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 μ M 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 $\pm$ 1 μ M 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 $\times$ 2 $\times$ 2 factorial design with temperature, melatonin, and oxygen concentration as main effects), and blastocysts were genotyped for a deletion (D) mutation (C $\rightarrow$ D) in the promoter region of *HSPA1L* associated with thermotolerance. Heat shock decreased percent of zygotes developing to the blastocyst stage independent of mela-

tonin 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-Ispuerto 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- μ L 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) CO₂. 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 μ L 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) CO₂.

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 μ L of HEPES-TALP containing 10,000 U/mL of hyaluronidase. Unless otherwise stated, embryos were cultured in groups of 25–30/50 μ L 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) O₂ and 5% (vol/vol) CO₂ with the balance N₂. When embryos were heat shocked, culture was performed under either high oxygen conditions [40.0°C in a humidified atmosphere of 6% (vol/vol) CO₂ and atmospheric oxygen] or low oxygen conditions [40.0°C

in a humidified atmosphere of 5% (vol/vol) O₂ and 6% (vol/vol) CO₂ with the balance as N₂. The increase in CO₂ for heat shock was to account for its reduced solubility at a higher temperature. Heat shock was initiated 8 h after insemination (at the end of fertilization) and proceeded for 3, 12, or 24 h. Afterward, heat-shocked embryos were examined for ROS production (3 h) or were returned to the same environment as the embryos not exposed to heat shock [38.5, 5% (vol/vol) O₂ and 5% (vol/vol) CO₂ in a humidified incubator with the balance N₂].

Percentage of putative zygotes that cleaved was determined at Day 3 of development (Day 0 = day of fertilization) and the percentage of putative zygotes and cleaved embryos that became blastocysts was determined at Day 7 of development.

Detection of Reactive Oxygen Species

Production of ROS by embryos was determined by incubating putative zygotes for 3 h in SOF-BE2 containing 5 μ M CellROX green reagent (Thermo-Fisher Scientific, Waltham, MA). This reagent is a cell-permeant dye that exhibits bright green photostable fluorescence upon oxidation and subsequent binding to DNA. After incubation with CellROX, embryos were washed 3 times in 50- μ L droplets of Dulbecco's phosphate-buffered saline (DPBS) containing 1% (wt/vol) polyvinylpyrrolidone (PVP), fixed in 4% (wt/vol) paraformaldehyde in DPBS, washed 3 additional times in DPBS-PVP and mounted in groups of 10 embryos on microscope slides using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA). Embryos were examined for fluorescence within 10 h after labeling by fluorescence microscopy using a green emission filter with a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Digital images of each embryo were acquired using AxioVision software (v. 4.8.2, Zeiss, Peabody, MA) and a high-resolution black and white Zeiss Axiocam MRM digital camera. Analysis of the images was performed using ImageJ V. 1.48 (National Institutes of Health, Bethesda, MD). Net fluorescent intensity was calculated by obtaining the average pixel intensity of each embryo (obtained after manually drawing a boundary around the embryo) and subtracting the background intensity obtained from a region of the image not containing the embryo.

Genotype Determination and Frequency

Day 7 blastocysts were genotyped for a SNP in the promoter region of *HSPA1L* (UMD3.1.1 chr 23:27334005) in which a C is replaced by a deletion (C/D mutation; Rosenkrans et al., 2010; Supplemental Figure S1;

