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Developmental Changes in Sensitivity of Bovine Embryos to Heat Shock and Use of Antioxidants as Thermoprotectants

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ABSTRACT: Experiments were conducted with in vitro-produced bovine embryos to determine whether 1) increased culture temperatures (i.e., heat shock) adversely affected embryonic development, 2) embryos become more resistant to heat shock as they advance in development, and 3) selective antioxidant molecules alleviate heat shock effects on embryonic development. Development of 2-cell embryos to ≥16-cell stage on d 5 after in vitro fertilization was not affected by a heat shock of 40°C for 3 h, but 41 or 42°C for 3 h decreased (P = .004) development. In a separate experiment, development of 2-cell embryos was decreased (P = .01) by exposure to 41°C for 3 h but not for 1 h. In contrast, development of morulae to blastocysts was not affected by heat shock of 41°C for 1 or 3 h. Medium supplementation with 50 nM glutathione or 50 mM taurine before heat shock did not reduce the effects of heat shock (41°C for 3 h) on 2-cell embryos. Likewise, addition of glutathione ester, a more membrane-permeable analog of glutathione, did not protect 2-cell embryos from heat shock. In conclusion, early bovine embryos are susceptible to disruption in development caused by heat shock. As embryos progress in development, they acquire resistance to heat shock. Glutathione, taurine, and glutathione ester were not effective in alleviating the effects of heat shock on development of 2-cell embryos. Consequently, molecules have yet to be identified that can protect early-stage bovine embryos from the adverse effects of heat shock.

Key Words: Cows, Bovidae, Embryos, Heat Shock, Glutathione, Taurine

Introduction

Establishment of pregnancy becomes less susceptible to disruption by heat stress as pregnancy proceeds (Dutt, 1963; Ealy et al., 1993). For example, heat stress in cows decreased embryonic development and survival if applied at d 1 of pregnancy but not if given at d 3 or later (Ealy et al., 1993). The embryo itself is susceptible to increased temperature; exposure of embryos to high temperatures in vitro (i.e., heat shock) disrupted embryonic development and viability (Alliston et al., 1965; Gwazdauskas et al., 1992; Ealy et al., 1992; Ealy and Hansen, 1994). Free radical production may be one mechanism by which heat shock alters cellular function (Loven, 1988). Depletion of the intracellular antioxidant, glutathione (GSH), prevented thermoprotective responses in murine morulas (Aréchiga et al., 1992). Also, supplementation of GSH (Ealy et al., 1992; Aréchiga et al., 1994), vitamin E (Aréchiga et al., 1994), or taurine (Ealy et al., 1992; Malayer et al., 1992) reduced effects of heat shock on murine and bovine morulas. The present objectives were to characterize culture temperatures that disrupt embryonic development in cattle, determine whether embryos gain resistance to heat shock as they progress from the 2-cell to morula stage, and evaluate whether specific antioxidants can block effects of heat shock.

Materials and Methods

Materials

Steer serum was purchased from Pel-Freez (Rogers, AR). Pituitary-derived follicle-stimulating hormone

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(FSH-P) was from Schering (Kenilworth, NJ). Frozen semen from various Holstein bulls was obtained from American Breeders Service (Madison, WI), and Buffalo rat liver cells (line BRL-3A) were obtained from American Type Culture Collection (Rockville, MD). Acetic acid and n-propanol were purchased from Fisher Scientific (Pittsburgh, PA). Tissue Culture Medium 199 (TCM-199), paraffin oil, and other culture reagents were obtained from Sigma Chemical (St. Louis, MO). The CZB medium was prepared as described by Chatot et al. (1989), and modified Tyrode's solutions (HEPES-TALP and IVF-TALP) were prepared as described by Parrish et al. (1986). Ovary transportation solution was 9% (wt/vol) NaCl containing 1% (vol/vol) antibiotic-antimycotic solution (ABAM; Sigma Chemical) so that there was 550 μg/L of amphotericin, 100,000 IU/L of penicillin-G, and 100 mg/L of streptomycin. Reduced GSH and taurine were prepared as described by Parrish et al. (1986). Tyrode's solutions (HEPES-TALP and IVF-TALP) were incubated at 39°C in an atmosphere of 5% CO₂ in air for 24 to 26 h. An average of 11.5 oocytes were collected per ovary.

