

# Actions of thermal stress in two-cell bovine embryos: oxygen metabolism, glutathione and ATP content, and the time-course of development

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## Abstract

The mechanism by which heat shock disrupts development of the two-cell bovine embryo was examined. The reduction in the proportion of embryos that became blastocysts caused by heat shock was not exacerbated when embryos were cultured in air (20.95% O<sub>2</sub>) as compared with 5% O<sub>2</sub>. In addition, heat shock did not reduce embryonic content of glutathione, cause a significant alteration in oxygen consumption, or change embryonic ATP content. When embryos were heat-shocked at the two-cell stage and allowed to continue development until 72 h post insemination, heat-shocked embryos had fewer total nuclei and a higher percentage of them were condensed. Moreover, embryos became blocked in development at the eight-cell stage. The lack of effect of the oxygen environment on the survival of embryos exposed to heat shock, as well as the unchanged content of glutathione, suggest that free radical production is not a major cause for the inhibition in development caused by heat shock at the two-cell stage. In addition, heat shock appears to have no immediate effect on oxidative phosphorylation since no differences in ATP content were observed. Finally, the finding that heat shock causes a block to development at the eight-cell stage implies that previously reported mitochondrial damage caused by heat shock or other heat shock-induced alterations in cellular physiology render the embryo unable to proceed past the eight-cell stage.

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## Introduction

Exposure of preimplantation embryos to elevated temperature causes disruption of continued development (Alliston *et al.* 1965, Elliot *et al.* 1968, Gwazdauskas *et al.* 1992, Rivera & Hansen 2001). Temperatures causing this effect represent a relatively mild elevation over hyperthermic temperatures. In cattle embryos, for example, 41.0°C can block development as compared with culture at 38.5°C (Rivera & Hansen 2001, Rivera *et al.* 2003). An elevation in culture temperature of only 2.0°C (from 37.0 to 39.0°C) reduced development of mouse embryos (Gwazdauskas *et al.* 1992). Since temperatures reducing development are within the range of temperatures experienced by heat-stressed females, it is likely that the disruption of embryonic development caused by thermal stress is of physiological relevance to the reduction in fertility experienced by heat-stressed females (Elliot & Ulberg 1971, Wettemmn & Bazer 1985, Putney *et al.* 1988, Ealy *et al.* 1993).

Little is known about the mechanisms by which mild elevations in temperature cause such catastrophic effects on the preimplantation embryo. Most of the literature dealing with effects of heat shock on cells involves much higher temperatures – commonly 43.0°C–45.0°C – and the molecular and cellular changes affected by these temperatures may not occur in embryos exposed to 41.0°C.

One possible action of heat shock is to increase free radical production by the embryo. Heat shock of cells has been shown to increase the flux of cellular free radicals (Flanagan *et al.* 1998) by uncoupling mitochondria, one of the main reactive oxygen species generators, as well as by activating enzymes which are involved in the generation of free radicals (Skibba *et al.* 1989). The major evidence that heat shock increases free radicals in embryos comes from the mouse, where thermal stress has been shown to increase intracellular hydrogen peroxide production (Ozawa *et al.* 2002) and decrease intracellular content of

the antioxidant glutathione (Aréchiga *et al.* 1995). In addition, mouse embryos (Aréchiga *et al.* 1995, Aréchiga & Hansen 1998) can be made more resistant to heat shock by addition of antioxidants in the culture medium. Induced thermotolerance in mouse embryos can be blocked by inhibition of glutathione synthesis (Aréchiga *et al.* 1995).

Exposure of bovine two-cell embryos to 41.0°C for 6 h resulted in a 15% increase in the number of mitochondria undergoing swelling (Rivera *et al.* 2003). Heat shock has also been demonstrated to cause mitochondrial swelling in other cell types (Welch & Suhan 1985, Cole & Armour 1988, Funk *et al.* 1999). Mitochondrial swelling is a phenomenon which results from loss of membrane potential as a result of the opening of the high conductance permeability transition pores on the inner mitochondrial membrane (Halestrap *et al.* 2002). Increased calcium and generation of reactive oxygen species promote permeability transition pore opening which, in turn, causes uncoupling of oxidative phosphorylation and prevents mitochondria from synthesizing ATP (Lemasters 1999, Halestrap *et al.* 2002). In non-embryonic cells, temperatures in the range of 41.0°C can impair electron transport along the respiratory chain (Floridi *et al.* 1987) and thereby decrease the amount of ATP and phosphorylation efficiency (Findly *et al.* 1983, Calderwood 1987, Macouillard-Pouilletier de Gannes *et al.* 1998). Oxygen consumption would be expected to decline in heat-shocked cells undergoing mitochondrial damage. In fact, the respiratory properties of microglial cells were compromised during and immediately after heat shock with an immediate drop in oxygen consumption (Macouillard-Pouilletier de Gannes *et al.* 2000).