<http://dx.doi.org/10.3168/jds.2016-11501>). Blastocysts were washed 3 times in PBS-PVP and the zona pellucida denuded using 0.1% (wt/vol) protease from *Streptococcus griseus*. After further washing, embryos were placed individually in wells of 96-well plates with 5 μ L of molecular biology-grade water for subsequent genotyping by PCR using an anchored KASP by design assay (LGC Genomics, Middlesex, UK). The assay is a PCR-based technique involving a common reverse primer and 2 allele-specific forward primers, with one bound to fluorescein amidite (FAM) and the second to hexachlorofluorescein (HEX) to produce genotype-specific PCR products. The primer sequences (5' $\rightarrow$ 3') to target each allele were CAAGTCCTGCCCCCTGC-FAM for the deletion allele and CTCAAGTCCTGCCCCCTGG-HEX for the C allele. The common reverse primer was GCATCCAGGGCGCTGATTG-GTT. Each PCR reaction included 5 μ L of embryo resuspended in molecular biology-grade water, 5 μ L of 2 $\times$ supermix with low ROX (LGC Genomics), and 0.14 μ L of KASP by design primer mix. Amplification and analysis were performed using a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA); thermal cycling conditions were 94°C for 15 min, followed by 10 cycles of 94°C for 20 s and 61°C for 60 s, where the second temperature was decreased 0.6°C per cycle to achieve a final annealing temperature of 55°C at the end of the 10th cycle. The reaction proceeded for an additional 26 cycles of 94°C for 20 s and 55°C for 60 s, and a read step of 37°C for 60 s. To improve genotype cluster visibility, 3 additional cycles of 94°C for 20 s and 55°C for 60 s and a final read step at 37°C for 60 s were performed. In each run, DNA samples from whole blood of cows of known genotypes were analyzed as controls. Determination of the genotype was performed using the allelic discrimination feature of the CFX96 machine. A representative output is shown in Figure 1.

Experiments

Experiment 1 was performed to determine (1) if exposure to heat shock increased ROS production in the embryo, and (2) whether melatonin blocked ROS production. The experiment was a 2 $\times$ 2 factorial design including 2 temperature treatments (38.5 and 40°C) and 2 melatonin concentrations (0 and 1 μ M). Putative zygotes were produced from a single in vitro production of embryos (IVP) procedure involving embryos produced by fertilization with semen pooled from 3 bulls. Groups of putative zygotes supplemented with 0 or 1 μ M melatonin were cultured during 3 h at either 38.5°C [5% (vol/vol) O₂ and 5% (vol/vol) CO₂ with the balance N₂], or 40°C [5% (vol/vol) O₂ and 6% (vol/vol) CO₂ with the balance as N₂] beginning 8 h after

Figure 1. Representative results for genotyping assay for the deletion mutation in *HSPA1L*. Representative example of results from the genotype assay for the SNP in *HSPA1L* using the KASP assay (LGC Genomics, Middlesex, UK). In the assay, PCR amplicons from embryos with the cytosine (C) allele hybridize to the hexachlorofluorescein fluorophore (HEX), whereas amplicons from embryos with the deletion (D) allele hybridize to the fluorescein amidite fluorophore (FAM). Embryos classified as homozygous for C are indicated by black (blue) circles, embryos homozygous for the deletion allele are indicated by light gray (orange) circles and heterozygotes are indicated by dark gray (green) circles. The 3 black circles at the bottom left of plot represent no signals (no genotype assigned). Color version available online.

insemination. After treatment, putative zygotes were assessed individually for ROS production as described above. The number of embryos evaluated varied from 91 to 117 per group.

Experiment 2 was conducted similarly to experiment 1 except the effect of oxygen concentration in the culture environment was also determined. The experiment involved a $2 \times 2 \times 2$ factorial design including 2 temperature treatments (38.5 and 40°C), 2 melatonin concentrations (0 and 1 μM), and 2 oxygen environments (5 and 21% vol/vol O_2). Putative zygotes were produced from a single IVP procedure involving fertilization with sperm from a pool of 3 bulls. Beginning 8 h after insemination, putative zygotes supplemented with 0 or 1 μM melatonin were cultured for 3 h in a low oxygen environment at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2] or in a high oxygen environment at either 38.5 in a humidified atmosphere of 5% (vol/vol) CO_2 or at 40°C in a humidified atmosphere of 5% (vol/vol) CO_2 . The number of putative zygotes per treatment ranged from 20 to 36.

Experiment 3 was performed to determine if melatonin blocks the negative effects of heat shock at the zygote stage on embryonic development to the blas-

tocyst stage. The experiment was a 2×2 factorial design which included 2 melatonin concentrations (0 and 1 μM) and 2 temperatures (38.5 or 40°C). Putative zygotes supplemented with 0 or 1 μM were cultured at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2]. Heat shock treatment started 8 h after insemination and lasted for 12 h, after which all groups were cultured at 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2] until Day 7 of development. Endpoints were percent of putative zygotes that cleaved by Day 3 of development, percent of putative zygotes that became a blastocyst at Day 7 of development, and percent of cleaved embryos that became a blastocyst at Day 7 of development. The experiment was conducted in 22 replicates using a total of 25 bulls for fertilization. The total number of putative zygotes per treatment ranged from 1,451 to 1,532.

