spleen TLR4 mRNA transcript expression was recorded in HS group. However, not much difference was there in spleen TLR4 mRNA transcript expression between CS and NS groups (Fig. 4).

Spleen TLR5 mRNA expression: The results revealed that spleen TLR5 mRNA expression was evident in C (1 fold), HS

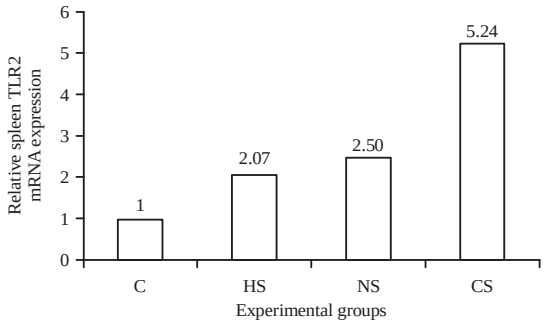

Fig. 2: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR2 mRNA expression in Osmanabadi goats

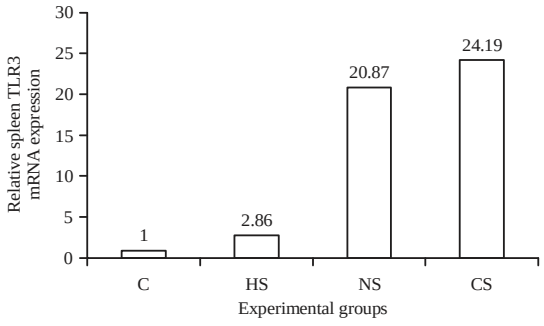

Fig. 3: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR3 mRNA expression in Osmanabadi goats

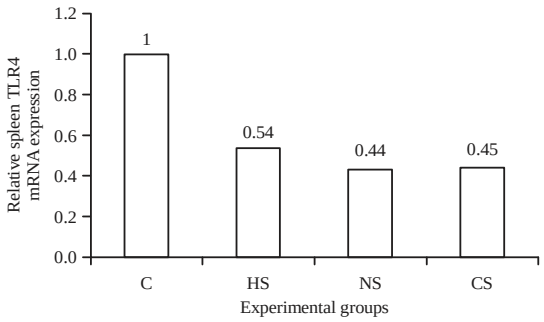

Fig. 4: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR4 mRNA expression in Osmanabadi goats

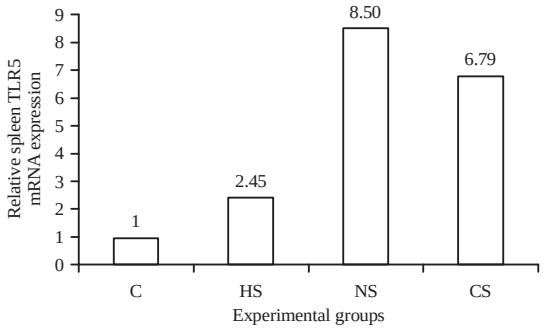

Fig. 5: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR5 mRNA expression in Osmanabadi goats

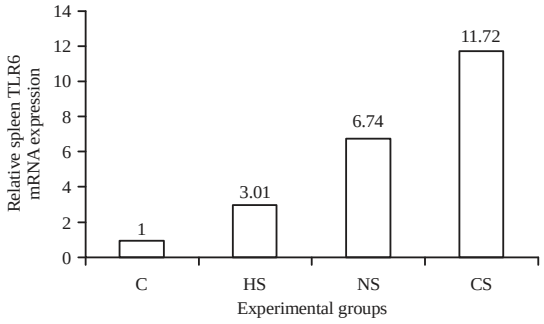

Fig. 6: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR6 mRNA expression in Osmanabadi goats

(2.54 fold), NS (8.50 fold) and CS (6.79 fold) groups (Fig. 5). Within the stress groups, the highest spleen TLR5 mRNA expression was also recorded in NS group followed by CS and HS groups, respectively (Fig. 5).

Spleen TLR6 mRNA expression: Spleen TLR6 mRNA expression was evident in C (1 fold), HS (3.01 fold), NS (6.74 fold) and CS (11.72 fold) groups (Fig. 6). On comparative basis, the higher expression of spleen TLR6 mRNA expression was recorded in CS group goats. Within the stress groups, the highest spleen TLR6 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 6).

