

# Involvement of Apoptosis in Disruption of Developmental Competence of Bovine Oocytes by Heat Shock During Maturation<sup>1</sup>

Z. Roth and P.J. Hansen<sup>2</sup>

Department of Animal Sciences, University of Florida, Gainesville, Florida 32611-0910

## ABSTRACT

Various pathological stimuli such as radiation, environmental toxicants, oxidative stress, and heat shock can initiate apoptosis in mammalian oocytes. Experiments were performed to examine whether apoptosis mediated by group II caspases is the cause for disruption of oocyte function by heat shock applied during maturation in cattle. Bovine cumulus-oocyte complexes (COCs) were cultured at 38.5, 40, or 41°C for the first 12 h of maturation. Incubation during the last 10 h of maturation, fertilization, and embryonic development were at 38.5°C and 5% (v/v) CO<sub>2</sub> for all treatments. In the first experiment, exposure of COCs to thermal stress during the first 12 h of maturation reduced cleavage rate and the number of oocytes developing to the blastocyst stage. In the second experiment, a higher percentage of TUNEL-positive oocytes was noted at the end of maturation for oocytes matured at 40 and 41°C than for those at 38.5°C. In addition, the distribution of oocytes classified as having high (>25 intensity units), medium (15–25 intensity units), and low (<15 intensity units) caspase activity was affected by treatment, with a greater proportion of heat-shocked oocytes having medium or high activity. In the third experiment, COCs were placed in maturation medium with vehicle (0.5% [v/v] DMSO) or 200 nM z-DEVD-fmk, an inhibitor of group II caspases. The COCs were matured at 38.5 or 41°C, fertilized and cultured for 8 days. The inhibitor blocked the effect of heat shock on cleavage rate and the percentage of oocytes and cleaved embryos developing to the blastocyst stage. In conclusion, heat shock during oocyte maturation can promote an apoptotic response mediated by group II caspases, which, in turn, leads to disruption of the oocyte's capacity to support early embryonic development following fertilization.

*apoptosis, environment, oocyte development*

## INTRODUCTION

The nuclear and cytoplasmic status of the oocyte at the time of fertilization is a major determining factor of the developmental program of the resultant embryo [1]. Perturbations in the physiology of the oocyte during the long period of follicular development in mammals (about 84 days from the primary follicle stage to ovulation in cattle [2, 3]), can potentially lead to an oocyte with reduced competence for fertilization and support of subsequent development. This seems to be the case for heat stress in dairy

cattle, which can have catastrophic effects on fertility [4–6] and embryonic survival [7, 8]. While heat stress causes infertility through a multiplicity of effects, including actions on hormonal secretion [9] and on the embryo itself [10, 11], damage to the oocyte is also involved. Oocytes harvested from cows during the summer can have reduced ability to develop to the blastocyst stage after invitro fertilization [12–14]. Ovarian recovery from summer thermal stress requires a period of 2–3 estrous cycles before competent oocytes are present [15], suggesting that the ovarian pool of oocytes can be damaged by heat stress during early stages of folliculogenesis. The process of oocyte maturation is also susceptible to disruption by heat stress. Exposure of heifers to heat stress between the onset of estrus and insemination increased the proportion of abnormal and retarded embryos [7]. Similarly, exposure of cultured cumulus-oocyte complexes (COCs) to elevated temperature during maturation decreased cleavage rate and the proportion of oocytes that became blastocysts [16].

The mechanism by which heat stress leads to a disruption in developmental competence of the oocyte remains unclear. One of the processes that may be involved in disruption of oocyte competence is apoptosis. In mammals, programmed cell death has been identified as the mechanism underlying the depletion of oocytes from the ovarian pool throughout life [17, 18]. Various pathological stimuli, such as radiation, chemotherapy, and environmental toxicants, can initiate apoptosis in the oocyte [19–21]. There is evidence that sphingomyelin metabolism regulates death of germ cells and ceramide, a second messenger produced from sphingomyelin, mediates apoptosis in oocytes exposed to chemotherapy drugs [19].

