Effect of thermal stress on expression profile of apoptosis related genes in peripheral blood mononuclear cells of transition Sahiwal cow

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Summary

The study was conducted to evaluate the effect of thermal stress on expression profile of genes related to apoptosis in peripartum Sahiwal cows. For this, twelve pregnant dry Sahiwal cows were selected from Livestock Research Centre at National Dairy Research Institute, Karnal. The cows were divided into two groups consisting of six Sahiwal cows each. Cows of group I calved during thermoneutral temperature conditions (THI=67.3) and cows of group II calved in summer season (THI=79.9). Blood samples were collected on -15, 0 and +15 days with respect to calving where day '0' represents the day of calving. The peripheral blood mononuclear cells (PBMC) were separated and total RNA was isolated for the BCL-2 (B-Cell Lymphoma-2), BAX (BCL-2 antagonist killer-1), BAK (Bcl-2-associated X protein), CASP-3 (cysteine-aspartic proteases-3) and P53 (tumour protien-53) mRNAs expression. It was found that there was up regulation of CASP-3 on the day of calving during both temperature conditions. Comparison between the two temperature conditions showed that expression of CASP-3, BCL-2, BAK, P53 and ratio of BAX/BCL-2 in PBMC increased during summer as compared to thermoneutral condition suggesting the susceptibility of these cells to apoptosis. Based on the above findings it can be concluded that during calving PBMC are more susceptible to apoptosis, and summer being more stressful potentiates the apoptosis of PBMC in Sahiwal cows.

Key words: Apoptosis, PBMC, Sahiwal, Thermal stress, Transition cattle

Introduction

The transition period in the dairy cow is the change from a gestational non-lactating to a non gestational lactating state and is characterized by changes in metabolism and host defense mechanisms which are associated with increased disease (Contreras and Sordillo, 2011; Morgante *et al*., 2012). Cows are physiologically at the most stressful time of their lives during the transition period and also experience environmental stressors (Mulligan and Doherty, 2008). Environmental factors like extreme temperature exert detrimental effects on the health and production of animals during periparturient period (Tao and Dahl, 2013). The ambient temperature above or below thermoneutral zone leads to thermal stress and impairs metabolism, health status, production, reproduction and immune response (Yousef *et al*., 1985; Nardone *et al*., 2010). Heat stress is a major stressor which occurs due to imbalance between heat production within the body and its dissipation affecting animals at cellular, molecular and ecological levels (Mehla *et al*., 2014). The upper critical temperature is 25-26°C for dairy cows and most likely remains unaltered irrespective of previous acclimatization or of their milk production (Berman *et al*., 1985). Heat stress affects expression of significant number of genes in peripheral blood leukocytes (Kolli *et al*., 2014). It has been previously reported that when thermal humidity index (THI) reaches 83.58, peripheral blood lymphocyte apoptosis rate is 17.79 (Li and Wang, 2009) also apoptotic rate of PBMC is higher during the transition period (Tharwat *et al*., 2012).

Apoptosis is a physiological process which involves a complex network of biochemical pathways where unwanted cells are eliminated during development and other normal biological processes to ensure a homeostatic balance between cellular proliferation and turnover in nearly all tissues (Elmore, 2007; Choudhury *et al*., 2012). Apoptosis is critical for normal tissue homeostasis and is involved in processes like immune clearance and development. Apoptosis is controlled by many factors, and dysregulation of apoptosis plays a significant role in the pathophysiology of many diseases (Kennedy *et al*., 2014). These factors are categorized as the BCL-2 family, the tumour necrosis factor (TNF) family and P53. More than two dozen BCL-2 family members have been discovered and categorized into two main subfamilies; the inhibitors or antiapoptotic (BCL-2, BCL-XL) members, and the promoters of apoptosis like BAX, BAK members (Danial and Krosmeyer, 2004). Cytochrome c release from mitochondria is a central event in apoptosis. Upon exposure to proapoptotic stimuli cytochrome *c* enters the cytosol and binds to the cytosolic Apaf-1 (apoptotic protease activating factor 1)

