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Effects of heat stress on development, quality and survival of *Bos indicus* and *Bos taurus* embryos produced *in vitro*

C.F. Silva [a], E.S. Sartorelli [a], A.C.S. Castilho [a], R.A. Satrapa [a], R.Z. Puelker [a,b], E.M. Razza [a], J.S. Ticianelli [a], H.P. Eduardo [b], B. Loureiro [a], C.M. Barros [a,*]

[a] *Department of Pharmacology, Institute of Bioscience, University of São Paulo State (UNESP), Botucatu, São Paulo, Brazil*
[b] *Progest Ltda., Botucatu, São Paulo, Brazil*

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ABSTRACT

Heat stress is an important cause of poor development and low survival rates in bovine embryos. Experiments were conducted to test the hypothesis that *Bos indicus* embryos are more resistant to heat stress than are *Bos taurus* embryos. In experiment 1, Nelore and Jersey embryos from oocyte pick-up-derived oocytes were submitted to heat stress (96 hours post-insemination, 41 °C, 6 hours), developmental ratios were assessed at Day 7 (Day 0 = day of fertilization), and blastocysts were frozen for RNA extraction. Experiment 2 evaluated expression of *COX2*, *CDX2*, *HSF1*, and *PLAC8* in previously frozen blastocysts. In experiment 3, Nellore and Angus embryos from oocyte pick-up-derived oocytes were submitted to heat stress (96 hours post-insemination, 41 °C, 12 hours) and transferred to recipients on Day 7. In experiment 4, embryos developed as in experiment 3 were fixed for Terminal deoxynucleotidyl transferase dUTP nick end labeling labeling and total cell counting. In experiment 1, heat stress decreased the percentage of Jersey oocytes that became blastocysts, but had no effect on Nellore embryos (34.6%, 25.0%, 39.5%, and 33.0% for Jersey control, Jersey heat-stressed, Nellore control, and Nellore heat-stressed oocytes, respectively; $P < 0.05$). In experiment 2, heat stress decreased ($P < 0.05$) expression of *CDX2* and *PLAC8*, with higher expression of these genes in Nellore embryos than in Jersey embryos. Heat stress also decreased ($P < 0.05$) expression of *COX2* in Jersey embryos, but had no effect on Nellore embryos. Expression of *HSF1* was decreased ($P < 0.05$) by heat stress in both breeds, with a greater effect in Nellore embryos. In experiment 3, heat stress tended ($P = 0.1$) to decrease the percentage of pregnancies among cows (Day 30 to 35) that received Angus embryos. In experiment 4, heat stress increased ($P < 0.05$) the percentage of apoptotic blastomeres, but had no breed-specific effects. In addition, Nellore embryos had fewer ($P < 0.05$) Terminal deoxynucleotidyl transferase dUTP nick end labeling- positive blastomeres than did Angus embryos. We concluded that the detrimental effects of heat stress were dependent upon embryo breed and were more evident in *Bos taurus* embryos than in *Bos indicus* embryos.

1. Introduction

Heat stress is a major problem that affects the cattle industry worldwide. Industry losses result especially from deleterious effects of heat stress on the animal's reproductive system. In the female reproductive tract, exposure to elevated temperatures can alter folliculogenesis [1], reduce uterine blood flow [2], and reduce circulating progesterone concentrations [3–6]. Oocytes can also become damaged by maternal hyperthermia, losing competence for fertilization and development [7–10]. Furthermore, heat stress is responsible for low pregnancy rates and high embryonic losses in embryos produced either *in vivo* [8] or *in vitro* [10,11]. However, different breeds respond differently to heat stress. For example,

* Corresponding author. Tel.: +55 14 38800236; fax: +55 14 38153744.
E-mail address: cmbarros@ibb.unesp.br (C.M. Barros).

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European breeds of cattle (*Bos taurus*) are particularly sensitive to heat stress when compared with zebu breeds (*Bos indicus*). In several *in vitro* studies, *Bos indicus* embryos were better able to survive heat stress (measured as the blastocyst rate) compared with *Bos taurus* embryos [12–15].

