

# Development of cultured bovine embryos after exposure to high temperatures in the physiological range

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Embryonic development is inhibited by exposure of cultured embryos to high temperatures. However, culture temperatures used to demonstrate the effects of heat on development have been higher than the body temperatures experienced typically by heat-stressed cows. The aim of this study was to determine whether exposing bovine oocytes and embryos to temperatures characteristic of body temperatures of heat-stressed cows would affect embryonic development *in vitro*. The CO<sub>2</sub> percentage of the gas phase was adjusted in all experiments to prevent pH changes in the medium caused by decreased solubility of CO<sub>2</sub> at high temperatures. Fertilization of oocytes at 41.0°C reduced cleavage rate and the percentage of oocytes that became blastocysts compared with at 38.5°C. There was no deleterious effect of fertilization at 40.0°C. When putative zygotes and two-cell embryos were exposed to a range of temperatures from 38.5 to 41.0°C for 3, 6, 9 or 12 h, heat shock reduced the number that

developed to the blastocyst stage but only after exposure to 41.0°C for 9 or 12 h. In addition, it was tested whether low O<sub>2</sub> tension would reduce the detrimental effects of heat shock. The deleterious effect of 41.0°C was not dependent upon oxygen content or the gas mixture used for culture (5% versus 20.95% O<sub>2</sub>), indicating that the deleterious effects of heat shock did not depend upon a high O<sub>2</sub> environment. In the final experiment, embryos were exposed to 24 h fluctuations in temperature designed to mimic the rectal temperatures of cows exposed to heat stress. Exposure of embryos to this pattern of temperatures starting after fertilization reduced development when embryos were exposed to this environment for 8 days but not when embryos were exposed for 1 day only. These findings indicate that embryonic development can be disrupted by a short-term severe or a prolonged mild heat shock and that the effects of heat shock are not artefacts of changes in pH or high oxygen tension.

## Introduction

Heat stress can reduce fertility in cattle (Dutt, 1963; Dunlap and Vincent, 1971; Turner, 1982; Ealy *et al.*, 1993; Ryan *et al.*, 1993) and other species (Shah, 1956; Alliston and Ulberg, 1961; Tompkins *et al.*, 1967). The mechanism by which heat stress causes embryonic mortality is multifactorial, as heat stress can alter several aspects of reproductive physiology, including blood flow to the reproductive tract (Roman-Ponce *et al.*, 1978), ovarian steroid concentrations (Gwazdauskas *et al.*, 1973; Roman-Ponce *et al.*, 1981; Badinga *et al.*, 1993; Trout *et al.*, 1998) and patterns of follicular development (Badinga *et al.*, 1993; Wolfenson *et al.*, 1995). One possibility is that embryos cannot survive the increase in oviductal and uterine temperature that is coincident with heat stress. Culture of embryos at high temperatures has been reported to reduce embryonic development (Ulberg and Sheean, 1973; Edwards and Hansen, 1996, 1997). Similarly, increased culture temperatures can compromise oocyte function

(Lenz *et al.*, 1983; Baumgartner and Chrisman, 1987; Edwards and Hansen, 1996) and fertilization rate (Ulberg and Burfening, 1967; Lenz *et al.*, 1983).

Studies in cattle have demonstrated the deleterious effects of heat shock on cultured embryos at 41.0–43.0°C (Ealy *et al.*, 1992; Ealy and Hansen, 1994; Edwards and Hansen, 1996, 1997). However, these experimental temperatures are higher than those generally experienced by heat-stressed cows with reduced fertility. For example, Dunlap and Vincent (1971) observed a reduction in fertility from 48% in control animals to 0% for heifers exposed to 32.2°C for 72 h immediately after mating; however, the mean rectal temperature of the heat-stressed heifers was 40.0°C. Ryan *et al.* (1992) reported that a heat shock of 40.0°C actually increased the development of cultured bovine embryos.

Heat shock in culture differs from the situation *in utero* in several other respects. For example, increases in temperature *in vitro* cause decreased CO<sub>2</sub> solubility in medium and an increase in medium pH. This confounding of pH with heat shock has not been considered previously in experiments evaluating the effects of heat shock on embryos. Experiments on heat shock have also been performed in a gaseous environment in which O<sub>2</sub> content is

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higher than in the oviduct (8.7% in rabbits; Fischer and Bavister, 1993). As the effects of heat shock on embryonic development also involve free radicals (Ealy *et al.*, 1992; Aréchiga *et al.*, 1995), it is not known whether heat shock would affect embryonic development at oxygen environments similar to those found *in vivo*.

The aims of the present study were: (i) to determine whether exposing bovine oocytes and embryos to temperatures characteristic of the body temperatures of heat-stressed cows would affect embryonic development *in vitro*; (ii) to determine whether culturing early bovine embryos in low oxygen concentrations would eliminate the adverse effects of heat shock on embryonic development; and (iii) to verify that the detrimental effects of heat shock were not due to changes in pH as a result of reduced solubility of CO<sub>2</sub> at high temperatures.

