Modification of Actions of Heat Shock on Development and Apoptosis of Cultured Preimplantation Bovine Embryos by Oxygen Concentration and Dithiothreitol

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ABSTRACT
Preimplantation embryos exposed to elevated temperatures have reduced developmental competence. The involvement of reactive oxygen species in these effects has been controversial. Here we tested hypotheses that (1) heat shock effects on development and apoptosis would be greater when embryos were cultured in a high oxygen environment (air; oxygen concentration = ~20.95%, v/v) than in a low oxygen environment (5% oxygen) and (2) that these effects would be reversed by addition of the antioxidant dithiothreitol (DTT). Heat shock of 41 °C for 9 hr reduced development of two-cell embryos and Day 5 embryos to the blastocyst stage embryos when in high oxygen. There was no effect of heat shock on development when embryos were in low oxygen. Furthermore, induction of TUNEL-positive cells in Day 5 embryos by heat shock only occurred when embryos were in high oxygen. Addition of DTT to two-cell embryos either did not reduce effects of a heat shock of 41 °C for 15 hr on development or caused slight protection only. In contrast, treatment of Day 5 embryos with DTT reduced effects of heat shock on development and apoptosis. In summary, oxygen tension was shown to be a major determinant of the effects of heat shock on development and apoptosis in preimplantation bovine embryos. Protective effects of the antioxidant DTT were stage specific and more pronounced at later stages of development.

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Key Words: embryo; heat shock; oxygen; apoptosis; development

INTRODUCTION
Exposure to elevated temperature reduces developmental competence of preimplantation embryos. This phenomenon has been shown in several species including mice (Arechiga et al., 1995; Arechiga and Hansen, 1998; Ozawa et al., 2002), rabbits (Makarevich et al., 2007), and cattle (Edwards and Hansen, 1997; Krininger et al., 2002; Paula-Lopes et al., 2003; Sakatani et al., 2004). The mechanism by which heat shock inhibits embryonic development is not completely understood. In the cow, exposure of two-cell embryos to elevated temperature causes a slight increase in the number of swollen mitochondria and changes in the cytoskeleton that lead to displacement of organelles away from the plasma membrane (Rivera et al., 2003, 2004b). At later stages of development (after the 8–16 cell stage), apoptosis can be induced by heat shock but this phenomenon does not occur for earlier embryos (Paula-Lopes and Hansen, 2002b).

Increased production of reactive oxygen species (ROS) may be one mechanism by which heat shock disrupts development of preimplantation embryos. Whether this is really the case is not clear from the literature and may depend on stage of development and degree of heat shock. In the mouse, effects of a heat shock of 42 °C for 2 hr on development were reported to be decreased by glutathione (Arechiga et al., 1995). Moreover, the phenomenon of induced thermotolerance, whereby exposure to a mild heat shock makes cells more resistant to a subsequent, severe heat shock, was dependent upon glutathione synthesis in the mouse morula (Arechiga et al., 1995). However, inhibition of glutathione synthesis did not alter effects of 41 °C for 1 hr on development of two-cell embryos or morulae (Arechiga and Hansen, 1998). Data are inconsistent as to whether heat shock increases production of ROS. One report indicates that exposure of mouse morulae to 41 °C for 1 hr tended to reduce intracellular glutathione (GSH) content (Arechiga et al., 1995). In contrast, heat shock had no direct effect on redox state of mouse zygotes, as assessed by embryonic content of GSH and hydrogen peroxide (Ozawa et al., 2002).

In cattle, there was no thermoprotective effect of the antioxidants GSH, glutathione ester or vitamin E on development (Ealy et al., 1995; Paula-Lopes et al., 2003) but administration of anthocyanins, a class of
antioxidant from purple sweet potato, reduced effects of heat shock at Day 2 after insemination on development to the blastocyst stage (Sakatani et al., 2007). Likewise, GSH was effective at reducing deleterious effects of heat shock in embryos at the morula stage (Ealy et al., 1992). There are also reports that exposure of Day 0 and 2 embryos (relative to insemination) to 41°C or 41.5°C for 6 hr increased free radical production as determined by oxidation of 2’,7’-dichlorodihydrofluorescein diacetate (Sakatani et al., 2004). Heat shock did not increase oxidation of 2’,7’-dichlorodihydrofluorescein diacetate later in development at Days 4 or 6 after insemination (Sakatani et al., 2004). Exposure of Day 2 embryos to 41.5°C for 6 hr also reduced GSH content (Sakatani et al., 2007) although a less severe heat (41°C for 6 hr) did not reduce intracellular GSH content of two-cell embryos (Rivera et al., 2004a).

