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Expression Pattern of Genes Related to Heat Shock Proteins, Apoptosis, Antioxidants and Interleukins in Growing Female Murrah Buffaloes Implanted with Melatonin during Summer Season

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ABSTRACT

Background: Heat stress has deleterious effect in the production trait and biological functions in animals. In tropical continents, melatonin has been shown to retain genomic actions and regulating the expression of several genes necessary for cell survival during stress. So, the study was carried out to observe the effect of melatonin implantation on mRNA gene expression for Heat Shock Proteins, Apoptosis, Antioxidants and Interleukins affected by heat stress during summer season.

Methods: Twelve healthy growing female Murrah buffaloes were selected and grouped into control and treatment (melatonin @ 18 mg/50 kg body weight subcutaneously). Peripheral Blood Mononuclear Cells were separated at fortnightly intervals and processed for quantitation of mRNA expression following the double delta Ct method.

Result: The fold change in expression pattern of Heat Shock Proteins 60, 70, 90, BAX, Caspase-3, IL-2 and IL-6 were found significantly (P≤0.05) higher in control group. Whereas *Bcl-2, Mn SOD* and *CuZn SOD* were found significantly (P≤0.05) higher in treatment group. Melatonin implantation reduces the stress levels by up-regulating the expression of the antioxidant genes, anti-apoptotic genes and lowering the expression of heat shock protein genes during heat stress. Therefore, melatonin can be implanted to growing buffaloes for coping during heat stress.

Key words: Antioxidants, Apoptosis, Buffaloes, Heat shock proteins, Interleukins, Melatonin, Summer season.

INTRODUCTION

Exposure to extreme temperature elicits a series of extreme changes in the animal's biological functions that include reduction in feed intake, efficiency and utilization, hormonal secretions, enzymatic reactions and blood metabolites. Heat shock causes mitochondrial damage and senescence is induced by exhilarated ROS levels by alters cell homeostasis in animals (Nir *et al*., 2022). The effect of heat stress is exaggerated when it is accompanied with high ambient humidity (Marai *et al*., 2007). Animal's adaptation to cacophonous environmental conditions and confrontation to stress has been associated with expression of HSPs (Dangi *et al*., 2012). In tropics, melatonin has been shown to retain genomic actions and regulating the expression of several genes necessary for cell survival during stress (Gechev *et al*., 2002). Melatonin accrues higher concentration in mitochondria of the cells and managed the mitochondrial homeostasis. Sharma *et al*. (2013) found that the melatonin reduces oxidative stress and enhance the HSPs transcription. Melatonin is widely tested in the context of regenerative medicine and cell therapy and as antioxidants, they are predicted to improve stem cell fitness and resistance to stress (Nir *et al*. 2022).

Apoptosis is a physiological process which is associated to a complex network of biochemical pathways where undesirable cells are disposing off during development and other biological processes to ensure a homeostatic balance between cellular proliferation and turnover in every tissue (Choudhury *et al*., 2012). *BAX* hasten programmed cell death by binding to alienate the apoptosis repressor *Bcl-2* or its adenovirus homolog E1B 19k protein. During stress, *BAX* undergoes to cause translocation to the mitochondrion membrane, leading to the release of cytochrome-C that then triggers apoptosis by activation of *Caspase-3* and thereby apoptosis. *Bcl-2* gene regulates cell death by inducing or inhibiting apoptosis (Cleary *et al*., 1986). Amidst of three superoxide dismutase responsible for destroying free

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superoxide radicals in the body, SOD-I bind with copper and zinc ions.

MATERIALS AND METHODS

The experiment was conducted at Livestock Research Station of ICAR-NDRI, Karnal, Haryana (India) during 2015-17. The dry and wet bulb temperature and relative humidity were recorded twice daily at 7.30 AM and 2.30 PM with dry and wet bulb hygrometer (Zeal, UK) every day during experimental period. The temperature humidity index (THI) was calculated as method described by Mc Dowell (1972).

