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Developmental competence and expression of the Hsp 70.1 gene in oocytes obtained from *Bos indicus* and *Bos taurus* dairy cows in a tropical environment

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Abstract

Bos indicus cows usually have better reproductive performance in tropical and subtropical regions than *Bos taurus* cows, presumably due to their better adaptation to tropical environments. The aim of this study was to evaluate the developmental competence and expression of the Hsp 70.1 gene in immature oocytes from *B. taurus* (Holstein) and *B. indicus* (Gyr) dairy cows raised in a tropical region. Cumulus–oocyte complexes were obtained by transvaginal ultrasound-guided follicle aspiration between spring and early autumn, and subjected to in vitro maturation and fertilization. Presumptive zygotes were co-cultured with their own cumulus cells in CR2aa media with 10% fetal calf serum; Grade 1 blastocysts were transferred to synchronized crossbred recipients. The total RNA was extracted from immature Holstein and Gyr oocytes (three pools for each breed) and relative quantification of the Hsp 70.1 transcripts was performed by real time PCR after reverse transcription. Cleavage and blastocyst rates were greater ($P < 0.05$) for Gyr ($n = 390$ oocytes) than Holstein ($n = 505$) breed (66.7% versus 53.1% of cleavage and 19.6% versus 10.8% of blastocysts, respectively), but pregnancy rates were not significantly different following transfer to recipients (44.5% for 36 Gyr embryos; 60% for 10 Holstein embryos). Holstein immature oocytes had a higher level ($P < 0.05$) of Hsp 70.1 relative expression (1.82 ± 0.22; mean ± S.E.M.) than Gyr oocytes (1.12 ± 0.11). In conclusion, Gyr oocytes obtained in a tropical region were less subject to stress and more likely to develop (after IVF) than Holstein oocytes.

Keywords: Heat stress; Oocyte; In vitro fertilization; Heat shock protein; *Bos indicus*

1. Introduction

European (*B. taurus*) dairy cows frequently have low conception rates during the summer and autumn seasons, presumably due to heat stress [1]. In Holstein cows, heat stress seems to decrease oocyte competence [2] and the fertilization rate [3], reducing embryo development and contributing to poor fertility during warmer months. This susceptibility to heat stress makes European dairy breeds unpopular in tropical and subtropical countries where special care and housing are needed to reduce the effects of heat on fertility and milk yield. In contrast, zebu cattle (*Bos indicus*) are well adapted to tropical environments, demonstrating a high tolerance to heat. *B. indicus* cattle have displayed better

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reproductive performance than *B. taurus* when in tropical and subtropical regions [4]. This adaptation may involve different mechanisms. A severe decline in oocyte quality of Holstein cows was observed in the warmer season when compared to cool season, whereas no difference was detected between seasons in oocytes obtained from Brahman, a *B. indicus* beef breed [5]. Furthermore, lymphocytes from Brahman and Senepol cows were less susceptible to heat-induced apoptosis [6], suggesting a protective mechanism in *B. indicus* cells.

Heat shock proteins (Hsp) are chaperones that promote cell protection against heat damage, preventing protein denaturation [7], and blocking apoptosis; its transcription is increased by heat shock as well other stress stimuli [8] and can be an indicator of stress in bovine embryos [9]. In mature oocytes exposed to heat stress, Hsp 70 mRNA did not increase, which may be result of an inactivity of their transcription [10], despite the fact that heat shock during maturation can lessen developmental competence after fertilization [11].

Among *B. indicus* cattle, Gyr is the main dairy breed used in the tropics. It is widely used in South America, mainly in Brazil, where genetic improvement has increased its average milk yield to ~3200 kg/lactation, with several cows producing >12,000 kg. Like other *B. indicus* breeds, Gyr cattle are well adapted to tropical regions. However, there is no study showing the competence of Gyr oocytes as well the expression of genes linked to thermotolerance. Studies of oocyte competence associated to gene expression may help to understand the mechanism used by thermotolerant breeds to maintain reproductive performance in tropical environments. Thus, the aim of this study was to compare in vitro development and expression of the Hsp 70.1 gene in oocytes obtained from Gyr and Holstein cows kept in a tropical environment.

2. Materials and methods

All chemicals used were from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise.