## Materials and Methods

### Materials

FSH was Folltropin®-V from Vetrep Pharm Canada Inc (London, Ontario) and was purchased from AgTech (Manhattan, KS). The culture media SP-TL, IVF-TL and Hepes-TL were prepared by Cell and Molecular Technologies Inc (Lavallete, NJ) using recipes described by Parrish *et al.* (1986). These media were used to prepare SP-TALP, Hepes-TALP and IVF-TALP as described by Parrish *et al.* (1986). The CR1aa medium was prepared as described by Rosenkrans *et al.* (1993). BSA fraction V, essentially fatty-acid free BSA fraction V (EFAF-BSA) and all other chemicals were from Sigma (St Louis, MO).

### In vitro production of embryos

Bovine ovaries were obtained from a local abattoir located at a travel distance of approximately 1.5 h from the laboratory. Ovaries were transported to the laboratory in 0.9% (w/v) NaCl at room temperature. The ovaries were sliced and oocyte-cumulus complexes were collected into a beaker containing oocyte collection medium (TCM-199 with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U heparin ml<sup>-1</sup>), 100 iu penicillin ml<sup>-1</sup> and 0.1 mg streptomycin ml<sup>-1</sup>). Cumulus-oocyte complexes (COCs) were cultured in 50 µl microdrops of oocyte maturation medium (TCM-199 with Earle's salts supplemented with 10% bovine steer serum 100 iu penicillin ml<sup>-1</sup>, 0.01 mg streptomycin ml<sup>-1</sup>, 2 µg oestradiol ml<sup>-1</sup>, 20 µg FSH ml<sup>-1</sup> and 0.2 mmol sodium pyruvate l<sup>-1</sup>) in groups of 10 for 20–23 h. After maturation, the COCs were washed in Hepes-TALP and placed in groups of about 30 in 600 µl IVF-TALP in four-well plates. Spermatozoa that had been purified by Percoll gradient centrifugation (Parrish *et al.*, 1986) and suspended in SP-TALP were added to the matured oocytes at a density of approximately 1 × 10<sup>6</sup> spermatozoa per well. Coculture of spermatozoa and COCs proceeded for 8–10 h, after which time the putative zygotes were denuded of cumulus cells by

vortexing in a 2.0 ml microcentrifuge tube containing 0.5 ml Hepes-TALP for 5 min. Putative zygotes were cultured in 50 µl microdrops of CR1aa. On day 5 after insemination, 5 µl fetal bovine serum was added to each culture drop.

All cultures were performed at 38.5°C in 5% CO<sub>2</sub> in humidified air unless specified otherwise. When cultures were performed at high temperatures, the CO<sub>2</sub> percentage of the gas phase was adjusted to prevent pH changes in the medium caused by decreased solubility of CO<sub>2</sub> at high temperatures. The CO<sub>2</sub> percentage needed to maintain a pH of approximately 7.4 in the CR1aa medium was determined experimentally for each temperature. These were 5.5, 6.0, 6.5 and 7.0% CO<sub>2</sub> for 39.5, 40.0, 40.5 and 41.0°C, respectively. The accuracy of the incubators was ± 0.2°C for one of the incubators and 0.1°C for two of the incubators. The temperatures of all incubators were calibrated routinely using mercury thermometers.

### Heat shock during fertilization

Coculture of spermatozoa and COCs was performed for 8 h at 38.5, 40.0 or 41.0°C. After fertilization, putative zygotes were cultured at 38.5°C for 8 days. The cleavage rate was determined on day 3 after insemination and development to the blastocyst stage was determined on day 8 after insemination. This experiment was replicated six times using a total of 179–180 oocytes per treatment.

### Heat shock at the one-cell stage

After coculture of spermatozoa and COCs was complete (9 h after insemination), putative zygotes were cultured in 50 µl microdrops in groups of 27–32 at 38.5°C continuously or were exposed to 40.0 or 41.0°C for 3, 6, 9 or 12 h. After this time, all embryos were cultured at 38.5°C for the duration of the culture. Cleavage rate was determined on day 3 after insemination and development to the blastocyst stage was determined on day 8 after insemination. The experiment was replicated four times using 117–121 putative zygotes per treatment.

### Heat shock at the two-cell stage

Two experiments were conducted. In both experiments, embryos at the two-cell stage were collected from culture drops at about 28 h after insemination and transferred to a new drop of CR1aa medium (8–25 embryos per drop). For the first experiment, embryos were then cultured for 3, 6, 9 or 12 h at 40.0 or 41.0°C or were maintained at 38.5°C. Furthermore, a group of embryos was left undisturbed in the original culture drops (two-cell embryos were not separated) to determine whether the handling itself would adversely affect development. After heat shock, the embryos were returned to 38.5°C for the duration of culture. Development to the blastocyst stage was determined on day 8 after insemination. Unlike most of the experiments, the type of BSA used to supplement CR1aa was fraction V.

The experiment was replicated five times with a total of 54–84 two-cell embryos per treatment.

The second experiment was conducted in a similar way except that heat shocks were at 39.5 and 40.5°C. Furthermore, the CR1aa was supplemented with EFAF-BSA. The experiment was replicated four times with a total of 52–68 embryos per treatment.