Twelve healthy growing female Murrah buffaloes (8 to 12 months) were selected and equally divided into control and treatment (melatonin implanted @ 18 mg/50 kg body wt.) group on the basis of their body weight and body condition score. The experimental animals were housed in a custom design animal shed throughout the study. Deworming of animals were done prior to experiment.

PBMC was collected at fortnightly interval for analysis and quantitation of mRNA expression for *HSP 60, 70, 90, IL2, IL6, Bcl2, Bax, Caspase3, MnSOD* and *CuZnSOD*. Isolation of RNA was done by Qiagen RNeasy minikit, U.S. Agarose Gel electrophoresis was run to check RNA bands and the gels were subsequently photographed by Gel Doc System (Bio-Rad, Hercules, CA, USA). Strand cDNA Synthesis was done using kit from Thermo Scientific, U.S.A. The reference and targeted genes were procured from Europhins Genomics India Pvt. Ltd. Relative expression of the studied genes with respect to the THI was transformed in terms of fold change for quantification of mRNA expression following the double delta Ct method according to Livak and Schnittgen (2001). β-actin used as reference gene for normalization of differential expression of mRNA for relative quantification.

The expression of different genes in different samples was estimated as:

$$\text{Relative expression, } R = 2^{-\Delta\Delta CT}$$

Where,

$^{\Delta\Delta C}{}_T = \Delta C_T$ sample- ΔC_T control.

ΔC_T sample= C_T of target gene in sample- C_T of reference gene in sample.

ΔC_T control= C_T of target gene in control- C_T of reference gene in control

The experiment was approved by IAEC constituted vide article no. 13 of CPCSEA rules (Reg. No. 1705/GO/ac/CPCSEA dt. 3/7/2013). Statistical analysis was done using Two-way ANOVA followed by post-hoc Tukey's test. Evaluation of the correlation between all the factors was made, using a correlation coefficient at the level of probability ($P \leq 0.05$). The analysis was performed using software version (9.1) SAS Institute Inc., Cary, NC, USA Copyright© (2011).

RESULTS AND DISCUSSION

Temperature and humidity index

Mean±SE of Temperature Humidity Index (THI) during the experimental period have been depicted in Fig 1. The 6th, 7th and 8th fortnights showed most stressful period. The higher value for THI is obtained in the 7th fortnight and lower value is obtained in the 1st fortnight. Lactating dairy cows experience heat stress when THI rises above 72 and severe when it exceeds 88 (Thatcher *et al*., 2010). Kumar and Singh (2021) reported significant positive association of THI with RR and RT in growing buffaloes. Factors like level of air movement, sun exposure and duration of these conditions may affect THI values, because animals may experience more severe heat stress at lower temperature and higher relative humidity (Thatcher *et al*., 2010).

Heat shock proteins

The mean values of *HSP60, 70* and *90* gene expressions in treatment group was significantly ($P \leq 0.05$) lower than the control group and mRNA expression pattern has been presented in Fig 2a, 2b and 2c respectively. HSP's expression showed positive correlation with *Bcl-2, IL-2, IL-*

