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Differences in Heat Tolerance Between Preimplantation Embryos from Brahman, Romosinuano, and Angus Breeds*

J. Hernández-Cerón,[1] C. C. Chase, Jr.,[2] and P. J. Hansen[3]
[1]Departamento de Reproducción,
Facultad de Medicina Veterinaria y Zootecnia,
Universidad Nacional Autónoma de México,
Apartado Postal 22-256 México D.F. 14000, México
[2]Subtropical Agricultural Research Station, USDA,
ARS, Brooksville, FL 34601-4672
[3]Department of Animal Sciences, University of Florida,
Gainesville 32611-0910

ABSTRACT

Exposure to 41°C reduces development of embryos of heat-sensitive breeds (Holstein and Angus) more than for embryos of the heat-tolerant Brahman breed. Here it was tested whether embryonic resistance to heat shock occurs for a thermotolerant breed of different genetic origin than the Brahman. In particular, the thermal sensitivity of in vitro produced embryos of the Romosinuano, a *Bos taurus*, Criollo-derived breed, was compared to that for in vitro produced Brahman and Angus embryos. At d 4 after insemination, embryos ≥ 8 cells were randomly assigned to control (38.5°C) or heat shock (41°C for 6 h) treatments. Heat shock reduced the proportion of embryos that developed to the blastocyst stage on d 8 after insemination. At 38.5°C, there were no significant differences in development between breeds. Among embryos exposed to 41°C, however, development was lower for Angus embryos than for Brahman and Romosinuano embryos. Furthermore, an Angus vs. (Brahman + Romosinuano) × temperature interaction occurred because heat shock reduced development more in Angus (30.3 ± 4.6% at 38.5°C vs. 4.9 ± 4.6% at 41°C) than in Brahman (25.1 ± 4.6% vs. 13.6 ± 4.6%) and Romosinuano (28.3 ± 4.1% vs. 17.5 ± 4.1%). Results demonstrate that embryos from Brahman and Romosinuano breeds are more resistant to elevated temperature than embryos from Angus. Thus, the process of adaptation of Brahman and Romosinuano breeds to hot environments resulted in both cases in selection of genes controlling thermotolerance at the cellular level.
(**Key words:** heat shock, embryo, cattle breed)

Abbreviation key: BE2 = bovine embryo 2, **IVF** = in vitro fertilization, **KSOM** = potassium simplex optimized medium, **PVP** = polyvinylpyrollidone, **TCM-199** = Tissue Culture Medium-199.

INTRODUCTION

Exposure to heat stress has a less adverse effect on reproductive function for breeds of cattle adapted to hot climates than for breeds from temperate climates (Turner, 1982; Madalena et al., 1990; Rocha et al., 1998). The basis for much of the thermotolerance displayed by adapted breeds is superior ability for thermoregulation (Finch, 1986; Hammond et al., 1996; Gaughan et al., 1999). In addition, certain heat-tolerant breeds of cattle have acquired mechanisms to protect cells against damage from high temperature. For example, the decrease in lymphocyte viability caused by heat shock was greater for lymphocytes from Angus cows than for lymphocytes from Brahman and Senepol cows (Kamwanja et al., 1994). Likewise, lymphocytes from Brahman and Senepol cows were more resistant to heat-induced apoptosis than lymphocytes from Angus and Holstein cows (Paula-Lopes et al., 2003).

This genetic difference in susceptibility to elevated temperature at the cellular level may be an important determinant of embryonic survival during heat stress. Cows exposed to heat stress have a high incidence of early embryonic mortality (Putney et al., 1988; Ealy et al., 1993), and some of this effect is due to the direct effects of elevated temperature on the embryo (Edwards and Hansen, 1997; Rivera and Hansen, 2001; Krininger et al., 2002). In a recent study (Paula-Lopes et al., 2003), it was observed that exposure of embryos to 41°C reduced development to the blastocyst stage more for em-

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Corresponding author: P. J. Hansen; e-mail: hansen@animal.ufl.edu.
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bryos of heat-sensitive breeds (Holstein and Angus) than for the heat-tolerant Brahman breed. Identification of the gene or genes conferring cellular thermotolerance offers the possibility of transferring this gene to heat-sensitive breeds to improve embryonic survival.