Experiment 4 was designed to test interactions between melatonin, oxygen concentration, and temperature on embryonic development. The experiment had a $2 \times 2 \times 2$ factorial design including 2 temperature treatments (38.5 and 40°C), 2 melatonin concentrations (0 and 1 μM), and 2 oxygen environments (5 and 21% vol/vol O_2). Beginning 8 h after insemination, putative zygotes supplemented with 0 or 1 μM melatonin were cultured for 24 h at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2] in a low oxygen (5% vol/vol) environment or at either 38.5 or 40°C in a humidified atmosphere of 5% (vol/vol) CO_2 in a high oxygen environment. Thereafter, all embryos were cultured at 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2] until Day 7 of development. The experiment was performed in 8 replicates with a total of 19 sires and the number of putative zygotes ranged from 510 to 578 per treatment. Endpoints were as for experiment 3. In addition, 542 blastocysts from the experiment were collected individually and genotyped for the deletion mutation in *HSPA1L*, as described above.

Statistical Analysis

Differences in ROS intensity were evaluated by ANOVA using the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). For experiment 1, the model included temperature, melatonin treatment, and the interactions as fixed effects. For experiment 2, the model included temperature, melatonin treatment, oxygen, and their interactions as fixed effects. In both experiments 1 and 2, each embryo was analyzed individually and considered an experimental unit. In experiment 1, the total amount of experimental units

was 416. In experiment 2, the total amount of experimental units was 205.

For experiments 3 and 4, data on percent cleavage, putative zygotes, and cleaved embryos becoming blastocysts calculated for each replicate were analyzed by ANOVA using the MIXED procedure of SAS. The main effects included in the model were temperature, melatonin, and their interactions for experiment 3; and temperature, melatonin, oxygen, and their interactions for experiment 4. In both cases, replicate was included as random effect in the model.

To determine effect of treatment on blastocyst genotype, the frequency of each genotype (CC, CD, DD) was calculated for each replicate. Treatment effects on frequency were determined by the ANOVA with the GLIMMIX procedure, using a Poisson distribution. Frequency was the response variable and the model included genotype, temperature, oxygen, and their in-

teractions as fixed effects, and replicate as a random effect.

RESULTS

ROS Production

In experiment 1, embryos were cultured in low oxygen. Representative images of ROS labeling are shown in Figure 2. When melatonin was added to culture medium, ROS intensity was reduced at both temperatures and embryos at 40°C did not have greater labeling intensity than embryos at 38.5°C (compare Figure 2C and Figure 2D).

Least squares means for ROS labeling intensity are shown in Figure 3. Significant effects were found for temperature ($P = 0.0012$), melatonin ($P = 0.0031$), and the temperature $\times$ melatonin interaction ($P <$

Figure 2. Representative images of zygotes labeled with CellROX (ThermoFisher Scientific, Waltham, MA) to assess production of reactive oxygen species as affected by incubation temperature and melatonin (experiment 1). Images were obtained by merging visualization of the embryo using differential interference and with a fluorescein isothiocyanate (FITC) fluorescent filter. Panels A and B represent embryos cultured without melatonin at 38.5°C (A) and 40°C (B). Panels C and D represent embryos cultured with melatonin at 38.5°C (C) and 40°C (D). Color version available online.

Figure 3. Effect of incubation temperature and melatonin on production of reactive oxygen species in the zygote as determined by CellROX (ThermoFisher Scientific, Waltham, MA) net intensity (experiment 1). Data are LSM $\pm$ SEM of arbitrary units of intensity. Intensity was affected by temperature ($P = 0.0012$), melatonin ($P = 0.0031$), and the temperature by melatonin interaction ($P < 0.0001$).