and procaspase-9 (Li *et al*., 1997). This results in activation of CASP-9 (cysteine-aspartic proteases-9) and processing of the main executioner CASP-3, thus reaching the "point of no return" (Enari *et al*., 1996). Proapoptotic proteins such as BAX and BAK oligomerize into the pores of the outer mitochondrial membrane, changing the mitochondrial permeability leading to release of cytochrome *c* (Tsujimoto and Shimizu, 2002; Hussein *et al*., 2003). The proapoptotic protein BAX counteracts the apoptosis-preventing effect of BCL-2. Hence imbalance of the BAX/BCL-2 ratio tilts the scales towards survival (Reed, 1994). The P53 gene product causes upregulation of the BAX gene (Miyashita and Reed, 1995). This P53 binds to the BAX gene promoter and directly transactivates proapoptotic gene. Apoptosis also plays a critical role in lymphocyte development and homeostasis. Enhanced apoptosis of lymphocyte can cause immunodeficiency due to cell loss (Rathmell and Thompson, 2002). To the best of our knowledge there is very little information available on expression profile of the genes related to apoptosis in the transition Sahiwal cows undergoing thermal stress. Therefore, based on information given above the present experiment was designed to investigate the effect of thermal stress on the expression level of BCL-2, BAX, BAK, CASP-3 and P53 in the peripheral blood of transition Sahiwal cattle. The present study has two main objectives, to compare expression profile of apoptosis related genes in PBMC during different calving periods (-15, 0, +15) of transition cow in two different seasons, and to compare apoptosis in summer season with thermoneutral condition.

Materials and Methods

Animals and experimental design

For the present study, 12 pregnant dry Sahiwal cows were selected at 15 days prepartum (±1 or 2 days) from the herd of Livestock Research Centre (LRC), National Dairy Research Institute (NDRI), Karnal. Animals were housed under loose housing system and fed according to the standard feeding practices followed at the NDRI farm. The cows were divided into 2 groups consisting of 6 Sahiwal cows each. Cows of group I calved during thermoneutral temperature conditions when THI was 67.3. Cows of group II calved in summer season when THI was 79.9. Recording of environmental parameters viz. maximum temperature (Tmax), minimum temperature (Tmin), dry-bulb temperature (Tdb), wet-bulb temperature (Twb), and relative humidity (RH) that was done throughout the study are given in detail in Table 1.

Blood collection and PBMC separation

Blood samples were collected from periparturient cows on the days -15, 0 (day of calving) and +15 with respect to day of parturition. Blood was collected from jugular vein in sterile EDTA vacutainer (BD Vacutainer™, UK) tubes, with minimum disturbance to animal. Vacutainers were transported to the laboratory at 4°C in ice boxes. All the animal experiments had prior approval of the animal ethics committee of NDRI, Karnal, India.

The collected blood samples were centrifuged at 2,300 rpm for 40 min and buffy coat was separated from plasma. The separated buffy coat was diluted with 1:1 v/v Dulbecco's phosphate-buffered saline (DPBS; pH = 7.4). It was carefully added over Histopaque 1077 (Sigma) in sterile 15 ml polypropylene centrifuge tube at 3:1 v/v and then centrifuged at 2,000 rpm for 30 min at room temperature. After centrifugation, the layer of PBMC at the interface between the plasma and the Histopaque 1077, was collected and washed twice with DPBS by centrifugation at 1,500 rpm for 10 min. From PBMC pellet, total RNA was prepared using RNeasy Mini Kit (Qiagen India Pvt. Ltd.) according to manufacturer's protocol. The possible genomic DNA contamination in RNA preparation was removed by using RNase-Free DNase Set (Qiagen India Pvt. Ltd.). Purity of RNA was checked in UV spectrometer with the ratio of the OD at λ260 and λ280 being >1.8. RNA integrity was assessed in 1.5% agarose gel electrophoresis by observing rRNA bands corresponding to 28S and 18S

cDNA synthesis and quantitative real time PCR (qPCR)