2.1. Animals and ovum-pick up

The experiment was performed using non-lactating adult Gyr ($n = 13$) and Holsteins ($n = 9$) cows raised in a tropical region of Brazil, located at 21°35″S latitude, 43°51″W longitude and 435 m altitude. All cows had regular reproductive cycles and a body condition score between three and four on a scale of one (thin) to five (fat). The Holstein cows were housed in free-stalls, but with free access to an open area with pasture and shade. Gyr cows were kept only in a pasture with shade. The cumulus–oocyte complexes (COCs) were harvested by transvaginal ultrasound-guided follicle aspiration (TGFA) between November (spring) and April (early autumn), with at least two TGFA sessions/cow for both breeds. Daily temperature and humidity data were recorded at a meteorological station on the same farm. Mean (±S.E.M.) average daily minimum and maximum temperatures were 19.1 ± 0.15 and 30.2 ± 0.2 °C, and the relative humidity was $78.2 \pm 1.4\%$.

Prior to TGFA, the cows received 20 mg i.m. of acepromazine maleate (Univet, Sao Paulo, SP, Brazil) and caudal epidural anesthesia (3–5 mL of 2% lidocaine; Eurofarma, Sao Paulo, SP, Brazil). Ovaries were visualized using an ultrasound scanner equipped with a 7.5 MHz sector transducer and a needle guide mounted in a vaginal probe (Scanner 100S, Pie Medical, Maastricht, Netherlands); follicles >2 to 3 mm were punctured using 19G disposable hypodermic needles. Follicular fluid and cumulus–oocyte complexes (COCs) were collected in a warmed 50 mL-tube with modified Dulbecco's PBS (Nutricell, Campinas, SP, Brazil) supplemented with 10% fetal calf serum (FCS; Nutricell) and 10 IU/mL sodium heparin (Liquemine, Roche, Basel, Switzerland). After TGFA, the COCs were analyzed and those classified as viable (with one or more layers of cumulus cells and homogenous cytoplasm) were transported to the laboratory in 1.5 mL tubes in TCM199 medium supplemented with 25 mM HEPES (Invitrogen–Gibco BRL) and 10% of FCS (temperature, 37–38 °C). In some TGFA sessions, the rate of viable COCs in relation to the total number of obtained COCs was recorded for both breeds ($n = 11$ TGFA sessions for Holstein and $n = 20$ for Gyr, respectively).

2.2. In vitro maturation and fertilization

The COCs were washed twice in TCM199 + Hepes medium and matured in vitro in TCM 199 (Invitrogen–Gibco BRL) with 10% inactivated estrous cow serum and 20 μg/mL of FSH (Pluset, Serono, Italy) for 22–24 h, in a humidified atmosphere of 5% CO_2 and 38.8 °C in air. Holstein and Gyr oocytes were in vitro fertilized by Holstein and Gyr semen, respectively. To reduce bull effect, semen with a similar cleavage rate, previously evaluated by in vitro fertilization with oocytes obtained from abbatoir-derived ovaries, was used. Spermatozoa were obtained by the swim-up method [12], using Sperm TALP [13] supplemented

with 6 mg/mL BSA fraction V. In vitro fertilization was performed in drops with 100 μL of fertilization medium [13] supplemented with 20 μg of heparin/mL and 6 mg/mL of fatty free-acid BSA fraction V covered with mineral oil for 18 h in a humidified atmosphere of 5% CO_2 and 38.8 °C in air. The sperm concentration during fertilization was 2×10^6 spermatozoa/mL.

2.3. In vitro embryo culture

After fertilization, oocytes were partially stripped by mechanical pipetting in TALP medium [13] until one or two cumulus cells layers were remaining. Groups of 12–18 presumptive zygotes with their respective cumulus cells were then cultured in 50 μL CR2aa medium [14] supplemented with 10% FCS and 1 mg/mL BSA, covered with mineral oil. Embryo culture was performed in 5% CO_2 and a humidified atmosphere at 38.8 °C in air. Half of the medium was replaced at 72 h post-insemination (hpi), when cleavage and 8 to −16-cell embryo rates were evaluated. The blastocyst rate was assessed at approximately 180 hpi.