It is known that heat stress affects the embryo through apoptosis [16–18], which might be influenced by expression of stress-related genes [19]. Perhaps *Bos indicus* embryos exhibit increased expression of stress-protective genes, and as a result, decreased apoptosis and increased survival rates. In recent reports, El-Sayed et al. [20], studying embryos produced *in vitro* (no breed defined), and Ghanem et al. [21], studying *Bos taurus* embryos derived *in vivo*, identified genes that were expressed at higher levels in embryos that successfully developed into calves compared with embryos that either did not induce pregnancy or resulted in pregnancy loss. In both studies, *PLAC8* expression was higher in cells from embryos that developed into calves. In the *in vitro* study, *COX2* and *CDX2*, genes that are necessary for implantation, were also upregulated in the calf delivery group. A stress-related gene, *HSPD1*, was most highly expressed in cells from the group of *in vivo* embryos that failed to induce pregnancy.

The goal of this study was to determine the effects of heat stress on embryo development (measured as blastocyst rate), quality (measured as rate of apoptosis and level of expression of stress- and survival-related genes), and pregnancy establishment success of *Bos taurus* (Jersey and Angus) and *Bos indicus* embryos (Nellore). Our hypothesis was that Nellore (*Bos indicus*) embryos are affected less by heat stress than are Angus and Jersey (*Bos taurus*) embryos.

2. Materials and methods

All reagents and media used were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Animals were housed and cared for in accordance with the guidelines described by the ethics committee of the University of São Paulo State (IB, Botucatu, Brazil).

2.1. Oocyte collection and grading

On random days of the estrous cycle, cumulus-oocyte complexes (COCs) were collected using transvaginal ultrasound-guided oocyte collection with a 5-MHz sectorial transducer equipped with a needle guide and attached to an Aloka 900 ultrasound scanner (Aloka, Tokyo, Japan). Cumulus-oocyte complexes were aspirated using a 17-ga needle and a vacuum pump set to a pressure of 70 mm Hg. Cumulus-oocyte complexes were collected from all visible follicles $\geq$3 mm. The contents of each follicle were collected into a 50 mL conical tube with 10 mL of oocyte collection medium. All COCs were washed in TCM-199 maturation medium for oocytes with HEPES supplemented with 10% (vol/vol) bovine fetal serum (Gibco, Langley, OK, USA), 2 μg/mL pyruvate, and 75 μg/mL gentamicin (wash media), and then placed in oocyte maturation medium for transport back to the laboratory in a portable incubator. The COCs were divided into four categories based on the compactness of the cumulus cells and the homogeneity and transparency of the ooplasm [22]: grade I, oocytes with homogeneous ooplasm and many tight layers of cumulus cells; grade II, oocytes with homogeneous ooplasm and two or three cumulus cell layers; grade III, oocytes with heterogeneous ooplasm surrounded by one cumulus cell layer; and grade IV, denuded oocytes. Grades I, II, and III oocytes were pooled across cows within the same breed.

2.2. In vitro embryo production

Embryo production was performed as described [15]. Briefly, COCs classified in categories I, II, and III were washed three times in wash media, placed in groups of 20 in 90 μL microdrops of TCM-199 supplemented with 10% (vol/vol) bovine fetal serum, 2 μg/mL pyruvate, 75 μg/mL gentamicin, 20 μg/mL FSH (Pluset; Hertape Calier, Juatuba, MG, Brazil), and 10 IU/mL LH (Choriomon; Vivimed, Ribeirão Preto, SP, Brazil) overlaid with mineral oil and matured for 22 to 24 hours at 38.5 °C in an atmosphere of 5% (vol/vol) CO_2 in humidifled air. Matured COCs were washed three times in TCM-199 with HEPES and once in IVF-Tyrode's albumin lactate pyruvate [23] supplemented with 6 mg/mL fatty acid-free bovine serum albumin (BSA), 2 μL/mL pyruvate, 75 μg/mL gentamicin, 11 μg/mL heparin, and 44 μL/mL 0.5 mM penicillamine, 0.25 mM hypotaurine, transferred in groups of 20 oocytes per 90-μL drop of IVF-Tyrode's albumin lactate pyruvate, overlaid with mineral oil, and fertilized with approximately 1×10^6 Percoll-purifled sperm from a pool of frozen-thawed semen. The day of fertilization was considered Day 0. After 10 to 12 hours at 38.5 °C in an atmosphere of 5% (vol/vol) CO_2 in humidifled air, putative zygotes were removed from the fertilization drops, denuded of cumulus cells by repeated pipetting in TCM-199 with HEPES, and placed in groups of 20 in 90-μL microdrops of synthetic oviduct fluid [15] supplemented with 5% (vol/vol) fetal bovine serum, 5% BSA, and 0.2% sodium pyruvate overlaid with mineral oil. Culture plates were placed in sealed bags with an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for the entire culture period.