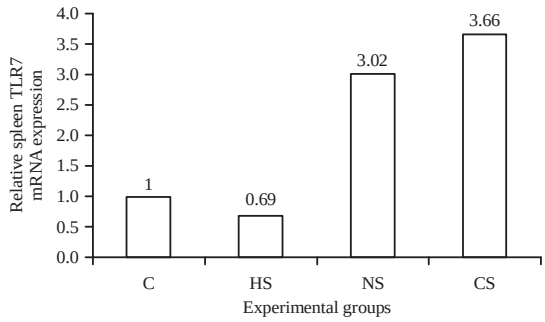

Fig. 7: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR7 mRNA expression in Osmanabadi goats

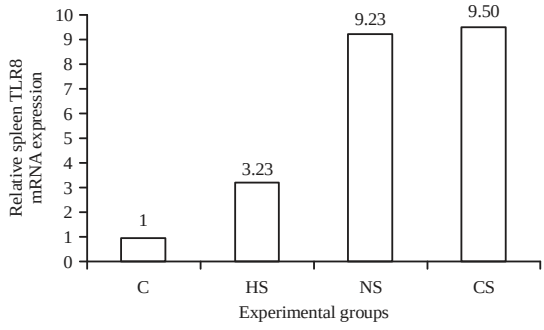

Fig. 8: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR8 mRNA expression in Osmanabadi goats

Spleen TLR7 mRNA expression: Spleen TLR7 mRNA gene expression also showed similar trend like that of TLR6. Spleen TLR7 mRNA expression was evident in C (1 fold), HS (0.69 fold), NS (3.02 fold) and CS (3.66 fold) groups (Fig. 7). The higher expression of spleen TLR7 mRNA expression was recorded in CS group goats. Within the stress groups, the highest spleen TLR7 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 7).

Spleen TLR8 mRNA expression: The results revealed that spleen TLR8 mRNA expression was evident in C (1 fold), HS (3.23 fold), NS (9.23 fold) and CS (9.50 fold) groups (Fig. 8). The higher expression of spleen TLR8 mRNA expression was recorded in CS group goats. Within the stress groups, the

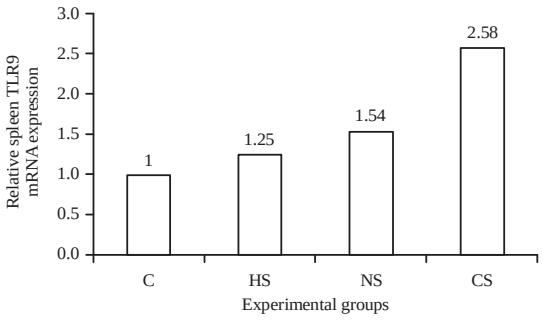

Fig. 9: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR9 mRNA expression in Osmanabadi goats

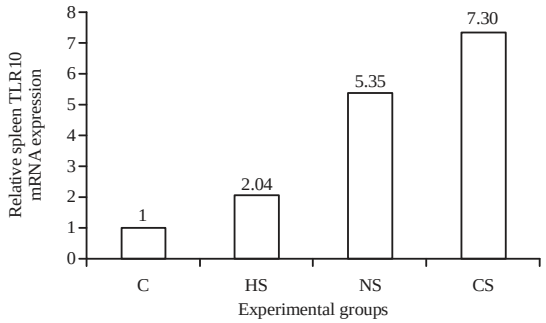

Fig. 10: Effect of heat stress, nutritional stress and combined (heat and nutritional) stresses on relative spleen TLR10 mRNA expression in Osmanabadi goats

highest spleen TLR8 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 8).

Spleen TLR9 mRNA expression: The results also revealed that spleen TLR9 mRNA expression was evident in C (1 fold), HS (1.25 fold), NS (1.54 fold) and CS (2.58 fold) groups (Fig. 9). The higher expression of spleen TLR9 mRNA expression was recorded in CS group goats. Within the stress groups, the highest spleen TLR9 mRNA expression was recorded in CS group followed by NS and HS groups, respectively (Fig. 9).

Spleen TLR10 mRNA expression: The results revealed that spleen TLR10 mRNA expression was evident in C (1 fold), HS (2.04 fold), NS (5.35 fold) and CS (7.30 fold) groups (Fig. 10). On comparative basis, the higher expression of spleen TLR10 mRNA expression was recorded in CS group goats (Fig. 10).

The relative spleen TLR1-10 expressions are described in Fig. 1-10. Except TLR4 (Fig. 4) rest all TLRs showed higher expression in stress groups in spleen. However, TLR4 was down regulated in all stress groups (HS, NS and CS) as compared to control (C) group. The TLR1, TLR2, TLR3, TLR6, TLR7, TLR8, TLR9 and TLR10 gene expression showed similar trend between the groups with highest expression recorded in CS group. However, TLR4 and TLR5 gene expression was found to be higher in C and NS group, respectively.