For each sample, about 200 ng of total RNA was used for cDNA synthesis by reverse transcription PCR using Revert Aid First strand cDNA synthesis kit (Fermentas, USA) according to the manufacturer's protocol. The RT reaction was carried out at 25°C for 10 min, 42°C for 60 min, 75°C for 5 min in a thermal cycler (Bio-Rad, USA). Primers for BCL-2, BAX, BAK, CASP3, P53 and housekeeping gene (GAPDH) were designed using primer 3.0 software (Table 2). The real time PCR reaction was carried out in Applied Biosystems 7500 real time PCR systems. 1.0 µL of cDNA template, 7.5 µL of Maxima SYBR green qPCR master mix and volume of BCL-2, BAX, CASP3, P53 and GAPDH sequence specific forward and reverse primers (10 pmol) were used and final volume of 15 µL was made with nuclease-free water (Table 2). The real time PCR program consisted of initial heating at 50°C for 2 min followed by 95°C for 10 min, and samples were amplified for 40 cycles (95°C for 30 s; Tm for BCL-2, BAX, CASP3, P53 and GAPDH for 30 s and 72°C for 30 s). The final extension at 72°C incubation was continued for a further 10 min. Relative quantification of a target gene was done by comparing

Table 1: Environmental variables during the experiment

Seasons	Tmin (°C)	Tmax (°C)	Tdb (°C)	Twb (°C)	R. H. (%)	THI
Thermoneutral	8.3	16.5	20.9	16.2	96	67.3
Summer	21.4	34.2	33.7	20.5	69	79.9

Table 2: Primer sequences and details of each gene target analyzed in bovine PBMC by quantitative real time PCR

Genes	Sequence (5′→3′)	Acc No.	Size (bp)	Annealing Temp (°C)
BAX	F-TTTGCTTCAGGGTTTCATCC R-CAGTTGAAGTTGCCGTCAGA	NM_173894.1	246	52.6
BCL-2	F-ATGACTTCTCTCGGCGCT R-CGGTTCAGGTACTCGGTCAT	NM_001166486.1	244	55.5
BAK	F-GCCTACTGACCCAGAGATGG R-CTCATAGGCGTTGTCTGCTG	NM_001077918.1	166	57
CASP-3	F-GACAGTGGTGCTGAGGATGA R-CGAGCCTGTGAGCGTGCTTT	NM_001077840.1	169	62
P53	F-ATTTACGCGCGGAGTATTTG R-CCAGTGTGATGATGGTGAGG	NM_174201.2	174	57
GAPDH	F-CCAACGTGTCTGTTGTGGATCTGA R-GAGCTTGACAAAGTGGTCGTTGAG	NM_001034034.2	218	52-62