The culture medium was partly replaced with 50 μL of fresh medium at 48, 96, and 144 hours after fertilization. Before each of these feedings, cleavage, morula, and blastocyst yields were evaluated.

2.3. Experiments

2.3.1. Effects of heat stress on embryo development

This experiment was designed to test the effects of heat stress on embryonic development. Cumulus-oocyte complexes were aspirated from five Nellore and seven Jersey cows, and embryos were produced as described above. Cows were aspirated within a 21-day interval between May and November. For this experiment, a pool of semen from two bulls (from a total of six bulls of each breed) were used in each replicate. All sires had previously been approved for IVF. On Day 1, putative zygotes were randomly distributed in two groups: (1) a control group, in which embryos were kept at 38.5 °C throughout the culture period; and (2) a heat stress group, in which embryos were cultured at 38.5 °C until Day 4, on which they were cultured at 41 °C for 6 hours. Thereafter, embryos were returned to

38.5 °C for the remainder of the culture period. At each feeding, degenerated structures were removed from each drop. The cleavage rate was assessed on Day 2, and the blastocyst rate was recorded on Day 7. On Day 7, embryo development was recorded and blastocysts were selected from the culture drops and frozen for RNA extraction.

2.3.2. Effects of heat stress on mRNA expression in Nellore and Jersey embryos

This experiment was designed to test the effects of heat stress on the expression of developmentally important genes in Nellore and Jersey blastocysts collected in the experiment described in section 2.3.1. Total blastocyst RNA was extracted from pools of five embryos (N = 9 pools in the Nellore control group, N = 7 pools in the Nellore heat stress group, N = 5 pools in the Jersey control group, and N = 5 pools in the Jersey heat stress group) using the RNeasy kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Total extracted RNA was stored at −80 °C until reverse transcription-polymerase chain reaction (RT-PCR) analysis; RNA samples (8 μL) were incubated with DNAse I (1 U/μg; Invitrogen) and reverse-transcribed with SuperScript III (Invitrogen) and oligo-dT primers.

Quantitative real-time RT-PCR analysis of four developmentally important genes (*COX2, CDX2, HSF1*, and *PLAC8*) and a housekeeping gene (peptidylprolyl isomerase A; *PPIA*) was performed on each pool of control or heat-stressed Nellore or Jersey embryos. Specific primers (Table 1) were designed using Integrated DNA Technologies software (http://idtdna.com). To choose the most stable housekeeping gene for analysis, we analyzed the amplification profiles of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *PPIA*, and histone H2AFZ (*H2AFZ*) using the geNorm applet for Microsoft Excel (http://medgen.ugent.be/jvdesomp/genorm/) [24]. The geNorm is a popular algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel. From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes (at least three). The software shows values for each one and that with the lower level is the most stable gene (in this case, *PPIA*). Power Sybr Green PCR Master Mix (Applied Biosystems) reaction chemistry and the ABI Prism 7500 Sequence Detection System (Applied Biosystems) were used to quantify mRNA concentrations, and the specificity of each PCR product was determined through melting curve analysis. Negative controls (in which water replaced cDNA) were run in each plate. Duplicate reactions of each sample were analyzed. Target gene mRNA abundance was expressed relative to the level of *PPIA* mRNA. The relative expression of each gene was determined using the ΔΔCt method with efficiency correction (Pfaffl's equation) [24].