There were three objectives for the present study. The first was to evaluate the possible role of free radicals in disruption of embryonic development caused by a heat shock. This question was evaluated by determining whether the magnitude of the reduction in development caused by heat shock was reduced by lowering oxygen tension and by determining whether heat shock reduced embryonic content of glutathione, the major cytoplasmic antioxidant in the cell. The second objective was to determine whether heat shock altered oxygen consumption and ATP content of the embryo in a manner consistent with a change in blastomere function caused by increased mitochondrial damage. The third objective of this study was to determine the time point in development at which heat shock at the two-cell stage blocks development, i.e. whether heat shock causes an immediate inhibition of development or rather compromises the ability of the embryo to proceed through the eight-cell stage, around the time when embryos begin major transcriptional activity (Memili & First 2000).

## Materials and Methods

### *In vitro* production of embryos

Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as described elsewhere (Rivera & Hansen 2001, Rivera *et al.* 2003, Soto *et al.* 2003). Except for one experiment described later, all incubations were performed in an atmosphere of CO<sub>2</sub> in humidified air. Briefly, ovaries were collected at a local abattoir located at a travel distance of approximately 1.5 h from the laboratory. Oocytes obtained by slashing the ovary were matured for approximately 21 h and then inseminated with a cocktail of Percoll-purified spermatozoa from three different Angus bulls; a different group of bulls was used for each replicate. At 12–18 hours post-insemination (hpi), putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium containing 1000 units/ml hyaluronidase type IV (Sigma, St Louis, MO, USA) and vortexing in a microcentrifuge tube. Presumptive zygotes were then placed in groups of ~30 in 50 µl microdrops KSOM. For most experiments, the specific formulation KSOM-BE1 was used (Soto *et al.* 2003). In one experiment, however, after the supplier changed the formulation for KSOM, a different formulation called KSOM-BE2 (Soto *et al.* 2003) was used. Rates of development and effects of heat shock were similar in both formulations (RM Rivera & PJ Hanson, unpublished observations).

### Heat shock treatment

Two-cell embryos were collected at 28–30 hpi and cultured in fresh microdrops of KSOM-BE1 or KSOM-BE2 at one of two temperatures: 38.5°C (i.e. homeothermic body temperature of the cow) or 41.0°C (characteristic temperature for heat-stressed cows; Rivera & Hansen 2001). After 6 h, embryos were either collected and analyzed for oxygen consumption, glutathione content or ATP content, or returned to the control temperature (38.5°C) until day 3 or 8 post insemination when subsequent development was assessed. All cultures at 38.5°C were performed in an environment of 5% CO<sub>2</sub> in humidified air except where otherwise specified. The percentage CO<sub>2</sub> was adjusted to 7% in the incubator used for heat shock to maintain the pH of the medium at a similar value as that of the control incubator (~7.4; Rivera & Hansen 2001). The temperatures of all incubators were calibrated routinely to assure accuracy of treatments.

### Effects of oxygen tension on inhibition of development caused by heat shock

This experiment was performed to determine if culture in a low oxygen environment (5%) would reduce the effects of heat shock on development as compared with culture in air (20.95% oxygen). The design was a 2 × 2 factorial in which two temperatures (38.5 and 41.0°C) and two

oxygen tensions (5 and 20.95%) were tested. Embryos were cultured in either a high or low oxygen environment for the duration of the experiment beginning after removal from fertilization drops. For cultures under a low oxygen environment, the gaseous phase was 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for the control incubator and 7% CO<sub>2</sub>, 5% O<sub>2</sub>, and 88% N<sub>2</sub> for the heat shock incubator. Approximately 30 hpi, two-cell embryos were placed in fresh microdrops of KSOM-BE2 and cultured for 9 h at one of two temperatures in high or low oxygen tension. Embryos cultured at 41.0°C were returned to the control temperature (i.e. 38.5°C) after 9 h. Development to the blastocyst stage was evaluated on day 8 post insemination. The experiment was replicated four times with a total of 53–77 two-cell embryos/treatment.