0.0001). These effects reflected the fact that heat shock increased labeling intensity and that melatonin reduced labeling intensity at both temperatures and prevented the increase in ROS labeling caused by culture at 40°C.

Experiment 2 was conducted similarly except that the experiment was performed under 2 gaseous atmospheres, high oxygen (21% O₂) and low oxygen (5% O₂) conditions. Representative images of ROS labeling are shown in Figure 4. Intensity of labeling was increased by culture at 40°C compared with 38.5°C for both oxygen environments (compare Figure 4A with 4B and 4E with 4F). Intensity was also greater for embryos in high oxygen than low oxygen (compare Figure 4A with 4E and 4B with 4F). Melatonin decreased ROS production in embryos exposed to heat shock under low oxygen (compare Figure 4D with 4C) and high oxygen conditions (compare Figure 4H with 4G).

Least squares means for ROS intensity are shown in Figure 5. Intensity was affected by oxygen concentration ($P = 0.004$) and the interactions of temperature with melatonin treatment ($P = 0.0003$) and oxygen with melatonin treatment ($P = 0.0054$). These interactions occurred because melatonin prevented the increase in ROS intensity caused by culturing embryos in a high oxygen environment or at 40°C.

Figure 4. Representative images of zygotes labeled with CellROX (ThermoFisher Scientific, Waltham, MA) to assess production of reactive oxygen species as affected by incubation temperature and melatonin (experiment 2). Images were obtained by merging visualization of the embryo using differential interference and with a fluorescein isothiocyanate (FITC) fluorescent filter. Panels A to D represent embryos cultured in low oxygen [5% (vol/vol) O₂] for embryos cultured without melatonin at 38.5°C (A) and 40°C (B) and with melatonin at 38.5°C (C) and 40°C (D). Panels E to H represent embryos cultured in high oxygen [21% (vol/vol) O₂] without melatonin at 38.5°C (E) and 40°C (F) or with melatonin at 38.5°C (G) and 40°C (H). Color version available online.

Development to the Blastocyst Stage

For experiment 3, embryos were cultured continuously in low oxygen. Results are shown in Figure 6. Culture at 40°C during the first 12 h of culture decreased the percent of putative zygotes that cleaved ($P = 0.0012$) and that progressed to the blastocyst stage ($P < 0.0001$) as well as the percent of cleaved embryos that became blastocysts ($P < 0.0001$). No effect was found of melatonin or temperature by melatonin interactions on any developmental trait measured.

Experiment 4 was conducted similarly except that heat shock was for 24 h and embryos were cultured either in low oxygen continuously or were moved to high oxygen during the period corresponding to temperature treatments. Results are presented in Figure 7. No effect was found of temperature treatment, oxygen concentration, or the interaction on the percent of putative zygotes that cleaved, although culture at 40°C tended to reduce cleavage ($P = 0.09$). Elevated temperature also reduced the percent of putative zygotes that became blastocysts ($P = 0.003$) and the percent of cleaved embryos developing to blastocysts ($P = 0.01$). Neither

oxygen, melatonin, nor interactions with these 2 main effects affected development to the blastocyst stage.

Genotype

The genotype of embryos that developed to the blastocyst stage was determined in experiment 4. As shown in Figure 8, the distribution of blastocysts according to the 3 genotypes (CC, CD, DD) was affected by temperature (genotype $\times$ temperature, $P < 0.0001$), oxygen environment (genotype $\times$ oxygen, $P < 0.0001$), and the interaction of temperature by oxygen (genotype $\times$ temperature $\times$ oxygen, $P = 0.0054$). These effects reflected the fact that frequency of the D allele was lower for blastocysts cultured at 38.5°C in low oxygen than for blastocysts produced under other conditions. For embryos cultured at 38.5°C in low oxygen (i.e., the least stressful environment for the embryo), the overall frequency of the D allele was 33%. In other groups, however, the frequency of the D allele was higher, ranging from 43 to 56%. No effect was found of melatonin or interactions of melatonin with other main effects on genotype.