2.3.3. Effects of heat stress on embryonic survival after transfer to recipients

This experiment was designed to test the effects of heat stress on embryonic survival after transfer. Cumulus-oocyte complexes were aspirated from six Nellore and seven Angus cows. Each cow was aspirated four times within a 15-day interval between the months of May and November. Embryos were produced as described above. In this experiment, oocytes were fertilized by semen from two bulls within each breed. Bulls had previously been tested for IVF. On Day 4, embryos ≥16 cells were randomly divided in two experimental groups: a control group, in which embryos were kept at 38.5 °C throughout the culture period (N = 122 Nellore embryos and N = 47 Angus embryos), and a heat stress group, in which embryos were cultured at 41 °C for 12 hours and then at 38.5 °C (N = 121 Nellore embryos and N = 40 Angus embryos). Blastocyst rate and quality were recorded on Day 7, when embryos were selected from culture drops, washed in HEPES and loaded into 0.25 mL straws. Grades 1, 2, and 3 embryos [25] were immediately transferred into recipients synchronized according to the PEPE protocol. In this procedure, cows received an intravaginal progesterone device (1.0 g, Primer; Tecnopec, São Paulo, SP, Brazil) and 2.5 mg of estradiol benzoate im (BER-BE; Syntex, Buenos Aires, Argentina) followed by an administration of an analog of $PGF_{2\alpha}$ im (150 mg dcloprostenol; Prolise; RARS SRL, Buenos Aires, Argentina). The Primer was removed and after 24 hours and a new application of estradiol benzoate was carried out. The embryos in the seventh day of culture (in the blastocyst stage) were transferred 7 days after estrus detection in female recipients. The recipients selected were crossbred heifers (*Bos taurus* × *Bos indicus*), previously ranked according to their health status and reproductive capacity (via transrectal ultrsonography). The experiment was replicated four times using 311 embryos. Pregnancy was diagnosed by ultrasound between Days 30 and 35.

Table 1
Primers used for quantitative polymerase chain reaction.

Accession number	Name	Sequence	Melting temperature (°C)	Product size
NM_174445	*COX2*	F 5′ AAGCCTAGCACTTTCGGTGGAGAA R 3′ R 5′ TCCAGAGTGGGAAGAGCTTGCATT 3′	60	168
NM_1206299	*CDX2*	F 5′ TGGAGCTGGAGAAGGAGTTTCACT 3′ R 5′ TCCTTCGCTCTGCGGTTCTGAAAT 3′	56	133
NM_1076809	*HSF1*	F 5′ AAGCACAGCAACATGGCTAGCTTC 3′ R 5′ AGTGGACACACTGGTCACTTTCCT 3′	60	189
NM_1076987	*PLAC8*	F 5′ GAC TGG CAG ACT GGC ATC TT 3′ R 5′ CTC ATG GCG ACA CTT GAT CC 3′	60	140
NM_178320	*PPIA*	F 5′ GCC ATG GAG CGC TTT GG 3′ R 5′ CCA CAG TCA GCA ATG GTG ATC T 3′	60	65

Abbreviations: F, forward; R, reverse.

