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HSPA1A relative gene expression in bovine leukocytes by *in vitro* heat shock

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Abstract: The thermotolerance is the cell adaptation caused by a single exposure to heat, severe but not lethal, which enables the organism to survive a subsequent lethal heat stress by the synthesis or accumulation of heat shock proteins. Therefore, this study aimed to identify cellular responses by the HSPA1A relative gene expression in cattle leukocytes by *in vitro* heat shock. The experiment was conducted in Biometeorology and Ethology Laboratory with 20 cows. Were collected blood samples at the insemination during the 2013's breeding season for relative gene expression analysis, after harvesting were initiated the thermal treatment in three different water baths calibrated for the temperatures 38°C, 40°C and 42°C during two consecutive hours. At the end of the heat treatment, was extracted the RNA, synthetized the cDNA and performed the qPCR using the StepOnePlus. The target gene was the HSPA1A and the endogenous were ACTB, RPL-15 and PPIA. To analyze the relative gene expression were done variance analyses by MIXED procedure using the SAS. There was a significant difference between the abundance of heat shock proteins transcripts between treatments 38°C and 40°C to 42°C. However, there was not an increase in HSPA1A gene expression with the increase of *in vitro* temperature. In conclusion, we observed the the Nelore leukocytes, in an *in vitro*, situation maintain the abundance of HSPA1A transcripts at treatments 38°C and 40°C and had a substantial decline at 42°C, because probably at this temperature occurred a protein instability which unfeasible the transcription.

Keywords: *Bos taurus indicus*, heat shock protein, Nellore cow.

Introduction

Cells are constantly exposed to various stressors and these challenges promote the activation of anti-stress mechanisms. These mechanisms involve a variety of molecules, including chaperones, also known as heat shock proteins (HSPs). There are evidences that cattle that have evolved in hot climate acquired genes that protect them from the adverse effects caused by elevated temperatures as observed in *Bos taurus indicus*, Senepol and Romosinuano (Hansen, 2004). According to the same author, little is known about the genes responsible for the heat resistance in Zebu cattle, as well as the understanding of the physiological basis for the thermotolerance. According to Moseley (1997), heat adaptation is divided into thermotolerance and acclimatization. The thermotolerance is the cell adaptation caused by a single exposure to heat, severe but not lethal, which enables the organism to survive a subsequent lethal heat stress by the synthesis or accumulation of heat shock proteins. In many organizations, the HSP70 (HSPA1A) is considered the largest family of proteins highly induced and its synthesis increase in response to various stressors. Therefore, it is necessary to know how the cells respond to heat stress and which adaptability characteristics would be relevant looking for genetic improvement. Understanding the heat shock proteins gene expression in different species and their origins will allow the identification of a thermotolerance marker and thus improve the reproduction and production at heat stress condition. The objective of this study is to identify cellular responses by the HSPA1A relative gene expression in cattle leukocytes by *in vitro* heat shock.

Material and Methods

The experiment was conducted in Biometeorology and Ethology Laboratory, at the Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga-SP, located at 21°80'00" South latitude and 47°25'42"West longitude, 634m altitude. Were used 20 Nellore cows with 30 to 90 days postpartum, cyclical, body condition score between 3 and 4 (0: thin, 5: obese). Were collected six heparin tubes (~ 8 mL each) BD Vacutainer ™ per animal (duplicate by heat treatment) at the breeding season of 2013 in the insemination moment. After the harvest, for greater cellular preservation, the blood samples were immediately transported to the Biometeorology and Ethology L3,aboratory where were initiated the thermal treatment in three different water baths calibrated for the temperatures 38°C, 40°C and 42°C during two consecutive hours. At the end of the heat treatment, was proceeded the centrifugation to remove the buffy coat which was transferred to a 1.5 mL microtube and the volume was completed with hemolysis solution.