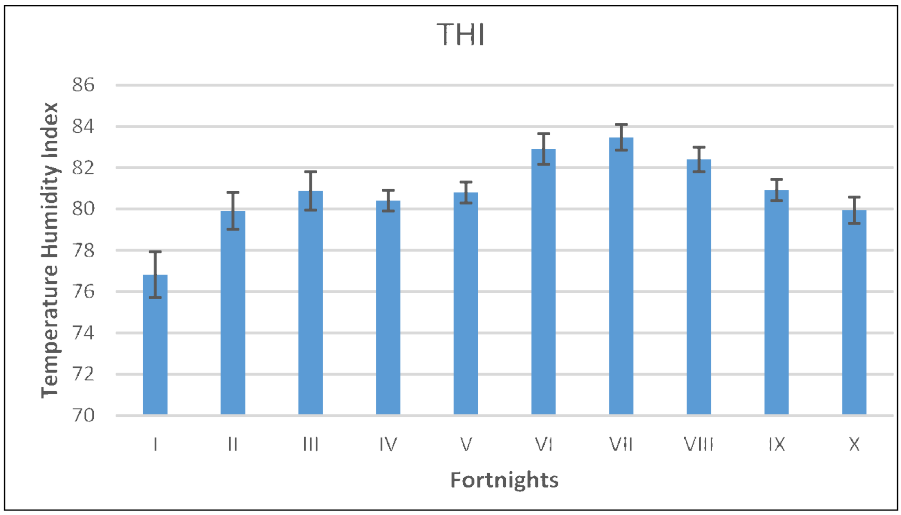

Fig 1: Fortnightly temperature humidity index during the experimental period.

6, Mn SOD and *CuZn SOD* and negative correlation with *BAX* and *Caspase-3*, presented in Table 1.

Melatonin interacts with numerous cellular proteins such as signalling molecules, transporters, channels and enzymes (Hemati *et al.,* 2020). During heat stress, regulation of expression of *HSP60* genes protects cell damage from heat stress through homeostatic mechanism (Vargas-Parada and Solis, 2001). *HSP60* mitochondrial protein helps in refolding of proteins and preventing aggregation of denatured proteins. Sharma *et al*. (2013) revealed increased mRNA expression of *HSP60* in melatonin treated group due to modulating effect under heat stress. The mRNA expression of *HSP70* was significantly one fold higher in control than the treatment during summer. *HSP70* among

