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Apoptosis is an adaptive response in bovine preimplantation embryos that facilitates survival after heat shock☆

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Abstract

Heat shock compromises development of preimplantation bovine embryos and the percentage of blastomeres labeled as TUNEL-positive. It was hypothesized that TUNEL labeling represents apoptosis and that apoptosis after heat shock is beneficial for continued embryonic development. To test these hypotheses, experiments were performed with z-DEVD-fmk, an inhibitor of group II caspases, on heat shock responses of embryos $\geqslant$ 16-cell stage at day 4 after insemination. Heat shock of 41 °C for 9 h increased group II caspase activity and the proportion of TUNEL positive cells; z-DEVD-fmk blocked these effects. The reduction in development of embryos exposed to heat shock for 6–9 h was magnified in the presence of z-DEVD-fmk. Results indicate that group II caspases mediate heat-induced apoptosis in bovine embryos and that inhibition of these caspases has a detrimental effect on embryonic resistance to heat shock. Apoptosis can be viewed as an adaptative mechanism to allow embryonic survival and development following stress.

Keywords: Embryo; Apoptosis; z-DEVD-fmk; Heat shock

Apoptosis is a highly conserved form of cell death that plays a major role in animal development and cellular homeostasis by acting as a quality control mechanism to remove cells that are damaged, nonfunctional, misplaced or supranumerary [1]. Cellular changes associated with death caused by apoptosis typically lead to the removal of cells without induction of inflammation that might otherwise compromise integrity of the surrounding cells or tissues [2]. Use of the TUNEL assay to detect oligonucleosomal DNA fragments has revealed the presence of apoptotic cells in preimplantation embryos from several species [3–6]. Spontaneous apoptosis is first observed in bovine embryos at the 8–16-cell stage [5,6] at a time coincident with major activation of an embryonic genome [7].

Exposure of preimplantation embryos to an adverse environment can increase the number of apoptotic cells. Both heat shock and arsenic, for example, increase the proportion of TUNEL-positive blastomeres and caspase activity in bovine embryos [8, Krininger, Stephens and Hansen, unpublished observations]. The induction of apoptosis by heat shock in cattle embryos is developmentally regulated and does not occur until day 4 after insemination [8]. This pattern of development parallels embryonic resistance to heat shock, since embryos acquire increased resistance to heat shock (as determined by ability to continue development following heat shock) as they progress from the 2-cell stage to the morula stage [9]. Such a correlation suggests that apoptosis may be one of the mechanisms preimplantation bovine embryos acquire to allow survival after heat shock. Given that heat shock induces apoptosis in only a fraction of cells (15–29%), and not the majority of blastomeres, apoptosis may ensure that damaged cells are removed from the heat shocked embryo so as to facilitate further development.

For the current studies, it was hypothesized that heat-induced apoptosis in bovine embryos is mediated by

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group II caspases and that the removal of heat-damaged cells by apoptosis is beneficial to embryonic development. Experiments were performed to (1) test whether the group II caspase inhibitor z-DEVD-fmk decreases caspase activity in bovine embryos exposed to heat shock of 41 °C; (2) test whether z-DEVD-fmk decreases TUNEL-positive cells in bovine embryos exposed to heat shock of 41 °C; and (3) evaluate whether blocking apoptosis with z-DEVD-fmk decreases development to the blastocyst stage in bovine embryos exposed to heat shock of 41 °C.

Materials and methods

Materials

Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare Hepes-Tyrode's Albumin Lactate Pyruvate (TALP), IVF-TALP, and Sp-TALP [10]. Potassium Simplex Optimized Medium (KSOM) was obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding an additional 2 mg/ml EFAF-BSA, 2.5 μg/ml gentamicin, and essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) purchased from Sigma. Oocyte collection medium was Tissue Culture Medium 199 (TCM-199) with Hanks' salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) steer serum, 22 μg/ml sodium pyruvate, 20 μg/ml FSH, 2 μg/ml estradiol 17-β, 50 μg/ml gentamicin, and an additional 1 mM glutamine. Other materials for in vitro fertilization were purchased as described by Paula-Lopes and Hansen [8].

In Situ Cell Death Detection Kit (fluorescein) and propidium iodide were from Roche Diagnostics (Indianapolis, IN) and Sigma, respectively. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY). Prolong Antifade Kit was obtained from Molecular Probes (Eugene, Oregon), RQ1 RNA-free DNase was from Promega (Madison, WI), RNase A was from Qiagen (Valencia, CA), PhiPhiLux-G_1D_2 was from OncoImmunin (Gaithersburg, MD), and z-DEVD-fmk (FMK004) was from R & D Systems (Minneapolis, MN). Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma.