In Vitro Maturation/Fertilization/Culture

The procedures used to produce in vitro-derived embryos were modifications of published procedures (Parrish et al., 1986; Xu et al., 1992; Hernandez-Ledezma et al., 1993). Bovine ovaries were obtained from a local abattoir and transferred to the laboratory in sterile transportation solution at 23 to 26°C. Ovaries were washed repetitively in fresh saline upon arrival and placed in a warm room (approximately 30°C) for oocyte collection. Checkerboard incisions (approximately 2 mm depth) were then made on the surface of each ovary using scalpel blades. Ovaries were washed vigorously in collection medium (TCM-199 with Hank's salts, 10 mM HEPES, 2% [vol/vol] steer serum, 40 units/L heparin, and 1% [vol/vol] ABAM). Oocytes containing at least one complete layer of surrounding cumulus cells and evenly granulated ooplasm (approximately 300 oocytes/10 mL of medium in 60-mm x 15-mm culture dishes) were placed in maturation medium (TCM-199 with Earle's salts, 10% [vol/vol] steer serum, 2 μg/mL of estradiol, 20 μg/mL of FSH-P, and 50 μg/mL of gentamicin) and were incubated at 39°C in an atmosphere of 5% CO₂ in air for 24 to 26 h. An average of 11.5 oocytes were collected per ovary.

Following maturation, oocytes containing expanded cumulus were washed in HEPES-TALP and placed in IVF-TALP (approximately 30 oocytes/600 μL of medium in four-well plates). Viable spermatozoa were recovered from frozen/thawed semen from two to four bulls by Percoll gradient centrifugation (Hernandez-Ledezma et al., 1993) and added to IVF-TALP at a final concentration of 1 x 10⁶ spermatozoa/mL of medium. Immediately after adding spermatozoa, epinephrine (2.5 μM), hypotaurine (10 μM), and penicillamine (20 μM) were added to wells to improve fertilization rate (Leibfried and Bavister, 1982). Oocytes were then incubated at 39°C for 16 to 18 h (d = day of in vitro fertilization [IVF]). Cleavage rate averaged 59.0% and rate of parthenogenesis averaged 5.8%.

At 16 to 18 h after fertilization (d 1 after IVF), putative zygotes were removed from wells, vortexed to remove cumulus cells and attached spermatozoa, and washed in HEPES-TALP. Zygotes were then placed either in 600 μL of CZB medium containing 10% (vol/vol) steer serum and 50 μg/mL of gentamicin (termed modified CZB; approximately 50 oocytes and zygotes/well) or in 50-μL microdrops of TCM-199 medium containing 10% (vol/vol) steer serum, and 50 μg/mL of gentamicin (termed modified TCM-199) covered in paraffin oil (approximately 50 oocytes and zygotes/well). Before use in embryo culture, both media were conditioned for 24 to 48 h by Buffalo rat liver cells. Conditioned medium was sterile-filtered and stored for up to 1 wk at 4°C.

Heat Shock Treatments

Two-Cell Embryos. Putative embryos (approximately 50/well) were collected on d 1 after IVF and placed into wells containing 600 μL of modified CZB medium for 18 to 24 h at 39°C in 5% CO₂. On d 2 after IVF, 2-cell embryos were placed in groups of 15 to 25 into wells containing 600 μL of modified CZB medium, incubated for 1 h at 39°C, and exposed to heat shock treatments of 41 or 42°C. Embryos were heat-shocked by placement in a humidified CO₂ incubator. When placed at 41°C, wells reached a temperature of 40°C within 13 min, 40.5°C within 26 min, and 41.0°C within 50 min. After heat shock, embryos were maintained at 39°C. Embryos were transferred to 600 μL of modified TCM-199 on d 3 after IVF and then incubated at 39°C until d 5, when development was assessed.

