ABSTRACT

The objective of this study was to determine whether bovine embryos become more resistant to deleterious effects of maternal heat stress as early embryonic development progresses. Superovulated, lactating Holstein cows were bred by AI and assigned to be heat stressed on d 1, 3, 5, or 7 of pregnancy (d 0 = day of estrus) or not heat stressed (control). Embryos were retrieved from the uterus on d 8 and evaluated for viability and stage of development. Compared with embryos of control cows, embryos of cows receiving heat stress on d 1 had decreased viability and development. Maternal heat stress on other days had no detrimental effect on embryonic viability or stage of development. Bovine embryos become more resistant to adverse effects of maternal heat stress as pregnancy progresses; substantial resistance develops by d 3. This information may be useful in design of environmental modification systems that provide cooling at critical periods of gestation to enhance pregnancy rates during summer in hot climates. (Key words: embryos, heat stress, hyperthermia)

INTRODUCTION

In hot climates, fertility of dairy cattle is depressed during summer (1, 19, 21, 26, 29). Heat generated from metabolic functions associated with lactation, growth, and maintenance are not exchanged readily in hot environments, and cows often become hyperthermic when they are exposed to heat stress. A major source for reduction in embryonic survival induced by heat stress may be adverse effects of elevated body temperatures on developing zygotes and embryos. Exposure of cattle to elevated temperatures during oocyte maturation and ovulation (24) or during the first 3 or 7 d of pregnancy (6, 23) decreased embryonic viability and development. In other species, embryos become more resistant to elevated temperature as development progresses. In particular, sheep and swine embryos are very sensitive to deleterious effects of maternal heat stress during the first 2 d of pregnancy but become more resistant to maternal heat stress effects by d 3 to 5 after breeding (7, 20). The increased resistance may be due to development of biochemical responses that limit deleterious effects of elevated temperature, i.e., the heat shock response (15, 18).

The objective of the current study was to test whether embryos from superovulated cows become more resistant to adverse effects of maternal heat stress as embryonic development progresses. Superovulated cows were used to increase the number of embryos examined per cow and thereby to improve the efficiency of the experimental design. Identification of stages at which embryos are most susceptible to heat stress will contribute to the knowledge of developmental changes in embryonic responses to stress and is of practical importance because pregnancy rate during periods of heat stress may be improved by provision of cooling to cows during critical periods of early pregnancy.
MATERIALS AND METHODS

Synchronization of Estrus and Superovulation

The experiment was conducted from June to September over 2 consecutive yr at the University of Florida Dairy Research Unit in Hague. Groups of 10 to 20 lactating, nonpregnant Holstein cows (50 to 150 DIM) were housed in a free-stall barn containing fans and sprinklers. Fans operated continuously from 0700 to 2000 h, and sprinklers operated for 3 min at 20-min intervals from 0700 to 2000 h daily. Estrous cycles were synchronized by administration of PGF$_{2\alpha}$ (25 mg; Lutalyse®; Upjohn Co., Kalamazoo, MI) twice at 11-d intervals. From 48 to 96 h after the second injection of PGF$_{2\alpha}$, cows were observed for estrus twice daily. Cows observed in standing estrus were superovulated by administration of 44 mg of pituitary-derived FSH (Schering Corp., Kenilworth, NJ) twice daily on d 10 (14 mg/d), 11 (12 mg/d), 12 (10 mg/d), and 13 (8 mg/d) postestrus (23). On d 12 postestrus, PGF$_{2\alpha}$ (25 mg) was administered twice and cows were inseminated artificially three times at 12-h intervals from the onset of estrus (onset of estrus = d 0 of pregnancy). A majority of cows demonstrated standing estrus on the morning of d 14; cows not demonstrating standing estrus by this time were administered 4 mg of pituitary-derived FSH and observed for estrous behavior for an additional 24 h.

Treatments

For yr 1, treatments were heat stress on d 1, 3, 5, or 7 of pregnancy or no heat stress (control). For yr 2, treatments were heat stress on d 1 or 3 or control. To induce heat stress, cows were placed in an unshaded lot from 0800 to 1500 h. If a cow experienced rectal temperatures >42°C, she was removed from the heat stress lot and placed under a shade structure until 1500 h. At all other times, except during milking (0700 and 1600 h for yr 1; 0200, 1000, and 1600 h for yr 2), cows were housed in a free-stall barn to maintain thermoneutral conditions.

Cows were assigned randomly to treatments at the onset of estrus. If environmental conditions on the assigned day of treatment apparently would not be conducive to impose heat stress (for example, rain or cool air temperatures), cows were not placed in the lot but were reassigned randomly to other available treatments.