### Effects of heat shock on glutathione content

#### Glutathione recycling assay

Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. Immediately after the 6-h culture, groups of 16–20 two-cell heat-shocked or control embryos were suspended in 5 µl KSOM-BE1 and stored at –20.0°C until analyzed for glutathione content. Glutathione content of embryos was determined as previously described (Aréchiga *et al.* 1995) by the use of the glutathione recycling assay (Baker *et al.* 1990). This method is based on the reduction of 5,5-dithiobis (2-nitrobenzoic acid; DNTB) to 2-nitro-5-thiobenzoic acid (a yellow colored product) by glutathione and the recycling of glutathione disulfide to glutathione by glutathione reductase. On the day of the assay, groups of embryos were suspended in 45 µl deionized water and pipetted into separate wells of a 96-well microtiter plate. In addition to the embryo samples, 50 µl of standard (0 – 100 pmol glutathione per well) were pipetted into duplicate wells. This was immediately followed by the addition of 100 µl reaction mixture (prepared by mixing 5.75 ml 100 mM NaPO<sub>4</sub> buffer with 1 mM EDTA, pH 7.5, 5 ml 1 mM DNTB, 5 ml 1 mM NADPH, and 0.1 ml 200 U/ml glutathione reductase dissolved in NaPO<sub>4</sub> –EDTA buffer) to the embryo samples or standards (all chemicals for the assay were from Sigma-Aldrich). Absorbance was read at 405 nm after ~6–8 min incubation using a microtiter plate reader (model EL 309; BIO-TEK Instruments Inc.). The experiment was replicated on 7 different days so that seven pools of embryos (16–20 embryos/pool) were assayed for each treatment.

### Effects of heat shock on oxygen consumption

#### Electrode preparation

All chemicals were obtained from Sigma-Aldrich at the highest available purity level unless otherwise stated. Solutions were made with deionized water from a Milli-Q Plus system water purifier (Millipore Co., Bedford, MA, USA). The epoxy Epon 828 with *m*-phenylenediamine as

the curing agent was purchased from Miller-Stephenson (Dandury, CT, USA). Oxygen sensing electrodes were manufactured following a previously described protocol (Jung *et al.* 1999a). Briefly, a 1.5 cm by 25 µm diameter platinum wire was attached to a 27-gauge syringe needle using silver epoxy (Epoxy Technology, Billerica, MA, USA). The needles were baked for 1 h at 100°C. After baking, the platinum wire was immersed in a solution of 3 M KCN and 1 M KOH while a 3.4 V square wave at a frequency of 250 Hz was applied for 6–8 min to etch the platinum wire to 1–3 µm diameter. The etched platinum wire and needle were then placed inside a pulled glass capillary that had been cut at the end such that the wire protruded from the tip. Next, the electrodes were dipped into epoxy (Epon 828 with *m*-phenylenediamine as the curing agent) and were allowed to dry overnight followed by curing at 100°C for 2 h and then at 150°C for an additional 2 h. After curing, a second etching procedure was undertaken under the same conditions as described above, with the exception that KOH was not present, to recess the electrode. The second etching step proceeded until the platinum wire was recessed 2–3 µm into the end of the glass pipette. The electrodes were then dipped into 7% (w/w) cellulose acetate in tetrahydrofuran for 60 s. The final diameter of the electrodes was 2–5 µm.

All electrodes were tested and calibrated by addition of N<sub>2</sub>, air, and O<sub>2</sub> to a solution at the electrodes. These solutions corresponded to partial pressures of oxygen of 0, 150 and 720 mm Hg respectively. Testing was performed with the electrodes immersed in Krebs' Ringer buffer (KRB) which consisted of 118 mM NaCl, 5.4 mM KCl, 2.4 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, and 20 mM Hepes at pH 7.4.

#### Oxygen consumption measurements

Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. Immediately after the 6-h culture, two-cell embryos were transferred to fresh KSOM-BE1 medium and transported in a portable incubator (Minitube, Verona, WI, USA) set at 39.0°C. Embryos were then incubated at 38.5°C in 5% CO<sub>2</sub> on a poly-L-lysine coated Petri dish for 20–30 min in KSOM-BE1. The Petri dish was transferred to a micro-incubator on the stage of a Zeiss Axiovert 35 microscope where the KRB was maintained at 37.0°C. Stabilization of the electrodes was performed by positioning the electrodes 100–200 µm away from the embryo. Once a stable baseline was observed for 2–5 min, the electrode was positioned 10–20 µm away from the embryo and measurements taken. The electrode was then inserted into the perivitelline space of the embryo and measurements recorded for 2 min. The electrode was repositioned outside the embryo (10–20 µm) and the measurement process repeated at least three times on each embryo (i.e. the electrode was inserted at least three times through the zona).

Oxygen measurements were performed by applying a potential with a battery to a saturated sodium calomel electrode and using a Keithley 428 current amplifier to measure the current from the working electrode. The potential at the working electrode was  $-0.6$  V vs sodium saturated calomel electrode. The data were collected via a data acquisition board (Axon Digidata 1200) from Axon Instruments, Inc. (Foster City, CA, USA) and a personal computer using Axoscope 8.1 software. Data were collected at 100 Hz and low-pass filtered at 20 Hz. The oxygen concentration was determined by using the calibration curve. Oxygen consumption was calculated for each embryo as the difference in partial pressure of oxygen between measurements taken inside and outside the zona pellucida. These values were converted to mol/s using an equation described by Mueller-Klieser (1984) and assuming the diameter of the embryo was  $120\ \mu\text{m}$ . The experiment was replicated three times with a total of 7–8 two-cell embryos/treatment.