Heat shock can induce apoptosis in many cell types, including preimplantation embryos [22–24]. Here, it was hypothesized that apoptosis involving actions of group II caspases is involved in disruption of oocyte function caused by heat shock during maturation. Group II caspases are those caspases that have substrate specificity for the amino acid motif DEXD [25, 26] and include the execution caspases 3 and 7 that are responsible for destruction of structural and regulatory proteins that lead to DNA damage and cell demise [27]. The other group II caspase is caspase 2, which has been implicated as an upstream initiator of mitochondrial permeability [28]. While the group III caspase, caspase 6, is also involved in execution [27], ablation of this gene in mice is without apparent effect [29]. Inhibition of Group II caspases with z-DEVD-fmk blocked induction of apoptosis by heat shock in bovine embryos [24]. To test our hypothesis, experiments were performed to examine whether heat shock induces apoptosis in maturing oocytes, whether apoptosis is mediated by group II caspases, and whether inhibition of apoptosis in the oocyte prevented the loss of developmental potential caused by heat shock.

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<sup>2</sup>Correspondence: P.O. Box 110910, Gainesville, FL 32611-0910. FAX: 352 392 5595; e-mail: Hansen@animal.ufl.edu

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## MATERIALS AND METHODS

### Materials

Oocyte collection medium (OCM) was Tissue Culture Medium 199 (TCM-199) with Hanks salts without phenol red (Hyclone, Logan, UT) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Roger, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (Gibco/Invitrogen, Grand Island, NY) with Earle salts supplemented with 10% (v/v) steer serum, 22 µg/ml sodium pyruvate, 20 µg/ml FSH (Follotropin-V; Vetrepharm Canada, London, ON), 2 µg/ml estradiol 17-β, 50 µg/ml gentamicin and 1 mM glutamine. HEPES-tyrode lactate (HEPES-TL), in vitro fertilization Tyrode lactate (IVF-TL), and sperm-TL (SPTL) were obtained from Cell and Molecular Technologies (Lavallette, NJ) and used to prepare HEPES-tyrode albumin lactate pyruvate (TALP), IVF-TALP, and SPTALP [30]. Bovine serum albumin (BSA) fraction V and essentially fatty-acid BSA were purchased from Sigma (St. Louis, MO). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Breeders Service (Wellborn, FL). Embryo culture medium was potassium simplex optimized medium (KSOM) from Cell and Molecular Technologies (Lavallette, NJ) modified for bovine embryos (KSOM-BE2) as described elsewhere [31].

The in situ cell death detection kit (fluorescein) was obtained from Roche Diagnostics (Indianapolis, IN). Hoechst 33342 dye (Sigma) was used for staining DNA of oocytes and embryonic cells. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY). The Prolong Antifade Kit was obtained from Molecular Probes (Eugene, OR), and RQ1 RNA-free DNase was from Promega (Madison, WI). The PhiPhiLux-G<sub>1</sub>D<sub>2</sub> assay was obtained from OncoImmunin Inc. (Gaithersburg, MD) and z-DEVD-fmk was from R & D Systems (Minneapolis, MN).

### In Vitro Production of Embryos

Embryos were produced using procedures described earlier [22, 31]. Briefly, ovaries were obtained from a local slaughterhouse and transferred in 0.9% (w/v) NaCl at room temperature. Ovaries were sliced and COCs were collected into a beaker containing OCM. COCs were washed and matured in groups of 10 in 50-µl drops of OMM overlaid with mineral oil for 22 h at 38.5°C in atmosphere of 5% (v/v) CO<sub>2</sub> in humidified air. Groups of 30 COCs were then transferred to four-well plates containing 600 µl IVF-TALP per well and fertilized with 25 µl (~1 × 10<sup>6</sup>) Percoll-purified spermatozoa supplemented with 25 µl PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl). After 18 h, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortexing COCs with 100 µl hyaluronidase (1000 U/ml in 1 ml HEPES TALP medium for 5 min), washed 2–3 times in HEPES-TALP, and placed in groups of 25–30 in 50-µl drops of KSOM-BE2 overlaid with mineral oil at 38.5°C and 5% (v/v) CO<sub>2</sub> in humidified air. The numbers of cleaved oocytes and blastocysts were examined on Days 3 and 8 postinsemination, respectively. Matured oocytes were harvested after 22 h of maturation and developing blastocysts on Day 8 after fertilization for further analysis.