In vitro production of embryos

Procedures were as described earlier [8]. Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2–10 mm follicles on the surface of the ovary into a beaker containing oocyte collection medium. Cumulus-oocyte complexes which had at least one layer of compact cumulus cells were washed two times and matured in groups of 10 in 50 μl drops of oocyte maturation medium overlaid with mineral oil for 22 h at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. For fertilization, groups of 30 COCs were transferred to four well plates containing 600 μl IVF-TALP per well. Oocytes were fertilized with 25 μl sperm suspension and 25 μl PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% (w/v) NaCl] added to each well. Presumptive zygotes were removed from fertilization wells, denuded of cumulus cells, washed 2–3 times in Hepes-TALP to remove remaining cumulus cells and associated spermatozoa and placed in groups of 25–30 in 50 μl drops of KSOM overlaid with mineral oil at 38.5 °C and 5% CO_2 (v/v) in humidified air. Embryos $\geqslant$ 16-cell stage were harvested from culture at day 4 after insemination and subjected to treatments.

TUNEL and propidium iodide labeling

Embryos fixed in 4% (w/v) paraformaldehyde were subjected to TUNEL analysis using one of two procedures—after embryos were fixed to microscope slides or while embryos were in solution. The procedure for TUNEL analysis on embryos affixed to slides was described previously [8]. For embryos in solution, the following procedure was performed. Embryos were washed once in PBS-PVP and permeabilized in 4-well plates containing 500 μl of 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at room temperature. Positive controls were incubated in 50 μl drop RQ1 RNase-free DNase (50 U/ml) at 37 °C for 1 h. Embryos were washed in PBS-PVP and incubated with 50 μl drop of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyltransferase), as prepared by the manufacturer, for 1 h at 37 °C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyltransferase. Embryos were then incubated in 4-well plates containing 500 μl RNase A (50 μg/ml) for 1 h at room temperature, followed by 500 μl of 0.5 μg/ml propidium iodide for 30 min at room temperature. Embryos were washed 3 times in PBS-PVP to remove excess propidium iodide, placed on microscope slides, and mounted with 16 μl mounting medium containing Antifade as recommended by the manufacturer. TUNEL labeling was observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter. Each embryo was analyzed for total number of nuclei and number of TUNEL-labeled nuclei.

Caspase activity

PhiPhiLux-G_1D_2 is a fluoroprobe that incorporates the group II caspase-recognition sequence DEVD into a bifluorophore-derivitized peptide that mimics the structural loop conformation present in native protease cleavage sites. Group II caspases include caspase 3, caspase 2, and caspase 7. To measure caspase activity, embryos were removed from culture medium and washed 3 times in 50 μl drops of prewarmed Hepes-TALP. Embryos were incubated in 25 μl microdrops of Hepes-TALP containing 5 μM PhiPhiLux- G_1D_2 at 39 °C for 40 min in the dark. Negative controls were incubated in Hepes-TALP only. Following incubation, embryos were washed twice in 50 μl drops of Hepes-TALP, placed on poly-L-lysine coated slides, and mounted with a coverslip. Caspase activity was assessed using a Zeiss Axioplan 2 fluorescence microscope with an X 45 objective. Images were obtained using a Spot camera and software (Diagnostic Instruments). Pictures were taken using a background subtract feature from the spot software and digital images were stored as .tiff files. Fluorescence intensity was quantified using IPLab for MacIntosh version 3.5 (BioVision Technologies, Exton, PA). Each embryo represented a region of interest (ROI). A circular draw was manually performed for each ROI and the pixel intensity per unit area was determined.

Experiments

Effect of z-DEVD-fmk on heat-induced caspase activity in bovine embryos. The experiment was designed as a 2×2 factorial arrangement of treatments to determine whether z-DEVD-fmk blocks heat-induced caspase activity. Embryos $\geqslant$ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of KSOM containing 200 μm z-DEVD-fmk reconstituted in 1% (v/v) DMSO or 1% (v/v) DMSO alone (vehicle) at 38.5 °C for 15 h. After this preincubation period, embryos were either maintained at 38.5 °C or exposed to 41 °C for 9 h. At the end of heat shock, embryos were washed 3 times in 50 μl drops of prewarmed Hepes-TALP and then incubated in 25 μl drop Hepes-TALP containing 5 μM PhiPhiLux-G_1D_2 at 39 °C for 40 min in the dark. Caspase activity was then determined. The experiment was replicated 4 times using 57–77 embryos/treatment.