#### *Effect of type of BSA on response to heat shock*

The experiment was designed as a 2 × 2 factorial with two types of BSA (fraction V and EFAF) and two temperatures (38.5 or 41.0°C). Two-cell embryos were collected at about 28 h after insemination and placed in CR1aa supplemented with either EFAF-BSA or fraction V BSA. Embryos were cultured continuously at 38.5°C or at 41.0°C for 12 h and 38.5°C thereafter. Development to the blastocyst stage was determined on day 8 after insemination. The experiment was replicated three times with a total of 57–58 embryos per treatment.

#### *Effect of oxygen concentration on response to heat shock*

This experiment was performed to determine whether the effects of heat shock in culture are exacerbated by high O<sub>2</sub> environment. A chamber was constructed from a 115 ml vacuum filter unit from Corning for regulation of the gaseous environment of cultured embryos. Tygon tubing (3.2 mm i.d. × 6.4 mm o.d.) with a stopcock fastened to the end was attached to the hose connector and a 00 size rubber stopper was inserted into the pour spout. The cellulose nitrate membrane in the filter was punctured in several places to facilitate movement of gases. Culture plates were positioned gently on top of the membrane. The lid of the filter system was taped securely with scotch tape and parafilm was placed around the lid to make the chamber airtight. Gases were injected for 3 min through the pour spout from a hose connected to the gas cylinder while the stopcock was in the open position. Once injection of gases was complete, the rubber stopper was fastened to the pour spout and the stopcock was dialled to the closed position. A filter (0.22 µm) was attached to the gas hose to prevent contamination during injection of gases. The chambers were airtight, as pH was maintained for 24 h in the presence of various gas mixtures.

The design was a 2 × 2 factorial with two O<sub>2</sub> tensions (5 or 20.95%) and two temperatures (38.5 and 41.0°C). Embryos were collected at the two-cell stage and placed in fresh microdrops (14–27 embryos per drop). The culture plates were placed into airtight chambers. The chambers containing the embryos cultured at 38.5°C were injected with a mixture of gases containing high (20.95%) or low (5%) O<sub>2</sub> with 5% CO<sub>2</sub> and a balance of N<sub>2</sub>. Chambers containing the embryos cultured at 41.0°C were injected with a similar mixture of gases except that CO<sub>2</sub> was 7% to maintain a pH of about 7.4 and the N<sub>2</sub> content was adjusted accordingly. After 12 h of culture, the plates were

removed from the chambers and returned to incubators at 38.5°C and 5% CO<sub>2</sub> in air until day 8 after insemination. The experiment was replicated four times with a total of 80–107 embryos per treatment.

#### *Rectal temperatures of heat-stressed lactating dairy cows*

This experiment was performed as a prelude to determining the effects of culture temperatures characteristic of those experienced by heat-stressed cows on embryonic development. Animals used were primiparous ( $n = 8$ ) or multiparous ( $n = 17$ ) lactating Holsteins (day 50–150 of lactation except for one cow at day 266 of lactation) located in North Florida (Hague, Florida; 29° 46' N 82° 25' W). The cows were milked three times per day and received injections of bovine somatotrophin (Posilac; Monsanto, Chesterfield, MO) according to the manufacturer's recommendations. Milk yields on the day of recording ranged from 17.2 to 33.6 kg day<sup>-1</sup>. The cows were kept in free-stall barns equipped with a cooling system using high-pressure foggers. On each of three separate days in August 1999, a group of nine cows was examined. Cows were chosen so that three cows were > 75% white in colour (white), three cows were 25–75% white (black and white) and three cows were < 25% white (black). Rectal temperatures were measured using mercury rectal thermometers (precision = 0.1°C) and respiration rates were determined at 1 h intervals for 24 h. In addition, relative humidity, dry bulb temperature and black globe temperature were recorded at 1 h intervals. For each replicate, a separate group of eight or nine cows in a different free-stall barn was used.

#### *Exposure of embryos to a pattern of temperatures similar to those experienced by heat-stressed cows*

Incubators were set at either 38.5, 39.5 or 40.5°C. The heat shock treatment consisted of sequential exposure to 38.5°C for 5 h, 39.5°C for 5 h, 40.5°C for 5 h and 39.5°C for 9 h. Given the limited number of incubators available, this pattern was found to mimic most closely the pattern of rectal temperatures experienced by cows in the previous experiment (see results). Putative zygotes were cultured continuously at 38.5°C (control group), heat shocked from day 0–1 after insemination (heat shock 0–1 group) or heat shocked on each day from day 0 to day 8 after insemination (heat shock 0–8). The experiment was replicated four times with 121–122 embryos per treatment.

#### *Statistical analysis*

For the embryo experiments, percentage cleavage and development was calculated for each microdrop of putative zygotes or embryos. Each experiment was replicated on several days with one or more microdrop per treatment on each day. Data were analysed by least-squares ANOVA using the PROC GLM procedures (SAS Institute, 1989). All

main effects were considered fixed. Embryo number per drop was also included as a covariate for experiments with two-cell embryos where there was variation in the number of embryos per drop. Percentage data were transformed by arcsine transformation before analysis. The analysis of transformed data was used for probability values but least squares means  $\pm$  SEM are presented from analysis of untransformed data. In some experiments, the SAS option pdiff was used to compare the mean of each treatment with the mean of the control group. Orthogonal contrast was used to determine whether the effects of duration of heat shock followed a linear, quadratic or cubic pattern.