To test the effect of antioxidants on embryonic development, antioxidant stock solutions were prepared on the day of use and administered after transfer of 2-cell embryos to wells containing 600 μL of modified CZB medium on d 2 after IVF. Cultures were equilibrated for 1 h (GSH and taurine) or 3 h (GSH ester) at 39°C before exposure to heat shock. Embryos were then maintained in CZB medium containing antioxidants until d 3 after IVF, when embryos were transferred to modified TCM-199 medium without additional antioxidants.

Morula-Stage Embryos. Putative embryos (approximately 20/drop) on d 1 after IVF were placed into...
50-μL drops of modified TCM-199 covered with paraffin oil and cultured at 39°C. On d 3 after IVF, an additional 50 μL of modified TCM-199 was added to each drop. Morulas were collected on d 5 after IVF. Groups of 9 to 15 morulas were placed either in 50-μL drops of modified TCM-199 medium or modified CZB medium, maintained at 39°C for 1 h, and exposed to heat shock treatments of 41°C for 1 or 3 h. Embryos were heat-shocked by placement in a humidified CO₂ incubator. When placed at 41°C, microdrops reached a temperature of 40°C within 9.5 min, 40.5°C within 18 min, and 41.0°C within 48 min. After heat shock, embryos were maintained at 39°C. For embryos placed in modified CZB medium on d 5 after IVF, embryos were transferred to modified TCM-199 microdrops on d 6 after IVF. Embryos placed in modified TCM-199 medium on d 5 after IVF were maintained in the same microdrop for the duration of culture. Stage of development was recorded on d 9 after IVF. Data from CZB and TCM-199 cultures were combined for analysis because the proportion of embryos that developed to the blastocyst stage was not affected by type of medium used on d 5 after IVF.

Statistical Analysis

Each experiment was performed on several days, and one or more wells or microdrops (replicates) of embryos per treatment were used on each day. Treatment effects on the number of embryos developing in each replicate (i.e., each well or microdrop) were analyzed with least squares analysis of variance (SAS, 1989). The independent variables were temperature, day, temperature × day, and, when appropriate, antioxidant treatment and interactions with antioxidant treatment. The total number of embryos in each replicate was used as a covariate. When applicable, orthogonal contrasts were performed to partition treatment effects into individual comparisons (SAS, 1989). Probability values represent results from these analyses. To calculate least squares means for percentage of embryos developing, percentage of development for each replicate was also analyzed with least squares analysis of variance. Each data set was tested for heterogeneity of variance; when present (Exp. 1 only), individual standard errors were calculated for each treatment. Otherwise, pooled standard errors generated from least squares analyses of variance were reported.

Results

Effects of Heat Shock on Two-Cell and Morula-Stage Embryos

In the first experiment, 2-cell embryos were exposed to a heat shock of 40, 41, or 42°C for 3 h (Table 1). Compared with controls incubated continuously at 39°C, exposure to 40°C did not influence the proportion of embryos that had developed to ≥16-cell stage on d 5 after IVF. However, heat shock of 41 or 42°C decreased subsequent development (P = .004) compared with embryos cultured at 39 or 40°C. Sensitivity of 2-cell embryos to heat shock was further determined by exposing 2-cell embryos to a 41°C heat shock for 1 or 3 h (Table 2). Compared with embryos cultured continuously at 39°C, exposure to 41°C for 3 h decreased (P = .01) subsequent development of 2-cell embryos, whereas heat shock of 41°C for 1 h did not affect development. Effects of a 41°C heat shock were also determined for morula-stage embryos (Table 2). In contrast to embryos at the 2-cell stage, there was no effect of 41°C for 1 or 3 h on subsequent development.