The fact that both Senepol and Brahman cattle exhibit superior cellular thermotolerance despite their different genetic origins suggests that either multiple genes for thermotolerance exist in cattle, the same gene was selected for in 2 different populations of cattle, or there was some introduction of *Bos indicus* genes into the Senepol population. The purpose of the present experiment was to examine how widespread is the phenomenon of cellular thermotolerance for preimplantation embryonic development in cattle. The approach was to compare resistance of embryos from the Angus, a heat-sensitive breed, with embryos from Brahman and Romosinuano cattle, two heat-tolerant breeds. The Romosinuano is a *B. taurus* developed in Colombia from the first cattle brought to the New World by Spain (Primo, 1990). Like the Brahman, Romosinuano is a tropically adapted breed that exhibits advantageous characteristics during periods of hyperthermia as compared to nonadapted breeds (Hammond et al., 1996). It is not known, however, whether this breed, distinct in origin from Brahman, has evolved to possess cellular adaptations to heat shock sufficient to provide thermoprotection to embryos. Heat shock was administered at d 4 after insemination on embryos ≥8 cells. We chose d 4 because of previous results indicating genetic differences in embryonic resistance to heat shock are apparent at this time (Block et al., 2002).

MATERIALS AND METHODS

Materials

Media from Cell and Molecular Technologies, Inc. (Lavallete, NJ) were used to prepare Sperm-TALP in vitro fertilization (**IVF**)-TALP, and HEPES-TALP as previously described (Parrish et al., 1986). Potassium Simplex Optimized Medium–Bovine Embryo 2 (**KSOM-BE2**) was prepared as described elsewhere (Soto et al., 2003) using KSOM from Cell and Molecular Technologies. Tissue Culture Medium 199 (**TCM-199**) with Hanks' salts and without phenol red was obtained from Atlanta Biologicals (Norcross, GA). The TCM-199 with Earle's salts was from Cell and Molecular Technologies. Bovine steer serum was purchased from Pel-Freez (Rogers, AR) and Percoll from Amersham Pharmacia Biotech (Uppsala, Sweden). Follicle stimulating hormone was Folltropin-V from Vetrepharm Canada (London, ON) and was purchased from Agtech (Manhattan, KS). Polyvinylpyrrolidone (**PVP**) was obtained from Eastman Kodak (Rochester, NY). Paraformaldehyde was from Fisher Scientific (Fair Lawn, NJ) and Prolong Antifade kit was purchased from Molecular Probes (Eugene, OR). Hoechst 33342 dye was from Sigma (St. Louis, MO). All other chemicals were obtained from Sigma or Fisher Scientific. Frozen semen was from USDA-STARS (Brahman and Romosinuano), ABS (De Forest, Wisconsin; Brahman), and Southeastern Semen Services (Wellborn, Florida; Angus).

Collection of Ovaries

Ovaries were obtained at slaughter from nonlactating Angus (n = 14), Brahman (n = 17), and Romosinuano (n = 15) cows. Cows were slaughtered in 5 (Angus) or 6 (Brahman and Romosinuano) replicates. Groups of 2 to 3 cows per breed were slaughtered for each replicate. After collection, ovaries were sorted by breed, placed into buckets containing sterile saline (0.9% (wt/vol) NaCl solution] supplemented with 100 U/mL of penicillin-G and 100 μg/mL of streptomycin, and transported to the laboratory at room temperature (a 1.5- to 2-h trip).

In Vitro Production of Embryos

After arrival in the laboratory, ovaries were washed several times with collection medium (prewarmed sterile saline supplemented with 100 units/mL of penicillin G and 0.1 mg/mL of streptomycin) at 38°C to remove blood and debris. Cumulus-oocyte complexes were harvested by slicing the surface of each ovary and agitating the ovary in a beaker containing oocyte collection medium [TCM-199 with Hanks' salts without phenol red and with the addition of 2% (vol/vol) bovine steer serum, 0.04 U heparin/mL, 100 U/mL of penicillin-G, 0.1 mg/mL of streptomycin, and 1 m*M* glutamine]. For each replicate, cumulus-oocyte complexes were collected separately from ovaries from each breed; oocytes from all ovaries from a specific breed were pooled together for subsequent steps.

Cumulus-oocyte complexes that had at least one layer of compact cumulus cells were washed 3 times and matured in groups of 10 in 50-μL drops of oocyte maturation medium [TCM-199 with Earle's salts supplemented with 10% (vol/vol) steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL of FSH, 22 μg/mL of sodium pyruvate, 50 μg/mL of gentamicin, and an additional 1 m*M* glutamine]. Drops were overlaid with mineral oil and cumulus-oocyte complexes incubated for 22 h at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air.