Rectal temperature data were analysed by least squares ANOVA. The mathematical model included the main effects of coat colour, replicate, cow (replicate  $\times$  coat colour) and time. All interactions were included in the model. Cow was considered a random effect and other main effects were considered as fixed.

## Results

### Effect of heat shock during fertilization

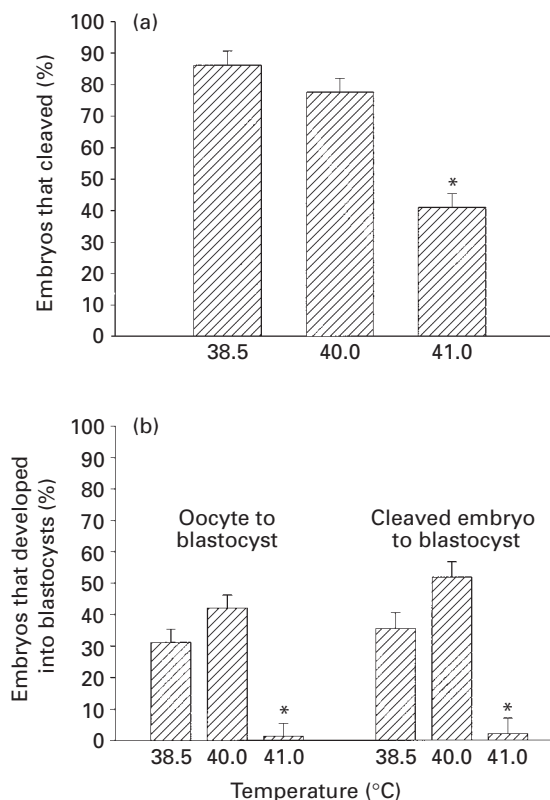
Bovine oocytes fertilized at 41.0°C had lower ( $P < 0.01$ ) cleavage rates than did oocytes fertilized at 38.5°C (Fig. 1a). The proportions of oocytes and cleaved embryos that developed to the blastocyst stage were also lower in oocytes fertilized at 41°C compared with those fertilized at 38.5°C ( $P < 0.01$ ) (Fig. 1b). A heat shock of 40.0°C during fertilization had no effect on cleavage rate and tended to increase the proportion of oocytes forming blastocysts compared with oocytes fertilized at 38.5°C.

### Heat shock at the one-cell stage

No reduction in cleavage rate or blastocyst formation was detected for one-cell embryos cultured at 40.0°C for 3, 6, 9 or 12 h, or 41.0°C for 3 or 6 h. However, a heat shock of 41.0°C for 12 h reduced cleavage rate ( $P < 0.01$ ) (Fig. 2a). Similarly, development to the blastocyst stage was affected adversely only by heat shocks of 41.0°C for 9 or 12 h ( $P < 0.01$ ; Fig. 2b).

### Heat shock at the two-cell stage

In the first part of this experiment, a heat shock of 40.0°C did not significantly reduce the percentage of two-cell embryos that developed into blastocysts by day 8 after insemination (Fig. 3a). In contrast, incubation at 41.0°C for 9 and 12 h significantly reduced the proportion of embryos that were blastocysts at day 8 after insemination ( $P < 0.01$ ). In the second experiment, exposure to either 39.5 or 40.5°C did not reduce the rate of blastocyst formation (Fig. 3b). The proportion of embryos reaching the blastocyst stage was greater for the second experiment, in which the medium was supplemented with EFAF-BSA, than for the first experiment, in which the medium was supplemented with fraction V BSA. Accordingly, another



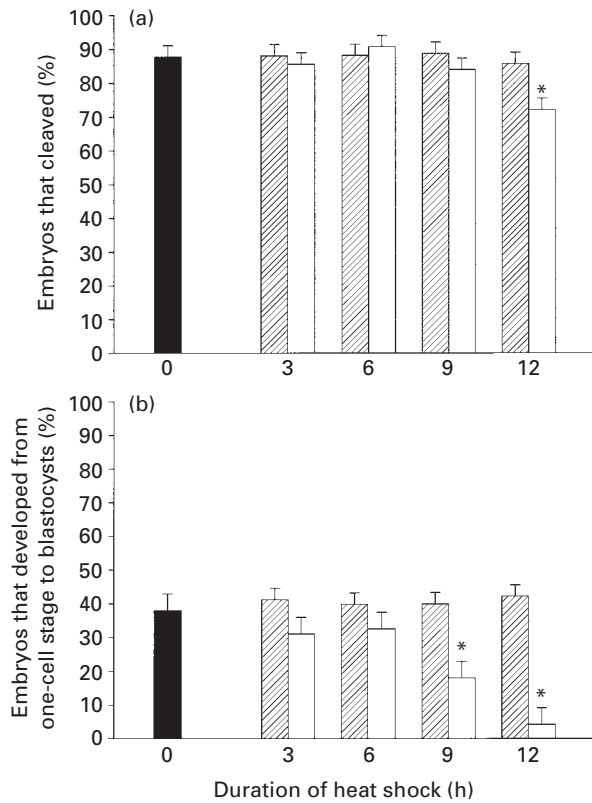
**Fig. 1.** Effect of temperature during fertilization on the percentage of oocytes that (a) cleaved and (b) developed into blastocysts on day 8 after insemination. Data are least squares means  $\pm$  SEM (mean of six replicates, each with 179–180 oocytes per treatment). Fertilization was for 8 h at 38.5, 40.0 or 41.0°C. \*Significantly different from control value (38.5°C) ( $P < 0.01$ ).