Antioxidant Treatment

Two experiments were performed to determine whether administration of selective antioxidants protected 2-cell embryos from heat shock. In the first experiment, effects of 50 nM GSH and 50 mM taurine were evaluated (Table 3). Heat shock of 41°C for 3 h decreased (P = .06) subsequent embryonic development. Although there were effects of antioxidant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of replicates</th>
<th>No. of embryos</th>
<th>Percentage of embryos developing to ≥16-cell stagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>39°C</td>
<td>6</td>
<td>109</td>
<td>31.4 ± 5.8b</td>
</tr>
<tr>
<td>40°C for 3 h</td>
<td>6</td>
<td>110</td>
<td>29.2 ± 8.3b</td>
</tr>
<tr>
<td>41°C for 3 h</td>
<td>6</td>
<td>107</td>
<td>10.6 ± 3.4c</td>
</tr>
<tr>
<td>42°C for 3 h</td>
<td>6</td>
<td>97</td>
<td>2.6 ± 1.8c</td>
</tr>
</tbody>
</table>

aData are least squares means ± SE for embryonic development on d 5 after IVF. Overall, temperature affected development (P = .01).
b Means with different superscripts differ as determined with orthogonal contrasts between 39 vs 40°C (not significant), 41 vs 42°C (not significant), and 39 and 40°C vs 41 and 42°C (P = .004).
Table 2. Effects of a 41°C heat shock on subsequent development of 2-cell and morula-stage bovine embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of replicates</th>
<th>No. of embryos</th>
<th>Percentage of embryos undergoing subsequent developmenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39°C</td>
<td>6</td>
<td>138</td>
<td>33.2 ± 4.2b</td>
</tr>
<tr>
<td>41°C for 1 h</td>
<td>6</td>
<td>137</td>
<td>27.3 ± 4.2b</td>
</tr>
<tr>
<td>41°C for 3 h</td>
<td>6</td>
<td>138</td>
<td>12.2 ± 4.2c</td>
</tr>
<tr>
<td>Morula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39°C</td>
<td>6</td>
<td>65</td>
<td>30.0 ± 4.9b</td>
</tr>
<tr>
<td>41°C for 1 h</td>
<td>6</td>
<td>64</td>
<td>22.8 ± 4.9b</td>
</tr>
<tr>
<td>41°C for 3 h</td>
<td>6</td>
<td>67</td>
<td>21.7 ± 4.9b</td>
</tr>
</tbody>
</table>

aData represent least squares means ± SE for percentage of 2-cell embryos developing to 216-cell stage on d 5 after IVF or percentage of morula developing to blastocysts on d 9 after IVF. Overall, heat shock decreased subsequent development of 2-cell embryos (P = .03) but not morulas.

Within each stage of development, different superscripts designate differences determined with orthogonal contrasts. For 2-cell embryos, differences were detected when comparing 41°C for 1 h vs 41°C for 3 h (P = .01) but not when comparing 41°C for 1 h and 41°C for 3 h vs 39°C. No orthogonal contrasts were significant for morulas.

treatment (P = .02) and heat shock × antioxidant treatment (P = .02), there were no thermoprotective effects of GSH or taurine. Indeed, taurine decreased development of embryos at 39°C. In a second experiment, administration of GSH ester, which penetrates cell membranes more readily than GSH, was examined for its ability to prevent the effects of heat shock at doses of 10 μM, 100 μM, 1 mM, and 10 mM. At the greatest concentration (10 mM), GSH ester prevented subsequent development at 39°C (0/38 developed) and 41°C for 3 h (0/26 developed). Therefore, this treatment was removed from subsequent statistical analysis (Figure 1). Heat shock of 41°C for 3 h decreased embryonic development (P = .002), and there was a heat shock × GSH ester interaction (P = .04). This interaction represents decreased development of control embryos administered 100 μM GSH ester. The GSH ester did not prevent heat shock effects on embryonic development at any concentration examined.