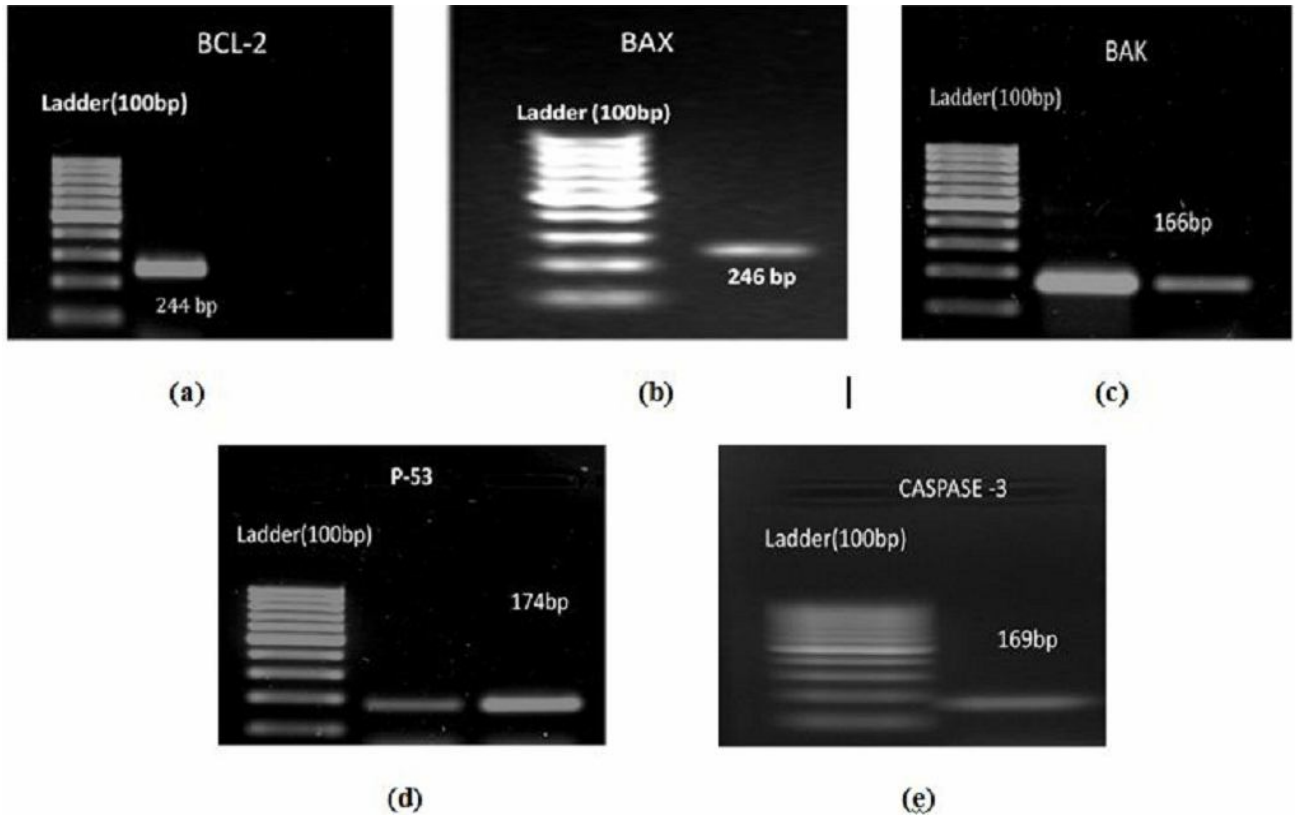

Fig. 1: Agarose gel electrophoretic profile of (a) BCL-2, (b) BAX, (c) BAK, (d) CASP-3, and (e) P53 gene products. Real time PCR amplified products on agarose gel (2%)

expression level of reference gene GAPDH (Livak and Schmittgen, 2001).

Statistical analysis

The data analysis was done using SAS software (2011). One Way ANOVA was done to find out the significant difference between periods of calving and for comparing season separately. Data from different experiments are presented as mean ± SE. The difference at $P \leq 0.05$ was considered to be statistically significant.

Results

The relative mRNA expression of five apoptosis genes, i.e. BCL-2, BAX, BAK, CASP3 and P-53 were quantified and compared by real time PCR on day of calving, 15 days pre and post calving in PBMCs of Sahiwal cows in summer and thermoneutral conditions. Melting curve analysis did not yield any non-specific peak from each primer set tested. Additionally, every PCR product generated a prominent band with an expected size in the gel electrophoresis analysis (Figs. 1a-e). These indicated that non-specific amplifications with the primer sets tested were not detected in the real time PCR analysis. The following is a descriptive and specific account of the genes which have been studied.

Relative mRNA expression of BCL-2 and BAX and BAX/BCL-2 ratio

The relative gene expression of BCL-2, BAX and

Table 3: Relative expression value of mRNA of different genes during different transition periods in Sahiwal

Gene	Season	Days around calving		
		-15	0	+15
BAX	Thermoneutral	2.41 ± 0.39^{a}	1 ± 0^{b}	2.25 ± 0.55^{a}
	Summer	0.57 ± 0.05^{b}	1 ± 0^{a}	1.06 ± 0.05^{a}
BCL-2	Thermoneutral	3.75 ± 0.45^{a}	1 ± 0^{b}	3.22 ± 0.40^{a}
	Summer	1.96 ± 0.30^{a}	1 ± 0^{b}	1.32 ± 0.16^{ab}
BAX/BCL-2	Thermoneutral	0.70 ± 0.15^{a}	1 ± 0^{a}	0.84 ± 0.23^{a}
	Summer	0.35 ± 0.06^{b}	1 ± 0^{a}	0.92 ± 0.16^{a}
BAK	Thermoneutral	0.26 ± 0.08^{b}	1 ± 0^{a}	0.20 ± 0.10^{b}
	Summer	0.92 ± 0.08^{a}	1 ± 0^{a}	0.85 ± 0.10^{a}
CASP-3	Thermoneutral	0.49 ± 0.11^{b}	1 ± 0^{a}	0.39 ± 0.06^{b}
	Summer	0.33 ± 0.10^{b}	1 ± 0^{a}	0.35 ± 0.05^{b}
P53	Thermoneutral	1.65 ± 0.36^{a}	1 ± 0^{a}	1.56 ± 0.15^{a}
	Summer	1.33 ± 0.21^{a}	1 ± 0^{a}	1.54 ± 0.18^{a}