experiment was performed to verify this effect of BSA type and to determine whether the BSA source would affect responses to heat shock (Fig. 4a). Two-cell embryos cultured at 38.5°C had a higher rate of blastocyst formation than did embryos exposed to 41.0°C for 12 h ( $P < 0.02$ ). In addition, the percentage of embryos that developed into blastocysts was higher ( $P < 0.01$ ) for embryos cultured in CR1aa medium containing EFAF-BSA than for those cultured in CR1aa medium containing Fraction V. However, there was no temperature  $\times$  BSA interaction, indicating that the effect of heat shock was similar for the two media.

### Effect of oxygen concentration on response to heat shock

The two-cell embryos cultured at 38.5°C had a higher ( $P < 0.01$ ) rate of blastocyst formation than did embryos exposed to a heat shock of 41.0°C for 12 h (Fig. 4b). Low  $O_2$  tended to increase the number of embryos that developed to the blastocyst stage, although the embryos were only in the presence of low  $O_2$  for 12 h at the two-cell stage. However, culturing two-cell embryos in low  $O_2$  tensions did not decrease the effect of heat shock on





**Fig. 2.** Effect of heat shock (■: 38.5°C; ▨: 40.0°C; □: 41.0°C) at the one-cell stage of development on the percentage of oocytes that (a) cleaved and (b) became blastocysts on day 8 after insemination. Data are least squares means  $\pm$  SEM (mean of four replicates, each with 117–121 oocytes per treatment). \*Significantly different from control value (38.5°C) ( $P < 0.01$ ).

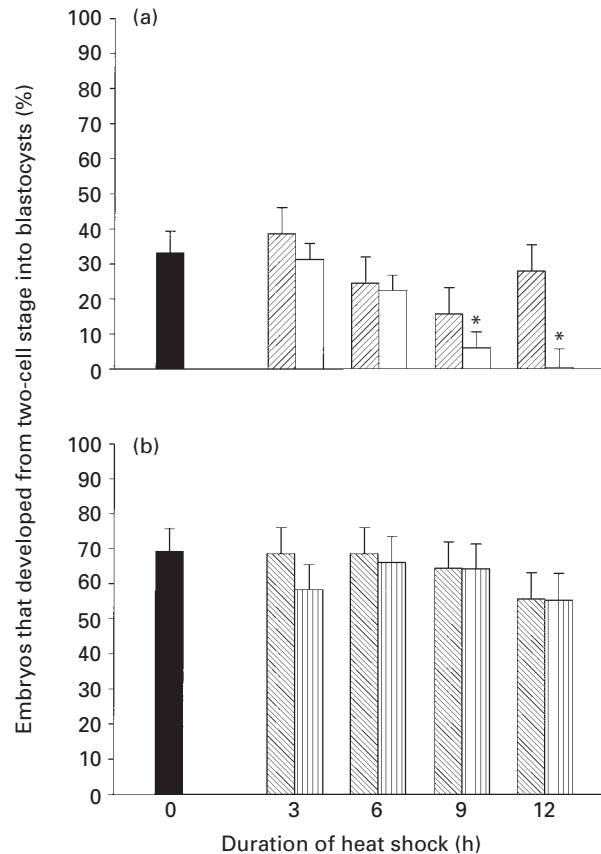
embryonic development, as indicated by the lack of oxygen  $\times$  temperature interaction. Heat shock reduced development from 53.5 to 27.0% at 20.95% O<sub>2</sub> and from 69.4 to 29.9% in 5% O<sub>2</sub>.

#### Rectal temperatures of heat-stressed lactating dairy cows

The mean peak air temperatures and peak black globe temperatures on the days when the experiment was performed were 34.5 and 33.7°C, respectively (Fig. 5a). Mean rectal temperatures ranged from 38.6°C (08:00 h) to 40.5°C (17:00 h) (Fig. 5b). Mean respiration rates ranged from 71.8 breaths min<sup>-1</sup> (08:00 h) to 105 breaths min<sup>-1</sup> (12:00 h; data not shown). Coat colour affected rectal temperatures of cows ( $P < 0.001$ ), with predominantly white cows having lower rectal temperatures than predominantly black cows (Fig. 5b).