DISCUSSION

Information pertaining to heat stress impact on immune response in goats are negligible. Hence, the current study is of high significance as it attempts to study the impact of heat, nutritional and combined stresses and the corresponding influence of these stresses on TLR expression was studied. The TLRs are a family of at least 10 proteins that function as central mediators of the innate immune response to diverse pathogens as well as to endogenous molecules released by injured or dying cells[9,11]. Paul *et al.*[9] explained that heat stress might alter TLRs expression in immune cells and then modulate immune responsiveness to PAMPs to the full activation of innate and adaptive immune system to fight against pathogenic microorganisms in goats. The higher expression of most of the TLR studied in all stress groups (HS, NS and CS) reveals that stress stimuli could able to alter the immune functions in goats. It is a well-established fact that heat stress response is mediated through glucocorticoid release in goats[12]. There are reports suggesting that under glucocorticoid influence, TLR expression and innate immune functions were enhanced in human peripheral blood mononuclear cells[13]. Hence, the increased cortisol concentration as a result of heat stress could have influenced the TLR expression pattern.

The higher expression of all the TLRs in CS group shows the severity of cumulative impact of environmental stresses. This could be attributed to the seveirity of stress response as in this particular group, animals were exposed to both heat and nutritional stress simultaneously. This cumulative effect could have stimulated the speenic innate immune receptors. The activated splenic innate immune functions during combined stress exposure also indicates the animals' different adaptative and disease resistance mechanisms under extreme environmental conditions in goats. Thus, the higher expression of majority of TLRs in CS group indicates that the cumulative stress effects could enhance multifold innate immune responses to PAMPs by promoting TLR expression. This shows that although CS group goats were compromised with nutrition, still they could mount better immune response. This indicates the extreme adaptive and disease resistance capability of Osmanabadi goats.

The multi-fold increase in TLR3 expression in CS group indicates that TLR3 may act as ideal biological marker to assess cumulative stress impact in goat. Similar higher expression of TLR3 in spleen was also reported in buffaloes[14]. However, in the present study, TLR4 expression in spleen got down regulated in all stress groups and this could be due to the suppressive effect of different environmental stresses in the current study. The higher expression of TLR4 mRNA during normal condition in goats as reported by Tirumurugaan *et al.*[15] justifies the argument of environmental stresses reduced TLR4 expression in the current study. However, Paul *et al.*[9] observed contrasting result of higher TLR4 receptor expression in goat during summer season.

The higher TLR5 mRNA expression in spleen of NS group as compared to HS and CS groups shows the sensitivity of TLR5 expression to heat stress condition as both CS and HS group goats are exposed to heat stress. Similar summer heat stress induced reduction in TLR5 expression in goat was reported by Paul *et al.*[9]. Further, Paul *et al.*[9] also observed that TLR5 expression was lower both during winter and summer season as compared to moderate season. In addition, Sophia *et al.*[16] also observed no significant effect on hepatic TLR5 expression in goats exposed to different environmental stresses. This shows the sensitivity of TLR5 to temperature stress in goats.

CONCLUSION

The study is the first of its kind to identify the impact of heat and nutritional stress individually and in combination on the different spleen TLR1-10 expression in goats. The significantly higher expression of majority of TLR receptors in all stress groups (HS, NS and CS) as compared to C group in spleen indicates immune response is compromised during environmental stress condition in goats. The significantly higher expression of majority of TLR genes in CS group of spleen indicates different mechanisms of controlling immune functions in spleen as compared to individual stresses. In addition, the significantly higher expression of several TLR receptors in CS group of spleen indicates the different adaptive ability of these goats to maintain immune response when environmental stresses occur simultaneously. The multi-fold expression of TLR3 in all stress groups indicates that spleen TLR3 may act as immunological markers for environmental stress conditions in goats.

SIGNIFICANCE STATEMENTS

The study is the first of its kind to identify the impact of heat and nutritional stress individually and in combination on the different spleen TLR1-10 expression in goats. Stress affects both innate and adaptive immune response in animals. The significantly higher expression of majority of TLR receptors in all stress groups (HS, NS and CS) as compared to C group in spleen indicates immune response is compromised during environmental stress condition in goats. The multifold expression of TLR3 in spleen shows that splenic TLR3 may act as immunological markers for environmental stress conditions in goats.

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