### **Effects of heat shock on ATP content**

#### *ATP assay*

Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at  $38.5$  or  $41.0^\circ\text{C}$  for 6 h. The ATP assay was performed on pooled groups of 3–5 two-cell embryos per treatment immediately after the 6-h culture period. The ATP determination kit, from Molecular Probes (Eugene, OR, USA), is a bioluminescence assay based on the requirement of firefly luciferase for ATP to produce light. The standard reaction solution was prepared following the manufacturer's specifications. A series of freshly prepared ATP standards (0.08 to 5 pmol/tube dissolved in distilled water) were used to generate a standard curve. The assay consisted of the addition of  $10\ \mu\text{l}$  embryos ( $8\ \mu\text{l}$  distilled water plus  $\sim 2\ \mu\text{l}$  embryos and culture medium) or  $10\ \mu\text{l}$  standards to  $100\ \mu\text{l}$  reaction mixture. Luminescence was detected with the use of a luminometer (AutoLumat LB953, Wallac Inc., Gaithersburg, MD, USA). Reaction mixture was added automatically by the luminometer immediately prior to measurement and each sample was measured for 5 min. The experiment was replicated on eight different days with a total of 21 pools ( $38.5^\circ\text{C}$ ) or 24 pools ( $41.0^\circ\text{C}$ ) of 3–5 two-cell embryos.

### **Effects of heat shock at the two-cell stage on subsequent development to day 3 post-insemination**

Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at  $38.5$  or  $41.0^\circ\text{C}$  for 6 h. Culture dishes containing heat-shocked embryos were returned to the control temperature until 72 hpi. Embryos were fixed in 4% paraformaldehyde (purchased as an 8% solution from Electron Microscopy Sciences, Fort Washington, PA, USA) in  $0.2\ \text{M}$   $\text{NaPO}_4$ , pH 7.4 containing  $0.15\ \text{M}$  NaCl (phosphate-buffered saline; PBS) for 1 h. Nuclei of embryos were then

stained with a  $1\ \mu\text{g/ml}$  solution of Hoechst 33342 dye in PBS supplemented with  $1\ \text{mg/ml}$  polyvinyl pyrrolidone (PBS + PVP) for 45 min. Embryos were washed three times in PBS + PVP prior to placing on glass slides containing  $5\ \mu\text{l}$  ProLong antifade solution (Molecular Probes). Total and condensed nuclei were counted by viewing with a Zeiss Axioplan2 epifluorescent microscope (Carl Zeiss Inc., Gottingen, Germany). Images were acquired using AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Carl Zeiss Inc., Thorwood, NY, USA). The experiment was replicated on four different days with 46 embryos/treatment.

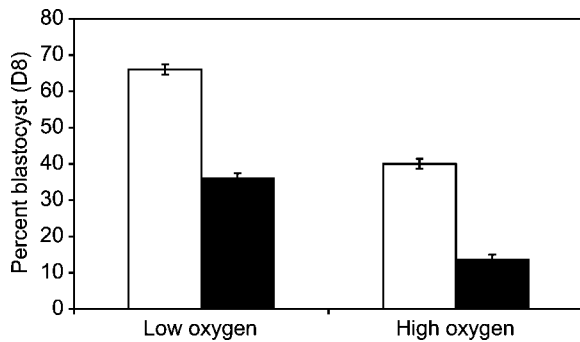
### **Statistical analysis**

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 8e, Cary, NC, USA). In each case, replicate was considered as a random effect and other variables were considered as fixed effects. Tests of significance were made using error terms derived from the calculation of expected means squares. For the experiment to test the effect of oxygen tension on the response to heat shock, data subjected to analysis were the percentage of two-cell embryos that developed to the blastocyst stage calculated for each replicate. The mathematical model included main effects of temperature, oxygen tension, replicate, and their interactions. Percentage data were analyzed before and after transformation by arcsine transformation. Analysis of transformed data was used to obtain probability values while analysis of untransformed data was used to obtain least-squares means  $\pm$  S.E.M. For the ATP, glutathione and oxygen consumption experiments, the mathematical model included effects of temperature, replicate and temperature  $\times$  replicate. For the experiment regarding effects of heat shock on development on day 3 after insemination, percentage data were calculated for each replicate and untransformed and arcsine-transformed data were analyzed using a mathematical model that included effects of temperature, replicate and temperature  $\times$  replicate. All means presented in the paper are shown as least-squares means  $\pm$  S.E.M.