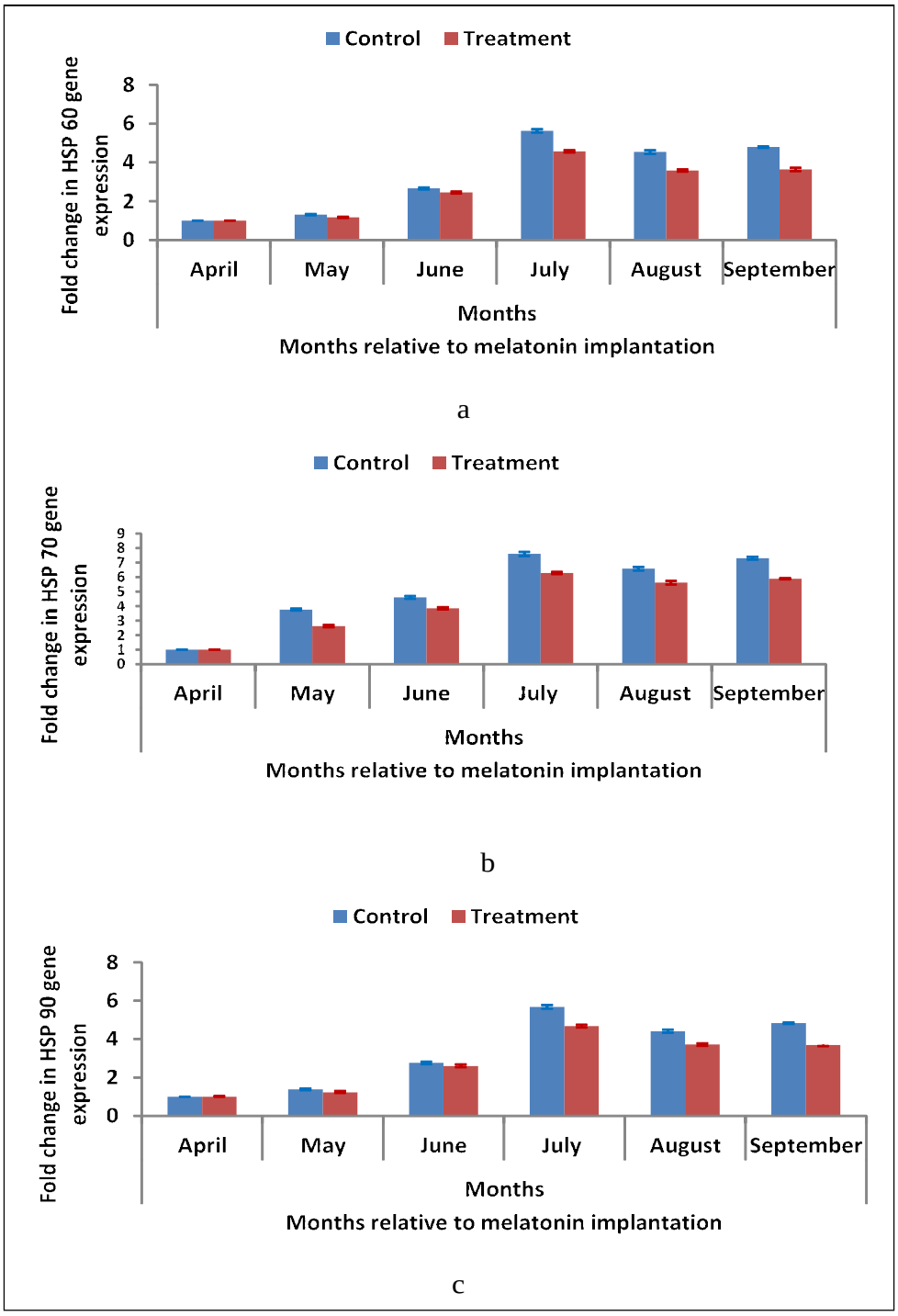

Fig 2: Changes in *HSP60, HSP70* and *HSP90* in control and treatment groups of growing Murrah buffaloes during summer season.

all the HSPs is most sensitive and positively correlated with heat tolerance. Patir and Upadhyay (2007) also observed *HSP70* arose in Murrah buffaloes after two hours of heat exposure due to change in the adaptive and physiological mechanism to cope up with the thermal stress and to attain the thermo-tolerance. Manjari *et al*. (2015) reported an increase in *HSP70* expression due to summer stress in different livestock species. *HSP90* acts as a co-chaperone of *HSP70* and restores the protein recognition (Pratt and Toft, 2003).

Apoptotic genes

The mean values of *Bcl-2* mRNA expression in treatment group was significantly (P≤0.05) higher than the control