2.4. Embryo transfer

To evaluate blastocyst viability, fresh Gyr ($n = 36$) and Holstein ($n = 10$) blastocysts were transferred to recipients. Blastocysts classified as Grade 1 (IETS Manual) were washed twice in DPBS supplemented with 0.4% BSA and loaded into a 0.25 mL straw. For both breeds, embryos were transferred non-surgically to the uterine horn ipsilateral to the CL of synchronized *B. indicus* versus *B. taurus* crossbred recipients, kept in the same nutritional and environmental conditions. Transfers were conducted monthly. Pregnancy was determined by transrectal ultrasonography between 40 and 50 days after embryo transfer.

2.5. RNA extraction, reverse transcription and real time PCR amplification

COCs with one or more layers of cumulus cells, classified as viable, were obtained by TGFA from Gyr ($n = 5$) and Holstein ($n = 4$) cows in late spring (December). Cumulus cells were removed by vortexing the COCs in TALP-HEPES plus 3 mg/mL BSA. Oocytes were washed three times and then pooled randomly (three pools of 12 immature oocytes for each breed); they were rapidly frozen in liquid nitrogen, stored at −80 °C, and subsequently thawed for RNA extraction. Total RNA extraction was performed using Rneasy Micro kit (Qiagen, Valencia, CA, USA), treated with DNAse, and the first strand was synthesized using Superscript™ III First Strand Synthesis kit (Invitrogen, Chicago, IL, USA). Relative quantification was performed in duplicate using real time PCR (ABI Prism® 7000, Applied Biosystem, Foster City, CA, USA) and reactions using a mixture of iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Waltham, MA, USA) with cDNA equivalent to 1.2 oocytes and gene specific primers. Template cDNA was denatured at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s; gene-specific primer annealing temperature for 30 s, and elongation at 72 °C for 45 s. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single specific product was generated. Amplicon size was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. Negative controls, comprised of the PCR reaction mix, without nucleic acid, were also run with each group of samples. Expression of the GAPDH gene was used as an endogenous reference. Calculations of relative quantification were performed by the comparative Ct method, using the lowest value found in *B. indicus* oocytes as a calibrator, and values are shown as *n*-fold difference relative to the calibrator. The primers quantifying Hsp 70.1 (GeneBank accession number U09861) were forward 5′-AACAAGATCACCATCACCAACG and reverse 5′-TCC TTC TCC GCC AAG GTG TTG with an annealing temperature of 59 °C to amplify a 275 bp fragment. The GAPDH (GeneBank accession number BC102589) primers were forward 5′-CAGGAGCACGAGAGGAAGAGTT and reverse 5′-GGCCTTAGAGATGGAAACATGTG with an annealing temperature of 55 °C to amplify a 102 bp fragment.

2.6. Statistical analysis

Data for the number of oocytes, cleaved embryos, blastocysts per TGFA session and relative quantification are reported as means (±S.E.M.), and were evaluated by ANOVA (SPSS 12.0 SPSS Inc., Chicago, IL, USA). Viable COCs, cleavage, 8–16 cell embryo, and blastocyst rates were assessed by Chi-square. Pregnancy and calving rates were evaluated by Fisher's exact test.

3. Results

3.1. In vitro embryo development

The number of oocytes, cleaved embryos and blastocysts were similar ($P > 0.05$) between Gyr and Holstein breeds (Table 1) as well as the rate of viable

Table 1
Mean (±S.E.M.) number of oocytes and embryos per transvaginal ultrasound-guided follicle aspiration (TGFA) session in dairy cattle (no significant difference between breeds for any end point)

Breed	No. TGFA sessions	No. oocytes (range)	No. cleaved embryos (range)	No. blastocysts (range)
Holstein ($n = 13$ donors)	24	16.2 ± 2.0 (2–54)	8.6 ± 2.1 (0–38)	1.75 ± 0.5 (0–8)
Gyr ($n = 9$ donors)	33	15.3 ± 1.3 (6–32)	10.2 ± 1.1 (0–24)	3.0 ± 0.6 (0–12)

COCs (71.2% {364/511} for Gyr and 75.1% {253/337} for Holstein) recorded from 20 TGFA sessions for Gyr and from 11 TGFA sessions for Holstein. However, the cleavage, the 8–16 cell stage and the blastocyst rate based on the number of oocytes were greater ($P < 0.05$) for Gyr than Holstein oocytes (Table 2). Although the proportion of cleaved embryos that developed to 8–16 cell stage was not different ($P > 0.05$) between breeds (33.2% {112/337} for Gyr 29.4% {62/207} for Holstein), the blastocyst rate based on those cleaved embryos was greater ($P < 0.05$) for Gyr than for Holstein (Table 2) as well as the blastocyst rate based on the number of 8–16 cell stage embryos at 72 hpi (88.4%; {99/112} for Gyr and 67.7% {42/62} for Holstein).