Molecular analyzes were performed in the Physiology and Molecular Endocrinology Laboratory, at Veterinary Medicine and Animal Science Faculty, University of São Paulo, Pirassununga-SP. Total RNA was isolated by Trizol Reagent method (Invitrogen, Carlsbad, CA), which was added to each sample, homogenizing until the complete pellet dissolution. After addition of chloroform and centrifugation, approximately 600µL of the aqueous layer was transferred into new RNA microtubes, which was added 500µL of isopropyl alcohol. After mixing, the microtubes were incubated at -80°C overnight. In the following day, the samples were centrifuged and was observed the RNA pellet at the microtube background. The supernatant was removed and added 1mL ethanol 75% to the microtube containing the RNA, proceeding again the centrifugation and the ethanol supernatant was carefully removed. Finally, the RNA was resuspended in 20µL with water treated with diethyl pirocarboneto (DEPC). Immediately after the resuspension was done the digestion with DNase I (deoxyribonuclease I Amplification Grade - Invitrogen - Cat No. 18068-015.). The concentration and purity of RNA were estimated by spectrophotometer NanoVue Plus (GE Life Sciences) by absorption 260nm and by the ratio 260/280 and 260/230 respectively (where the ratio should be between 1.6 and 1, 8). Finally, the samples were stored at -80°C until cDNA synthesis.

Before the cDNA synthesis, RNA was diluted in DEPC water to achieve concentrations of 1 micrograms in 10 microliters of water. For cDNA synthesis was used Superscript III reverse transcription kit (Invitrogen). The reverse transcription reaction was responsible for transcribing RNA into cDNA. In a microtube of 0.2mL free of DNase and RNase were added 10.0µL RNA sample (1g) and 1.00uL pd(T) 12-18 (0.5µg.µL^{-1}). Then the tubes were incubated at 65°C for 5 minutes and then cooled to 4°C. One mix solution was prepared containing: 4.0µL of 5X buffer, 2.0µL DTT, 1.0µL dNTPs (10 mM each one), 1.0µL of RNase Out, and 1.0µL Superscript III. Were added 9.4µL of the mix solution to each tube and incubated for 1 hour at a temperature of 50°C, followed by 15 minutes at 70°C. Afterwards the tubes were cooled and stored at -20°C.

The target gene was the HSPA1A and the endogenous were ACTB, RPL-15 and PPIA (Table 1). The primers previously designed arrived lyophilized at the laboratory. The first resuspension was made with DEPC treated water free of nucleases. An aliquot of this solution was diluted with water to make a working solution with a concentration of 20 mM. This solution was also stored at -20°C.

To obtain the gene expression data analyzes were performed a qPCR using the StepOnePlus® Applied Biosystem equipment (Life Technologies, Carlsbad, CA) using as fluorescent marker the SYBR® Green (Power SYBR® Green PCR Master Mix, catalog number : 4367659). Each reaction had a total volume of 20µL. Using a 96 well plates and transparent adhesive tape to seal them. The qPCR race condition is characterized by a waiting stage: 95°C for 10 minutes; 40 cycles: denaturation temperature increase and separation of double-stranded (95°C for 15 seconds), annealing, primers bind to its homologous region in the cDNA (60°C for 1 minute); dissociation curve.

Table 1: Characteristics of primers designed for the transcripts' quantification.

Gene	GeneBank number	Forward Primer (3' - 5')	Reverse Primer (5' - 3')
HSPA1A	NM_203322.2	ACCCGCAGAACACGGTGTT	AGGCTTGTCTCCGTCGTTGA
ACTB	NM_173979.3	GGATGAGGCTCAGAGCAAGAGA	TCGTCCCAGTTGGTGACGAT
RPL-15	AY786141.1	TGGAGAGTATTGCGCCTTCTC	CACAAGTTCCACCACACTATTGG
PPIA	NM_178320.2	GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT

After primers' validation by concentration and standard curves tests, were initiated the comparisons related to the abundance of each transcript of the 10 animals with the respective heat treatment (38°C, 40°C, 42°C) using cDNA in 1:40 dilution, in triplicate. The efficiency of qPCR and Cq values (quantification cycle) were determined for each sample using the Software LinRegPCR (V2014.2; http://linregpcr.nl/). Quantitation was obtained after normalization of target gene expression values (Cq values) by the geometric mean of the expression values of endogenous ACTB, PPIA and RPL15.