One potential approach to test the importance of ROS for mediating effects of heat shock is to modify the free radical status of embryos by manipulating the oxygen content of the atmosphere used for culture. Elevated oxygen concentration has been shown to increase hydrogen peroxide production in somatic cell culture systems (Kwak et al., 2006) as well as in culture of preimplantation embryos (Goto et al., 1993; Kitagawa et al., 2004). Under normothermia (38–39°C), the proportion of embryos successfully developing in culture is increased when bovine embryos are cultured in a gas environment of 5% (v/v) oxygen as compared to culture in air (Thompson et al., 1990; Lim et al., 1999). This difference in development is due in large part to ROS because addition of antioxidants and pro-antioxidants to culture medium of embryos cultured in air improves embryonic development (Takahashi et al., 1993, 2002).

In the present study, the role of oxidative stress in mediating deleterious effects of heat shock on the bovine preimplantation embryo was assessed. First, experiments were carried out to determine if effects of heat shock were reduced in a low oxygen environment as compared to a high oxygen environment (5% vs. ~20.95%). Secondly, experiments were performed to determine if effects of heat shock to reduce development and induce apoptosis can be prevented by the antioxidant dithiothreitol (DTT).

**MATERIALS AND METHODS**

**Materials**

Materials for production of HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF–TALP, Sperm–TALP, oocyte collection medium (OCM); Tissue Culture Medium-199 with Hank’s salts without phenol red supplemented with 2% (v/v) bovine serum containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine), oocyte maturation medium (OMM; Tissue Culture Medium-199 with Earle’s salts supplemented with 10% (v/v) bovine serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone, 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine) and KSOM-BE2 were as described previously (Soto et al., 2003; Jousan and Hansen, 2007). Percoll was from GE Biosciences (Uppsala, Sweden) and frozen semen from various bulls of different breeds was donated by Southeastern Semen Services (Wellborn, FL).

**OXYGEN AND DTT EFFECTS ON HEAT-SHOCKED EMBRYOS**

The In Situ Cell Death Detection Kit (fluorescein or TMR red) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Hoechst 33342 was purchased from Sigma–Aldrich. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI). All other reagents were purchased from Sigma–Aldrich or Fisher Scientific (Pittsburgh, PA).

**In Vitro Production of Embryos**

Embryo production was performed as previously described (Jousan and Hansen, 2004, 2007). Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-μl drops of OMM overlaid with mineral oil and matured for 20–22 hr at 38.5°C in an atmosphere of 5% (v/v) CO2 in humidified air. Matured COCs were then washed once in HEPES–TALP and transferred in groups of 30 to 4-well plates containing ~1 × 106 Percoll-purified spermatozoa (from a pool of frozen-thawed semen from three bulls) in 600 μl of IVF–TALP and 25 μl per well of a solution of 0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl. A different pool of three bulls was generally used for each replicate. After 10–12 hr at 38.5°C in an atmosphere of 5% CO2 in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1,000 U/ml hyaluronidase in HEPES–TALP, and placed in groups of 30 in 50-μl drops of KSOM-BE2. All drops of embryos were overlaid with mineral oil and cultured as described in for individual experiments. The CO2 environment of culture was maintained at 5% (v/v) for culture at 38.5°C and was raised to 6% for cultures at 41°C to correct for reduced solubility of CO2 and to maintain the pH of the medium at 41°C to a value similar to that for cultures at 38.5°C (pH~7.4) (Rivera and Hansen, 2001). A total of ~10–20 embryos were placed in each drop; a similar number for all drops of a given replicate of each experiment was used.

