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Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in *HSPA1L*

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ABSTRACT

The objectives were to test whether (1) melatonin blocks inhibition of embryonic development caused by heat shock at the zygote stage, and (2) the frequency of a thermoprotective allele for *HSPA1L* is increased in blastocysts formed from heat-shocked zygotes as compared with blastocysts from control zygotes. It was hypothesized that melatonin prevents effects of heat shock on development by reducing accumulation of reactive oxygen species (ROS) and that embryos inheriting the thermoprotective allele of *HSPA1L* would be more likely to survive heat shock. Effects of 1 μM melatonin on ROS were determined in experiments 1 and 2. Zygotes were cultured at 38.5 or 40°C for 3 h in the presence of CellROX reagent (ThermoFisher Scientific, Waltham, MA). Culture was in a low [5% (vol/vol)] oxygen (experiment 1) or low or high [21% (vol/vol)] oxygen environment (experiment 2). Heat shock and high oxygen increased ROS; melatonin decreased ROS. Development was assessed in experiments 3 and 4. In experiment 3, zygotes were cultured in low oxygen ± 1 μM melatonin and exposed to 38.5 or 40°C for 12 h (experiment 1) beginning 8 h after fertilization. Melatonin did not protect the embryo from heat shock. Experiment 4 was performed similarly except that temperature treatments (38.5 or 40°C, 24 h) were performed in a low or high oxygen environment (2 × 2 × 2 factorial design with temperature, melatonin, and oxygen concentration as main effects), and blastocysts were genotyped for a deletion (D) mutation (C→D) in the promoter region of *HSPA1L* associated with thermotolerance. Heat shock decreased percent of zygotes developing to the blastocyst stage independent of melatonin or oxygen concentration. Frequency of genotypes for *HSPA1L* was affected by oxygen concentration and temperature, with an increase in the D allele for blastocysts that developed in high oxygen and following heat shock. It was concluded that (1) lack of effect of melatonin or oxygen concentration on embryonic development means that the negative effects of heat shock on the zygote are not mediated by ROS, (2) previously reported effect of melatonin on fertility of heat-stressed cows might involve actions independent of the antioxidant properties of melatonin, and (3) the deletion mutation in the promoter of *HSPA1L* confers protection to the zygote from heat shock and high oxygen. Perhaps, embryonic survival during heat stress could be improved by selecting for thermotolerant genotypes.
Key words: heat shock, melatonin, reactive oxygen species, *HSPA1L*

INTRODUCTION

In lactating dairy cattle, environmental temperatures as low as 23 to 29°C can lead to hyperthermia (Sartori et al., 2002; Dikmen and Hansen, 2009). Increases in body temperature to around 39°C are associated with reduced pregnancy rates (Gwazdauskas et al., 1973) and therefore pregnancy rates per AI decline during period of heat stress (Hansen and Aréchiga, 1999; Flamenbaum and Galon, 2010). One of the actions of heat stress responsible for lower fertility are actions on oocyte maturation (Putney et al., 1989), fertilization (Sartori et al., 2002), and early embryonic development (Ealy et al., 1993). Embryos are particularly susceptible to the effects of heat stress in vivo and exposure to elevated temperature in vitro during early stages of development, from the 1-cell through 4-cell stages of development (Ealy et al., 1995; Edwards and Hansen, 1997; Sakatani et al., 2012).

One of the proposed mechanisms by which heat stress can damage the oocyte and the embryo is through pro-

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duction of reactive oxygen species (**ROS**). Exposure of the oocyte and embryo to elevated temperatures can increase ROS production (Sakatani et al., 2004; Nabenishi et al., 2012b). Some antioxidants have been reported to reduce effects of heat shock in cultured embryos including anthocyanin (Sakatani et al., 2007) and dithiothreitol (de Castro e Paula and Hansen, 2008), whereas others such as glutathione (Ealy et al., 1995) and vitamin E (Paula-Lopes et al., 2003a) had little effect on embryo susceptibility to heat shock. Recently, it has been reported that supplementation of melatonin to oocytes exposed to heat shock during the maturation period reduced negative effects of heat shock (Cebrian-Serrano et al., 2013). Moreover, administration of slow-release implants of melatonin to lactating cows exposed to heat stress improved fertility (Garcia-Ispierto et al., 2013b).