Figure 5. Effect of oxygen environment, incubation temperature (Temp.), and melatonin on production of reactive oxygen species in the zygote as determined by CellROX (ThermoFisher Scientific, Waltham, MA) net intensity (experiment 2). Data are LSM $\pm$ SEM of arbitrary intensity units. Intensity was affected by oxygen concentration ($P = 0.004$) and the interaction of temperature with melatonin treatment ($P = 0.0003$) and oxygen with melatonin treatment ($P = 0.0054$).

DISCUSSION

Results of these experiments in which embryos were exposed to heat shock confirm that exposure to elevated temperatures during the zygote stage of development can compromise the competence of the embryo to progress to the blastocyst stage (Sakatani et al., 2012). However, although heat shock resulted in increased ROS production, this action of elevated temperature is not a major cause for the reduced development. This conclusion is based on 2 observations. First, melatonin did not alleviate effects of heat shock on development despite causing a decrease in ROS production. Second,

effects of heat shock on development were similar for embryos cultured in a low or high oxygen environment, despite the fact that embryos cultured in high oxygen had higher production of ROS than embryos cultured in low oxygen. The other major conclusion of these experiments is that the genotype for *HSPA1L* affects survival of embryos in a manner that depends upon culture environment. This is because the proportion of blastocysts with a deletion mutation in *HSPA1L* previously related to cellular thermotolerance (Basiricò et al., 2011) was higher for blastocysts derived from heat-shocked zygotes or for embryos cultured at 38.5°C in high oxygen than for those derived from zygotes

Figure 6. Effect of incubation temperature from 8 to 20 h after fertilization on cleavage and development to the blastocyst stage as affected by melatonin for embryos cultured in low oxygen (experiment 3). Data are LSM $\pm$ SEM. The timing and design and timing of treatments is shown in panel A, cleavage rate is shown in panel B, the percent oocytes developing to blastocyst are shown in panel C, and the percent of cleaved embryos developing to blastocyst are shown in panel D. Temperature affected the percent of putative zygotes that cleaved ($P = 0.0012$), the percent that became blastocysts ($P < 0.0001$), and the percent of cleaved embryos that became blastocysts ($P < 0.0001$). No effects were found of melatonin or the interaction between heat shock and melatonin.

not exposed to heat shock and cultured in low oxygen. Thus, there are allelic variants of genes that confer the embryo with protection against adverse environments such as heat shock or high oxygen.

Intracellular ROS are formed by incomplete reduction of oxygen during oxidative phosphorylation and as byproducts of specific enzymatic reactions (Takahashi, 2012). Because of their propensity to strip electrons

Figure 7. Effect of incubation temperature from 8 to 20 h after fertilization on cleavage and development to the blastocyst stage as affected by oxygen environment during 8 to 20 h after fertilization and melatonin (experiment 4). Data are LSM ± SEM. The timing and design and timing of treatments is shown in panel A, cleavage rate is shown in panel B, the percent oocytes developing to blastocyst is shown in panel C, and the percent of cleaved embryos developing to blastocyst is shown in panel D. Temperature affected the percent of putative zygotes that cleaved ($P = 0.09$), the percent of putative zygotes that became blastocysts ($P = 0.003$), and the percent of cleaved embryos that became blastocysts ($P = 0.01$). Oxygen, melatonin, and interactions with these 2 main effects did not affect any developmental trait.

from other molecules in the cell, ROS can cause cellular damage (Guérin et al., 2001; Ray et al., 2012). As shown here and elsewhere (Sakatani et al., 2004; Nabenishi et al., 2012b), elevated temperature can increase ROS production in embryos. As a result, it has been proposed that increased ROS production is one of the mechanisms by which heat shock reduces embryonic development (Sakatani et al., 2004; Hansen, 2009; Nabenishi et al., 2012a; Hansen, 2013). However, current results are inconsistent with this idea, at least for the bovine zygote. Melatonin did not increase embryonic resistance to heat shock, despite causing a large decrease in ROS production. Likewise, effects of heat shock on development to the blastocyst stage were similar for embryos at high and low oxygen environments, even when ROS production was greater for embryos in a high-oxygen environment. Perhaps the reason why negative effects of high oxygen were not observed in this study is because the exposure to the high oxygen conditions was only for 12 to 24 h and was not enough time to affect embryonic development to the blastocyst stage. Prolonged exposure to high oxygen (24 h or greater) can decrease embryonic development (Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012). Under prolonged exposure to high oxygen, melatonin can protect the embryo from the damaging consequences of high oxygen (Papis et al., 2007).