#### Exposure of embryos to a pattern of temperatures similar to those experienced by heat-stressed cows

The results of the previous experiment were used to design a pattern of culture temperatures that would mimic

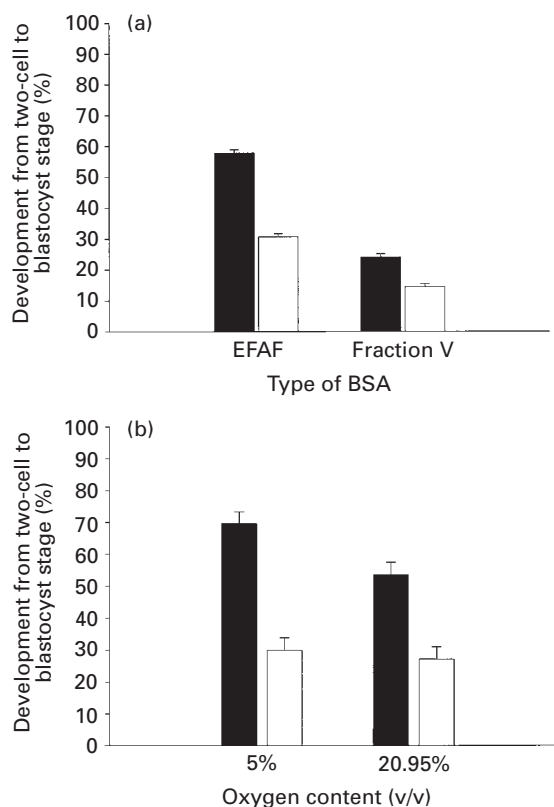


**Fig. 3.** Effect of heat shock at (a) 38.5 (■), 40.0 (▨) and 41.0°C (□) and (b) 38.5 (■), 39.5 (▨) and 40.5°C (□) at the two-cell stage of development on the percentage of two-cell embryos that became blastocysts on day 8 after insemination. Embryos were heat shocked for 3–12 h starting at about 28 h after insemination. Data are least squares means  $\pm$  SEM (mean of four replicates, each with 52–84 oocytes per treatment). There was a cubic effect ( $P < 0.1$ ) of duration of heat shock at 40°C. There was a linear effect ( $P < 0.001$ ) of duration of heat shock at 41.0°C. \*Significantly different from control value (temperature 38.5°C) ( $P < 0.01$ ).

the 24 h variation in body temperatures. Applying heat shock in this pattern from 52 to 84 h after insemination did not affect embryonic development to the blastocyst stage (Fig. 6b). In contrast, application of this pattern of fluctuating temperatures for 192 h (day 0–8 after insemination) reduced development of embryos to the blastocyst stage ( $P < 0.05$ ).

## Discussion

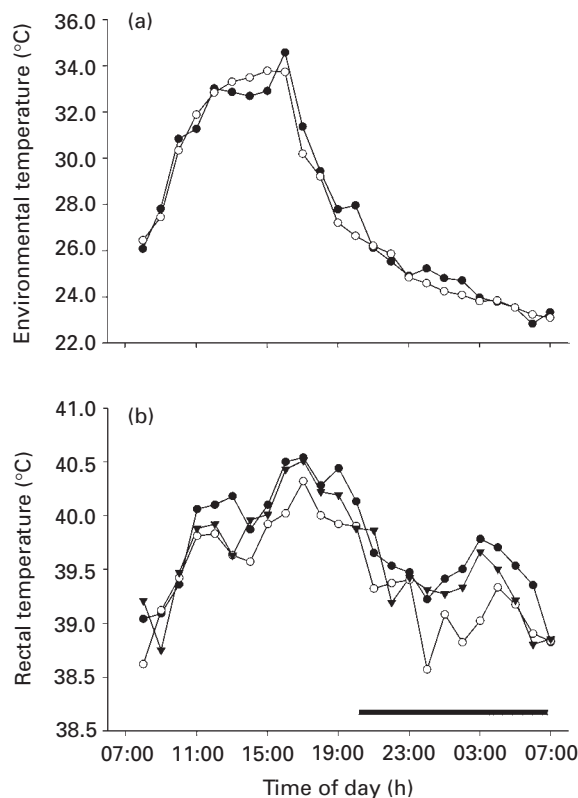
The results of the present study indicate that embryonic development can be disrupted by heat shock of 41.0°C applied during fertilization and at the one- and two-cell stages of development. Furthermore, exposing early embryos to temperatures similar to those experienced by heat-stressed dairy cows can reduce development to the blastocyst stage. Thus, under certain circumstances,



**Fig. 4.** Effect of culture conditions on responses of two-cell embryos to heat shock. (a) Effect of type of BSA used to supplement the culture medium on subsequent development to the blastocyst stage. Results are the percentage of two-cell embryos that became blastocysts on day 8 after insemination. The percentage of embryos becoming blastocysts was significantly different ( $P < 0.02$ ) between embryos cultured at 38.5 (■) and 41.0°C (□) for 12 h and between embryos cultured in media containing essentially fatty acid free (EFAF) BSA versus fraction V BSA ( $P < 0.01$ ). However, there was no temperature  $\times$  BSA type interaction. Data are least-squares means  $\pm$  SEM (mean of three replicates, each with 57–58 oocytes per treatment). (b) Effect of O<sub>2</sub> concentration (5 or 20.95%) during heat shock of two-cell embryos on subsequent development to the blastocyst stage. Results are the percentage of two-cell embryos that became blastocysts on day 8 after insemination. The percentage of embryos that developed into blastocysts was affected by incubation temperature ( $P < 0.01$ ) and there was a tendency for O<sub>2</sub> concentration to affect the percentage. Data are least squares means  $\pm$  SEM (mean of four replicates, each with 80–107 oocytes per treatment).