Genetic effects also occur on resistance of the embryo to heat shock, as is evidenced by the observation that heat shock reduced development of bovine embryos from thermotolerant breeds less than for less adapted breeds (Paula-Lopes et al., 2003b; Eberhardt et al., 2009; Silva et al., 2013). One family of genes involved in cellular thermoprotection is the heat shock protein 70 (**HSP70**) family (Christians et al., 2003). In the cow, 2 HSP70 genes exist: *HSPA1A* and *HSPA1L*. Reverse-transcription PCR using primers that do not distinguish between the 2 genes indicated that heat shock can increase transcription of *HSPA1A/A1L* as early as the 2-cell stage (Chandolia et al., 1999; Sakatani et al., 2012). A mutation in the promoter region of *HSPA1L* (Rosenkrans et al., 2010; Ortega et al., 2016) that results in a deletion of a cytosine (**C**) has been associated with thermotolerance in peripheral blood mononuclear cells exposed to heat stress (Basiricò et al., 2011). The same mutation has been associated with superior embryonic development to the blastocyst stage in cultured embryos (Cochran et al., 2013). It is possible, therefore, that inheritance of this allele can increase embryonic resistance to heat shock.

The objective of this study was to determine if the deleterious effects of exposure of bovine embryos to heat shock at the zygote stage of development is modified by melatonin or inheritance of the deletion (**D**) allele of *HSPA1L*. The effect of melatonin was tested by evaluating whether its addition to culture medium would reduce effects of heat shock on production of ROS and inhibition of embryonic development. The effect of *HSPA1L* genotype was determined indirectly by testing whether the allele frequency of the D allele (the putative thermotolerant allele) would be greater in blastocysts developing after exposure to heat shock than for blastocysts not exposed to heat shock.

MATERIALS AND METHODS

In Vitro Production of Embryos

Ovaries were obtained from Central Packing Co. (Center Hill, FL) from cattle of *Bos taurus* and various admixtures of *B. taurus* and *Bos indicus* breeds. The surface of each ovary was cut with a scalpel to harvest immature cumulus-oocyte complexes (**COC**) from follicles 2 to 8 mm in diameter into oocyte washing medium (BoviPRO), which contained salts, bicarbonate, HEPES, DL-lactic acid, and BSA and was purchased from MOFA Global (Verona, WI). The COC were washed and those having uniform cytoplasm and at least 3 layers of cumulus cells were matured in groups of 10 in 50-μL droplets of oocyte maturation medium (composition of all media is presented in Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-11501) covered with mineral oil for 21 h at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2. For each replicate, up to 300 COC were matured. After maturation, COC were washed 3 times in HEPES-TALP (Tyrode's albumen lactate pyruvate) medium and placed in a 35-mm dish containing 1.7 mL of fertilization medium (IVF-TALP). Insemination of each replicate of fertilization was performed with semen pooled from 3 individual bulls of various taurine breeds (the total number of bulls were 29). Sperm were purified from frozen-thawed straws of extended semen using an Isolate gradient [Irvine Scientific, Santa Ana, CA; 50% (vol/vol) and 90% (vol/vol) isolate] and diluted in IVF-TALP to achieve a final concentration of 1×10^6/mL in the fertilization dish. In addition, 80 μL of penicillamine-hypotaurine-epinephrine solution was added to each fertilization well to improve sperm motility and promote fertilization. Fertilization proceeded for 8 to 9 h at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2.

Putative zygotes (i.e., oocytes exposed to sperm) were denuded from the surrounding cumulus cells at the end of fertilization by vortexing groups of 200 to 300 putative zygotes for 5 min in 600 μL of HEPES-TALP containing 10,000 U/mL of hyaluronidase. Unless otherwise stated, embryos were cultured in groups of 25–30/50 μL microdrops of culture medium (SOF-BE2) prepared with or without 1 μ*M* melatonin (Santa Cruz Biotechnologies, Dallas, TX), which was dissolved directly in SOF-BE2. The microdrops were covered with mineral oil at 38.5°C in a humidified atmosphere of 5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2. When embryos were heat shocked, culture was performed under either high oxygen conditions [40.0°C in a humidified atmosphere of 6% (vol/vol) CO_2 and atmospheric oxygen] or low oxygen conditions [40.0°C