Environmental measurements (black globe temperature, dry bulb temperature, and relative humidity) were recorded on the day of heat stress at 1200 and 1500 h in the heat stress lot (both years) and in the free-stall barn (yr 2). Rectal temperatures were measured at 1200 and 1500 h on the day of heat stress for all heat-stressed cows (both years) and for control cows at either d 1 or 3 (yr 2).

Determination of Embryonic Survival

On d 8, each uterus was flushed nonsurgically to retrieve embryos, which were classified according to stage of development (5). Viability was determined using the vital stain 4',6'-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO). Embryos were incubated in Dulbecco’s PBS (pH 7.4, 25°C) containing 0.0001% DAPI for 15 to 20 min at room temperature (25°C), washed in Dulbecco’s PBS, and examined using an epifluorescence microscope with a 490-nm emission filter. Embryonic viability was scored on a four-point scale according to the proportion of cells within embryos that stained positive for DAPI; DAPI score was 1 when no cells stained, 2 when fewer than one-third of the cells stained, 3 when one- to two-thirds of the cells stained, and 4 when more than two-thirds of the cells stained (10, 28). Embryos were considered to be live when fewer than one-third of the cells stained positive for DAPI (scores of 1 and 2).

Statistical Analyses

Data were analyzed using both categorical procedures (CATMOD) and least squares ANOVA (GLM) through procedures of SAS (27). One-cell embryos were considered to be unfertilized oocytes and were removed from all analyses except for the stage of embryonic development at d 8 and percentage of embryos at the blastocyst stage. Number of cows and embryos for data including and excluding 1-cell embryos and unfertilized oocytes are presented in Table 1. For categorical procedures (DAPI score, percentage of embryos at each stage of development, and distribution of embryos within stage), the model included
components of treatment, year, and treatment × year interactions. Stages of embryonic development included 1 cell, 2 to 8 cells, 9 to 16 cells, morula, and blastocyst. Data were analyzed using all treatments (complete data set) and then reanalyzed after excluding treatments on d 5 and 7 (reduced data set) because these treatments were not represented during yr 2. For ANOVA (rectal temperature, DAPI score, percentage of live embryos, and percentage of blastocysts), cow was used as the experimental unit for effects of treatment, year, treatment × year, and experiment (treatment × year). These analyses were accomplished by analysis of mean embryonic responses for each cow. Preplanned orthogonal contrasts were performed to separate treatment effects and consisted of control, d 1 and 3 versus d 5 and 7, d 5 versus 7, control and d 3 versus d 1, and control versus d 3. For the reduced data set, contrasts were control and d 3 versus d 1 and control versus d 3. For rectal temperature, Duncan's multiple range test was used to evaluate differences among treatments.

RESULTS

Environmental Conditions

Peak black globe temperature, dry bulb temperature, and relative humidity in the unshaded lot averaged 42.1°C, 34.3°C, and 53.7% for yr 1 and 41.5°C, 34.7°C, and 64.4% for yr 2, respectively. During yr 2, black globe temperatures, dry bulb temperatures, and relative humidity in the control environment averaged 31.8°C, 30.5°C, and 64.4%, respectively. Rectal temperatures were higher (P < .05) for cows placed in the unshaded lot than for controls (40.9 to 41.7 vs. 39.1°C; Table 1) and are comparable with rectal temperatures in previous studies (23, 24). Variation in rectal temperatures among cows was not significant in embryonic responses because use of rectal temperature as a covariate did not affect results.

Embryonic Survival

A CATMOD analysis revealed an effect of treatment on DAPI score when all data were analyzed (P = .07) or after embryos from cows heat stressed on d 5 and 7 were excluded (P = .03). As shown in Figure 1, maternal heat stress on d 1 resulted in fewer embryos with DAPI scores of 1 and 2 and more embryos with DAPI scores of 4. Least squares means for DAPI scores are presented in Table 1. As determined by ANOVA of orthogonal contrasts, DAPI score was greater for embryos from cows heat stressed on d 1 than for embryos from control cows and cows heat stressed on d 3 (complete data set, P = .07; reduced data set, P = .05). Day of heat stress also affected the proportion of embryos classified as live or dead based on CATMOD analysis of DAPI scores (Table 1; P = .03 for complete and reduced data sets). The percentage of live embryos was also calculated for each cow, and data were analyzed by least squares ANOVA to consider variation among cows in the analysis. Least squares means for the percentage of live embryos were 70.9% for embryos from control cows and 54.9, 60.3, 62.6, and 82.0% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively (P = .07, d 1 versus control and d 3 for the reduced data set; SEM = 12%). No year × treatment interactions were detected for any of the models used to analyze mean DAPI score or percentage of live embryos.

![Figure 1. Effect of maternal heat stress on 4',6'-diamidino-2-phenylindole (DAPI) score of embryos (≥2 cells). Results represent the percentage of embryos within each treatment with DAPI scores of 1 (no cells stained), 2 (fewer than one-third of the cells stained), 3 (one-third to two-thirds of the cells stained), and 4 (more than two-thirds of the cells stained).](image-url)
TABLE 1. Effects of maternal heat stress for 1 d during early pregnancy on rectal temperature and embryonic viability.