In contrast to results with the zygote reported here, melatonin has been reported to protect cultured bovine oocytes exposed to 41.5°C for 24 h in high oxygen matu-

ration from effects of heat shock (Cebrian-Serrano et al., 2013). It is possible that oocytes are more susceptible to ROS than zygotes or that the higher temperatures and incubation periods used increase ROS production beyond what was induced with the lower, but more physiological, heat shock temperatures used here. The effective concentration of melatonin in the experiment of Cebrian-Serrano et al. (2013) was 100 μM versus a concentration of 1 μM in the present experiment. It is unlikely, however, that absence of melatonin effects in the present experiment was because concentrations were too low. Indeed, ROS was greatly reduced by treating heat-shocked zygotes with 1 μM melatonin.

Other antioxidants have been used to reduce effects of heat shock on bovine embryos in vitro and of heat stress in vivo on embryonic survival. Results, which have involved a range of stages of development and culture conditions, have been mixed. No protective effect was found of vitamin E, glutathione, or taurine on preimplantation bovine embryos exposed to heat shock (Ealy et al., 1995; Paula-Lopes et al., 2003a). Similarly, injection of cows with vitamin E (Ealy et al., 1994; Aréchiga et al., 1998b), vitamin E and selenium (Aréchiga et al., 1998a; Paula-Lopes et al., 2003a), or β -carotene (Aréchiga et al., 1998a) did not improve fertility in summer. In contrast, cultured bovine embryos were protected from heat shock by anthocyanin (Sakatani et al., 2007) and dithiothreitol (de Castro e Paula and Hansen, 2008) and fertility of heat-stressed cows was improved by feeding supplemental β -carotene (Aréchiga et al., 1998a).

Although a wide range of responses to altering redox status have been observed, it is likely that the lack of involvement of ROS in the negative effects of heat shock with cultured embryos seen here reflects the situation with respect to heat-stressed cows. That is because, as compared with some other studies, the magnitude of heat shock in terms of duration and temperature reflects body temperatures experienced by heat-stressed cows. Rectal temperatures in lactating dairy cows in the summer in Florida are usually below 40°C (Dikmen and Hansen, 2009; Dikmen et al., 2015). Also, the stage of development studied here, the zygote, is more relevant to the heat-stressed cows because fertility is more likely to be disrupted when cows are exposed to heat stress at d 1 after estrus than later in development (Ealy et al., 1993).

Administration of subcutaneous implants of melatonin have been reported to increase fertility of lactating cows during heat stress (Garcia-Ispuerto et al., 2013a). The concentrations of melatonin in the blood in that study ranged from 30 to 65 pg/mL (0.13 to 0.28 nM), which is much lower than tested here (1 μM) and too low to inhibit ROS production (Mayo et al., 2003; Tan

Figure 8. Genotype frequencies of the SNP in *HSPA11L* for blastocysts formed following exposure to different temperatures, oxygen concentrations, and melatonin concentrations (experiment 4). The distribution of blastocysts according to the 3 genotypes [CC (black), CD (gray), and DD (white)] was affected by the following interactions: genotype by temperature ($P < 0.0001$), genotype by oxygen ($P = 0.000184$), and genotype by temperature by oxygen ($P = 0.0054$).

et al., 2003; Rodriguez et al., 2004). Rather it is likely that the effect of melatonin seen by Garcia-Ispuerto et al. (2013a) reflects actions of melatonin mediated by specific receptors located in the brain, cumulus cell and oocyte, placenta, and other peripheral tissues (Cardinali et al., 1979; Iwasaki et al., 2005; El-Raey et al., 2011; Slominski et al., 2012). Melatonin has been reported to increase ipsilateral uterine artery blood flow in pregnant Holstein heifers (Brockus et al., 2016), increase circulating progesterone concentration, and decrease serum prolactin in ewes (Kennaway et al., 1982; Abecia et al., 2002), and evidence indicates that melatonin favors cumulus cell expansion in the cow (El-Raey et al., 2011).