### TUNEL Labeling

DNA fragmentation was determined by means of the TUNEL procedure, whereby free 3'-OH ends of DNA are labeled with fluorescein isothiocyanate-conjugated dUTP by means of the enzyme terminal deoxynucleotidyltransferase. Oocytes and embryos were removed from culture medium, washed three times in 100-µl drops of 10 mM KPO<sub>4</sub>, pH 7.4, containing 0.9% (w/v) NaCl (PBS) and with 1 mg/ml PVP (PBS-PVP), fixed in 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature, and stored in PBS-PVP at 4°C for up to 2–3 wk before assay. The TUNEL assay was initiated by permeabilizing oocytes or embryos in 100-µl drops of 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate in PBS for 30 min at room temperature. Samples were then incubated in 50-µl drops of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37°C in the dark. Oocytes and embryos were then washed in PBS-PVP, transferred to 50-µl drops of 1 µg/ml Hoechst 33342 in PBS-PVP for 30 min at room temperature, washed three times in PBS-PVP, placed on 10% (w/v) polylysine-coated slides, and coverslips mounted using 5 µl mounting medium containing Antifade (Molecular Probes). Each TUNEL procedure contained oocytes or embryos treated with RQ1 RNase-free DNase (50 U/ml) at 37°C for 1 h as a positive control and oocytes or embryos

incubated in the absence of the terminal deoxynucleotidyl transferase as a negative control. TUNEL labeling was observed using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Göttingen, Germany). Images were acquired using AxioVision software and an AxioCam MRm digital camera (Zeiss). Each embryo was analyzed for total number of nuclei and the number of TUNEL-labeled nuclei.

### Group II Caspase Activity

Denuded oocytes and embryos were washed three times in 50-µl drops of HEPES-TALP (prewarmed at 38.5°C) and incubated in 25-µl microdrops of HEPES-TALP containing 5 µM PhiPhiLux-G<sub>1</sub>D<sub>2</sub> at 38.5°C for 40 min in the dark. The negative control was incubated in HEPES-TALP only. Following incubation, oocytes and embryos were washed twice in 50-µl drops of HEPES-TALP and placed on two-well slides (catalog number 12-560B; Fisher) containing 100 µl of prewarmed HEPES-TALP. Caspase activity was determined using a Zeiss Axioplan microscope. Images were acquired using AxioVision software and an AxioCam MRm digital camera. Using the computer mouse, a circular draw function was manually performed around the internal side of the zona-pellucida and intensity per unit area was determined.

### Experiments

**Heat shock during maturation on oocyte competence.** Cumulus-oocyte complexes were collected and placed in maturation drops as described earlier and then cultured for 22 h at either 38.5°C for 22 h or at 40 or 41°C for 12 h followed by 38.5°C for 10 h. The gaseous atmosphere was CO<sub>2</sub> in humidified air. The percent CO<sub>2</sub> was adjusted to ensure that the concentration of dissolved CO<sub>2</sub> was similar between treatments and to maintain pH at ~7.4 (5%, 6%, and 7% for 38.5, 40, and 41°C, respectively). Fertilization and embryo culture were performed at 38.5°C for all treatments. Cleavage rate was recorded on Day 3 after insemination and the percentage of oocytes and cleaved embryos becoming blastocysts was recorded on Day 8 after insemination. The experiment was replicated 10 times using 434–471 oocytes/treatment. In addition, a subset of blastocysts produced from oocytes at 38.5 or 41°C was subjected to TUNEL analysis (20–27 embryos/treatment in three replicates) and group II caspase activity assay (26–28 embryos/treatment in three replicates).

**Heat-induced apoptosis in oocytes.** To determine effects of heat shock on apoptosis, COCs were matured at either 38.5°C for 22 h or at 40 or 41°C for 12 h followed by 38.5°C for 10 h. At the end of maturation, COCs were denuded of cumulus cells with hyaluronidase and denuded oocytes were washed 2–3 times in HEPES-TALP to remove remaining cumulus cells. Oocytes were fixed in 4% (w/v) paraformaldehyde and saved at 4°C in PBS-PVP until analysis by TUNEL. A total of 65–90 oocytes/treatment was analyzed in eight replicates.

**Activity of group II caspase in oocytes.** Cumulus-oocyte complexes were matured at either 38.5°C for 22 h or at 40 or 41°C for 12 h followed by 38.5°C for 10 h. At the end of maturation, COCs were denuded of cumulus cells with hyaluronidase and washed 2–3 times in HEPES-TALP to remove remaining cumulus cells. Group II caspase activity was then performed immediately as described before. The experiment was replicated three times using 105–138 oocytes/treatment.