<table>
<thead>
<tr>
<th>Day of heat stress</th>
<th>≥ 1-Cell embryos</th>
<th>≥ 2-Cell embryos</th>
<th>Peak rectal temperature</th>
<th>Mean DAPI score</th>
<th>Live embryos</th>
</tr>
</thead>
</table>
|                   | Cows | Embryos | Cows | Embryos | (°C) | (%)
| Control           | 20   | 118    | 18   | 94    | 39.1 | 2.2 | 70.2 |
| 1                 | 11   | 50     | 9    | 40    | 41.3 | 2.5 | 55.0 |
| 3                 | 8    | 50     | 8    | 50    | 40.9 | 2.0 | 68.0 |
| 5                 | 10   | 23     | 9    | 20    | 41.7 | 2.2 | 65.0 |
| 7                 | 5    | 31     | 5    | 27    | 41.0 | 1.7 | 88.9 |

1Data for cows from which at least one embryo at 1-cell (unfertilized oocyte) or subsequent stages was retrieved.
2Data for cows from which at least one embryo at 2-cell or subsequent stages was retrieved.
3Least squares means (SEM = .08°C). Controls differed from other treatments (P < .05) as determined by Duncan’s multiple range test.
4Least squares means (SEM = .11). The CATMOD analysis demonstrated an effect of treatment on 4',6'-diamidino-2-phenylindole (DAPI) score (P = .07 for complete data set; P = .03 for reduced data set). By ANOVA, DAPI score differed for embryos from cows heat stressed on d 1 compared with embryos from control cows and cows heat stressed on d 3 (P = .07 for complete data set; P = .05 for reduced data set). Contrasts for embryos from control cows versus embryos from cows heat stressed on d 3; embryos from cows heat stressed on d 3 versus embryos from cows heat stressed on d 7; and control, d 1 and d 3 versus d 5 and d 7 were not significant.
5Represents percentages calculated from the number of live embryos divided by the total number of embryos (2-2-cell). The CATMOD analysis demonstrated an effect of treatment on the percentage of live embryos (P = .03 for complete and reduced data set). By ANOVA, the percentage of live embryos differed on d 1 compared with control and d 3 (P = .10 for complete data set; P = .07 for reduced data set). Other orthogonal contrasts (control versus d 3; d 5 versus d 7; and control, d 1 and d 3 versus d 5 and d 7) were not significant.

Embryonic Development

Alterations in the distribution of stage of embryonic development (Table 2) were evident among treatments as determined by CATMOD analysis of all treatments (P = .10) or after exclusion of embryos from cows heat stressed on d 5 and 7 (P = .03). Individual analysis of the percentage of embryos at each stage showed that heat stress on d 1 decreased the percentage of embryos at the blastocyst stage of development (P = .02 for the complete data set; P = .05 for the reduced data set) and increased the percentage of embryos at the 9- to 16-cell stage of development (P = .04 for the complete data set; P = .01 for the reduced data set). In addition, day of heat stress affected percentage of 1-cell embryos and unfertilized oocytes (P = .03 for complete data set; P = .05 for the reduced data set) and was lower for cows treated at d 3. No treatment effects were observed at other stages. Least squares means for the percentage of blastocysts, when 1-cell embryos were removed from the data sets, were 60.6% for embryos from control cows and 37.4, 54.8, 62.6, and 64.5% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively; the percentage of blastocysts was decreased only on d 1 (P = .07 for complete data set; P = .06 for reduced data set when results from d 1 were compared with those for controls and d 3; SEM = 13%). When 1-cell embryos and unfertilized oocytes were included in the analysis, least squares means for the percentage of blastocysts were 42.1% for embryos from control cows and 28.4, 54.8, 63.0, and 58.5% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively. With this analysis, heat stress on d 1 decreased percentage of blastocysts compared with embryos from control cows and from cows heat stressed on d 3 (P = .03 for complete data set; P = .05 for reduced data set; SEM = 11%). Year × treatment interactions were not significant for any statistical analysis of embryonic development.

DISCUSSION

These results demonstrate that bovine embryos become more resistant to deleterious effects of maternal heat stress as they proceed through development. Thus, the cow is similar to the sheep (7) and pig (20) in this regard. A minimum period after heat stress before evaluation of embryonic viability and development could preclude the ability to detect adverse
TABLE 2. Effect of maternal heat stress during early pregnancy on stage of embryonic development on d 8 of pregnancy.