2.3.4. Effects of heat stress on total cell number and apoptosis

This experiment was designed to test the effects of heat stress on embryo quality measured as a percentage of apoptotic blastomeres. Cumulus-oocyte complexes were aspirated twice at 15-day intervals from each of six Nellore and five Angus cows. Embryos were produced as described for the experiment in section 2.3.3. On Day 4, embryos ≥16 cells were randomly divided into two experimental groups: (1) control group, in which embryos were kept at 38.5 °C throughout the culture period; and (2) heat stress group, in which embryos were cultured at 41 °C for 12 hours and then at 38.5 °C. On Day 5, embryos were removed from culture drops, washed, and submitted to a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay [26]. Briefly, embryos were washed twice in 50 μL microdrops of PBS–Polyvinylpyrrolidone (PVP). Zona pellucida-intact embryos were fixed in 50 μL microdrops of 4% (wt/vol) paraformaldehyde in PBS for 1 hour and then permeabilized in 0.5% (vol/vol) Triton X-100 containing 0.1% (wt/vol) sodium citrate for 1 hour. Positive controls for the TUNEL assay were incubated in DNase (50 U/mL) at 37 °C in the dark for 1 hour. Positive controls and experimental embryos were washed in PBS–PVP and incubated with 25 μL TUNEL reaction mixture (prepared following the guidelines of the manufacturer) for 1 hour at 37 °C. Negative controls were incubated without the enzyme. Each embryo was washed thrice in PBS–PVP and incubated in a 25 μL microdrop of Hoescht 33342 (1 μg/mL) for 15 minutes. Embryos were then mounted in glycerol. Labeling was observed using an epifluorescence microscope (Leica Dx, São Paulo, SP, Brazil). Each embryo was analyzed for total cell number and TUNEL-positive blastomeres using 4′,6-diamidino-2-phenylindole and fluorescein isothiocyanate filters, respectively. This experiment was replicated one time with 17 to 20 embryos in each treatment group.

2.4. Statistical analysis

Data on the percentage of oocytes that cleaved and became morulae and blastocysts, total cell number, and the percentage of cells that were TUNEL-positive were analyzed by least-squares ANOVA, using the General Linear Models procedure of SAS (SAS for Windows, Version 9.0; SAS Institute Inc., Cary, NC, USA). The data were arcsin-transformed before analysis. The mathematical model included two main effects (temperature and breed) and all interactions. Replicate was considered as a random effect, and other main effects were considered fixed. Differences in individual mean values were analyzed through pair-wise comparisons (SAS probability of difference analysis). All values were reported as the least-squares mean ± SEM.

Data regarding the percentage of cows that became pregnant after embryo transfer were analyzed by logistic regression using the LOGISTIC procedure of SAS. The same technician conducted embryo transfer and pregnancy diagnosis throughout the experiments, so these effects were not considered in the analysis. Initial analysis indicated that the effects of replicate were not significant. Treatment effects (temperature and breed) were also compared using orthogonal contrasts.

For gene expression analysis, the effects of both breed and temperature were tested by ANOVA and the levels of target gene expression were compared using orthogonal contrast. Data were presented as the mean ± SEM. Analyses were performed using the JMP (7.0) program of SAS.

Differences were considered significant when $P < 0.05$, and values of $P \geq 0.05$ and ≤ 0.1 were regarded as a tendency.

3. Results

3.1. Effects of heat stress on in vitro embryo production

Least-squares mean ± SEM of the percentages of cleaved embryos, morulae, and blastocysts are shown (Table 2). There was a difference ($P < 0.05$) between the Nellore and Jersey breeds in the percentage of oocytes that cleaved. However, there was no significant difference between breeds or temperatures in the percentage of cleaved embryos that became morulae.

Heat stress decreased ($P < 0.05$) the percentage of cleaved embryos that became blastocysts in the Jersey breed, but had no effect in Nellore embryos. There was no significant interaction between temperature and breed for cleaved embryos, morulae, or blastocyst per cleaved embryo.

3.2. Effects of heat stress on mRNA expression in the bovine blastocyst

In both Nellore and Jersey breeds, RNA levels for the *PLAC8* and *CDX2* genes were lower ($P < 0.05$) in pools of blastocysts submitted to heat stress than in the control groups. In addition, expression of these genes was higher in the blastocysts of the Nellore control group than in those of the Jersey control group (Fig. 1).

Heat stress decreased ($P < 0.05$) the levels of mRNA of the *HSF1* gene in the Nellore heat stress group relative to those in all other groups (Fig. 1).

The levels of mRNA for the *COX2* gene differed ($P < 0.05$) between the Jersey heat stress group and the Nellore groups (control and heat stress; Fig. 1), but did not differ within Jersey groups.

3.3. Effects of heat stress on embryonic survival

Overall cleavage rates were 72% and 56% for Nellore and Angus embryos, respectively. In both breeds, heat stress at 41 °C decreased ($P < 0.05$) the percentage of ≥16-cell embryos that became blastocysts when compared with

Table 2
Effect of heat stress (41 °C) on oocyte cleavage, morulae, and blastocyst per cleaved embryo rates.