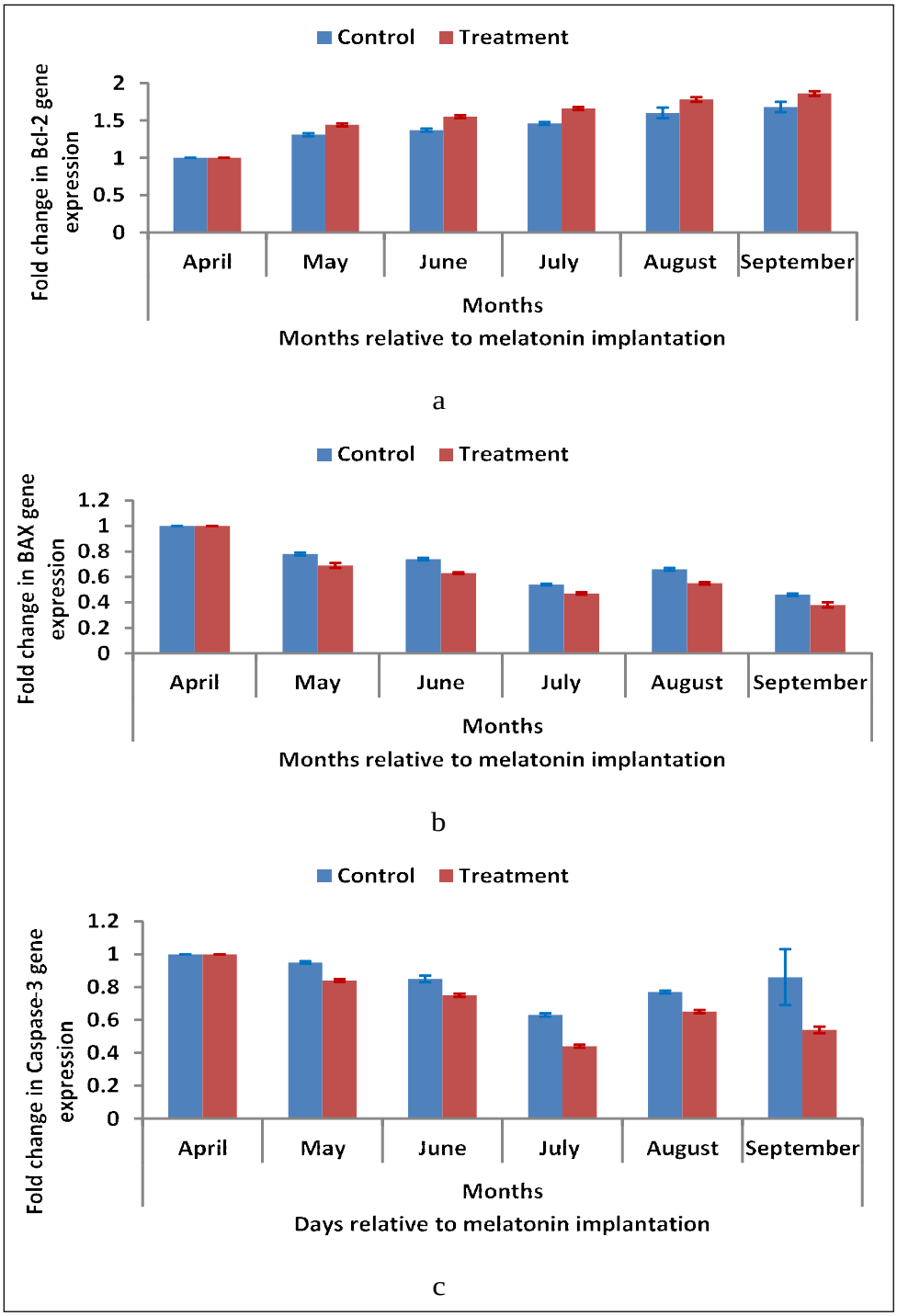

Fig 3: Changes in *Bcl-2, BAX* and *caspase-3* in control and treatment groups of growing Murrah buffaloes during summer season.

group and has been presented in Fig 3a. The mean values of *BAX* and *Caspase-3* gene expression in treatment was significantly (P≤0.05) lower than the control group and are presented in Fig 3b and 3c respectively. Apoptosis is a normal regulation in the cell, which ensures the normal development of the cell and maintains a balanced internal environment. The elimination of damaged cells or senescent after activation of a cell death program involving participation of pro-apoptotic molecules. Anti-apoptotic molecules block the emergence and evolution of these cell changes and prevent cell death. Melatonin acts as an important anti-apoptotic agent in various tissues by reducing cell calcium uptake, mitigating the production of ROS and decreasing pro-apoptotic proteins. Joubert *et al*. (2009) reported anti-apoptotic action of melatonin in nervous and renal tissues. Pedreanez *et al*. (2004) found that treatment with melatonin causes a decrease in the expression levels of pro-apoptotic proteins along with an increase in the expression of *Bcl-2*. Activation of *Caspase-3* is a sign of irreversible apoptosis stage (Fesik and Shi, 2001). Kireev *et al*. (2013) reported that mitochondrial DNA deletion induced oxidative stress and apoptosis and melatonin was found to protect the damage of neuronal cells (Jou *et al*., 2007). Juknat *et al*. (2005) found that melatonin down-regulate the expression of *BAX* and inhibit *Caspase-3* activation in the dentate gyrus of old male rats by inhibiting H_2O_2 induced apoptosis in cultured rat astrocytes. Ortiz *et al*. (2001) suggested that anti-apoptotic action of melatonin is one of the mechanisms by which it protects neuronal cells from neurotoxic insults. Espino *et al*. (2010) reported that melatonin is capable of reducing the activity in *caspases-3* and induced by increased concentration of cytoplasmic calcium in human leukocytes, due to the activation of *BAX* with release of cytochrome-C, thus leading to reduced apoptotic activity.