Columns with different superscripts in a parameter differed significantly (P<0.05) between periods

BAX/BCL-2 ratio during different transition periods has been provided in Table 3. During both seasons the relative mRNA expression of BCL-2 was significantly (P<0.05) higher on day 15 pre and post partum compared to day of calving. The relative expression of BAX also increased significantly (P<0.05) on day 15 pre- and postpartum in thermoneutral condition but in summer the relative expression significantly decreased (P<0.0001) on day 15 prepartum and no significant change was found in expression on day 15 postpartum with respect to day of calving. The BAX/BCL-2 ratio during the thermoneutral conditions showed no significant change 15 days pre and post partum with respect to calving whereas in summer season the ratio was less (P<0.05) 15 days prepartum but no significant change on day 15 post calving. While comparing seasons, the BAX mRNA expression of was significantly (P<0.0001) higher in all periods during the summer compared to thermoneutral conditions (Fig. 2). BCL-2 mRNA expression also increased during the summer significantly (P<0.05) in all periods (Fig. 3). The ratio of BAX/BCL-2 increased significantly (P<0.01) in summer on -15, 0, +15 days compared to thermoneutral conditions (Fig. 4).

Relative mRNA expression of BAK

The BAK mRNA expression during the thermoneutral condition was significantly (P<0.0001) lower at day 15 pre- and post partum, compared with the

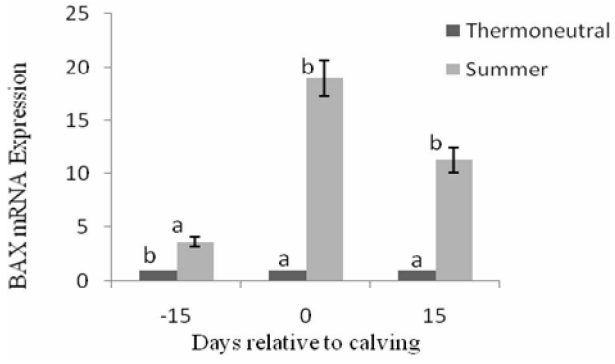

Fig. 2: Relative mRNA expression of BAX. Comparison between two seasons with superscripts significant at (P<0.0001)

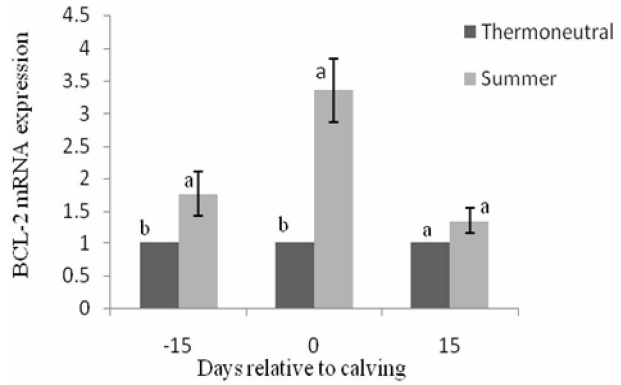

Fig. 3: Relative mRNA expression of BCL-2. Comparison between two seasons with superscripts significant at (P<0.05)