3.2. Pregnancy and calving rate

The viability of embryos was estimated transferring single Grade 1 embryo to the uterus of synchronized recipients. There were no differences ($P > 0.05$) in pregnancy and calving rates between in vitro produced blastocysts derived from Holstein and Gyr oocytes (60.0% {6/10} and 50.0% {5/10} for Holstein and 44.4% {16/36} and 38.9% {14/36} for Gyr, respectively).

3.3. Hsp 70.1 transcript relative quantification

Relative quantification of the Hsp 70.1 gene was performed to detect differential expression between Gyr and Holstein immature oocytes. Oocytes from Holstein cows had a higher level ($P < 0.05$) of Hsp 70.1 expression than oocytes obtained from Gyr cows (1.82 ± 0.22 for Holstein and 1.12 ± 0.11-fold difference for Gyr oocytes).

4. Discussion

The present experiment compared the development of oocytes obtained from Holstein (*B. taurus*) and Gyr (*B. indicus*) cows in a tropical environment; immature oocytes obtained from Gyr cows were more likely to develop to blastocysts after in vitro maturation and fertilization than immature oocytes from Holstein cows. Moreover, the expression of Hsp 70.1 transcripts differed between Holstein and Gyr immature oocytes; this may be associated with their developmental competence and adaptation to the tropical environment.

Besides differences between subspecies regarding thermo-tolerance, beef and dairy genotypes may also influence in vitro embryo development. Oocytes from beef cows were more competent to develop to blastocysts than those from dairy cows [15]. Therefore, we used only dairy breeds to compare oocyte development between *B. taurus* and *B. indicus*. We also used sperm from the same breed of oocyte to produce purebred embryos, since there are data showing that *B. taurus* versus *B. indicus* crossbred embryos can have lower rates of development than purebred embryos, yielding a negative heterosis [15]. However, to reduce the bull effect on the fertilization rate, sperm previously shown to have similar potential for fertilization in vitro was used.

In tropical environments, *B. indicus* have better reproductive performance than *B. taurus* cattle. That performance may be linked to a greater competence of *B. indicus* oocytes developing to blastocysts. In the present experiment, oocytes were obtained from spring to early autumn, with an average daily maximum temperature at 30.2 °C, which is above the upper critical threshold temperature for Holstein cattle [16]. The environmental conditions in these seasons may have

Table 2
Effect of oocyte donor breed on subsequent embryo development

Breed	No. oocytes	Cleavage no. (%)	8–16 cell stage no. (%)	Blastocysts no. (%)	Blastocysts/cleaved embryos[a] no. (%)
Holstein	390	207 (53.1) a	62 (15.9) c	42 (10.8) a	42 (20.3) c
Gyr	505	337 (66.7) b	112 (22.2) d	99 (19.6) b	99 (29.4) d

Within a column, values with different letters differ (a and b) ($P < 0.01$); (c and d) ($P < 0.05$).
[a] Percentage based on total number of cleaved embryos.

impaired the competence of oocytes from Holstein cows, in contrast to Gyr oocytes. Indeed, the proportion of Holstein oocytes developing to the 8–16 cell and the blastocyst stage was lower than that observed in Gyr oocytes (Table 2), as well as the proportion of cleaved and embryos at 8–16 cell stage developing to blastocyst stage, suggesting that the lower blastocyst rate in Holstein may be not only due to a low fertilization rate but also due to a reduced capacity for development of Holstein embryos. In previous studies, heat stress on bovine oocytes held at the germinal vesicle (GV)-stage interfered in subsequent embryo development, decreasing the proportion of both 8–16 cell embryos and blastocysts [17]. The effect of the season on the competence of immature Holstein oocyte reduced the development of blastocysts [2], whereas the effect was less pronounced in Brahman oocytes [5]. The elevated temperature may also have an effect on follicle cells, altering steroid synthesis [18,19], and contributing to a reduction of oocyte competence. Perhaps the low proportion of Holstein oocytes developing to 8–16 cells and blastocyst stages in the present experiment were due to an effect of environmental temperature on oocytes at the GV-stage and/or on cells inside the follicle, in contrast to Gyr oocytes.