## **Results**

### **Effects of oxygen tension on inhibition of development caused by heat shock**

Incubation of two-cell embryos at  $41.0^\circ\text{C}$  for 9 h reduced ( $P = 0.06$ ) the proportion of embryos that became blastocysts at day 8 after insemination (Fig. 1). The reduction in development occurred for embryos cultured under both low and high oxygen environments. There was a tendency ( $P = 0.08$ ) for a greater proportion of embryos to form blastocysts under low oxygen tension than embryos cultured under the high oxygen environment. There was a temperature  $\times$  oxygen tension interaction



**Figure 1** Effects of oxygen environment on response of two-cell embryos to heat shock. Data represent the proportion of two-cell embryos that became blastocysts at day 8 (D8) after insemination for embryos cultured in air (i.e. 20.95% oxygen; high oxygen) or under a low oxygen (5%) environment. Open bars represent control embryos and solid bars represent heat-shocked embryos. Values are least-squares means  $\pm$  S.E.M. of data from four replicates with 53–77 two-cell embryos per treatment.

( $P < 0.03$ ) that reflects the numerically greater reduction in development caused by heat shock for embryos under low oxygen tension.

#### Effect of heat shock on glutathione content

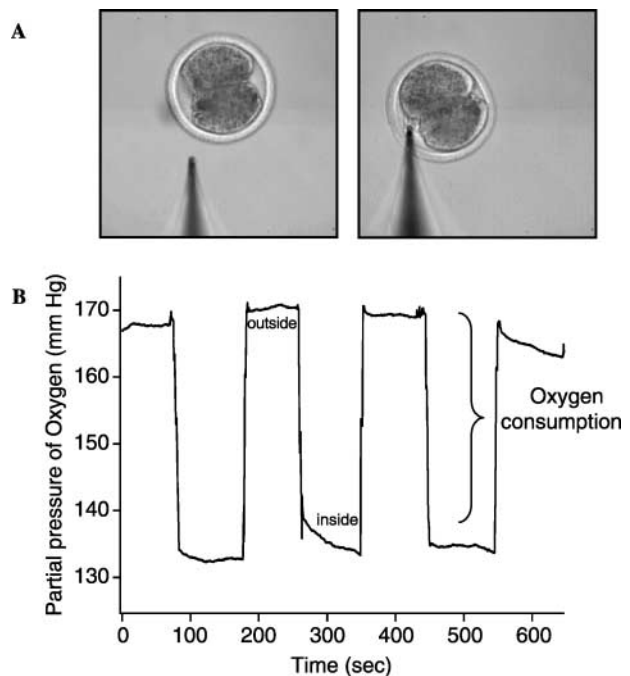
There were no differences in glutathione content between control and heat-shocked embryos ( $3.42 \pm 0.42$  vs  $3.12 \pm 0.42$  pmol/embryo for embryos cultured at  $38.5^\circ\text{C}$  and  $41.0^\circ\text{C}$  respectively).

#### Effect of heat shock on ATP content

Heat shock did not decrease embryonic content of ATP. Values were  $0.26 \pm 0.06$  pmol/embryo for embryos cultured at  $38.5^\circ\text{C}$  and  $0.30 \pm 0.05$  pmol/embryo for embryos at  $41.0^\circ\text{C}$ .

#### Effect of heat shock on oxygen consumption

Representative photomicrographs illustrating the placement of the electrode inside and outside the perivitelline space are shown in Fig. 2A while a representative pattern of changes in oxygen concentration as the electrode is moved from outside the zona pellucida to inside the perivitelline space is shown in Fig. 2B. There was no statistically significant effect of heat shock on concentrations of oxygen outside or inside the zona pellucida or on oxygen consumption (calculated as the difference between oxygen concentrations inside and outside the zona pellucida). Oxygen consumption was, however, numerically lower for heat-shocked embryos. Least-squares means  $\pm$  S.E.M. for oxygen consumption were  $25.58 \pm 5.4$  mmHg ( $\sim 2.8 \times 10^{-13}$  mol/s) and  $12.25 \pm 5.12$  mmHg ( $1.3 \times 10^{-13}$  mol/s) for  $38.5$  and  $41.0^\circ\text{C}$  respectively ( $P = 0.18$ ).