For fertilization, groups of 30 cumulus-oocyte complexes were transferred to 4-well plates containing 600 μL of IVF-TALP per well. Frozen-thawed sperm from

2 bulls of the same breed were pooled together, purified by centrifugation on a Percoll gradient (Parrish et al., 1986), and resuspended in IVF-TALP to give an approximate concentration of 4 to 6 million spermatozoa/mL. A different pair of bulls was used for each replicate to avoid confounding bull and breed effects. Oocytes were fertilized with semen from the same breed by adding 25 μL of sperm suspension and 25 μL of a mixture of 0.5 mmol/L penicillamine, 0.25 mmol/L of hypotaurine, and 25 μmol/L of epinephrine in 0.9% (wt/vol) NaCl to each well. After 8 h at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air, presumptive zygotes were removed from the fertilization wells and denuded of cumulus cells by vortex mixing in 1 mL of 1000 U/mL hyaluronidase in HEPES-TALP. After washing 3 times (twice in HEPES-TALP and once in KSOM-BE2), equal groups of 20 to 25 presumptive zygotes were cultured at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air in preequilibrated 50-μL drops of modified KSOM-BE2 overlaid with mineral oil. Within a replicate, the number of embryos per drop was kept constant for all breeds and one or more drops of embryos were prepared for each breed.

At d 4 after insemination, cleavage rate and stage of embryonic development was determined and embryos ≥8 cells were harvested and placed in fresh microdrops of KSOM. For each replicate, the number of embryos per drop was kept constant. When the number of embryos harvested resulted in groups of 5 to 10 embryos per drop, embryos were placed in 15-μL drops of KSOM. For groups >10 per drop, embryos were placed in 25-μL drops. Microdrops were randomly assigned to control (38.5°C) or heat shock (41°C for 6 h) treatments. After heat-shock, embryos were returned to 38.5°C until d 8 after insemination, when development to the blastocyst stage was determined and blastocysts were harvested to determine cell number.

Determination of Embryo Cell Number

Embryos were removed from culture drops and stored in paraformaldehyde solution [4% (wt/vol) in phosphate-buffered saline [**PBS**; 10 m*M* potassium phosphate, 0.9%, wt/vol, NaCl, pH 7.4] for 6 to 10 wk at 4°C. Embryos were removed from paraformaldehyde solution and washed 2 times in 100-μL drops of PBS containing 1 mg/mL PVP (PBS-PVP) by transferring the embryos from drop to drop. For staining, embryos were transferred to a drop of 50 μL of Hoechst 33342 dye for 10 min at room temperature and washed 2 times in 100 μL of PBS-PVP by transferring embryos from drop to drop. Embryos were transferred to poly-L-lysine coated slides in a minimal volume and allowed to dry for 15 min. Coverslips were mounted using Prolong Antifade mounting medium. During examination under a dissecting microscope, coverslips were pressed down with a pencil to spread the embryos. Fluorescent staining of nuclei was visualized with a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) and number of nuclei counted.

Statistical Analyses

Percentage data were analyzed 2 ways: as the untransformed data (used for reporting least squares means ± SEM) and after arcsine transformation (used for reporting probability values). Data on percent cleavage and percent development on d 4 were calculated within each replicate for each breed group of cows used to collect oocytes. The number of replicates was 5 (Angus) or 6 (Brahman and Romosinuano). Effects of breed on these variables were tested by least squares analysis of variance using the general linear models procedure of the Statistical Analysis System (SAS for Windows, Version 8, Cary, NC).

Data on the percentage of embryos developing to the blastocyst stage was calculated within replicate for each temperature-breed subset. For 1 replicate of Brahman embryos, insufficient numbers of embryos were available for subsequent culture. Therefore, the number of replicates was 5 (Angus and Brahman) or 6 (Romosinuano). Effects of temperature, breed, and the interaction between breed and heat shock were evaluated by least squares analysis of variance using the GLM procedure of SAS. In addition, breed effects for subsets of data for each temperature were also determined by least squares analysis of variance. Data on blastocyst cell number were analyzed as for percent development to the blastocyst stage except embryo was considered the experimental unit. In all analyses, variance associated with effects of breed and breed × temperature were partitioned into individual degree-of-freedom comparisons using orthogonal contrasts.

RESULTS

As shown in Figure 1, the proportion of oocytes that had cleaved at d 4 after insemination tended to be highest for Romosinuano (Romosinuano vs. other breeds, $P = 0.07$). The proportion of cleaved embryos that were ≥8 cells at d 4 after insemination was lowest for Brahman (Brahman vs. others, $P = 0.05$).