Discussion

Exposure of 2-cell bovine embryos to a heat shock of as little as 41°C for 3 h decreased embryonic development as determined on d 5 after IVF. This incubation temperature is similar to body temperatures measured in cattle during periods of heat stress (Putney et al., 1989; Ealy et al., 1993). Results support the idea that a major cause of decreased embryonal survival from maternal heat stress is the direct effect of increased body temperature on embryonic development and survival. In contrast to embryos at the 2-cell stage, morulas were not affected by heat shock of 41°C for 1 or 3 h. There was a slight reduction in development of morulas caused by heat shock, but this effect was not significant and was smaller in magnitude than the reduction in development caused by heat shock of 2-cell embryos. Thus, bovine embryos seem to acquire increased thermal resistance during embryonic development. This conclusion is tentative because certain aspects of culture conditions and the end point measured (development to 16-cell stage vs development to blastocyst) differed...
between the two stages of development. Also, it is possible that the apparently greater resistance of the morula-stage embryos is because embryos that possess thermal resistance capabilities were favored during in vitro development. Despite these caveats, the change in thermal resistance with increased development in vitro development. Despite these caveats, the change in thermal resistance with increased development in vitro seen in the present study parallels changes in vivo. In particular, deleterious effects of maternal heat stress on embryonal survival were less on d 3 of pregnancy than on d 1 of pregnancy in cattle (Ealy et al., 1993) and sheep (Dutt, 1963).

Although mechanisms responsible for the developmental resistance of embryos to heat shock are not defined in cattle, embryonal resistance to heat shock may be related to heat-inducible changes in synthesis of heat shock proteins or other thermoprotective molecules. The ability to undergo induced thermotolerance (i.e., increased resistance to a severe heat shock with prior exposure to a mild heat shock) in murine embryos increases as development progresses (Muller et al., 1985; Ealy and Hansen, 1994). Early data indicated that the acquisition of thermotolerance in mouse embryos is related to the inability to produce 70-kDa heat shock proteins (HSP70; Morange et al., 1984; Hahnel et al., 1986). More recent data indicate that heat shock-induced synthesis of HSP70 can precede induced thermotolerance (Howell et al., 1994). Future experiments will be required to determine whether the acquisition of thermal resistance in bovine embryos coincides with activation of heat shock protein synthesis or expression of additional thermoprotective mechanisms in response to heat. Increased thermal sensitivity of embryos during initial cleavage stages may also be due to the potentially more deleterious effect of loss of a few blastomeres caused by heat shock in early embryos than in embryos at later stages of development.

Present results that morulae were resistant to 41°C does not mean that development cannot be disrupted by heat shock at this stage. Other experiments indicate that a more severe heat shock of 42°C can decrease developmental capacity of bovine morulas. However, such a temperature is higher than usually experienced by heat-stressed cows (Putney et al., 1989; Ealy et al., 1993).

Antioxidants tested in the present study were taurine, a weak antioxidant (Wright et al., 1986; Green et al., 1991) present in high concentrations in oviducts (Fahning et al., 1967; Casslén, 1987), GSH, a prominent intracellular antioxidant that is membrane-impermeable (Meister, 1983), and a membrane-permeable form of GSH, monoethyl GSH ester (Anderson and Meister, 1989). None of the antioxidants tested protected 2-cell embryos from the adverse effects of heat shock. This is in contrast to previous studies demonstrating a thermoprotective effect of taurine (Ealy et al., 1992; Malayer et al., 1992), GSH (Ealy et al., 1992; Aréchiga et al., 1994), and vitamin E (Aréchiga et al., 1994) on morula-stage murine and bovine embryos. This discrepancy is likely due to differences in stage of embryonic development. Perhaps GSH and taurine were not effective at the 2-cell stage because the presence of other protective responses that occur later in development (for example, heat shock protein synthesis) are necessary for GSH or taurine to be effective thermost protectants. Also, it is possible that embryos are damaged by heat shock through processes that do not involve free radical formation. For example, temperature-dependent changes in protein and membrane structure (Lepock et al., 1983; Konings, 1988) may be sufficient to prevent development of early embryos. Even in mouse morulas, antioxidants were more effective in preventing the effects of heat shock on percentage of live embryos (determined by live/dead stain) than on subsequent development (Malayer et al., 1992; Aréchiga et al., 1994).