For sequencing of the amplified portion of the reaction, the PCR products were purified using QIAquick® PCR Purification Kit (Qiagen Laboratories). The PCR products were quantified by NanoVue (GE Life Sciences). The samples were diluted and added the solutions of the respective primers and sent to the Center of Studies of the Human Genome, University of São Paulo. The sequences of the results were accessed through Chomas software and tested for their specificity using the BLAST1 software. Definitely confirming the identity of the PCR product.

To analyze the relative gene expression was done variance analyses by MIXED procedure using the Statistical Analysis System (SAS Institute, 1995). For *in vitro* heat shocks were only included fixed effects of treatment (38°C, 40°C and 42°C). All tests were conducted at 5% probability and the amounts presented by the mean and standard error of the mean.

For the determinations of relative gene expression was done variance analyzes by MIXED procedure using the Statistical Analysis System (SAS Institute, 1995). For the *in vitro* thermal shocks was included fixed effects of treatment (38°C, 40°C and 42°C). All tests were conducted at 5% probability and the values were presented by the mean and standard error of the mean.

Results and Discussion

There was a significant difference between the abundance of heat shock proteins transcripts between treatments 38°C and 40°C to 42°C (Table 2). However, there was not an increase in HSPA1A gene expression with the increase of *in vitro* temperature. It was expected at cellular level that the thermal stress would induce the denaturation and protein breakdown, compromising cytoskeletal components, the permeability of membranes and inhibition of protein synthesis (Somna et al., 2002) and for consequence the synthesis of heat shock proteins to stabilize such disorders. Therefore, the data do not corroborate with those of Kishore et al. (2013) in *Bos taurus indicus*, *Bos taurus taurus* and *Bubalus bubalis*.

Under the experimental conditions of this study, the results suggest that the heat shock did not stimulate greater expression of heat shock proteins, with partial maintenance of the abundance of transcripts between treatments 38°C and 40°C. However, there is substantial decline in the abundance of mRNA in a thermal shock at 42°C. In this study exposure time factor was not taken into consideration, but it is assumed that the acute shock at 42°C produced a protein instability which unfeasible the transcription. Agnew & Colditz (2008) found that there is a reduction in HSP70 expression by intensity interference of temperature, and Kishore et al. (2013) found reduction in the expression by the interference of time in heat treatment. However, the time and temperature relationship is contradictory. Lepock (2005) states that cell stress

response is related to the amount of aggregated denatured proteins and the effect of thermal stress, but not in relation to time in a certain temperature.

Table 2. Means followed by standard error of HSPA1A relative gene expression in leukocytes for treatments.

	Treatments				
Gene	38°C	40°C	42°C	*P-value*	SEM
HSPA1A	1,30[a]	1,28[a]	0,84[b]	<0,0001	0,02

Lower case letters in the same line does not differ by t test (P> 0.05).

According to Moseley (1997), acclimatization not only reduce the body temperature by increasing the heat flow for the skin and the ability to dissipate heat, but also allows the organism to tolerate high temperatures. Thus, the heat acclimatization allow the animal to produce in stressful conditions by being in heat balance by physiological thermoregulation mechanisms. According to the same author, HSPs are related to the adaptation of the body beyond the heat thermotolerance. There inferences regarding interspecies differences in HSPs accumulation patterns associated with dwelling in hot climates. Ulmasova et al. (1992) reports that lizards who live in different regions hold different amounts of HSP70, and as higher is the temperature of ecological niche higher is the amount constitutive HSP70, without stress condition. Such information can not be extrapolated to the endothermic but allows us to understand that the HSP accumulation is related to a characteristic of adaptation. The same was described in ants (*Cataglyphis bombycina*) who inhabit the Sahara desert (Gehring; Wehner, 1995). Therefore, Moseley (1997) affirms the importance of HSPs in thermotolerance and the different accumulation of HSPs in animals adapted to hot environments suggest a possible role of HSPs in acclimatization.

Conclusions

In an *in vitro* situation, the Nellore leukocytes maintained the abundance of HSPA1A transcripts at treatments 38°C and 40°C and had a substantial decline at 42°C. It is suggested for a future studies to better understand the thermal tolerance *in vivo* conditions and even its variation throughout the year and what is the expression profile of other heat shock proteins.

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