Effect of z-DEVD-fmk on heat-induced changes in TUNEL-positive blastomeres and embryo cell number. Embryos $\geqslant$ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of KSOM containing 200 μm z-DEVD-fmk reconstituted in 1% (v/v) DMSO or 1% (v/v) DMSO alone (vehicle) at 38.5 °C for 15 h. After preincubation, embryos were either maintained at 38.5 °C for 24 h or exposed to 41 °C for 9 h and 38.5 °C for 15 h. Embryos were then processed for analysis by TUNEL. The experiment was replicated 2 times using 27–37 embryos/treatment.

Effect of z-DEVD-fmk on development of embryos after heat shock. Embryos $\geqslant$ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of KSOM containing 200 μm z-DEVD-fmk reconstituted in 0.5–1% (v/v) DMSO or 0.5–1% (v/v) DMSO alone (vehicle) at 38.5 °C for 15 h. After preincubation, embryos were either maintained at 38.5 °C continuously or exposed to 41 °C for 6–9 h followed by culture at 38.5 °C continuously. The percentage of embryos developing to the blastocyst stage was determined on day 8 after insemination. The experiment was replicated 15 times using 315–324 embryos/group.

Statistical analysis. Data were analyzed by least-squares analysis of variance. Independent variables were temperature, treatment, and replicate. The mathematical model included main effects and all interactions. Two analyses were performed: by the General Linear Model (GLM) procedure of SAS [11], where replicate (i.e., day of IVF procedure) was considered a fixed variable, and by the Mixed Model procedure of SAS, where replicate was considered a random variable. Probability values were similar for both methods and probability values reported herein are derived from the analysis by GLM. Percentage data were analyzed without transformation and after being subjected to the arcsin transformation. Unless stated otherwise, probability values were similar for both analyses; least-squares means reported here are derived from the analysis of untransformed data while probability levels are derived from the analysis of transformed data.

Results

Effect of z-DEVD-fmk on heat-induced caspase activity in bovine embryos

Representative digital images illustrating caspase activity in embryos $\geqslant$ 16-cell stage are shown in Fig. 1. For control embryos, heat shock of 41 °C for 9 h increased caspase activity (Figs. 1A and B). However, heat shock did not increase caspase activity in embryos cultured in z-DEVD-fmk (Figs. 1C and D). Caspase activity was quantified and subjected to analysis of variance (Fig. 2). There was a treatment × temperature interaction ($p < 0.01$) affecting caspase activity. Exposure of control embryos to 41 °C for 9 h increased caspase activity as compared to 38.5 °C. However, culturing embryos in the presence of z-DEVD-fmk reduced caspase activity caused by heat shock of 41 °C for 9 h.