**TUNEL and Hoechst 33342 Labeling**

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The labeling procedure was as described elsewhere (Jousan and Hansen, 2007). The TUNEL reaction mixture included either fluorescein isothiocyanate- or TAM red-conjugated dUTP. Labeling was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-
positive blastomeres (green or red nuclei) with DAPI and FITC or rodamine filters, respectively, using a 20× objective. Digital images were acquired using AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera.

**Modification of Heat Shock Effects by Oxygen Environment**

**Effect of heat shock at the two-cell stage on development to the blastocyst stage as modified by oxygen environment throughout culture.** After insemination, putative embryos were randomly assigned to be cultured in either high (5% CO₂ in humidified air) or low oxygen (5% O₂, 5% CO₂ and N₂ in a humidified chamber). Two-cell embryos were harvested at ~28 hr after insemination and randomly assigned to culture at 38.5°C or 41°C for 9 hr under high or low oxygen in a 2 × 2 factorial design. After the heat shock period, all embryos were further cultured at 38.5°C in either high or low oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. Embryos were maintained in the same oxygen environment throughout culture. The experiment was replicated three times with a total of 220 embryos.

**Acute effect of oxygen environment on development of heat shocked two-cell embryos.** Putative embryos were cultured in high oxygen. Two-cell embryos were harvested at ~28 hr after insemination and randomly assigned to culture in either high or low oxygen at either 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C for 15 hr. Thereafter, all embryos were cultured at 38.5°C in high oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. The experiment was replicated nine times using a total of 672 two-cell embryos.

**Acute effect of oxygen environment on development of heat shocked Day 5 embryos.** Putative embryos were cultured in high oxygen after insemination. Embryos ≥16 cells were harvested on Day 5 after insemination and were randomly assigned to be cultured at either 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C for 15 hr in either high or low oxygen using a 2 × 2 factorial arrangement of treatments. Thereafter, embryos were cultured at 38.5°C in high oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. The experiment was replicated seven times using a total of 447 embryos.

**Heat shock effects on apoptosis of Day 5 embryos as modified by oxygen environment throughout culture.** Putative embryos were randomly assigned to be cultured in either high or low oxygen beginning after insemination. On Day 5, embryos ≥16 cells were harvested and placed in new drops and cultured at 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C for 15 hr. During this period, embryos were cultured in the same oxygen environment that they were exposed to earlier. Thus, the design was a 2 × 2 factorial design with two temperatures and two oxygen environments. All embryos were fixed 24 hr after initiation of the heat shock period, on Day 6 after insemination, and stored in PBS–PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated five times with a total of 217 embryos.

**Effects of heat shock in low oxygen on apoptosis of Days 4 and 5 embryos.** Putative embryos were cultured in low oxygen after insemination. Embryos ≥16 cells were harvested at either Days 4 or 5 after insemination. Separate drops of embryos were used for selection on each day. Harvested embryos were placed in fresh drops of culture medium and randomly assigned to be cultured in low oxygen at 38.5°C for 24 hr or 41°C for 9 hr followed by 15 hr at 38.5°C. Embryos were harvested at the end of culture and stored in 10 mM KPO₄, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml polyvinylpyrrolidone (PBS–PVP) at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated three times using a total of 239 embryos.

**Experiments on Thermoprotective Actions of DTT**

**Protective action of DTT on development of embryos heat shocked at the two-cell stage and cultured in high oxygen continuously.** After insemination, putative embryos were cultured in high oxygen. Two-cell embryos were harvested at ~28 hr post insemination and randomly assigned to be cultured in the presence of 0, 50, or 500 μM DTT at 38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr using a 3 × 2 factorial arrangement of treatments. The duration of heat shock in this experiment and other experiments with DTT was lengthened to 15 hr to increase the magnitude of heat shock effects and thereby increase the magnitude of the potential thermoprotective effect of DTT. Afterwards, embryos were washed three times in 100-μl drops of HEPS–TALP and cultured in fresh drops of KSOM-BE2 at 38.5°C in high oxygen. Blastocyst development was determined at Day 8. The experiment was replicated six times using a total of 431 embryos.