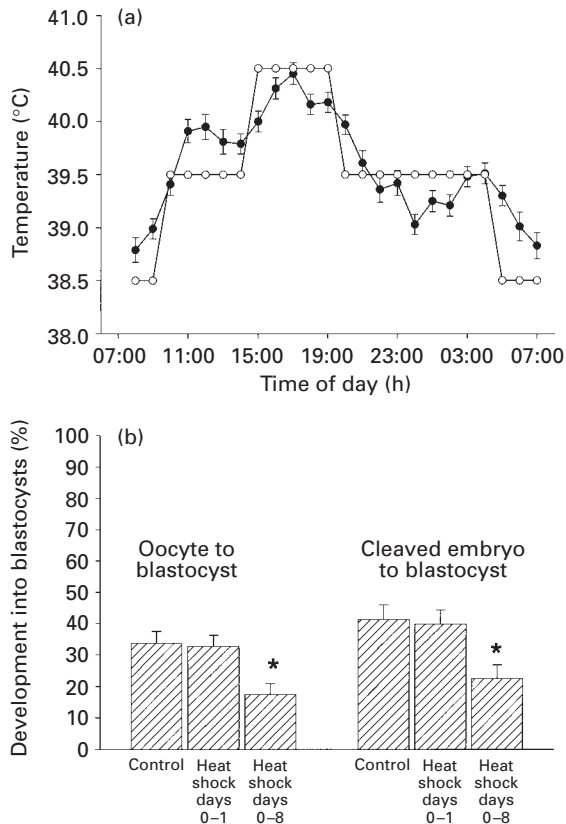
embryonic development is likely to be compromised by the direct actions of increased temperature on the oocyte and embryo. The finding that short-term heat shock ( $\leq 12$  h incubation) only affected development at very high temperatures (41.0°C) may indicate that certain causes of heat-associated infertility associated with mild maternal hyperthermia are the result of effects other than the direct actions of increased temperature on embryonic survival.

Many previous reports have indicated the deleterious effects of increased temperature on oocytes and early em-



**Fig. 5.** Rectal temperatures of lactating dairy cows under heat stress conditions in North Florida. (a) Dry bulb (●) and black globe (○) environmental temperatures. (b) Rectal temperatures for cows that are predominantly black (●;  $n = 9$ ), about equally black and white (▼;  $n = 8$ ) and predominantly white (○;  $n = 8$ ). Rectal temperature was affected by coat colour ( $P < 0.001$ ). Data are least square means. The pooled SEM in (b) is 0.19. The black bar shows the period from sunset to sunrise.

bryos (Shah, 1956; Alliston and Ulberg, 1961; Dutt, 1963; Tompkins *et al.*, 1967; Dunlap and Vicent, 1971; Turner, 1982). However, in these experiments, the effects of temperature were confounded with changes in pH because CO<sub>2</sub> solubility in the medium decreases as temperature increases. In the present study, it is shown that, even when controlling for pH changes, decreased development occurs after exposure of oocytes, or one- or two-cell embryos to increased temperature. Moreover, this effect of heat shock is not simply an artefact caused by high oxygen concentrations in culture. Embryos are most commonly cultured in air, which has an O<sub>2</sub> content of about 20.95%, which is higher than that found in the oviduct or uterus ( $< 10\%$ ; Fischer and Bavister, 1993). As formation of free radicals can increase as a result of exposure to high O<sub>2</sub> tensions (Fowler and Callingham, 1978; Goto *et al.*, 1993) and high temperatures (Flanagan *et al.*, 1998), it is possible that the detrimental effects of heat shock in cultured embryos involves the formation of free radicals. In addition, heat shock of cultured embryos decreases concentrations of glutathione, which is a free radical scavenger (Aréchiga



**Fig. 6.** Effect of exposing cultured embryos to a pattern of temperatures similar to that experienced by heat stressed cows. (a) Rectal temperatures of heat-stressed lactating dairy cows in North Florida (●); least square means  $\pm$  SEM;  $n = 9$ ) and culture temperatures for heat-shocked embryos (○). (b) Percentage of oocytes and percentage of cleaved embryos that developed into blastocysts after continuous exposure of embryos to 38.5°C (control), heat shock for 24 h starting 9 h after fertilization (heat shock days 0–1) or heat shock throughout the culture period (heat shock days 0–8). Data are least squares means  $\pm$  SEM (four replicates, each with 121–122 oocytes per treatment). \*Significantly different from control ( $P < 0.05$ ).