To determine the association between the morphology of matured oocytes and intensity of caspase activity, a subset of oocytes (36–42 oocytes per treatment in two replicates) was also evaluated for morphology using bright-field microscopy after the caspase assay. Another subset of oocytes subjected to the caspase assay was also evaluated by the TUNEL procedure to determine whether the degree of caspase activity in matured oocytes corresponds with TUNEL status of the pronuclei. In this case, oocytes were washed three times in PBS-PVP after measurement of caspase activity, fixed in 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature, and stored in PBS-PVP at 4°C until the TUNEL assay was performed as described above. Thus, the same set of oocytes was analyzed for both caspase and TUNEL activity although identity of individual oocytes was not maintained. The dual analysis was performed once using 18–42 oocytes/treatment.

**Effect of z-DEVD-fmk on competence of heat-shocked oocytes.** The experiment was designed as a 2 × 2 factorial arrangement of treatments to determine whether z-DEVD-fmk, an inhibitor of group II caspases, disrupts the deleterious effect of heat shock on oocyte competence. Oocytes were matured in the presence of 200 nM z-DEVD-fmk reconstituted in 0.5% (v/v) dimethyl sulfoxide (DMSO) in OMM or 0.5% (v/v) DMSO in OMM (vehicle). Maturation was performed with z-DEVD-fmk or vehicle at either 38.5°C for 22 h or at 41°C for 12 h followed by 38.5°C for 10 h. Cleavage rate and development to blastocyst was recorded on Days

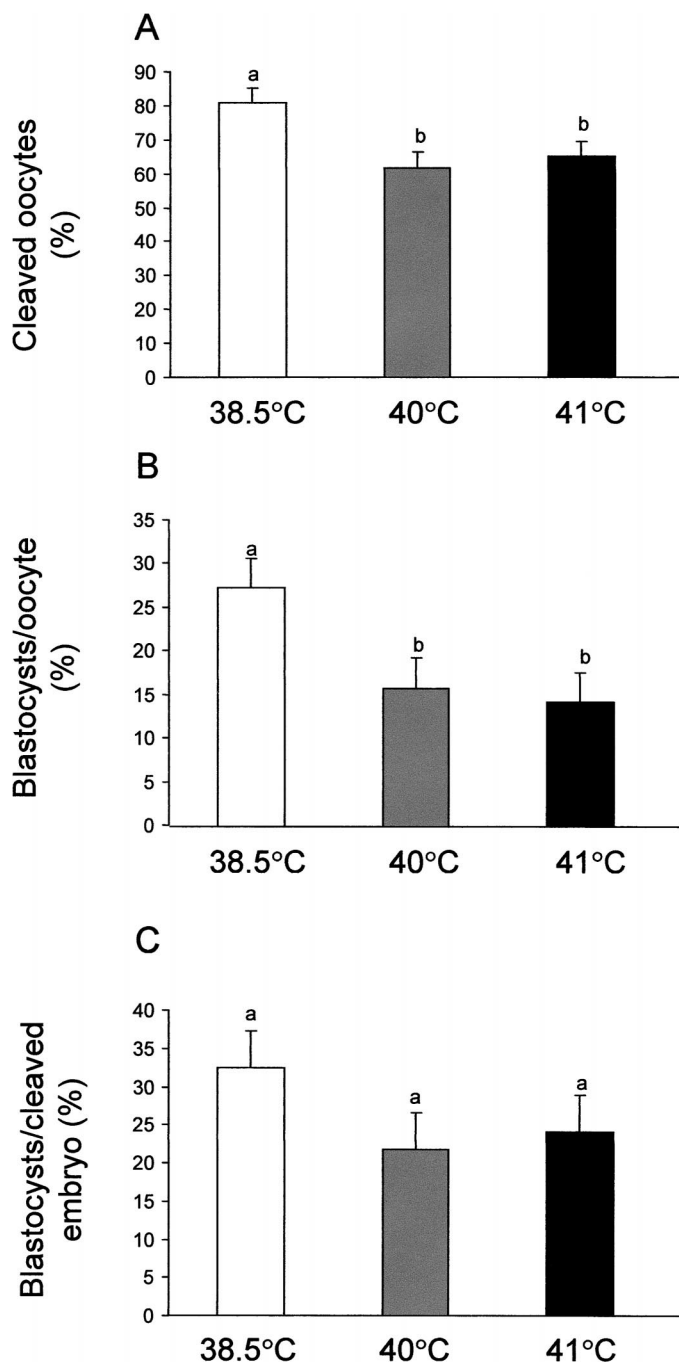


FIG. 1. Effect of incubation temperature during the first 12 h of maturation on cleavage of oocytes at Day 3 after insemination (A) and the percentage of oocytes (B) and cleaved embryos (C) that became blastocysts at Day 8 after insemination. Data represent least-squares means  $\pm$  SEM. Superscripts above each bar represent means that differ ( $P < 0.05$ ).

