Heat shock response in PBMCs of Nellore cows after *in vitro* thermal stress

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Abstract Adaptation is an important feature to be studied in animals when thinking about maintenance and raise of productivity. Although Nellore breed is widely disseminated in Brazil, the knowledge related to its thermotolerance in tropical climate conditions remains unknown. Hence, the aim of this study was to understand the Nellore breed cellular adaptation when exposed to heat shock in vitro, using peripheral blood mononuclear cells (PBMCs). The comprehension of the most expressed and sensible heat shock proteins in *Bos taurus indicus* may elucidate a molecular marker for genetic improvement related to thermotolerance. Previous to blood sampling, the physiological parameters of 16 cows were measured in order to classify them in efficient and non-efficient on heat loss. For this experiment, the blood was collected in three different heparin tubes by jugular venipuncture only in 10 cows, 5 efficient and 5 non-efficient, at morning. The molecular analyzes were performed in Physiology and Molecular Endocrinology Laboratory, Faculty of Veterinary Medicine and Animal Science, University of São Paulo. After sampling, each blood tube were placed in three different water bath devices calibrated for: 38°C, 40°C and 42°C for two consecutive hours. The PBMCs were separated, washed with hemolysis solution, the RNA extracted, the cDNA synthetized and performed the qPCR for the genes HSP60, HSP70 and HSP90. There was a maintenance on HSPs transcripts on 38°C and 40°C and a decrease on all HSPs transcripts at 42°C. Among all, the HSP70 was the most expressed at 38°C and 40°C, elucidating its protective importance. No difference was observed between gene expression and heat loss efficiency. In conclusion, the Nellore cell adaptability was confirmed by the maintenance of heat shock proteins 60, 70 and 90 kDa. The pathway for understanding responses involving thermotolerance is still long and requires more knowledge of cell signaling, either *in vitro* or *in vivo* conditions. Taken together, these informations may contribute in future keys for genetic selection of adapted animals.

Keywords: *bos taurus indicus*, heat shock proteins, thermotolerance

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Introduction

Climate changes are becoming increasingly common with increase of global temperatures, hot air waves, droughts or shifting in precipitation and glacier reduction. These changes negatively affect animal and vegetable production, while it is necessary to increase food production. Thus, adaptation is an important feature to be studied in animals and plants when thinking about maintenance and raise of productivity. At the cellular level, heat adaptation is termed thermotolerance which is the response caused by a single, severe but non-lethal heat exposure that allows the organism to survive subsequent and lethal heat stress by the synthesis and accumulation of heat shock proteins (HSPs), involved in anti-stress mechanisms.

During heat stress, cells generally reduce gene transcription, translation and processing of RNA, alter the activity of expressed proteins and for a short time increase the expression of HSPs. These molecular chaperones are proteins that help others in folding, refolding and transport to their destinations within cells (including translocation through the membranes of organelles). They also participate in the dissolution of protein aggregates leading them to degradation by proteolytic machines.

There are evidences that *Bos taurus indicus*, Senepol and Romosinuano cattle evolved in hot climates had acquired genes related to protection when exposed to temperature elevation (Hansen 2004). According to the same author, little is known about the genes responsible for heat resistance in Zebu, as well as the physiological basis for thermotolerance. Kumar et al. (2015) have suggested that genes involved in the synthesis of heat shock proteins can be used as biomarkers in the study of stress in cattle and buffaloes. Although Nellore breed is widely disseminated in Brazil, the knowledge related to its thermotolerance in tropical climate conditions remains unknown. Hence, the aim of this study was to understand the Nellore breed cellular adaptation when exposed to heat shock *in vitro*, using peripheral blood mononuclear cells (PBMCs). The comprehension of the most expressed and sensible heat shock proteins in *Bos taurus indicus* may elucidate a molecular marker for genetic improvement related to thermotolerance.

Material and Methods

The molecular analyzes were performed in Physiology and Molecular Endocrinology Laboratory, Faculty of Veterinary Medicine and Animal Science, University of São Paulo. The Institutional Ethics Committee of Animal Use approved all animal care, handling and procedures in this project with the protocol number nº13.1.2186.74.2. The animals used belongs to the Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo, Pirassununga (21°80' latitude South and 47°25'42'' longitude West). The Nellore cows were kept in pastures with mineral supplementation and water *ad libitum*.

Previous, the vaginal temperature and sweating rate of 16 cows were measured during 6 continuous days in order to classify them in efficient and non-efficient on heat loss. The most efficient cows were the ones with lower body core temperature throughout continuously measurement and higher heat loss by sweating. After classification, 10 cows, 5 more efficient and 5 non-efficient had the blood collected in three different heparin tubes by jugular venipuncture at morning, the period of less interference of climate variables. After sampling, each blood tube were placed in three different water bath devices calibrated for: 38°C, 40°C and 42°C for two consecutive hours (Wang et al. 2003).