**Protective action of DTT on development of embryos heat shocked at the two-cell stage in different oxygen environments and otherwise cultured in low oxygen.** After insemination, putative embryos were cultured in low oxygen. Two-cell embryos were harvested at ~28 hr after insemination and randomly assigned to one of eight treatments in a 2 × 2 × 2 factorial design with main effects of incubation temperature (38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr), oxygen environment (high or low oxygen) and DTT (0 or 500 μM). After 24 hr, embryos were washed three times in 100-μl HEPES–TALP drops and placed in fresh drops of KSOM-BE2 and cultured in low oxygen at 38.5°C until Day 8 when blastocyst development was determined. The experiment was replicated 16 times using a total of 1,264 two-cell embryos.

For 5 of the above-mentioned 16 replicates, additional treatments were included that were analyzed as a separate data set. These embryos were in high oxygen...
for the entire culture period. Two-cell embryos were harvested and randomly assigned to one of four treatments in a 2 × 2 factorial design with main effects of incubation temperature (38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr) and DTT (0 or 500 μM). A total of 184 two-cell embryos were used. After 24 hr, embryos were washed three times in 100-μl HEPES–TALP drops and placed in fresh drops of KSOM-BE2 and cultured in high oxygen at 38.5°C until Day 8 when blastocyst development was determined.

**Protective action of DTT on development of heat-shocked Day 5 embryos cultured in high oxygen continuously.** Putative embryos were cultured in low oxygen after insemination. On Day 5, embryos ≥16 cells were harvested and randomly assigned to one of six treatments in a 2 × 3 factorial design with main effects of incubation temperature (38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr) and DTT (0, 50, or 500 μM). All embryos were cultured in high oxygen. Afterwards, embryos were washed three times in 100-μl HEPES–TALP and cultured in fresh KSOM-BE2 at 38.5°C in high oxygen until Day 8 when blastocyst development was determined. The experiment was replicated 10 times using a total of 545 embryos.

**Protective action of DTT on apoptosis of heat-shocked Day 5 embryos cultured in high oxygen continuously.** Putative embryos were cultured in high oxygen after insemination. On Day 5, embryos ≥16 cells were harvested and randomly assigned to culture in the presence of 0, 50, or 500 μM DTT at 38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr. Afterwards, were fixed in paraformaldehyde for TUNEL assay. The experiment was replicated four times using a total of 287 embryos.

**Statistical Analysis**

Data on percent of embryos becoming blastocysts were calculated for each replicate and analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC). Replicate was considered a random replicate and other main effects were considered fixed. Effects of specific doses of DTT in protecting embryos from heat shock were calculated using contrasts. When necessary to determine the effects of one main effect within one factor of a second main effect, the first main effect was fixed using the Sort procedure from SAS. For example, to determine if there was an effect of 500 μM DTT at 41°C, the main effect of temperature was fixed using the Sort procedure and effects of each DTT concentration was determined separately for embryos at 38.5 and 41°C.

In some experiments, the percent reduction in blastocyst development induced by heat shock was calculated for each replicate according to the equation: 100 − [percent development at 41°C/percent development at 38.5°C] × 100. For apoptosis, the percent of blastomeres that were TUNEL-positive (i.e., assumed apoptotic) were determined for each embryo. Total cell number for each embryo was also analyzed. For all of these variables, data were analyzed by least-squares analysis of variance as described above.

All values reported are least-squares means ± SEM.

**RESULTS**

**Effect of Heat Shock at the Two-Cell Stage on Development as Modified by Oxygen Environment**

In the first experiment, embryos cultured continuously in either high or low oxygen were heat-shocked by exposure to 41°C for 9 hr at the two-cell stage (Fig. 1A). As shown in Figure 1B, a smaller proportion of two-cell embryos developed to the blastocyst stage at Day 8 after insemination when cultured in high oxygen than when cultured in low oxygen (P < 0.05). Heat shock decreased development of two-cell embryos to the blastocyst stage in high oxygen but not in low oxygen (temperature × oxygen, P < 0.05).