Breed	°C	Oocytes, N	CL (%)	MO/CL (%)	BL/CL (%)
Nellore	38.5	148	84.5 ± 2.3^{a}	70.8 ± 3.7	39.5 ± 2.5^{a}
	41	144	85.6 ± 2.1^{a}	71.5 ± 3.7	33.0 ± 2.5^{a}
Jersey	38.5	118	67.9 ± 4.2^{b}	63.6 ± 3.7	34.6 ± 2.5^{a}
	41	163	56.5 ± 2.8^{b}	61.0 ± 3.7	25.0 ± 2.5^{b}

Effect of temperature on: CL = not significant; MO/CL = not significant; BL/CL = 0.02. Effect of breed on CL; MO/CL; BL/MO ≤0.05. There was no interaction temperature × breed for CL, MO/CL, or BL/CL. Within a column, means without a common superscript letters differed ($P < 0.05$).
Abbreviations: BL, blastocyst; CL, cleavage; MO, morulae.

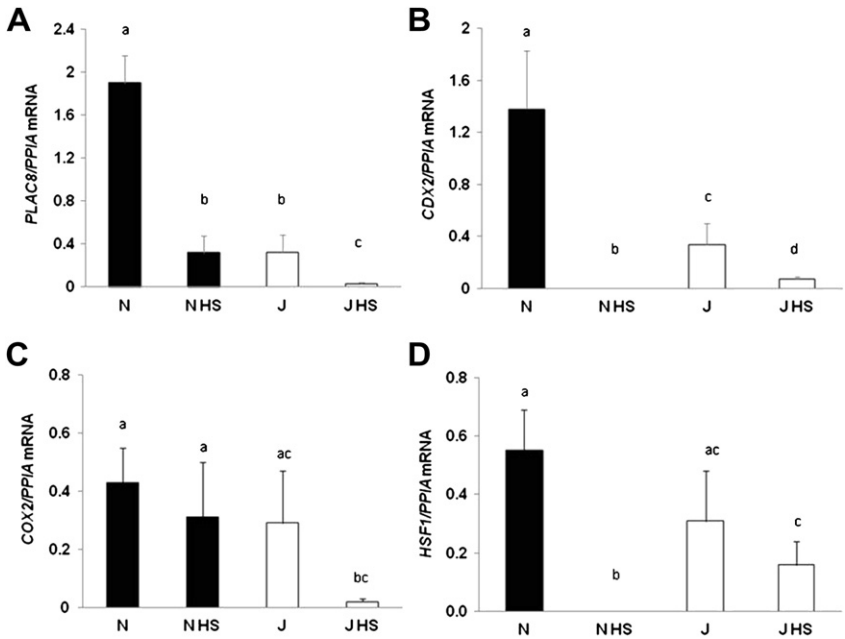

Fig. 1. Expression of *PLAC8* mRNA (A), *CDX2* mRNA (B), *COX2* mRNA (C), and *HSF1* mRNA (D), relative to *PPIA* (mean ± SEM) in pools of five Nellore (N; solid bars) and Jersey (J; open bars) blastocysts submitted to heat stress (HS) or control groups. Within a gene, means without a common lowercase letter differed ($P < 0.05$).

embryos cultured at 38.5 °C (Table 3). Logistic regression analysis detected a tendency ($P = 0.1$) for heat stress to decrease pregnancy rates at Days 30 to 35 for Angus embryos. There was no significant interaction between breed and temperature in the percent of blastocyst or pregnancy rate. When pregnancy rates were compared using contrasts, recipients that received heat-stressed Angus embryos had lower pregnancy rates ($P = 0.08$) than all other groups.