After heat treatment, blood samples were centrifuged to separate the peripheral blood mononuclear cells (PBMCs). The PBMCs were used as a model to understand the heat shock response in a systemic view without the interference of other external factors, in a control situation equally than other studies (McClung et al. 2008; Deb et al. 2013; Kishore et al. 2013; Mohanarao et al. 2014). The PBMCs were transferred to a microtube and the volume was completed with hemolysis solution. The hemolysis washing procedure was repeated until the PBMCs'pellet had been clear for finally be storaged at -80°C. The total RNA was isolated by Trizol Reagent method (Invitrogen, Carlsbad, CA). The RNA obtained was treated with DNAse I (Invitrogen, Carlsbad, CA) following the manufactures procedures to exclude genomic DNA contaminations. The RNA concentration and purity were estimated by NanoVue Plus spectrophotometer (GE Life Sciences) at 260nm absorbance and the 260/280 and 260/230 ratios respectively. Samples were stored at -80°C until cDNA synthesis. The cDNA synthesis was performed using the Superscript III kit (Invitrogen, Carlsbad, CA) and stored at -20°C.

The gene sequences were found in the NCBI database (http://www.ncbi.nlm.nih.gov/gene) and synthesized by outsourced laboratory (Table 1). Primarily, each primer was validated and the efficiency tested (Table 2). The qPCR analyzes were performed in Applied Biosystem equipment StepOnePlus® (Invitrogen, Carlsbad, CA) with SYBR® Green (Power SYBR® Green PCR Master Mix, Invitrogen, Carlsbad, CA) as the fluorescent label. In order to select the reference genes was used the GeNorm Microsoft Excel, which provides a measure of gene expression stability (M) (Vandesompele et al. 2002). The genes: Beta actin (ACTβ), Ribosomal protein L15 (RPL-15), Histone 2 and Cyclophilin A (PPIA) were converted to a quantitative expression scale using the delta Ct method and were processed in GeNorm. These genes have been classified based on M values, in which the genes with the most stable expression had the smallest M values. The gene expression of HSP60, HSP70 and HSP90 were normalized with the geometric mean of the most stable housekeeping genes: ACTβ, RPL15 and PPIA. The efficiency of qPCR and the quantification cycle (Cq) were determined for each sample using the Software LinRegPCR.

Table 1 Primers sequences forward (3'-5') and reverse (5'-3') used in the experiment.

Gene	Simbol	GeneBank number	Sequence	Amplicon	Efficiency	Reference
Bos taurus heat shock 60kDa protein 1	*HSP60*	NM001166610.1	3'CTCATCTCACTC GGGCTTATG5' 5'GGCTACAGCATC GGCTAAA3'	98	1.996	PrimerQuest
Bos taurus heat shock 70kDa protein 1A	*HSP70*	NM203322.2	3'ACCCGCAGAACA CGGTGTT5' 5'AGGCTTGTCTCC GTCGTTGA3'	118	1.994	PrimerQuest
Bos taurus heat shock protein 90kDa alpha	*HSP90*	NM001012670.2	3'GTCCATACATCG GGCTTGTT5' 5'TACCTTTCCAGC GGCTTTAC3'	96	1.995	PrimerQuest
Bos taurus actin, beta	*ACTB*	NM173979.3	3'GGATGAGGCTCA GAGCAAGAGA5' 5'TCGTCCCAGTTG GTGACGAT3'	77	2.006	BETTEGOWDA(2006)
Ribosomal protein L15	*RPL-15*	AY786141.1	3'TGGAGAGTATTG CGCCTTCTC5' 5'CACAAGTTCCAC CACACTATTGG3'	64	1.944	BETTEGOWDA(2006)
Bos taurus histone H2A	*HISTONA 2*	AY835842.1	3'GAGGAGCTGAAC AAGCTGTTG5' 5'TTGTGGTGGCTC TCAGTCTTC3'	103	1.928	BETTEGOWDA(2006)
Peptidylprolyl isomerase A	*PPIA*	NM_178320.2	3'GCCATGGAGCGC TTTGG5' 5'CCACAGTCAGCA ATGGTGATCT3'	64	1.997	BETTEGOWDA(2006)

The gene expression was analyzed by MIXED procedure of SAS 9.2 software (SAS Inst., Inc., Cary, NC) considering as fixed effects the heat shock treatments (38°C, 40°C and 42°C). On the statistical analyses relating *in vitro* gene expression and heat loss efficiency, the heat treatment at 42°C was not considered because it is biologically unbearable. Means were compared by F-test and Student's T-test (pdiff). All tests were performed at 5% probability and the values presented by mean and standard error of the mean.