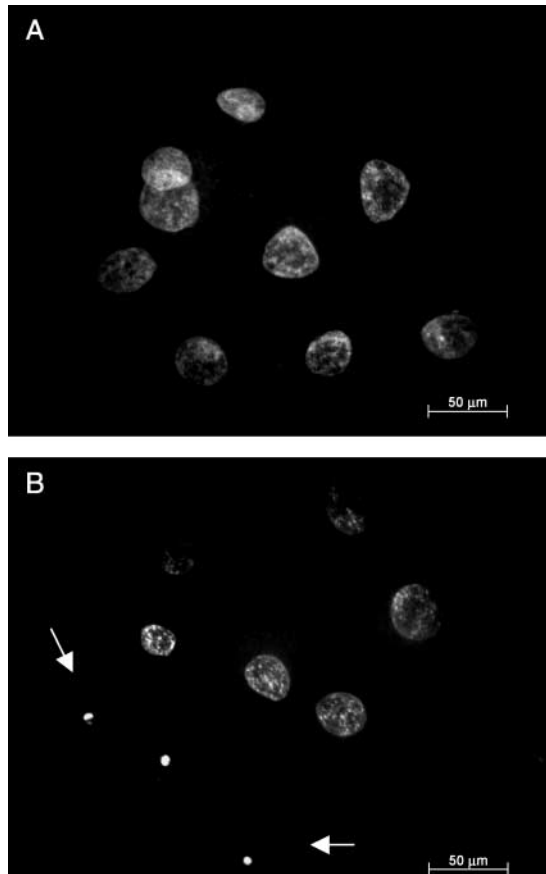


**Figure 2** Method for measuring oxygen consumption of two-cell bovine embryos. (A) Photomicrographs illustrating the placement of oxygen electrodes outside and in close proximity to the zona pellucida (left) or inside the perivitelline space (right). (B) Typical pattern of changes in oxygen concentration as the electrode is moved from outside the zona pellucida to inside the perivitelline space.

#### Effects of heat shock at the two-cell stage on subsequent development to day 3 post-insemination

Representative images illustrating formation of condensed nuclei in embryos are shown in Fig. 3 while least-squares means  $\pm$  S.E.M. for number of nuclei and number and percent of condensed nuclei are shown in Fig. 4A. Heat shock at the two-cell stage reduced ( $P < 0.05$ ) the number of total nuclei from  $10.3 \pm 0.6$  to  $7.6 \pm 0.6$ . Heat shock tended to increase ( $P = 0.08$ ) the number of condensed nuclei ( $0.43 \pm 0.12$  and  $1.12 \pm 0.12$  for  $38.5$  and  $41.0^\circ\text{C}$  respectively) and increased ( $P < 0.01$ ) the percentage of nuclei that were condensed ( $4.5 \pm 4.6$  vs  $21.8 \pm 4.5\%$  condensed for  $38.5$  and  $41.0^\circ\text{C}$  respectively).

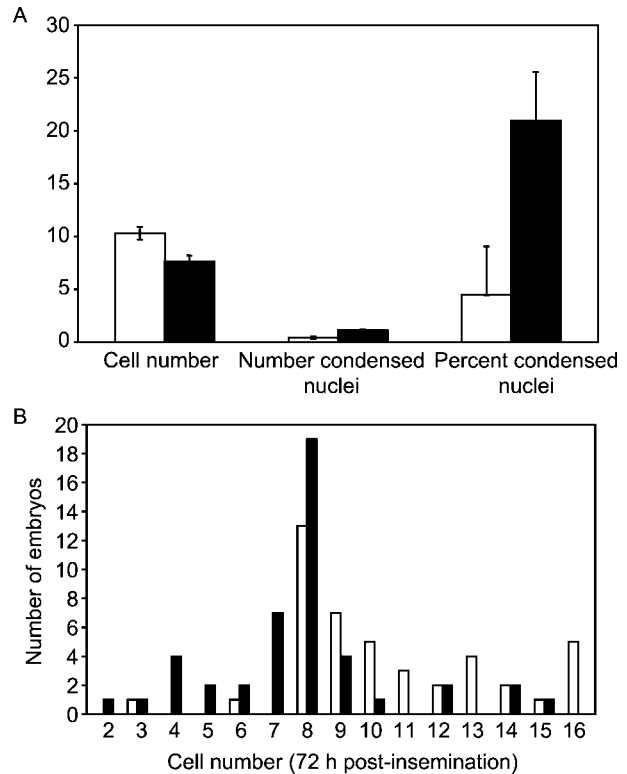
The stage of development at 72 h after insemination was evaluated to determine whether embryos subjected to heat shock at the two-cell stage became blocked in development at a specific stage. The distribution of cell number at 72 hpi is shown in Fig. 4B. It is apparent that heat-shocked embryos became blocked in development at the eight-cell stage. Only  $25 \pm 7\%$  of the heat-shocked embryos developed past the eight-cell stage versus  $67 \pm 7\%$  of control embryos ( $P < 0.05$ ). That the block to development occurred at this stage and not earlier was evident by the fact that heat shock did not affect the proportion of embryos developing past the two-cell stage ( $100 \pm 0$  vs  $98 \pm 15\%$ ) or four-cell stage ( $98 \pm 15$  vs  $96 \pm 21\%$ ).



**Figure 3** Effects of heat shock at the two-cell stage on condensed nuclei 72 h after insemination. The photomicrographs are representative images of control (A) or heat-shocked (B) embryos stained with Hoechst nuclear stain at 72 h post insemination. Arrows point to condensed nuclei. The sperm heads on the zona pellucida were digitally removed from the pictures.