3.4. Effects of heat stress on blastocyst total cell number and apoptosis

In this experiment, cleavage rates were 69% and 58% for Nellore and Angus embryos, respectively. Culture at 41 °C for 12 hours did not affect the total cell number in either Nellore or Angus embryos. However, the total cell number was greater in Nellore embryos than in Angus embryos (27.32 vs. 21.72; $P < 0.05$). The percentages of blastomeres that were TUNEL-positive were 3.75 ± 0.006 and 4.25 ± 0.016 in the Nellore control and heat stress groups, respectively, and 5.17 ± 0.001 and 6.22 ± 0.018 in the Angus control and heat stress groups, respectively ($P > 0.05$). Heat stress increased ($P < 0.05$) the percentage of apoptotic blastomeres in embryos cultured at 41 °C relative to embryos cultured at 38.5 °C (5.75 vs. 3.95). In general, there was a higher percentage ($P < 0.05$) of apoptotic blastomeres in Angus embryos than in Nellore embryos (5.7 vs. 4.0), but no there was no significant interaction between breed and treatment for either total cell number or apoptosis rate.

Table 3
Percent of ≥16-cell embryos that develop to blastocyst and pregnancy rate (percent) at Days 30 to 35 after transfer from control and heat stressed Nellore and Angus embryos.

	Embryos ≥16 cells	Blastocysts (%)	Pregnancy (%)
Nellore			
Control	122	$46.2 \pm 6.9^{a,b}$	21.8 ± 10
Heat stress	121	31.6 ± 6.9^{b}	21.3 ± 10
Angus			
Control	47	59.5 ± 6.9^{a}	18.2 ± 10
Heat stress	40	31.6 ± 6.9^{b}	12.5 ± 10

Effect of breed was not significant in the percent of blastocyst or pregnancy rate. Effect of temperature was $P = 0.008$ for percent of blastocyst and not significant for pregnancy rate. There was no significant breed × temperature interaction in the percent of blastocyst or pregnancy rate. Within a column, means without a common superscript letters differed ($P < 0.05$).

4. Discussion

Embryos subjected to heat stress at 41 °C had lower rates of development that advanced to the blastocyst stage and reduced quality, i.e., increased apoptosis and poor expression of important survival genes. In all cases, Nellore (*Bos indicus*) embryos were less affected by heat stress than were Jersey and Angus embryos (*Bos taurus*).

Heat stress decreased the percentage of embryos developing to the blastocyst stage, especially in taurine embryos, in agreement with reports by Paula-Lopes et al. [13] and Hernández-Cerán et al. [27]. In those studies, Brahman [13,27] and Romosinuano [27] embryos were more resistant to heat stress (41 °C, 6 hours) than Holstein [13] and Angus [13,27] embryos. Similarly, Barros et al. [28], Eberhardt et al. [14], and Satrapa et al. [15] reported that embryos of both pure and mixed *Bos taurus* breeds were

more sensitive to heat stress (41 °C, 12 hours) than *Bos indicus* embryos.

Overall, Jersey embryos had poorer development when compared with Nellore and Angus embryos. It is well known that *Bos taurus* cows have lower reproductive performance in tropical and subtropical regions than *Bos indicus* cows. Furthermore, oocytes from dairy breeds are less competent to develop to blastocysts than those from beef breeds [29]. The sum of these two factors might explain the poorer development in the Jersey oocytes when compared with the Nellore and Angus oocytes, two breeds with beef genotypes.

There is evidence that *Bos indicus* and some *Bos taurus* breeds (Senepol and Romosinuano) have developed genes that protect their cells from the deleterious effects of high temperatures [30]. For example, Brahman and Senepol lymphocytes were more resistant to cellular apoptosis caused by elevated temperatures than were Angus and Holstein lymphocytes [13]. Nevertheless, most of the genes that confer resistance to heat stress in zebu breeds have not yet been identified.

In the present study, differential mRNA expression between the control and heat stress groups occurred in both Nellore embryos and Jersey embryos.

When cells are exposed to a stressor such as heat, a cellular response is initiated that minimizes the deleterious effects of the stressor. In mammals, this response includes an alteration in the expression of genes within the heat stress transcription factor system (HSF) [31]. Heat stress factor 1 (*HSF1*) is the main heat stress transcription factor; it binds to the promoter region of the heat stress protein (HSP) genes, resulting in rapid induction of HSP expression in cells that are submitted to a stressor [32]. In the present study, there was a decrease in *HSF1* mRNA expression in the groups submitted to heat stress in both breeds. Although HSF1 protein levels were not investigated, it is possible that during heat stress, *HSF1* mRNA was used in the formation of the protein through posterior binding in the promoter region of the HSP genes, and therefore, in the induction of these proteins to protect the cells.