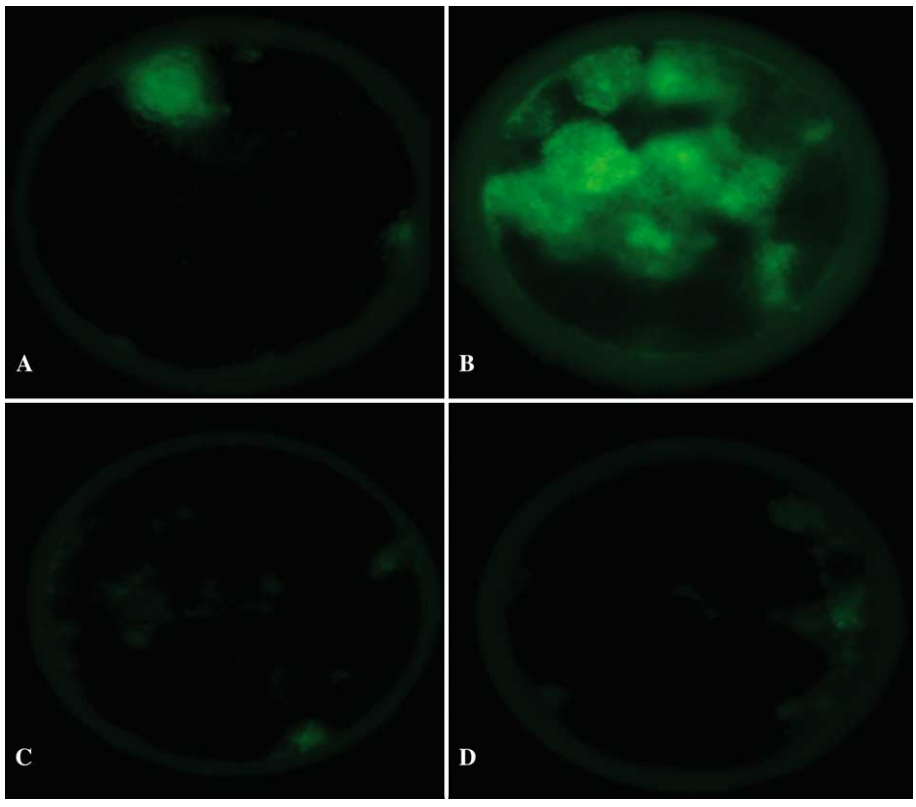

Fig. 1. Representative digital images illustrating group II caspase activity in embryos $\geqslant$ 16-cell stage. Panels A and B are control embryos cultured in medium containing the DMSO vehicle. Panels A and C represent embryos at 38.5 °C and panels B and D represent embryos at 41 °C. Panels C and D are embryos cultured in z-DEVD-fmk. Note that exposure of control embryos to heat shock of 41 °C for 9 h increased caspase activity. This increase did not occur for embryos cultured in z-DEVD-fmk.

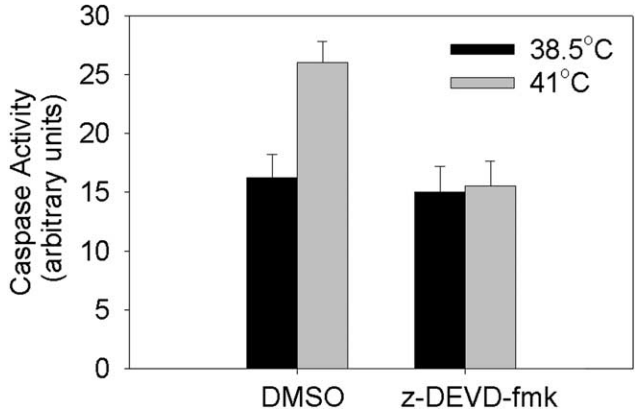

Fig. 2. Effect of z-DEVD-fmk on heat-induced caspase activity in bovine embryos. Results are least-squares means ± SEM of 4 replicates using 57–77 embryos/treatment. Exposure of control embryos (DMSO) to 41 °C for 9 h increased caspase activity as compared to 38.5 °C. Culture of embryos in the presence of z-DEVD-fmk, however, blocked the increase in caspase activity caused by heat shock (treatment × temperature, $p < 0.01$).

Effect of z-DEVD-fmk on heat-induced changes in TUNEL-positive blastomeres and embryo cell number

Heat shock of 41 °C for 9 h increased the percentage of TUNEL-positive cells in control embryos. However, there was no increase in the percentage of TUNEL-positive cells after heat shock in embryos cultured in z-DEVD-fmk (treatment × temperature, $p < 0.05$ for untransformed data; Fig. 3A). Moreover, heat shock of 41 °C for 9 h reduced ($p < 0.001$) total cell number in control (vehicle) or z-DEVD-fmk embryos. There was no effect of z-DEVD-fmk on total cell number (Fig. 3B).

Effect of z-DEVD-fmk on development of embryos after heat shock

Heat shock of 41 °C for 9 h reduced the percentage of embryos developing to the blastocyst stage for both control (vehicle) and z-DEVD-fmk embryos. However, the decrease in development was greater for z-DEVD-fmk embryos (treatment × temperature, $p < 0.05$; Fig. 4).

Discussion

During the course of preimplantation development, disruption in the microenvironment of the embryo can lead to inhibition of development and embryonic death. For at least two embryonic stresses, heat shock [8] and arsenic [Krininger et al., unpublished observations], affected embryos undergo an increase in the proportion of cells that are labeled with the TUNEL reaction. While the presence of TUNEL-positive cells is usually indicative of apoptosis, necrosis and artifacts caused by sample fixation can also lead to TUNEL labeling [4]. The present study provides strong evidence that the increase in TUNEL-positive cells in bovine preimplantation embryos caused by heat shock is in fact indicative of apoptosis. This is so because heat shock caused an increase in group II caspase activity and because preincubation of embryos in z-DEVD-fmk blocked heat-induced caspase activity and prevented the increase in TUNEL-positive blastomeres observed after heat shock. Thus, activation of the apoptotic machinery is one consequence of embryonic exposure to heat shock. Moreover, results indicate that heat shock-induced apoptosis represents an adaptive, thermotolerizing response in preimplantation bovine embryos because inhibition of the capacity for apoptosis with z-DEVD-fmk compromised ability of embryos to continue development after heat shock.