Antioxidant genes

The mRNA expression of Mn SOD and CuZn SOD have been presented in Fig 4a and 4b respectively and revealed that differential expression in treatment group was significantly (P≤0.05) higher than the control group. Antolin *et al*. (1996) reported that in the lacrimal glands of hamsters,

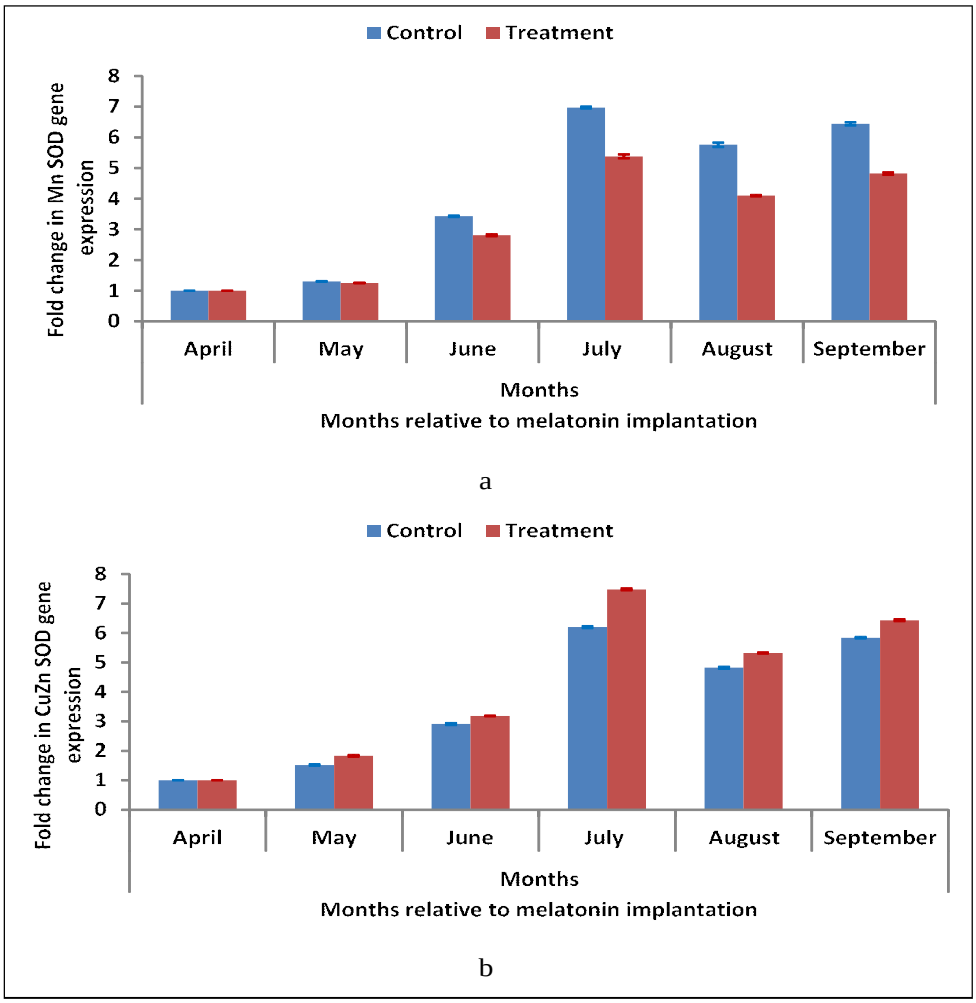

Fig 4: Changes in *Mn SOD* and *CuZn SOD* in control and treatment groups of growing Murrah buffaloes during summer season.