As described by Li et al. [33] and Bulman and Nelson [34], after the detection of stress, HSF1 acquires a high level of transcriptional activity, which is then gradually lost upon removal of the stress. The gradual loss of activity after the removal of stress appeared to be promoted by post-translational modifications that contributed to the repression of HSF1 transcriptional activity. Perhaps if we had measured *HSF1* mRNA right after heat stress, we would have found an opposite response, with a higher expression in heat stressed embryos.

The *CDX2* is a transcription factor that is exclusively expressed in the trophectoderm and is important for implantation and placental development [35]. The *CDX2* also stimulates *IFNT2* promoter activity, which is important for pregnancy recognition [36]. An invasion gene expressed in the trophectoderm, *PLAC8*, is important for placental development and maternal–fetus communication [37]. In the present study, the expression of *CDX2* and *PLAC8* was reduced by heat stress in both Nellore and Jersey breeds. In addition, expression of these two genes was higher in Nellore embryos from the control group than in embryos from all other groups.

El-Sayed et al. [20], studying embryos produced *in vitro*, reported that *CDX2* and *PLAC8* were expressed at higher levels in bovine embryos that successfully resulted in pregnancy than in embryos that did not result in pregnancy after transfer to recipients. In a study using embryos derived *in vivo*, Ghanem et al. [21] also reported *PLAC8* to be highly expressed in cells from embryos that became calves. Furthermore, Lazzari et al. [38] detected higher levels of expression of *CDX2* and *PLAC8* in bovine embryos derived from mixed breeds than in purebred taurine embryos. Perhaps zebu embryos have a greater capacity to establish a pregnancy because they express higher levels of *CDX2* and *PLAC8*.

Based on RNA analysis of *COX2*, a gene that transcribes an enzyme related to prostaglandin synthesis and is expressed in the trophectoderm of preimplantation embryos [20], heat stress tended to decrease ($P = 0.1$) the expression of this gene in Jersey embryos but had no effect in Nellore embryos. Because *COX2* is more abundant in blastocysts that become calves after transfer than in those that result in abortion [20], perhaps Jersey embryos submitted to heat stress were less likely than Nellore embryos to result in pregnancy after transfer.

Heat stress did not affect the percentage of embryos that survived after transfer in either the Nellore group or the Angus group, although there was a tendency toward decreased pregnancy rates for Angus embryos. It might be that the relatively low number of embryos transferred resulted in a lack of statistical power.

In the present study, neither Nellore nor Angus embryos exhibited a reduction in the total number of cells 12 hours after heat stress. In contrast, Jousan and Hansen [39] and Loureiro et al. [26] reported a reduction in the total number of blastomeres at 9 and 15 hours, respectively, after heat stress. Those two studies differed from the present study in the stage of development at which the embryos were stressed. The present study used early Day 4 embryos with 16 or more cells (approximately 22 cells). Jousan and Hansen [39] used Day 5 embryos with 16 or more cells (approximately 60 cells), and Loureiro et al. [26] heat-stressed Day 6 embryos with more than 48 cells. At the 16-cell stage (fourth to fifth cell cycles), mitosis takes 40 to 50 hours, whereas subsequent cell cycles last for approximately 20 to 24 hours [40,41]. Perhaps the time given to the embryos in this study was not sufficient to reveal a decrease in cell number.

4.1. Conclusions

We concluded that heat stress affects the bovine embryo by decreasing developmental rates, increasing the percentage of apoptotic blastomeres, and decreasing the expression of important developmental genes. Furthermore, these effects of heat stress were more evident in *Bos taurus* embryos than in *Bos indicus* embryos.

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Angus Bela Vista Farm and Pinheiros Farm provided the cattle used in the experiments.

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