Heat shock caused a reduction ($P < 0.001$) in the proportion of embryos that developed to the blastocyst stage (Figure 2). There was a trend for an Angus vs. (Brahman + Romosinuano) × temperature interaction ($P = 0.09$) affecting development. This interaction occurred because heat shock reduced development more

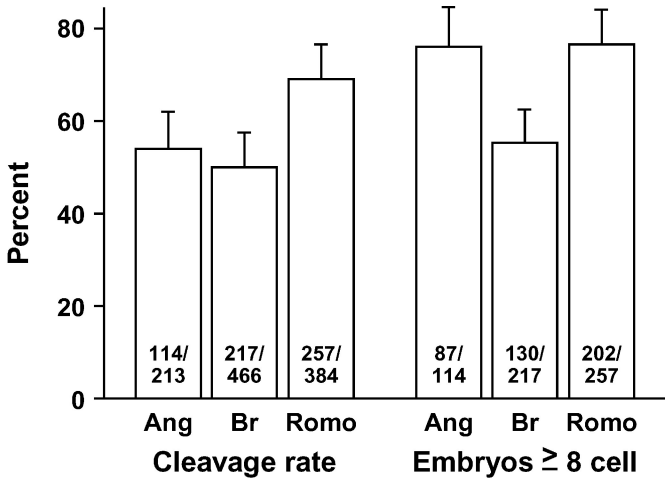

Figure 1. Breed effects on the proportion of oocytes that cleaved (cleavage rate) and the proportion of cleaved embryos that were ≥8 cells (embryos ≥8 cell). Bars represent least-squares means ± SEM of results from 5 (Angus; Ang) or 6 [Brahman (Br) and Romosinuano (Romo)] groups of cows. The numbers inside each bar are the fraction of oocytes that cleaved and that were ≥8 cells. The proportion of oocytes that had cleaved at d 4 after insemination tended to be highest for Romosinuano (Romosinuano vs. other breeds, $P = 0.07$). The proportion of cleaved embryos that were ≥8 cells at d 4 after insemination was lowest for Brahman (Brahman vs. others, $P = 0.05$).

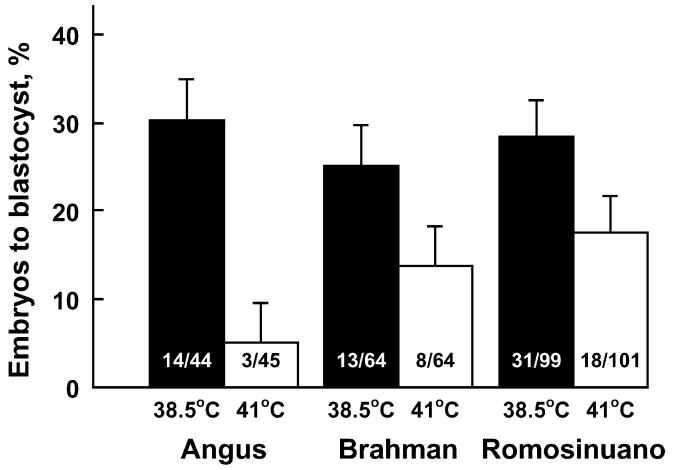

Figure 2. Breed × temperature interactions affecting development. Embryos ≥8 cells were collected at d 4 after insemination and cultured continuously at 38.5°C or exposed to 41°C for 6 h followed by 38.5°C thereafter. Bars represent least-squares means ± SEM of results from 5 (Angus or Brahman) or 6 (Romosinuano) groups of cows. The numbers inside each bar are the fraction of embryos that developed to the blastocyst stage. Note that the number of Angus embryos (89) is 2 greater than the number of embryos reported for this breed in Figure 1 because two 6-cell embryos were inadvertently harvested at d 4 and used for subsequent culture. Heat shock caused a reduction ($P < 0.001$) in the proportion of embryos that developed to the blastocyst stage. There was an Angus vs. (Brahman + Romosinuano) × temperature interaction ($P = 0.09$) affecting development. Among embryos exposed to 41°C, development was lower ($P < 0.05$) for Angus embryos than for Brahman and Romosinuano embryos. At 38.5°C, there were no significant differences in development between breeds.

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While heat shock reduced the proportion of embryos that became blastocysts, there was no effect of breed, temperature, or breed × temperature on the cell number of blastocysts recovered on d 8 (Figure 3).