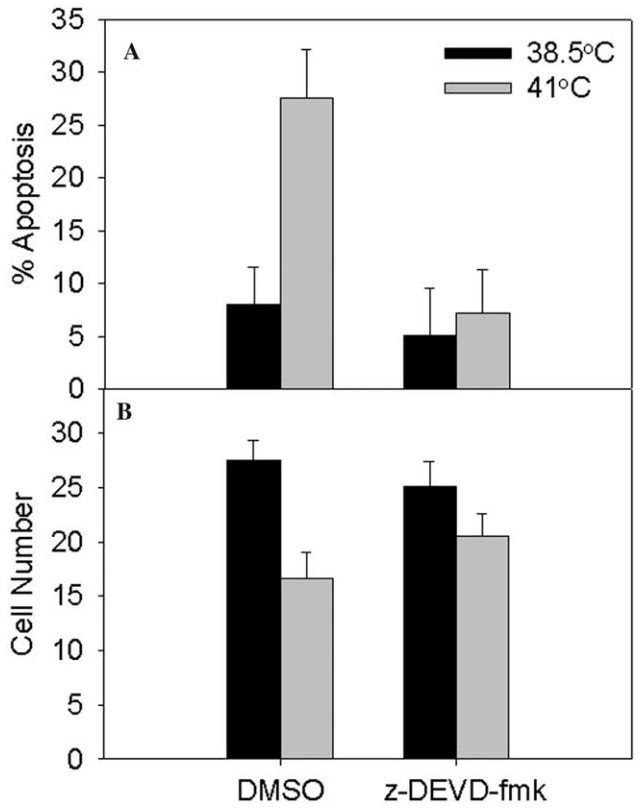

Fig. 3. Effect of z-DEVD-fmk on heat-induced changes in TUNEL-positive blastomeres and embryo cell number. Results are least-squares means ± SEM of 2 replicates using 27–37 embryos/treatment. For control embryos (DMSO), heat shock of 41 °C for 9 h increased the percentage of cells positive for TUNEL (% apoptosis). For embryos cultured in z-DEVD-fmk, however, there was no increase in the percentage of TUNEL-positive cells after heat shock (treatment × temperature, $p < 0.05$ for untransformed data) (A). Heat shock of 41 °C for 9 h reduced ($p < 0.001$) embryo total cell number but there was no treatment × temperature interaction (B).

This is the first report to indicate a beneficial role for apoptosis in allowing the preimplantation embryo to

overcome stress. While others have reported apoptosis in embryos [3–6], it is generally assumed that apoptosis represents an embryo at risk. This may be true and certain treatments which increase development also decrease apoptosis [3,12,13]. However, it is simplistic to view apoptosis as solely a marker of embryo stress without realizing that the process itself can contribute to the survival of the embryo in response to stress. Cell loss itself does not inexorably lead to embryonic death and numerous experiments have demonstrated the production of offspring resulting from that were hemi-sectioned to produce identical twins (see [14] for example). The mechanism by which inhibition of apoptosis reduces development in heat-shocked embryos remains unknown. One possibility is that cells that are not removed by apoptosis subsequently undergo death more typical of necrosis [15,16]. Also, apoptosis may prevent a damaged cell from contributing abnormal daughter cells to the embryo.

Given that apoptosis represents a mechanism to limit effects of heat shock on embryos, it is likely that the capacity of an embryo for apoptotic responses determines, at least in part, its resistance to heat shock. Heat shock cannot induce apoptosis in embryos less than the 8-cell stage [8] and embryos before this time experience a greater inhibition of development following heat shock than do embryos after the 8-cell stage [9]. Thus, one reason that early bovine embryos are more sensitive to heat shock than preimplantation embryos at later stages of development may be the inability of early embryos to undergo heat-induced apoptosis.

One of the features of heat-induced apoptosis in preimplantation bovine embryos is that only a limited proportion of cells undergo apoptosis following heat shock. In the present study, for example, 27.6% of blastomeres were positive for TUNEL labeling after exposure to 41 °C for 9 h. It is possible that more extensive apoptosis, such as might occur following more severe stress, would compromise embryonic development. Thus, the degree of the apoptotic response may be an important determinant in embryonic fate. While limited apoptosis is a mechanism for embryonic homeokinesis to remove damaged cells without disturbing the embryonic potential to further develop, more extensive apoptosis may leave the embryo with too few cells to sustain development. Given the importance of apoptosis in determining embryonic survival in response to stress, manipulation of apoptosis responses in embryos may lead to new methods for improving systems for in vitro production of embryos or for reducing embryonic mortality in female animals.