In the second experiment, embryos were cultured in high oxygen throughout culture except for the 24-hr period coincident with heat shock (Fig. 2A). During this time, embryos were cultured in either high or low oxygen and at either 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C for 15 hr. Data on the proportion of embryos that became blastocysts at Day 8 after insemination are presented in Figure 2. The percentage of embryos becoming blastocysts did not differ between high and low oxygen at 38.5°C. Heat shock decreased the proportion of two-cell embryos becoming blastocysts in high oxygen but not in low oxygen (temperature × oxygen, P < 0.05).

**Effect of Heat Shock on Development of Day 5 Embryos as Modified by Oxygen Environment**

Embryos were cultured in high oxygen until Day 5 when those ≥16 cells were harvested and cultured at either 38.5°C for 24 hr or 41°C for 9 hr and 38.5°C for 15 hr in high or low oxygen (Fig. 3A). Thereafter, all embryos were cultured in high oxygen and blastocyst development was determined on Day 8. Results are shown in Figure 3B. The percentage of embryos becoming blastocysts did not differ between high and low oxygen at 38.5°C. Heat shock decreased development of embryos to the blastocyst stage when culture was in high oxygen but not when culture was in low oxygen (temperature × oxygen, P < 0.05).

**Induction of Apoptosis by Heat Shock in Day 4 and 5 Embryos as Modified by Oxygen Environment**

Two experiments were conducted to test whether oxygen environment altered the induction of apoptosis in Day 4 and 5 embryos. In the first study, embryos were cultured continuously in either high or low oxygen (Fig. 4A). At Day 5, embryos ≥16 cells were cultured at 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C. As shown in Figure 4B, total cell number at the end of culture was lower for embryos cultured in high oxygen
compared to embryos cultured in low oxygen ($P < 0.05$). Neither temperature nor temperature × oxygen affected total cell number ($P > 0.1$). Results for percent of cells undergoing apoptosis as determined by TUNEL labeling is presented in Figure 4C. The proportion of cells that were TUNEL-positive was higher for embryos cultured at 41°C than for embryos at 38.5°C if culture was in high oxygen but not if culture was in low oxygen (temperature × oxygen; $P < 0.05$).

In the second experiment, embryos ≥16 cells cultured in low oxygen were harvested at either Days 4 or 5 after insemination (Fig. 5A). Thereafter, embryos continued to be cultured in low oxygen. Embryos were cultured at 38.5°C for 24 hr or 41°C for 9 hr followed by 38.5°C for 15 hr. Embryos were then fixed and stained for TUNEL and Hoechst 33342. As expected, Day 4 embryos had fewer cells than Day 5 embryos ($P < 0.05$) but heat shock had no effect on total cell number at either Days 4 or 5 (Fig. 5B; $P > 0.1$). Heat shock increased apoptosis for embryos collected at both Days 4 and 5 (Fig. 5C; temperature, $P < 0.05$). This increase, although significant, was much lower than the increase observed in the previous experiment (compare Figs. 4 and 5).

**Protective Actions of DTT on Development of Heat-Shocked Two-Cell Embryos**

Two experiments were performed to determine whether DTT blocked deleterious effects of heat shock on development of two-cell embryos. In the first experiment, embryos were cultured continuously in high oxygen (Fig. 6A). At the two-cell stage, embryos were cultured in the presence of 0, 50, or 500 μM DTT at either 38.5°C for 24 hr or at 41°C for 15 hr followed by 38.5°C for 9 hr. After the heat shock period, all embryos were further cultured at 38.5°C in fresh medium without DTT until Day 8 after insemination. The overall percentage of two-cell embryos becoming blastocysts was higher for embryos cultured in the presence of DTT (Fig. 6B; $P < 0.05$). Heat shock reduced development of two-cell embryos to the blastocyst stage in the presence or absence of DTT (temperature; $P < 0.05$). While there was no significant DTT × temperature interaction (Fig. 6B), the percent reduction in development caused by heat shock, as compared to development for nonheat shocked embryos, was less for embryos cultured in the presence of DTT (Fig. 6C; $P < 0.05$). Nonetheless, even...
at the highest dose of DTT, development was reduced 78.9% by culture at 41°C.