melatonin prevents from the porphyrins which are being produced due to the decreased mRNA synthesis of amino-levulinate synthetase caused cell damage and also it tends to increase the levels of *Mn SOD* and *CuZn SOD*. The synthesis of glutathione peroxidase enzyme eliminates the free radicals in an organism increases in the brains of mice when treated with melatonin (Weishaupt *et al*., 2006) and thus this hormone evident to act over other enzymes that provide protection against toxic reactive. Melatonin's antioxidant actions derive from its stimulatory effect on Glutathione Peroxide and Glutathione Reductase and its inhibitory action on NOS (Reiter, 2003).

Interleukins

The mean values of *IL-2* and *IL-6* expression in control group was significantly (P≤0.05) higher than the treatment group and have been presented in Fig 5a and 5b. Melatonin has been reported to be pro-inflammatory (Sutherland *et al*., 2002) whereas anti-inflammatory properties have also been attributed with some workers Cuzzocrea *et al*. (1999). The results are in accordance with Wang *et al*. (2015) who reported that heat exposure significantly (P<0.05) elevated the level of *IL-2* and *IL-12*. Carrillo-Vico *et al*. (2003) reported that melatonin regulates production of cytokine and acts mostly on the immune system. Melatonin activates the production of *IL-2, IL-6* and *IFNã* by T helper cells and monocytes Garcia-Maurino *et al*. (1997). *IL-6* is a potent pro-inflammatory cytokine which induces the expression of reactants in the acute phase and the differentiation and migration of the activated macrophages Perez-Fernandez and Kaski, (2002). This suggests that there is a down-regulation of these genes in antioxidant supplemented group and control animals express severe stress. Vargas and Marino, (2016) found that during severe heat stress or exercise there was efflux of LPS from G.I. tract which switch acute phase inflammatory response, including the release of *IL-6* which acts during endotoxemic surrounding by debilitating the response through signaling the hypothalamic pituitary adrenal (HPA) axis. Hence, during the torrent of events, there could be release of LPS and provoked response to *IL-6* during substantial heat stress.