3 and 8 after insemination, respectively. The experiment was replicated six times using 168–183 oocytes/treatment.

#### Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS System for Windows, Release 8.20; Cary, NC). Percentage data (calculated for each replicate) were transformed using the arcsine transformation before analysis. Probability values reported are based on the analysis of transformed data while least-squares means are from the untransformed analyses. The mathematical model included main effects and all interactions. Replicate was considered a random effect and all other main effects were considered fixed.

A means separation procedure of SAS called pdiff was performed when appropriate to determine differences between levels of individual treatments.

## RESULTS

### Disruption of Oocyte Competence by Heat Shock

Heat shock at both 40 and 41°C reduced ( $P < 0.05$ ) the percentage of oocytes that cleaved by Day 3 after insemination and the percentage of oocytes that developed to the blastocyst stage on Day 8 after insemination (Fig. 1, A and B). While not significant, the percentage of cleaved oocytes that became blastocysts was also reduced by maturation at elevated temperature (Fig. 1C).

Characteristics of blastocysts derived from heat-shocked oocytes were evaluated by determining total cell number and percent TUNEL-positive cells in one subset of blastocysts (see Fig. 2, A and B, for representative images) and by determining group II caspase activity in another subset (see Fig. 2, E–G, for representative images). The total cell number of blastocysts formed from oocytes matured at 41°C was higher than for blastocysts formed from oocytes matured at 38.5°C ( $P < 0.03$ ) but there was no significant difference in the percentage of apoptotic (TUNEL-positive) cells (Table 1). For caspase activity, blastocysts were categorized as having low ( $<15$  intensity units), medium (15–25 intensity units), or high ( $>25$  intensity units) caspase activity. The proportion of embryos in each category did not differ between blastocysts from oocytes matured at 38.5°C vs. those matured at 41°C (Table 2). The least-squares means  $\pm$  SEM for caspase activity were  $21 \pm 1.6$  vs.  $19 \pm 1.6$  intensity units for blastocysts from oocytes at 38.5 vs. 41°C, respectively.

### Induction of Apoptotic Processes in Oocytes by Heat Shock During Maturation

Representative images illustrating analysis of oocytes for TUNEL labeling are shown in Figure 2, C and D, while representative images of oocyte caspase activity are shown in Figure 2, H–J. The percentage of oocytes that were TUNEL-positive at the end of maturation was higher ( $P < 0.05$ ) for oocytes exposed to 40 or 41°C for the first 12 h of maturation than for oocytes matured at 38.5°C (Fig. 3). Similarly, incubation temperature affected group II caspase activity (Table 3). The percentage of oocytes expressing low amounts of caspase activity ( $<15$  intensity units) was higher for oocytes at 38.5°C than for those at 40°C ( $P < 0.06$ ) and 41°C ( $P < 0.05$ ). Conversely, the percentage of oocytes with medium caspase activity (15–25 intensity units) was lower ( $P < 0.05$ ) for oocytes at 38.5°C than for oocytes matured at 40 and 41°C. The same was true for high caspase activity, with a lower ( $P < 0.05$ ) percentage of oocytes matured at 38.5°C having high caspase activity ( $>25$  intensity units) as compared with oocytes at either 40 or 41°C.