<table>
<thead>
<tr>
<th>Day of heat stress</th>
<th>Embryos (n)</th>
<th>1 Cell1</th>
<th>2 to 8 Cells</th>
<th>9 to 16 Cells2</th>
<th>Morula</th>
<th>Blastocyst3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>118</td>
<td>20</td>
<td>11</td>
<td>5</td>
<td>15</td>
<td>49</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>20</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0</td>
<td>10</td>
<td>6</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>13</td>
<td>4</td>
<td>4</td>
<td>22</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
<td>13</td>
<td>0</td>
<td>6</td>
<td>17</td>
<td>64</td>
</tr>
</tbody>
</table>

1Treatment effect by CATMOD (P = .03 for complete data set; P = .05 for reduced data set).
2Treatment effect by CATMOD (P = .04 for complete data set; P = .01 for reduced data set).
3Treatment effect by CATMOD (P = .02 for complete data set; P = .05 for reduced data set). By ANOVA, the percentage of blastocysts was reduced on d 1 compared with control and d 3 (P = .07 for complete data set; P = .06 for reduced data set). Other orthogonal contrasts (control versus d 3; d 5 versus d 7; and control, d 1 and d 3 versus d 5 and d 7) were not significant.

Effects for embryos on d 5 and 7 treatments. Because no effects of heat stress on embryos were observed on d 3, however, embryos apparently became more resistant to heat stress as development progresses. The magnitude of adverse effects of maternal heat stress on embryonic survival was less severe in the present study than for studies in other species. Differences may be attributed to method of heat stress; studies in sheep and pigs (7, 20) were performed in environmentally controlled chambers for 17 to 24 h, but the present study was performed under more variable environmental conditions for 7 h. Present findings do not imply that bovine embryos are completely resistant to effects of maternal heat stress by d 3 of pregnancy; heat stress of greater severity or duration than that used in this study could possibly decrease embryonic survival before or after d 1. Maternal heat stress during final oocyte maturation and ovulation has deleterious effects on subsequent embryonic development that is more severe than was observed in the present study (24). In addition, conceptus development was decreased by maternal heat stress from d 8 to 16 of pregnancy (12). Nonetheless, these results demonstrate that embryos respond differentially to maternal heat stress depending on their stage of development, and, by d 3 of pregnancy, embryos have acquired some resistance to adverse effects of maternal heat stress.

Mechanisms that are responsible for the ontogeny of embryonic resistance to thermal stress are not defined but could reflect changes in embryonic function or in the microenvironment of the embryo. Embryos may develop the capacity to produce molecules that limit effects of heat on cellular function. In many cells, the synthesis of heat shock proteins (HSP) during elevated temperature limited deleterious effects of elevated temperatures (14, 15, 18, 25). Through induced thermotolerance, cultured bovine blastocysts can gain tolerance to 43°C if they are first exposed to a mild elevation in temperature (9). In mouse embryos, induced thermotolerance is regulated developmentally; 2- and 4-cell embryos cannot undergo induced thermotolerance, but later stages can (11). Moreover, induction of at least one HSP by elevated temperature, HSP68, may first occur at the 8-cell stage (4). The 8-cell stage of development in mouse embryos coincides with full activation of the mouse embryonic genome (22). The development of bovine embryonic resistance to maternal heat stress in cattle may be due to the ability of embryos to produce HSP in response to elevated temperatures. Consistent with this theory, the bovine embryonic genome is activated between the 8- to 16-cell stage (2) [i.e., d 3 of pregnancy (3)].

Developmental resistance of embryos to maternal heat stress may also involve interactions between the embryo and reproductive tract. Heat stress altered protein secretion from the oviduct and uterus (16, 17). The increase in
resistance of embryos to heat stress is not likely changed by a shift in the location of embryos from oviduct to uterus because embryos are present in the oviduct at d 1 and 3 of pregnancy (3).

This study may have practical implications for improving fertility during summer in hot climates. Hansen et al. (13) proposed that, although cows should receive cooling at all times during heat stress, summer pregnancy rates may be improved during periods of heat stress through strategic cooling, in which cows are placed in an environment that allows maximal cooling during times when embryos are most sensitive to heat stress effects. These times have been defined as the period of final oocyte maturation and ovulation (24) and the first few days of pregnancy (present study). Also, administration of agents that protect embryos from adverse effects of heat stress may be a useful approach to improve pregnancy rates during summer (13). One such agent, glutathione, improved embryonic viability and development in cultured bovine embryos exposed to elevated temperatures (8). Present results indicate that the most beneficial time to administer such agents is before d 3 of pregnancy.

CONCLUSIONS

Embryos are sensitive to deleterious effects of maternal heat stress at d 1 of pregnancy but become more resistant to heat stress effects by d 3 of pregnancy. These results suggest that the embryo acquires thermotolerance during development and that improved fertility may be possible during periods of heat stress if cooling is provided for a limited period when embryos are most sensitive to thermal stress.

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REFERENCES