## Discussion

While it is well established that exposure to a mild heat shock decreases development of preimplantation embryos (Alliston *et al.* 1965, Elliot *et al.* 1968, Gwazdauskas *et al.* 1992, Aréchiga *et al.* 1995, Aréchiga & Hansen 1998, Rivera & Hansen 2001), the mechanisms by which development is inhibited are not known. Increased production of free radicals has been implicated in the preimplantation mouse embryo (Aréchiga *et al.* 1995, Aréchiga & Hansen 1998, Ozawa *et al.* 2002) but present results do not support a critical role for free radicals in the two-cell bovine embryo. Other effects of heat shock in bovine two-cell embryos include an increase in the number of swollen mitochondria and cytoskeletal changes that lead to redistribution of organelles away from the periphery (Rivera *et al.* 2003, 2004). Here it was shown that these ultrastructural changes do not lead to an immediate alteration in ATP content or to inhibition of embryonic growth. Rather, a heat-shocked two-cell embryo can continue to undergo an additional two cleavage divisions until it



**Figure 4** Effects of heat shock at the two-cell stage on the number of total and condensed nuclei 72 h after insemination. Data in panel A are least-squares means  $\pm$  S.E.M. for total number of nuclei and number and percent of condensed nuclei as affected by heat shock. (B) The distribution of cell number at 72 h post insemination. Open bars represent control embryos and solid bars represent heat-shocked embryos. The experiment was replicated four times with 45 embryos per treatment.

becomes blocked in development at the eight-cell stage. Thus, the partial loss of mitochondria and cytoskeletal alterations caused by heat shock (Rivera *et al.* 2003, 2004) do not appear to lead to critical alterations in embryonic physiology until the stage of development coincident with embryonic genome activation (Memili & First 2000) and heat shock-induced apoptosis (Paula-Lopes & Hansen 2002).

Recently, exposure of bovine embryos to heat shock at day 0 and 2 relative to fertilization has been reported to increase peroxide production (Sakatani *et al.* 2004). Nonetheless, results from the present experiments fail to support a critical role for free radicals in actions of heat shock on embryo development in cattle. This conclusion is based primarily on the finding that low oxygen content did not reduce the effects of heat shock. If an increase in free radicals was important for the inhibition of development, one would expect that effects of heat shock would be greater in the presence of high oxygen. Many oxidases that produce reactive oxygen species are known to be oxygen dependent (Fridovich 1964, Cohen & Fridovich 1971) and the amount of oxygen radicals within mouse embryos is dependent upon the oxygen environment

(Goto *et al.* 1993). Embryos cultured in low oxygen were not less sensitive to heat shock, however. In fact, the decrease caused by heat shock was slightly greater for embryos cultured in low oxygen than in air because overall rates of development at 38.5°C were greater. In an earlier study, also, exposure of embryos to a 5% O<sub>2</sub> environment during heat shock did not lessen the detrimental effects of heat shock when compared with control embryos (Rivera & Hansen 2001). The lack of difference in the content of glutathione between heat-shocked and control embryos is additional evidence that free radicals are not a major source of damage to the two-cell embryo, at least in the cytosol where glutathione is the major antioxidant. It will be instructive to determine antioxidant status in the lipid compartment of the cell to determine whether the increased peroxide production caused by heat shock (Sakatani *et al.* 2004) depletes antioxidant stores in that compartment. Consistent with the idea that free radical production is not a crucial determinant of the effects of heat shock on bovine embryos are results from experiments in which addition of various antioxidants did not reduce the effects of heat shock on embryonic development (Ealy *et al.* 1995, Paula-Lopes *et al.* 2003).

In an earlier study, 15% of mitochondria of two-cell embryos exposed to 41.0°C for 6 h had become swollen (Rivera *et al.* 2003). Mitochondrial swelling is the result of a change in the conductance of the mitochondrial permeability transition pore to allow nonselective diffusion of large molecules into the matrix (Ichas & Mazat 1998). A major consequence of the mitochondrial permeability transition is the uncoupling of oxidative phosphorylation as a result of a collapse of the membrane potential and pH gradient (Duchen 1999, Halestrap *et al.* 2002, Skulachev 2002) and a drop in the ATP/ADP ratio (Duchen 1999). Eventually, the swollen mitochondrion undergoes death in a process called mitoptosis (Skulachev 2002). Given these effects of heat shock on mitochondrial function, it was hypothesized that heat shock would result in a reduction in oxygen consumption. Since the contribution of ATP from glycolysis is low in the early preimplantation embryo and energy is largely generated by oxidative phosphorylation (Houghton *et al.* 1996, Thompson 2000), it was also expected that heat shock would cause the intracellular content of ATP to decline. Heat shock did not significantly reduce oxygen consumption, although it was numerically lower for embryos at 41.0°C and the high variation in measurements may have precluded a real effect being significant. A portion of the oxygen consumption represents reactions not involved in oxidative phosphorylation (Trimarchi *et al.* 2000). In any case, heat shock did not affect ATP content and so it can be concluded that heat shock did not cause major changes in energy availability at this stage. One possibility is that the loss of mitochondria by mitoptosis (Rivera *et al.* 2003) did not cause a reduction in ATP content either because ATP utilization was also reduced by heat shock or because heat shock increased the activity of enzymes

involved in oxidative phosphorylation in unaffected mitochondria. Also, a decrease in mitochondrial ATP synthesis might have been compensated for by increased production of ATP by glycolysis, as suggested for some other cells types exposed to heat shock (Macouillard-Poulletier de Gannes *et al.* 2000).