An analysis was performed to determine whether oocytes with high caspase activity had a different morphology than oocytes with low caspase activity (Fig. 4). Oocytes with low expression of caspase activity were characterized by condensation and shrinkage of the cytoplasm while oocytes with medium and high expression of caspase activity had expanded cytoplasm with a small perivitelline space. To determine whether the expression of high caspase activity is related to the likelihood of a TUNEL-positive pronucleus, groups of oocytes matured at 38.5, 40, and 41°C were analyzed consecutively for caspase activity and TU-



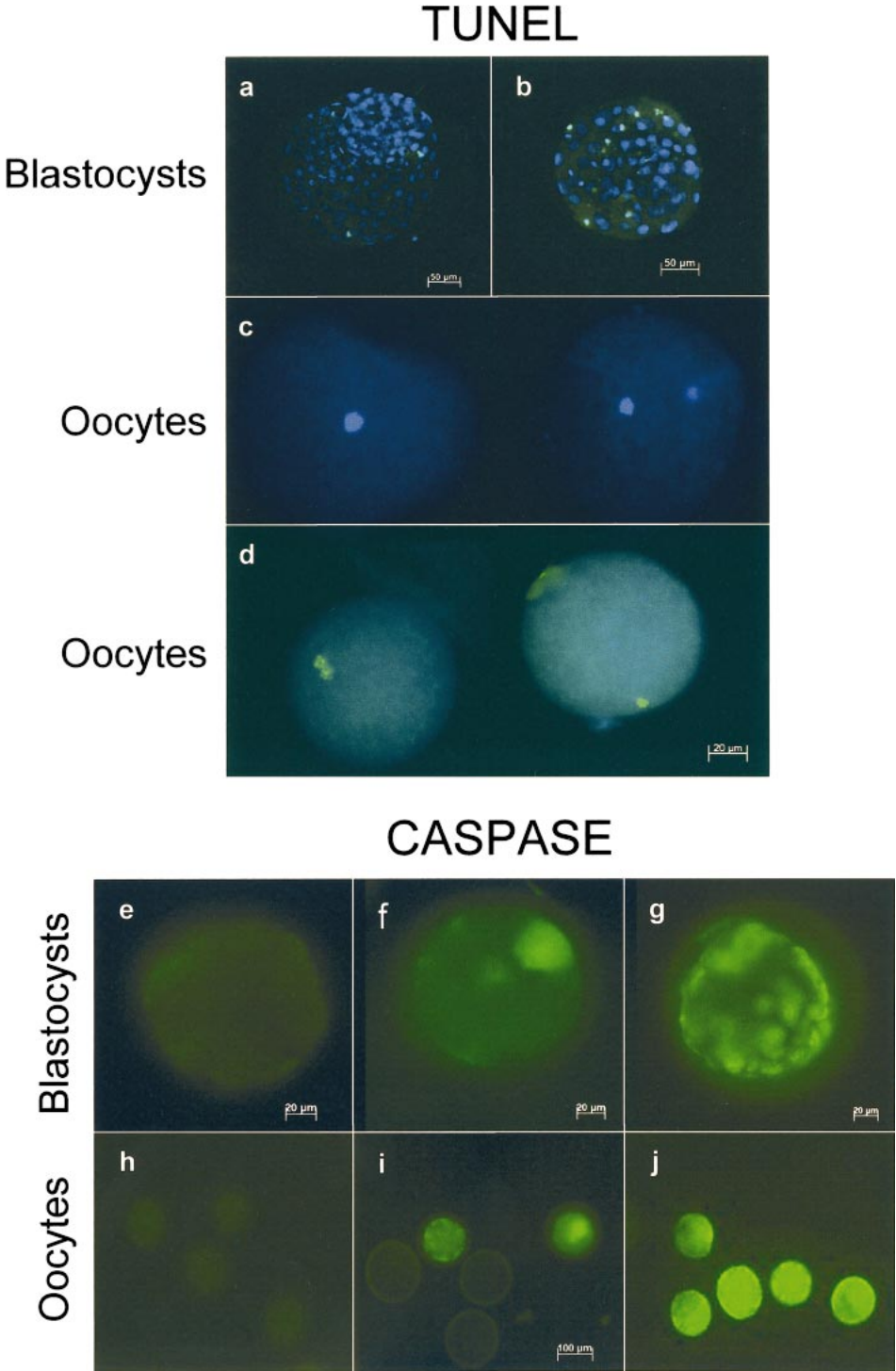


FIG. 2. Representative images of TUNEL labeling (a–d) and caspase activity (e–j). Shown in the top panels are representative blastocysts with a high (a) and low (b) frequency of apoptosis and matured oocytes with TUNEL-negative pronuclei (c) and TUNEL-positive pronuclei (d). Note that green nuclei and pronuclei are TUNEL positive while blue represents staining of DNA with Hoechst 33258. Also shown are blastocysts exhibiting low (e), medium (f), and high (g) caspase activity and matured oocytes exhibiting low (h), medium (i), and high (j) expression of group II caspase activity. (a and b) Bar = 50  $\mu$ m. (c–g) Bar = 20  $\mu$ m. (h–j) Bar = 100  $\mu$ m.