Results and Discussion

There was a maintenance of transcripts from 38°C to 40°C, a decrease of HSPs transcripts abundance from 38°C to 42°C and from 40°C to 42°C (Table 2). The time of exposure was not taken into account, although it was assumed that the acute shock at 42°C generated a protein instability that made transcription unfeasible.

Table 2 Means followed by standard error of the mean for peripheral blood mononuclear cell's gene expression of heat shock proteins 70, 60 and 90 at 38°C, 40°C and 42°C *in vitro* treatments.

Genes	38°C	40°C	42°C	*P-valor*	EPM
HSP70	1.30^{aA}	1.28^{aA}	0.84^{bB}	<0.0001	0.02
HSP60	1.19^{aB}	1.20^{aB}	0.93^{bA}	<0.0001	0.02
HSP90	1.02^{aC}	0.98^{aC}	0.78^{bC}	<0.0001	0.0018

*Different lowercase letters at the same line and capital letters in the column statically differ by T test (P<0.05).

The descending order of HSPs at 38°C and 40°C is the following: HSP70> HSP60> HSP90, however at 42°C was HSP60> HSP70> HSP90, the profile transcript amount changed between treatments probably due to the increase of mitochondrial activity at 42°C related to cell death. The HSP60 participates in mitochondrial membrane bidirectional transport and in heat stress occur an increase on its gene expression (Baraja-Vásquez et al. 2005). The HSP70 has been known as highly induced chaperone and are important in stabilizing native protein conformation, maintaining cell survival and integrity when thermal stressed (Moseley 1997). The HSP90 function is generally associated with HSP70 activity, as a co-chaperone complex, to restructure protein conformation.

At cellular level, it was expected that the thermal stress increase the protein denaturation and degradation, compromising the cytoskeletal components, membrane permeability and inhibition of protein synthesis (Sonna et al. 2002). Consequently, to stabilize such disorders there was the synthesis of heat shock proteins. Therefore, our data did not corroborate with the results observed by Bañuelos-Valenzuela and Sánchez-Rodríguez (2005); Agnew and Colditz (2008); Mishra et al. (2011); Kishore et al. (2013). The relation between temperature and time of exposure is contradictory, Lepock (2005) states that the cellular response to stress is related to the amount of proteins denatured and aggregated by the effect of thermal stress.

There was no difference of gene expression between efficient and non-efficient cows, however, it was observed a numerical variance between heat loss efficiency (Table 3). We speculate that the efficient cows with better thermoregulatory ability had less deleterious effects at cellular level and did not need to recruit the synthesis of heat shock proteins, consequently, with a small pool of HSPs. Therefore, when PBMCs of efficient cows were heat stressed there were *de novo* synthesis of HSPs. On the other hand, the non-efficient cows with worse thermoregulatory ability, probably holds a bigger pool of HSPs because cells needed constantly cytoprotective mechanisms, consequently the PBMCs *in vitro* were not stimulated for de novo synthesis of HSPs.

Table 3 Means and standard error of the mean of heat shock proteins 60, 70 and 90 gene expression with heat loss efficiency and interaction (efficiency x treatment). For this analysis the heat treatment at 42°C was not considered.

Gene	Heat loss efficciency				Efficiency x Treatment
	Efficient	Non-Efficient	*P-valor*	SEM	*P-valor*
HSPA1A	1,32	1,26	0,1635	0,02	0,7932
HSPD1	1,21	1,18	0,3260	0,02	0,1290
HSP90AA1	1,01	1,00	0,7515	0,018	0,6482

Moseley (1997) affirms that acclimatization not only reduces body temperature by increasing the heat flow to skin and the dissipation ability, but also allows the body to tolerate high temperatures and the HSPs are involved in this process. In hot climate conditions, there are implications about interspecies differences in the patterns of HSPs' accumulation associated with thermotolerance. Lizards in higher temperature ecological niches has greater amounts of HSP70, even in non-stressful conditions (Ulmasov et al. 1992). The same was described in ants (*Cataglyphis bombycina*) inhabiting the Sahara Desert (Gehring and Wehner 1995) and with humans living in Asia and others living in Europe (Lyashko et al. 1994).

Conclusions

In conclusion, the Nellore cell adaptability was confirmed by the maintenance of heat shock proteins 60, 70 and 90 kDa from 38°C to 40°C. However, *in vitro*, the heat treatment at 42°C reduced the transcripts amount. Among heat shock proteins the HSP70 was the one with higher expression in treatments 38°C and 40°C elucidating its protective importance, and at 42°C the higher expression was observed for HSP60. It was assumed that, the signaling for HSPs synthesis was probably influenced by the pre-existing amount of HSPs, stimulating or not to *de novo* synthesis. The pathway for understanding responses involving thermotolerance is still long and requires more knowledge of cell signaling, either *in vitro* or *in vivo* conditions. Taken together, this information may contribute in future keys for genetic selection of adapted animals.

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