The partial loss of mitochondria at the two-cell stage may not have a large effect on the energy-generating machinery of the cell because mitochondria are largely immature in the early stages of development (Van Blerkom *et al.* 1990, Plante & King 1994), have a low ATP synthetic ability (Rozell *et al.* 1992, Khurana & Niemann 2000), and do not replicate (Smith *et al.* 2000, Cummins 2001). Consequently, oxygen consumption is low during the early preimplantation stages (Leese 1991, Thompson *et al.* 1996). Perhaps embryos heat shocked at the two-cell stage become blocked in development at the eight-cell stage because increased cellular demands brought on by genome activation (Memili & First 2000) cannot be met because of earlier damage to mitochondria or other organelles. However, there does not seem to be a major increase in oxygen consumption in the bovine embryo until compaction (Thompson *et al.* 1996).

An alternative explanation for the eight-cell block in embryos exposed to heat shock at the two-cell stage involves developmental changes in embryonic capacity for apoptosis. Heat shock-induced apoptosis first occurs between the eight-cell and the sixteen-cell stages in cattle (Paula-Lopes & Hansen 2002). Perhaps, signals from mitochondria that were damaged at the two-cell stage were sufficient to induce apoptosis once the embryo reached the eight-cell stage. Blastomeres with a deficient number of mitochondria may overproduce ATP to meet the metabolic need of the blastomere, and this overproduction could itself trigger mitochondrial demise and send the cell through apoptosis (Skulachev 2002, Waterhouse 2003). The increase in cells with condensed nuclei could be evidence of apoptosis triggered as a result of mitochondrial damage (Burgoyne 1999). That only some nuclei were condensed could, in part, reflect asymmetrical distribution of mitochondria during cleavage. Such a phenomenon has been reported for human cleavage-stage embryos, resulting in some blastomeres having reduced mitochondrial inheritance and diminished ATP capacity (Van Blerkom *et al.* 2000).

The values for ATP content per embryo obtained in the present study (0.26–0.30 pmol) are not directly comparable to the literature because there are no reports on ATP content in bovine embryos at the two-cell stage. The values are similar to those reported for matured oocytes in one study (0.18–0.38 pmol; Hashimoto *et al.* 2000) but lower than the values for matured oocytes of 0.85 pmol found by Rieger (1997) and 1.6–2.5 pmol found by Stojkovic *et al.* (2001). In the cat, at least, ATP content is lower for two-cell embryos than for matured oocytes (Friestedt *et al.* 2001), and it is possible that a reduction in ATP content following fertilization and cleavage also

occurs in cattle. The rate of oxygen consumption in embryos at 38.5 °C was  $\sim 2.8 \times 10^{-13}$  moles/s. This value is closer to the value of  $0.14 \times 10^{-13}$  moles/s for bovine morula reported by Shiku *et al.* (2001) than for values reported by Thompson *et al.* (1996) of  $\sim 9 \times 10^{-11}$  moles/s for bovine embryos from day 0–4 of culture. Reported values for bovine blastocysts range from 0.4– $1.5 \times 10^{-9}$  moles/s (Thompson *et al.* 1996, Kaidi *et al.* 2001, Donnay *et al.* 2002). Reasons for the differences in absolute amounts are not known but could include methodology since the lower values in the present study and by Shiku *et al.* (2001) are based on electrochemical measurements of oxygen, while other papers cited above rely on quenching of pyrene fluorescence by oxygen. The electrochemical method employed here has been used extensively to characterize oxygen consumption in cultured islets of Langerhans (Jung *et al.* 1999a,b, 2000).

In summary, exposure of bovine embryos at the two-cell stage did not appear to cause a block to development by increasing oxygen free radicals or causing an immediate reduction in oxidative phosphorylation. Embryos did not become inhibited in development immediately after heat shock but rather progressed to the eight-cell stage before becoming blocked in development and developing condensed nuclei. It is likely that mitochondrial damage or other alterations in the embryo brought about by heat shock at the two-cell stage makes the embryo unable to proceed past the eight-cell stage, a critical time in embryo development when there is a surge in embryonic transcription (Barnes & Eystone 1990, Memili *et al.* 1998), and heat shock-induced apoptosis becomes possible (Paula-Lopes & Hansen 2002).

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