TABLE 1. Total cell number and percentage of blastomeres that were TUNEL positive in Day-8 blastocysts developed from oocytes matured at 38.5 or 41°C.<sup>a</sup>

Temperature (°C)	Number of blastocysts	Total cell number <sup>b</sup>	TUNEL-positive blastomeres (%)
38.5	20	81.9 $\pm$ 5.2	12.2 $\pm$ 3.2
41.0	28	98.6 $\pm$ 5.2	20.9 $\pm$ 3.2

<sup>a</sup> Data represent least-squares means  $\pm$  SEM.

<sup>b</sup> Temperature effect,  $P < 0.05$ .

TABLE 2. Distribution of Day-8 blastocysts developed from control (38.5°C) and heat shocked (40 or 41°C) oocytes with respect to low (<15 intensity units), medium (15–25 intensity units), and high (>25 intensity units) expression of group II caspase activity.<sup>a</sup>

Temperature (°C)	Number of blastocysts	Caspase activity <sup>b</sup>		
		Low	Medium	High
38.5	26	34.6 $\pm$ 5.5	40.3 $\pm$ 3.2	25.3 $\pm$ 3.2
41.0	28	31.6 $\pm$ 5.5	57.0 $\pm$ 3.2	11.3 $\pm$ 3.2

<sup>a</sup> Data represent least-squares means  $\pm$  SEM.

<sup>b</sup> Percentage of blastocysts in category.

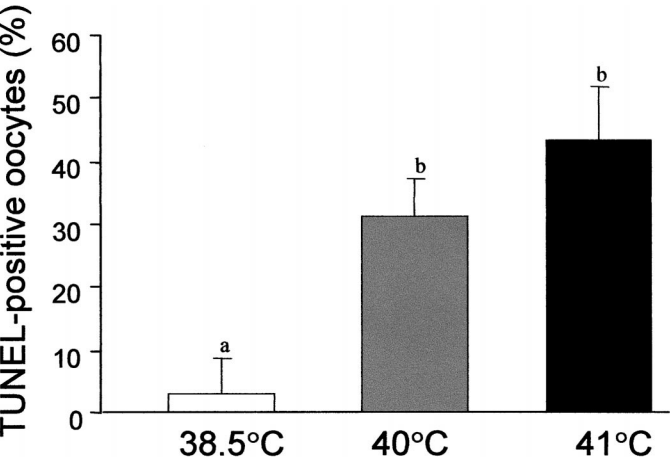


FIG. 3. Effect of heat shock during the first 12 h of maturation on the percentage of oocytes that were TUNEL positive. Data represent least-squares means  $\pm$  SEM. Superscripts above each bar represent means that differ significantly.

NEL labeling. The percent of matured oocytes with high caspase activity ( $>25$  intensity units) was 0% for oocytes matured at 38.5°C, 7% for oocytes matured at 40°C, and 21% for oocytes matured at 41°C. Similarly, the percent of oocytes that were TUNEL-positive was 1%, 5%, and 18% for oocytes matured at 38.5, 40, and 41°C, respectively. Thus, the percent of oocytes with high caspase activity paralleled the percent of oocytes that were TUNEL-positive.

Blocking the Disruption of Oocyte Competence for Cleavage and Subsequent Development with z-DEVD-fmk

In the absence of z-DEVD-fmk, maturation for the first 12 h at 41°C reduced the proportion of oocytes that cleaved

TABLE 3. Effect of incubation temperature during the first 12 h of maturation on distribution of oocytes into low ( $<15$  intensity), medium (15–25 intensity), and high ( $>25$  intensity) categories based on group II caspase activity.<sup>a</sup>

Temperature (°C)	n	Low	Medium	High
38.5	105	78.1 $\pm$ 6.3 <sup>b</sup>	20.3 $\pm$ 4.2 <sup>d</sup>	1.6 $\pm$ 2.1 <sup>e</sup>
40.0	110	44.8 $\pm$ 6.3 <sup>bc</sup>	48.3 $\pm$ 4.2 <sup>c</sup>	6.9 $\pm$ 2.1 <sup>e</sup>
41.0	138	19.4 $\pm$ 6.3 <sup>d</sup>	56.8 $\pm$ 4.2 <sup>c</sup>	23.8 $\pm$ 2.1 <sup>d</sup>

<sup>a</sup> Data represent least-squares means  $\pm$  SEM.  
<sup>b–e</sup> Means with different superscripts within a column differ at  $P < 0.05$ .