In the second experiment, two-cell embryos cultured in low oxygen were harvested and then cultured at either high or low oxygen in the presence or absence of 500 μM DTT and at either 38.5°C for 24 hr or 41°C for 15 hr followed by 38.5°C for 9 hr (Fig. 7A). Embryos were then washed and cultured in low oxygen at 38.5°C without DTT until Day 8 after insemination when blastocyst development was determined. Results are in Figure 7B. The proportion of two-cell embryos that became blastocysts was affected by the temperature × oxygen interaction (P < 0.05). This interaction existed because temperature reduced development in all groups except for the embryos cultured in low oxygen without DTT. Thus, unlike the previous experiment, DTT did not reduce effects of heat shock on development in high oxygen and, in fact, exacerbated the effects of heat shock in low oxygen.

To verify that the difference in response to DTT depended upon whether embryos were cultured in high oxygen (Fig. 6) or cultured in low oxygen for most of development (Fig. 7B), additional treatments were included in the second DTT experiment for some replicates. In particular, some embryos were cultured continuously in high oxygen but otherwise treated as for Figure 7A (see Fig. 7C for design). In this subset, DTT conferred partial protection against heat shock (Fig. 7D; DTT × temperature, P < 0.05). The percent reduction in blastocyst development by heat shock tended (P = 0.08) to be lower for DTT-treated embryos (77.3 ± 6.5%) than for control embryos (95.5 ± 6.5%).

**Protective Actions of DTT on Development of Heat-Shocked Day 5 Embryos**

This experiment was performed with embryos cultured continuously in high oxygen (Fig. 8A). On Day 5, embryos ≥16 cells were cultured in the presence of 0, 50, or 500 μM DTT at 38.5°C for 24 hr or 41°C for 15 hr and 38.5°C for 9 hr. Afterwards, all embryos were cultured at 38.5°C without DTT until Day 8 after insemination. The percent of embryos developing to blastocysts was reduced by heat shock (P < 0.01) and increased by DTT (P < 0.01) and there was no significant temperature × DTT interaction (Fig. 8B). However, the percent reduction in development caused by heat shock was less for embryos...
cultured in the presence of 50 μM (P = 0.06) and 500 μM DTT (P < 0.01) than for embryos cultured without DTT (Fig. 8C).

Protective Actions of DTT on Apoptosis of Heat-Shocked Day 5 Embryos

This experiment was done as for the previous experiment except that embryos were analyzed for apoptosis 24 hr after initiation of heat shock using the TUNEL assay (Fig. 9A). There was no effect of temperature or DTT on total cell number (Fig. 9B; temperature, P > 0.1). Overall, the proportion of cells that were apoptotic was affected by DTT (Fig. 9C; P < 0.01). Among embryos cultured at 38.5°C as well as for embryos at 41°C, DTT 500 μM decreased the percent of TUNEL positive nuclei (DTT, P < 0.01). Although there was no overall effect of temperature in the percent of TUNEL positive nuclei, heat shock increased the percent of TUNEL positive nuclei among embryos treated with 0 μM DTT (temperature, P < 0.01) but not for embryos treated with 50 or 500 μM DTT (temperature, P > 0.1).

DISCUSSION

Use of the high oxygen versus low oxygen model in the present study yielded clear evidence that oxygen environment is an important determinant of continued embryonic development following heat shock. Experiments using DTT revealed that some of the actions of heat shock on development involve ROS production in a stage-specific manner and that ROS-independent actions of heat shock are also likely. Given that actions of DTT to prevent heat-shock effects on development were incomplete, it is possible that some of the effects of oxygen environment on the magnitude of the inhibition of development caused by heat shock involves actions independent of ROS production. For apoptosis, however, clear evidence was obtained to demonstrate that ROS are indispensable triggers for induction of the apoptotic cascade during heat shock. In particular, heat-shock induced apoptosis was blocked by culture in low oxygen and by addition of DTT to culture medium.