DISCUSSION

Earlier results (Block et al., 2002; Paula-Lopes et al., 2003) established the concept that genotype differences in cellular resistance to heat shock, originally described for oviduct, endometrium, and lymphocytes (Malayer and Hansen, 1990; Kamwanja et al., 1994), also occur for preimplantation embryos. In the studies of Block et al. (2002) and Paula-Lopes et al. (2003), Brahman embryos or crossbred embryos derived from Brahman oocytes (Angus × Brahman), were more resistant to heat shock than Angus or Holstein embryos or crossbred embryos from Holstein oocytes. From these studies, it was not possible to determine whether embryonic tolerance to heat shock was a phenomenon limited to Brahman cattle or was present in other genotypes. The current results are significant because they indicate that

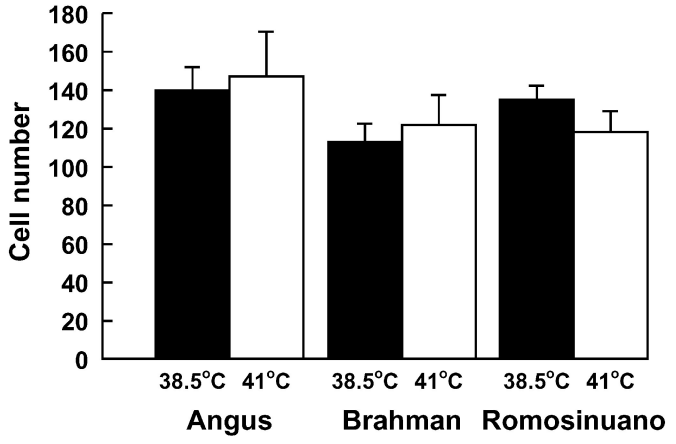

Figure 3. Cell number of blastocysts collected at d 8 after insemination. Bars represent least-squares means ± SEM of results from 8 (Angus), 11 (Brahman), and 21 embryos (Romosinuano) at 38.5°C and 2 (Angus), 5 (Brahman), and 9 embryos (Romosinuano) at 41°C. The low number of embryos at 41°C, especially for Angus, reflects the effects of heat shock on development. There was no effect of breed, temperature, or breed × temperature on the cell number of blastocysts recovered on d 8 ($P > 0.10$).

genetic differences in cellular resistance of preimplantation embryos to heat shock occur in two thermotolerant breeds of different genetic and geographical origin. The Brahman is a *B. indicus* of Indian origin while the Romosinuano breed, like other Criollo cattle, was developed in Latin America from *B. taurus* cattle stocks derived largely from importations from Spain in the colonial period (Primo, 1990).

Both Brahman and Romosinuano breeds are tropically adapted cattle that exhibit superior ability for thermoregulation during heat stress compared with nonadapted breeds (Hammond et al., 1996). While the process of adaptation to hot environments resulted in selection of genes controlling thermotolerance at the cellular level in both breeds, it is not known whether the same gene or genes conferring cellular thermotolerance is present in both breeds or whether distinct genes were selected. The phenomenon of cellular thermotolerance exists in other breeds as well. In particular, it has been shown that lymphocytes from Senepol cattle are more resistant to heat shock than lymphocytes from Holstein or Angus (Kamwanja et al., 1994; Paula-Lopes et al., 2003). It is not known whether the cellular thermotolerance displayed in the Senepol is sufficient to protect embryonic cells from heat shock.

Exposure to heat shock did not reduce blastocyst cell number. Conclusions are tentative because numbers of blastocysts were low. In particular, the increased sensitivity of Angus embryos to heat shock resulted in few blastocysts being available in this group. Accordingly, conclusions regarding breed × temperature interactions on blastocyst cell number are not warranted. However, the observation that there was no overall effect of heat shock on blastocyst cell number parallels similar observations from Block et al. (2002). Such a result is suggestive that an embryo which escapes the effects of heat shock at d 4 after insemination is similar to an embryo not exposed to heat shock. Other aspects of embryonic function need to be examined before definitive conclusions can be reached.

The large amounts of heat produced by the lactating dairy cow makes it more at risk to infertility caused by heat stress than other types of cattle. In Florida and South Georgia, for example, the summer depression in 90-d nonreturn rate increased in magnitude as milk yield increased (Al-Katanani et al., 1999). Identification of genes that confer cellular thermotolerance represents one potential strategy for reducing effects of heat stress on fertility in dairy cows. Once these genes are identified, they could potentially be readily transferred to Holstein cattle and other dairy breeds through conventional breeding schemes. Perhaps, the presence of these genes in dairy cows could reduce effects of heat stress on fertility or other physiological functions.

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