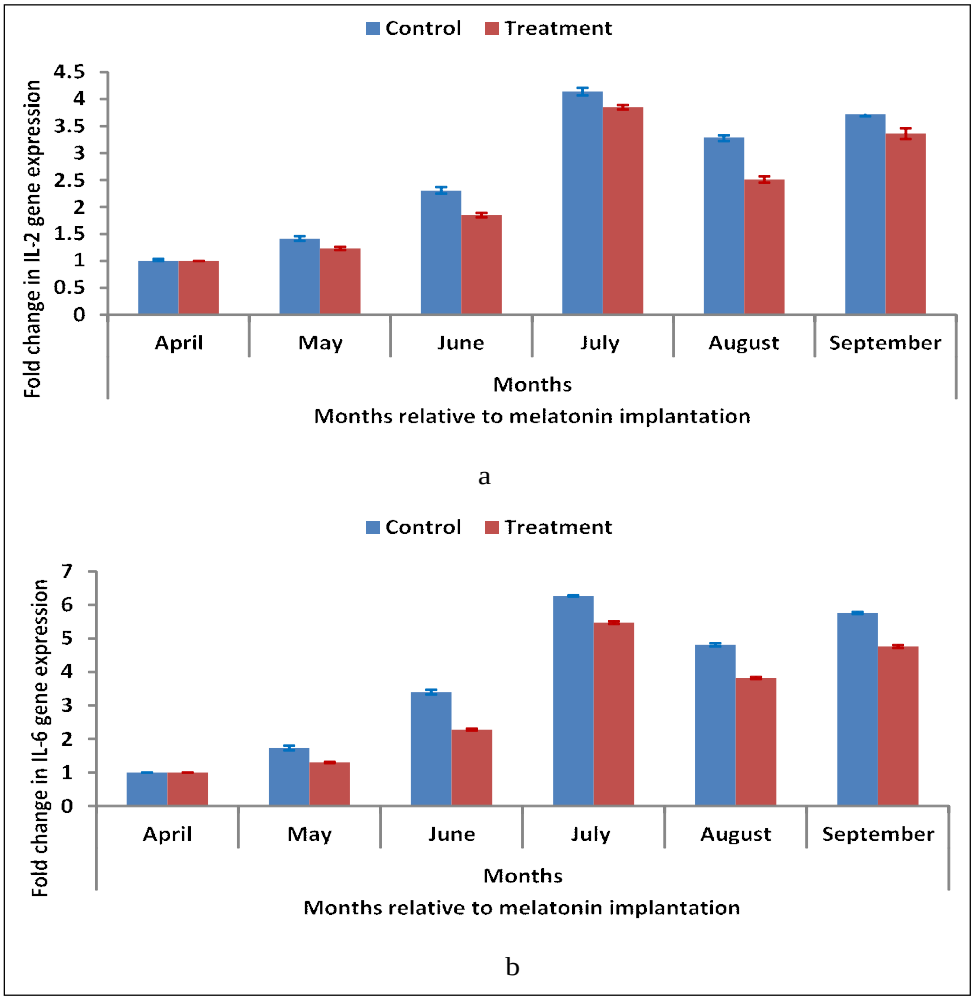

Fig 5: Changes in *IL-2* and *IL-6* in control and treatment groups of growing Murrah buffaloes during summer season.

Table 1: Correlation coefficient among different gene expressions of growing Murrah buffaloes.

	HSP70	HSP90	HSP60	IL-2	IL-6	Bcl-2	BAX	Caspase-3	MnSOD	CuZnSOD
HSP70	1									
HSP90	0.952**	1								
HSP60	0.948**	0.996**	1							
IL-2	0.943**	0.971**	0.970**	1						
IL-6	0.959**	0.980**	0.980**	0.988**	1					
Bcl-2	0.839**	0.738**	0.724**	0.737**	0.737**	1				
BAX	-0.831**	-0.739**	-0.722**	-0.731**	-0.734**	-0.889**	1			
Caspase-3	-0.568**	-0.596**	-0.583**	-0.617**	-0.590**	-0.638**	0.664**	1		
MnSOD	0.836**	0.872**	0.862**	0.884**	0.877**	0.789**	-0.749**	-0.757**	1	
CuZnSOD	0.894**	0.926**	0.919**	0.945**	0.937**	0.793**	-0.767**	-0.730**	0.980**	1

**Correlation is significant at the 0.01 level.

CONCLUSION

Melatonin implantation increased the levels of *Bcl-2, Mn SOD* and *CuZn SOD* in treatment group, which appears to be helpful in scavenging free radicals. The higher levels of *BAX, Caspase-3, IL-2* and *IL-6* in control group indicating the level of heat stress experienced by the animals, while lower levels in treatment group is associated with increased THI might be due to antioxidant activity of melatonin, which ameliorate the impact of summer stress and reduced metabolic heat production. Altogether, the relative mRNA expression of *HSP 60, 70* and *90* was significantly higher in control than treatment group. The findings of the study reveal that expression of HSPs were up-regulated during exposure of heat and *HSP70* was found to be most induced. These HSPs safeguards the protein folding in a systematic manner and regulation of apoptosis during stressful physiological conditions. An exogenous melatonin administration ameliorated the adverse effects of thermal stress and potentiated the immunity and improved the antioxidant status and abated the apoptosis rate which battens cell survival during heat stress.

Conflict of interest: None.

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