We did not determine consequences of heat shock at the 1-cell stage on characteristics of the blastocyst (i.e., cell numbers, incidence of apoptosis, gene expression, and so on). Heat shock during early development can reduce cell number of the resultant blastocysts (Sakatani et al., 2004, 2012). Therefore, it remains possible that, even though melatonin did not block the effects of heat shock on the proportion of embryos that developed to the blastocyst stage, it might have improved the quality of the embryos that did become a blastocyst. Even if this were the case, however, one would not expect that such an action would appreciably improve fertility in heat-stressed cows because most embryos would not reach the blastocyst stage.

The extent to which heat shock negatively affects embryonic development is also controlled in part by the genetics of the embryo. Embryos of thermotolerant breeds are more resistant to the anti-developmental effects of heat shock than embryos of more thermosensitive breeds (Paula-Lopes et al., 2003b; Hernández-Cerón et al., 2004; Eberhardt et al., 2009). Here we show that *HSPA1L* is one gene that contains mutations that confers resistance of embryos to 2 environments: heat shock and high oxygen. The HSP70 family is involved in stabilization of protein structure and inhibition of apoptosis during cellular stress (Radons, 2016). In cattle, 2 genes in the HSP70 family were formed by gene duplication: *HSPA1A* and *HSPA1L* (Grosz et al., 1992). Most studies examining expression of *HSP70* genes have not distinguished between these 2 genes, but a recent study examining the transcriptome of bovine embryos indicates that transcripts for both *HSPA1A* and *HSPA1L* are present in the 2-cell embryo (Jiang et al., 2014). Although embryonic gene activation in cattle occurs for most genes at the 8-cell stage (Graf et al., 2014), transcription of *HSPA1A/L* in response to heat shock occurs as early as the 2-cell stage (Chandolia et al., 1999). The mutation studied here is a deletion in the promoter of *HSPA1L* that, in bovine mononuclear

cells, increases upregulation of transcription of the gene in response to elevated temperature and increased viability after heat shock (Basiricò et al., 2011). In Holsteins, the frequency of the deletion mutation has been estimated at 29% (Ortega et al., 2016). In the current study, a similar frequency of the deletion mutation (33%) was observed for blastocysts formed after culture of embryos at 38.5°C in a low oxygen environment. In contrast, frequency of the deletion mutation was increased in blastocysts formed following heat shock under either low or high oxygen conditions or for embryos cultured at 38.5°C in the presence of a high oxygen environment. Such a finding suggests that inheritance of the deletion mutation increased the probability that embryos could develop to the blastocyst stage after exposure to conditions that compromised development (heat shock) or that has been reported to reduce development in other experiments (high oxygen; Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012). Even though the effect of oxygen on blastocyst development was not significant in experiment 4, the percent of embryos becoming blastocysts was numerically lower in the high oxygen group in the absence of melatonin. A negative effect of high oxygen on development has been observed in multiple experiments (Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012).

This finding has 2 implications. First, culture of embryos may lead to a skewing of frequency of genes that enhance development under suboptimal culture conditions. In addition, it may be possible to improve embryonic survival during heat stress by selecting for alleles such as the deletion mutation of *HSPA1L* that confer protection from elevated temperature.

CONCLUSIONS

Exposure of bovine zygotes to elevated temperatures reduced competence to develop to the blastocyst stage. This effect is independent of the increased generation of ROS caused by heat shock because reduced development caused by elevated temperature was not alleviated by melatonin or culture in a low oxygen environment even though both treatments reduced generation of ROS. Thus, the previously reported effect of melatonin on fertility of heat-stressed cows might involve actions independent of the antioxidant properties of melatonin. Nevertheless, the effect of melatonin on the oocyte or further embryonic developmental stages should be examined further. Inheritance of a deletion mutation in the promoter region of *HSPA1L* increased the probability that embryos became blastocysts after heat shock or exposure to high oxygen. Thus, genotype may affect whether an embryo survives or not in response

to adverse environments. Perhaps embryonic survival during heat stress could be improved by selecting for thermotolerant genotypes.

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