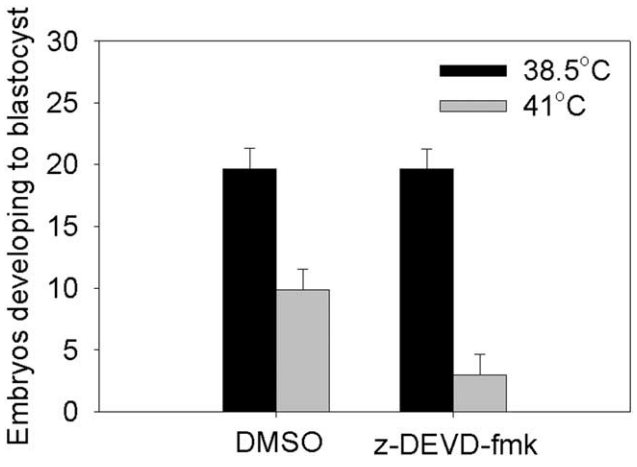

Fig. 4. Effect of z-DEVD-fmk on development of embryos after heat shock. Results are least-squares means ± SEM of 15 replicates using 315–324 embryos/group. Heat shock of 41 °C for 9 h reduced development of embryos to the blastocyst stage. Moreover, the deleterious effect of heat shock on development was exacerbated by culturing embryos in z-DEVD-fmk (treatment × temperature, $p < 0.05$).

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References

[1] M.D. Jacobson, M. Weil, M.C. Raff, Programmed cell death in animal development, Cell 88 (1997) 347–354.
[2] P. Meier, A. Finch, G. Evan, Apoptosis in development, Nature 407 (2000) 796–801.
[3] D.R. Brison, R.M. Schultz, Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor α, Biol. Reprod. 56 (1997) 1088–1096.
[4] K. Hardy, Apoptosis in the human embryo, Rev. Reprod. 4 (1999) 125–134.
[5] A.T. Byrne, J. Southgate, D.R. Brison, H.J. Leese, Analysis of apoptosis in the preimplantation bovine embryo using TUNEL, J. Reprod. Fertil. 117 (1999) 97–105.
[6] C. Matwee, D.H. Betts, W.A. King, Apoptosis in the early bovine embryo, Zygote 8 (2000) 57–68.
[7] E. Memili, N.L. First, Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species, Zygote 8 (2000) 87–96.
[8] F.F. Paula-Lopes, P.J. Hansen, Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon, Biol. Reprod. 66 (2002) 1169–1177.
[9] J.L. Edwards, P.J. Hansen, Differential responses of bovine oocytes and preimplantation embryos to heat shock, Mol. Reprod. Dev. 46 (1997) 138–145.

[10] J.J. Parrish, J.L. Susko-Parrish, E.S. Cristser, W.H. Eyestone, N.L. First, Bovine in vitro fertilization with frozen-thawed semen, Theriogenology 25 (1986) 591–600.
[11] SAS, SAS User's Guide, Cary, NC, Statistical Analysis System Institute, Inc., 1989.
[12] S. Kolle, M. Stojkovic, G. Boie, E. Wolk, F. Sinowatz, Growth hormone inhibits apoptosis in in viro produced bovine embryos, Mol. Reprod. Dev. 61 (2002) 180–186.
[13] A.V. Makarevich, M. Markkula, Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during in vitro maturation and culture, Biol. Reprod. 66 (2002) 386–392.
[14] S. Martinod, R.R. Maurer, B. Siegenthaler, G. Gerber, P.J. Hansen, The effects of recombinant bovine interferon-alpha on fertility in ewes, Theriogenology 36 (1991) 231–240.
[15] J. Xiang, D.T. Chao, S.J. Korsmeyer, BAX-induced cell death may not require interleukin-1β-converting enzyme-like proteases, Proc. Natl. Acad. Sci. 93 (1996) 14559–14563.
[16] M. Woo, R. Hakem, M.S. Soenas, G.S. Duncan, A. Shahinian, D. Kagi, A. Hakem, M. McCurrach, W. Khoo, S.A. Kaufman, G. Senaldi, T. Howard, S.W. Lowe, T.W. Mak, Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes, Genes Dev. 12 (1998) 806–819.