In each of four experiments, deleterious effects of heat shock on continued development of embryos was only observed when embryos were in a high oxygen environment during heat shock. This was true for both two-cell embryos and Day 5 embryos and was true whether embryos were cultured continuously in different oxygen environments or whether oxygen environment differed during the 24 hr after initiation of heat shock only. There are three possible explanations for this finding. One is...
that effects of heat shock on embryonic development are mediated by increased ROS production and that the magnitude of this increase is greater when embryos are cultured in high oxygen. Indeed, embryos cultured in a high oxygen environment have greater production of ROS (Goto et al., 1993; Kitagawa et al., 2004). Alternatively, the deleterious effect of heat shock may not be a direct result of increased production of ROS but rather heat shock is more likely to inhibit development of embryos when their physiology is already compromised by the increased ROS production caused by culture in high oxygen. A third possibility is that high oxygen does not increase susceptibility to heat shock but that culture in low oxygen results in changes in cellular function that increase embryonic resistance to heat shock. This latter explanation is deemed less likely. There are genes in the bovine blastocyst whose expression is dependent upon oxygen concentration but the full range of oxygen-induced transcription is attenuated by the lack of hypoxia-inducible transcription factor-1 (Harvey et al., 2004, 2007a,b). Also, effects of oxygen were seen at the two-cell stage, a time before embryonic genome activation has occurred (Memili and First, 2000).

If the effects of oxygen environment on embryonic development reflect altered ROS production, antioxidants should reduce effects of heat shock on development. Dithiothreitol was used as an antioxidant in the present study because it is a strong reducing agent that contains two thiol groups for sequential thiol-disulfide exchange reactions for direct scavenging of ROS as well as regeneration of reduced GSH from oxidized GSH (Rothwarf and Scheraga, 1992). Treatment of mouse embryos with DTT blocked effects of the thiol-oxidizing agent diamide (Liu et al., 1999). In the absence of heat shock, DTT increased the proportion of two-cell embryos and Day 5 embryos maintained continuously in high...
When two-cell embryos were cultured in low oxygen except during heat shock, there was no protective effect of DTT (Fig. 7). For the Day 5 embryo, in contrast, the degree of inhibition of development caused by heat shock in the presence of 500 μM DTT was 26.6% compared to 70.8% of that in the absence of DTT (Fig. 8).

The differential effect of DTT on two-cell embryos versus embryos at Day 5 could reflect several differences in cell physiology. The two-cell embryo is more susceptible to heat shock than the Day 5 embryo and the reduced effectiveness of DTT at the two-cell stage may reflect the increased heat susceptibility of the two-cell embryo. In another experiment in which heat shock was of reduced duration (41.5 °C for 6 hr) compared to the study here, anthocyanins from purple sweet potato completely blocked actions of heat shock on continued development of embryos at Day 2 after fertilization (Sakatani et al., 2007). Another possible explanation for the minimal effect of DTT at the two-cell stage relates to GSH status of the embryo. GSH content is higher for embryos at Day 5 than at the two-cell stage (Lim et al., 1996) and DTT might be more effective at altering redox status later in development as a result. Effectiveness of DTT as an antioxidant is reduced in cells depleted of GSH (Zou et al., 2001).

Indeed a deficiency in GSH could be one reason why DTT did not completely reverse effects of heat shock on development. It could also be postulated that DTT, which is water soluble, did not prevent oxidative damage in the lipid compartment of the cell. However, DTT can block lipid peroxidation in several models of oxidation (see e.g., Fariss et al., 1997; Zou et al., 2001) and GSH, which is regenerated by DTT, plays an important role in preventing lipid peroxidation (Blair, 2006). Another interpretation for the failure of DTT to completely block effects of heat shock is that there are effects of heat shock that are independent of ROS production. Among the possible ROS-independent effects of heat shock are protein denaturation and increased membrane fluidity.

The one unexpected result was that addition of DTT exacerbated effects of heat shock of two-cell embryos on development when embryos were cultured continuously in low oxygen (Fig. 7). Under certain conditions, thiol reagents such as DTT can be toxic to cells as a result of their oxidation and subsequent generation of lipid peroxides mediated by the Fenton reaction (Held et al., 1996). It is possible that similar toxic effects of DTT on two-cell embryos cultured in high oxygen obscured thermoprotective effects of DTT.