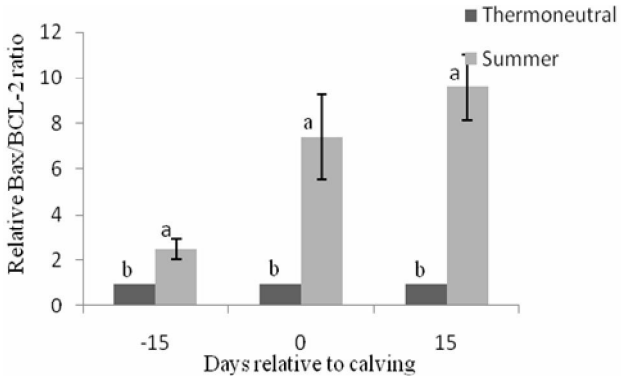

Fig. 4: Relative BAX/BCL-2 ratio. Comparison between two seasons with superscripts significant at (P<0.01)

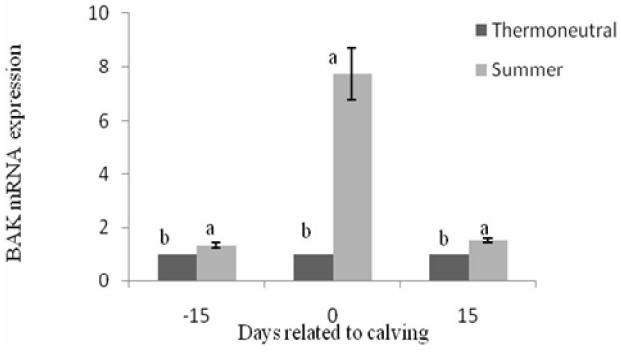

Fig. 5: Relative mRNA expression of BAK. Comparison between two seasons with superscripts significant at (P<0.01)

day of calving. But in summer no significant change was found (Table 3). On comparing the two seasons the expression in Sahiwal increased significantly (P<0.01) on -15 day, 0 day and +15 day during summer season (Fig. 5).

Relative mRNA expression of CASP-3

The CASP-3 mRNA expression in Sahiwal cows under thermoneutral condition and summer (thermal stress) decreased significantly (P<0.0001) on 15 day pre and post partum in comparison to the day of calving indicating up regulation of CASP-3 on the day of calving (Table 3). Comparison between the two temperature conditions showed that there was significant (P<0.01) upregulation of CASP-3 -15 day, 0 day and +15 day during summer season (thermal stress) as compared to thermoneutral condition (Fig. 6).

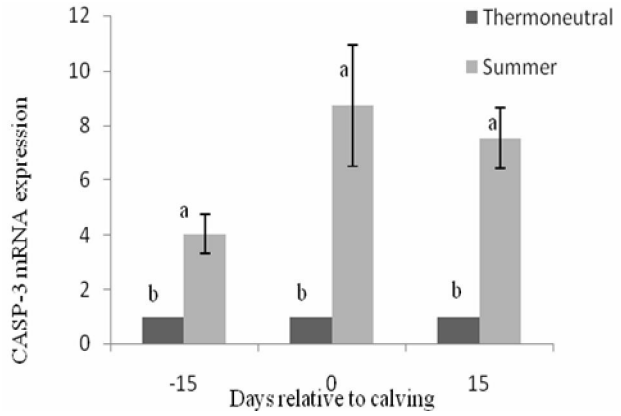

Fig. 6: Relative mRNA expression of CASP-3. Comparison between two seasons with superscripts significant at (P<0.01)

Relative mRNA expression of P53

The expression P53 mRNA in PBMC of Sahiwal cows showed no significant difference on day 15 pre- and post calving with respect to day of calving during both the seasons (Table 3), but while comparing the two seasons it was found that P53 mRNA expression was significantly (P<0.05) higher on -15, 0, +15 days, respectively during summer as compared with thermo-neutral conditions but significant difference was only found on day 0 and 15 (Fig. 7).

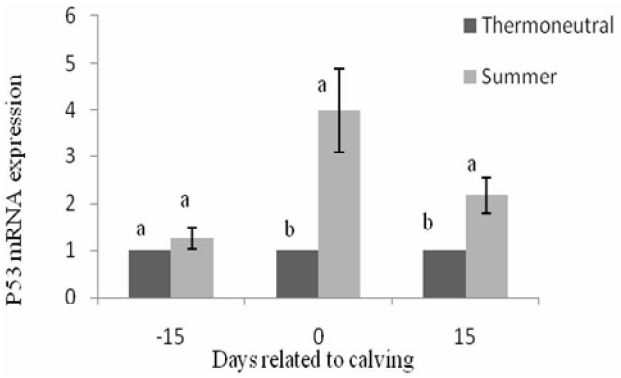

Fig. 7: Relative mRNA expression of P-53. Comparison between two seasons with superscripts significant at (P<0.05)