*et al.*, 1995), and the adverse effects of heat on embryonic development can be minimized by various antioxidants (Ealy *et al.*, 1992; Malayer *et al.*, 1992; Aréchiga *et al.*, 1994, 1995). Accordingly, in the present study, it was tested whether low  $O_2$  tension would reduce the detrimental effect of heat on two-cell embryos, presumably by lowering the exposure of the embryos to free radicals. There was no temperature by  $O_2$  interaction, which indicates that the deleterious effects of heat shock did not depend upon a high  $O_2$  environment.

It has been demonstrated that bovine embryos develop better when cultured in 5%  $O_2$  than in air (Dumoulin *et al.*, 1995; Takahashi *et al.*, 1996; Fujitani *et al.*, 1997; Lim *et al.*, 1999). In the present study, a beneficial effect of low  $O_2$  was observed when embryos were exposed to low  $O_2$  for only 12 h at the two-cell stage of development. The modified media filters used in the present study offer a

practical method for controlling oxygen concentration in embryo cultures to increase blastocyst yield.

The type of BSA used to supplement the culture medium also had an effect on development of embryos to the blastocyst stage. In particular, embryos developed better in media containing EFAF-BSA than in media containing fraction V BSA. However, the embryos that developed in EFAF-BSA were no more resistant to heat shock than were embryos that developed in fraction V BSA. The better development of embryos in EFAF-BSA could be due in part to the removal of fatty acids and other impurities from fraction V BSA. The differences between development in media containing fraction V or EFAF BSA may also have reflected simple batch-to-batch variation (McKieman and Bavister, 1992) rather than any purification process-related effect.

In the experiment in which rectal temperatures of cows were determined, cows that were mostly white had lower rectal temperatures than did cows that were mostly black. This finding is in accordance with previous observations that white cows exposed to intense solar radiation have lower rectal and surface temperatures than do black cows (Hansen, 1990). In addition, King *et al.* (1988) reported that predominantly white cows that are mated in warm months had a shorter interval from calving to conception than did predominantly black cows. In contrast, Godfrey and Hansen (1996) failed to observe any effect of coat colour on reproduction of Holstein cows in the Caribbean.

In the present study, as has been reported previously (Ulberg and Burfening, 1967; Lenz *et al.*, 1983), heat shock during fertilization reduced not only cleavage rate but also the ability of cleaved embryos to develop. Again, a high temperature (41.0°C) was necessary to achieve this effect. The fact that exposure to 41.0°C affected fertilization rates may have been due in part to the effects of temperature on the oocyte (Lenz *et al.*, 1983; Baumgartner and Chrisman, 1987; Edwards and Hansen, 1997; Rocha *et al.*, 1998) or spermatozoa (Lenz *et al.*, 1983; Chandolia *et al.*, 1999). Heat shock could also affect syngamy or the first cleavage division. These processes involve microtubule and microfilament assembly (Kim *et al.*, 1997), and cytoskeletal elements are known to collapse and aggregate after heat shock (Welch and Suhuan, 1985). Embryos formed from fertilization at 41.0°C had impaired developmental potential, perhaps because the damage to the cytoskeleton or other intracellular organelles persists. In addition, embryos formed via fertilization with heat-damaged spermatozoa had lower developmental potential (Ulberg and Burfening, 1967). Exposure of oocytes and spermatozoa to a temperature of 40.0°C during fertilization did not affect cleavage rate but did increase subsequent embryonic development. It is possible that 40.0°C was too low a temperature to damage gametes or embryos and that the increased thermal energy at 40.0°C increased the metabolic rate of the embryos.

The data obtained in the present study confirm that heat shocks such as those experienced by the cells of heat-

stressed cows can compromise embryonic development. *In vivo*, heat-stressed cows typically experience daily fluctuations in body temperature that persist for many days. Results from the final experiment in the present study indicated that exposure of embryos to fluctuating temperatures similar to the rectal temperatures of cows experiencing heat stress could decrease embryonic development *in vitro* if the fluctuating temperatures occurred for several days. Thus, a longer more chronic heat shock may reduce embryo survival through the direct action of increased temperature on embryos. It is important to note that the temperatures chosen to perform this experiment were the mean rectal temperatures of cows experiencing heat stress, whereas uterine temperatures are typically about 0.2°C higher than rectal temperatures (Gwazdauskas *et al.*, 1973). Thus, the actual temperatures experienced by embryos may be higher than those used in the present study.

Despite the fact that heat shock can disrupt embryonic development, a striking finding was that embryonic development was resistant to disruption by heat shocks of 39.5–40.5°C for up to 12 h. Thus, it is possible that infertility associated with mild hyperthermia (Dunlap and Vincent, 1971) involves more chronic effects of heat shock exposure on embryos or is the result of other changes in oocyte, reproductive tract or embryonic function, independent of the direct effects of heat shock on embryos.

In conclusion, embryonic development can be disrupted by short-term (9 and 12 h) heat shock applied during fertilization and at the one- and two-cell stages of development, but only when the heat shock is severe ( $\geq 41.0^\circ\text{C}$ ). Furthermore, exposing embryos throughout the 8 days of culture *in vitro* to a pattern of temperatures similar to those experienced by heat-stressed lactating dairy cattle affects their development to the blastocyst stage. In addition, the effects of heat shock are not due to artefacts caused by changes in pH or high oxygen concentrations.

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