Present results clearly indicated involvement of ROS in induction of apoptosis by heat shock. Heat shock-induced apoptosis in preimplantation embryos is a developmentally regulated phenomenon with the acquisition of apoptosis responses first occurring for embryos greater than 8–16 cells on Day 4 after fertilization (Paula-Lopes and Hansen, 2002b; Jousan and Hansen, 2004, 2007). The role of ROS in induction of apoptosis was studied in Day 5 embryos because these embryos had acquired capacity for heat-shock induced
apoptosis. The increase in apoptotic cells caused by heat shock was reduced or absent when embryos were cultured in low oxygen. Furthermore, DTT decreased apoptosis in embryos cultured at 38.5°C and completely blocked the increase in apoptosis induced by heat shock. In other cells as well, induction of apoptosis by heat shock is mediated by oxidative stress (Sreedhar et al., 2002; Zhao et al., 2006).

It is important to point out that the block to development caused by heat shock was not the result of the increase in apoptosis. This conclusion is based on previous observations that inhibition of apoptosis responses with the caspase inhibitor, z-DEVD-fmk, exacerbates effects of heat shock on development (Paula-Lopes and Hansen, 2002a) as well as present observations that DTT completely blocked induction of apoptosis caused by heat shock but was only partially effective in blocking effects of heat shock on development.

The reduction in development caused by heat shock at the two-cell stage in the presence of high oxygen was greater if embryos were cultured in high oxygen continuously (95.5–100% reduction in development as compared to culture in low oxygen) after 15 hr at 41°C (Figs. 6 and 7B) than if they were cultured in low oxygen before and after heat shock (37.6% reduction in development after 15 hr at 41°C; Fig. 7A). Thus, oxygen environment before and/or after heat shock is also a determinant of embryonic survival to heat shock. One possible explanation for these effects is that free radical formation occurs beyond the 24-hr period following initiation of heat shock. Another possibility is that heat shock is more likely to arrest development when the embryo is already compromised in developmental potential. Perhaps, heat shock affected development more for embryos maintained in high oxygen continuously because competence of these embryos to become blastocysts was reduced. If there is a relationship between embryonic capacity for development and the magnitude of heat-shock induced arrest of development, the consequences of heat shock in vivo will depend upon the characteristics of the embryo.

Oxygen content of the reproductive tract is low, ranging from 1.5% to 8.7% depending on segment of the tract and the species (Fischer and Bavister, 1993). Given the importance of oxygen tension and ROS
for heat shock-induced changes in embryonic function, the question remains as to whether an increase in temperature of the oviduct or uterus would result in reduced embryonic development and increased apoptosis. Heat stress during early embryonic development retards embryonic growth (Putney et al., 1988; Ealy et al., 1993) but this effect could involve direct effects of heat shock on the embryo or alterations in reproductive tract environment. It is hypothesized that, despite the low oxygen concentration present in the reproductive tract, the redox state of the reproductive tract would be sufficient to allow effects of heat shock on the embryo. This is because the reproductive tract itself can generate ROS during heat shock. This interpretation is supported by results obtained in mice. In particular, maternal heat stress increased free radical production in the oviduct (Ozawa et al., 2004; Matsuzuka et al., 2005b) and administration of the antioxidant melatonin reduced the effects of maternal heat stress on embryonic development in vivo (Matsuzuka et al., 2005a). Thus, the ROS environment of the heat-shocked embryo in vivo may be greater than for the embryo heat-shocked in vitro under low oxygen conditions.

ACKNOWLEDGMENTS

The authors express their appreciation to William Rembert for collecting ovaries; Marshall, Adam, and Alex Chernin and employees of Central Beef Packing Co. (Center Hill, FL) for providing ovaries; and Scott A. Randell of Southeastern Semen Services (Wellborn, FL) for donating semen. Research was supported in part by Research Grant Award No. US-3551-04 from BARD, The United States—Israel Binational Agricultural
OXYGEN AND DTT EFFECTS ON HEAT-SHOCKED EMBRYOS

Research and Development Fund and Grant No. 2004-34135-14715 from the U.S. Department of Agriculture T-STAR program. Luiz Augusto de Castro e Paula was supported by fellowship award No. 134202-9 from CAPES, Brazil.

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