Discussion

Elevated temperature is a well-known inducer of the heat shock response and to the best of our knowledge, no studies have been designed so far to gain insight into the seasonal variation effects on Apoptosis gene expression profile in transition Sahiwal cows. In the present investigation, the effects of thermal stress on the mRNA expression profiles of BCL-2, BAX, CASP3, BAK and P-53 in PBMCs of transition Sahiwal cows were studied *in vivo* during thermoneutral condition and summer seasons. The THI during thermoneutral temperature conditions and summer was 67.3 and 79.9 respectively.

The homologous BCL-2 and BAX proteins are intracellular membrane-bound proteins with opposing effects, where BCL-2 extends the cellular survival and BAX promotes cell death following an apoptotic stimulus (Cory, 1995). In the present study it was found that compared to thermoneutral conditions the expression of BAX was significantly (P<0.05) higher in summer during all periods. These findings are consistent with previous reports where experimental thermal trauma elevates BAX protein levels and it translocates to mitochondria causing apoptosis (Reiter, 2000) and thermal trauma increased the BAX expression in sinusoidal endothelial cells (SECs) of burn-treated rats (Bekyarova *et al*., 2013). Similarly there was upregulation of BAX in thermally exposed embryos (Yadav *et al*., 2013). BAX/BCL-2 ratio of cell population is a good predictor of cell death following an apoptosis stimulus and apoptotic rheostat is formed on balance between BCL-2 and BAX, which determines sensitivity to apoptosis (Vliet *et al*., 1997). In the present study, comparison between the two seasons showed higher BAX/BCL-2 ratio in summer season (thermal stress). Hence high temperature causes higher BAX/BCL-2 ratio, which was similar to previous findings and also elevated BAX/BCL-2 ratio suggesting the susceptibility of these cells to apoptosis (Bekyarova *et al*., 2013; Gao *et al*., 2013). BAX/BCL-2 ratio was found to be higher on day of calving only in summer season and not in thermoneutral conditions. The relative mRNA abundance of anti-apoptotic genes BCL-2 was also found to be increased during summer season. These results were consistent with previous results where higher temperature causes over expression of BCL-2 (Pernice *et al*., 2011) and also expression of anti-apoptotic genes BCL-xl was higher in embryos exposed to high temperature (Yadav *et al*., 2013). Whether this is part of the thermotolerance response or the onset of a delayed protective response which involves overexpression of Bcl-2 in surviving cells needs to be examined.

During the study there was significant (P<0.01) upregulation of BAK and CASP-3 during summer season compared to thermoneutral condition. These results are similar to the finding where BAK and CASP 3 mRNA were significantly increased in the heat shocked embryos (Yadav *et al*., 2013; Jin *et al*., 2007). It was also concluded in previous study (Pernice *et al*., 2011) that

increasing temperatures lead to activation of caspase 3-dependent apoptosis in cells. There was also upregulation of Caspase-3 on the day of calving in both temperature conditions, indicating higher cell losses on day of calving. Relative mRNA expression of BAK was higher on day of calving only in thermoneutral condition and not in summer.

Expression of P53 was also elevated during the thermal stress though there was no significant difference in expression on different days of transition period. It was also found that the expression of P53 protein was significantly higher under the high temperature conditions than under the normal temperature conditions which reduced cell turnover (Peng *et al*., 2011). It has been reported that activation of the P53 protein initiates a programmed cell cycle arrest, apoptosis or cellular senescence (Harris and Levine, 2005).

The results hereby presented take us a significant step forward in understanding apoptosis of PBMC with transition period in different temperature condition. Upregulation of CASP-3 on the day of calving in PBMC in both seasons indicates susceptibility of these cells to apoptosis and higher mRNA expression of CASP-3, BCL-2, BAK, P53 and BAX/BCL-2 ratio in summer indicate that apoptosis of PBMC which were further aggravated by thermal stress.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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