### Table 3. Effect of glutathione (GSH) and taurine on subsequent development of 2-cell bovine embryos exposed to heat shock (41°C for 3 h)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of replicates</th>
<th>No. of embryos</th>
<th>Percentage of embryos developing to ≥16-cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>39°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>99</td>
<td>46.3 ± 3.7</td>
</tr>
<tr>
<td>50 nM GSH</td>
<td>5</td>
<td>80</td>
<td>40.9 ± 4.1</td>
</tr>
<tr>
<td>50 mM taurine</td>
<td>4</td>
<td>58</td>
<td>13.8 ± 4.6</td>
</tr>
<tr>
<td>41°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>97</td>
<td>24.6 ± 3.7</td>
</tr>
<tr>
<td>50 nM GSH</td>
<td>6</td>
<td>98</td>
<td>30.0 ± 3.7</td>
</tr>
<tr>
<td>50 mM taurine</td>
<td>4</td>
<td>61</td>
<td>26.6 ± 4.6</td>
</tr>
</tbody>
</table>

aData represent least squares means ± SE for embryonic development on d 5 after IVF. Overall, subsequent embryonic development was affected by heat shock (P = .06), antioxidant treatment (P = .02), and heat shock × antioxidant treatment (P = .02). When only glutathione and control treatments were analyzed, there was an effect of heat shock (P = .006), but glutathione or heat shock × glutathione effects were not significant. When taurine and control treatments were analyzed, there were effects of taurine (P = .01) and heat shock × taurine (P = .006).
Lack of thermoprotection by administration of the membrane-permeable GSH ester indicates that the ineffectiveness of GSH in preventing the effects of heat shock was not caused by failure of entry into the cell. At least for GSH, it is also unlikely that the concentrations were inadequate for enhancing antioxidant status of embryos because a wide range of concentrations were tested. It is possible that GSH or taurine diffused into the oil covering each microdrop, although this seems unlikely given the poor lipid solubility of these molecules.

Culture of embryos in air rather than in a reduced-oxygen environment may increase the possibility for free radical damage in response to heat shock or even in the absence of heat shock. A role for free radicals in inhibiting development of embryos not exposed to heat shock is suggested by results from previous studies in which taurine (Dumoulin et al., 1992) and GSH (Legge and Sellens, 1991) enhanced murine embryo development at 37°C. The lack of beneficial effects of antioxidants at 39°C for bovine embryos in the present study can perhaps be attributed to the short duration of antioxidant treatment. In the present study, the only effects of antioxidants at 39°C were adverse. High concentrations of GSH ester reduced embryonic development. This effect may have been due to an increase in osmolarity or to increased acidity of the medium. Such a change in pH was noted in wells receiving 10 mM GSH ester based on the color of the pH indicator in culture medium. Additionally, 50 mM taurine inhibited development at 39°C, even though similar concentrations are present in uterine fluids (Fahning et al., 1967; Casslén, 1987). Perhaps, toxicity of taurine was caused by increased osmolarity of medium or another unidentified type of cellular stress. In contrast to results at 39°C, there was no toxic effect of taurine on 2-cell embryos exposed to 41°C for 3 h. This difference may have occurred if thermoprotective effects of taurine compensated for any toxic effects. Perhaps lower concentrations of taurine may be effective in protecting early stage bovine embryos from heat shock.

The low rate of development for the present experiments probably was caused by the additional manipulation of embryos associated with treatments. For embryos in the current studies not exposed to treatments until the morula stage, 37.9% of cleaved embryos progressed to the morula stage on d 5 after IVF. In other experiments with in vitro-produced embryos conducted concurrently with the present studies, in which embryos were not manipulated during development, an average of 31.2% of cleaved oocytes developed to blastocysts by d 8 after IVF.

**Implications**

Results indicate that increased temperatures disrupt embryonic development in cattle and that embryos become more resistant to heat shock as they proceed through development. Identification of mechanisms by which embryos acquire thermal resistance may lead to development of novel methods for increasing embryonal survival in heat-shocked embryos. Such strategies may include manipulation of antioxidant systems, but evidence from current studies does not support this scheme.

**Literature Cited**


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