Transcription of Hsp 70 may be increased by stressful conditions like heat shock [8]; its expression can be used as an indicator of stress in bovine embryos [9]. Based on analysis of the Hsp 70.1 mRNA expression, this transcript was more abundant in immature oocytes from Holstein versus Gyr cattle, suggesting that the Holstein oocytes were under higher stress, which may have resulted in the lower embryo development after IVF.

It is not clear why *B. indicus* cows are more resistant to higher temperatures and how this may affect oocyte quality. *B. indicus* cattle have some characteristics that favor regulation of body temperature under elevated environmental temperatures, e.g. less subcutaneous fat, lower body volume/surface area proportion, shorter hair, and larger sweat glands [20–22]. Such characteristics are likely useful for regulating the temperature of the whole body, reducing increases in core-body temperature that would affect the ovaries.

It has been shown that some species can regulate follicle temperature through endothermic reactions within follicles and maintenance of local temperature by counter-current heat exchange mechanisms [23], keeping it cooler than ovary stroma [24,25]. This kind of local thermoregulation mechanism may help to reduce the temperature inside the follicle when in a warm environment. Nevertheless, whether such a mechanism of cooling and maintenance of temperature inside the follicle has different behavior between *B. taurus* and *B. indicus* cows needs to be confirmed. A role of cumulus cells on oocyte thermotolerance has also been suggested, allowing an increase of Hsp protein synthesis in oocytes after heat shock [26]. The protective action of cumulus cells could differ between breeds, since Gyr oocytes had a lower level of Hsp 70.1 mRNA than Holstein, despite their higher developmental competence in a tropical environment.

A cellular mechanism may also be responsible for the thermotolerance of Gyr oocytes. Lymphocytes and embryos of *B. indicus* exposed to heat stress were more resistant than *B. taurus* [6]. It was shown that in vivo produced two- to four-cell stage Brahman embryos were as susceptible to heat shock as Holstein ones [27], whereas eight-cell stage embryos were more resistant [6], suggesting that thermal resistance in *B. indicus* embryos may be caused by differential expression of embryonic genome after major activation [22]. Oocyte competence in *B. indicus* may also involve differential expression of genes during oocyte growth in response to the environment, maintaining better oocyte viability (compared to *B. taurus* breeds). Expression of other genes besides Hsp 70.1 can be stimulated by the environment and may play a role as mediators and/or effectors of the cell stress response [28]. For instance, expression of ubiquitin can be induced by heat stress, avoiding accumulation of heat-denatured proteins that reduce cell viability [29]. In yeast cells, over-expression of ubiquitin might overcome the lack of Hsp expression [30]. Perhaps transcription of other thermotolerance-associated genes besides Hsp could be efficiently stimulated in *B. indicus* immature oocytes under elevated temperatures, allowing greater developmental competence than *B. taurus* oocytes.

It has been suggested that not only the oocyte but also the spermatozoa of *B. indicus* cattle contribute to embryonic resistance to heat shock at pre-implantation stages [31]. Although the present study used Gyr and Holstein sperm with the same cleavage potential and had not exposed the embryos to heat shock, the greater cleavage rate and development after cleavage of Gyr embryos might also be a result of an association between a less stressed oocyte with a *B. indicus* spermatozoa. However, the role of sperm is not clear and results between studies are contradictory [32,33].

Although the number of Holstein blastocysts transferred to crossbred recipients was low, the pregnancy and calving rate obtained with these embryos suggested that the Holstein immature oocyte that overcomes the effect of a tropical environment may

become a blastocyst with a similar competence as a Gyr blastocyst.

Gyr is the main *B. indicus* dairy breed, and is likely to show similar ability to regulate body temperature during heat stress observed in other *B. indicus* cows. Their thermotolerance is likely to be one of the mechanisms accounting for the differences in embryo development between breeds in this study, manifested as oocyte quality, and assuring embryo development in a warm environment.

In conclusion, Gyr immature oocytes obtained in the warm season in a tropical region were more competent to develop to blastocysts after IVD and seemed to be under less stress than Holstein oocytes, indicating that the donor breed affected subsequent in vitro embryo development in the tropics. This developmental competence may account for the better reproductive performance of Gyr cattle in such environmental conditions.

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