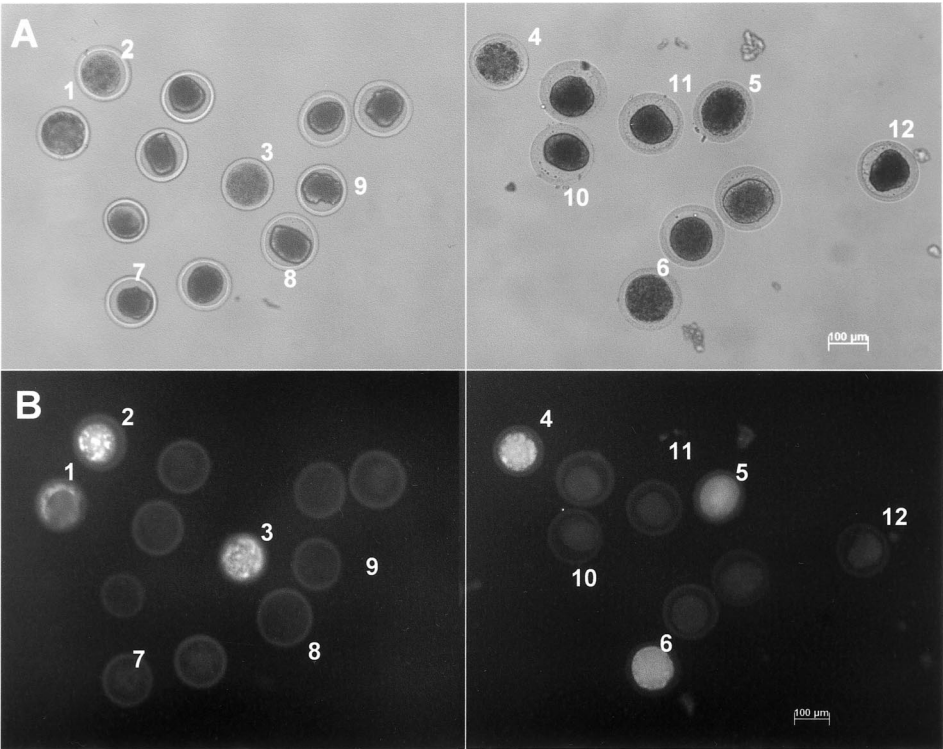
( $P < 0.01$ ; Fig. 5A) and that developed into blastocysts ( $P < 0.01$ ; Fig. 5B). Heat shock also caused a decrease in the proportion of cleaved embryos that became blastocysts ( $P < 0.05$ ; Fig. 5C).

Addition of 200 nM z-DEVD-fmk to maturation medium blocked the effect of heat shock on oocyte cleavage and subsequent embryonic development (Fig. 5). There were significant treatment  $\times$  temperature interactions for cleavage rate ( $P < 0.05$ ) and the proportion of oocytes that developed to the blastocyst stage ( $P < 0.05$ ) that reflect the lack of effect of heat shock in oocytes cultured with z-DEVD-fmk. Treatment with z-DEVD-fmk also blocked effects of heat shock on the proportion of cleaved embryos becoming blastocysts. Although the treatment  $\times$  temperature interaction was not significant, heat shock reduced the proportion of cleaved embryos becoming blastocysts in the absence of z-DEVD-fmk ( $P < 0.05$ ) but not in the presence of z-DEVD-fmk (Fig. 5C).

DISCUSSION

Heat shock during the first 12 h of maturation disrupted oocyte function as determined by a reduction in cleavage rate and in the percent of oocytes and cleaved embryos that developed into blastocysts. Similar deleterious effects of

FIG. 4. Representative images of morphology (A) and caspase activity (B) of oocytes matured during the first 12 h of maturation at 41°C. Note that oocytes (1–6) with swollen cytoplasm express high amounts of caspase activity while oocytes with condensed cytoplasm (7–12) express low caspase activity. Bar = 100  $\mu$ m.



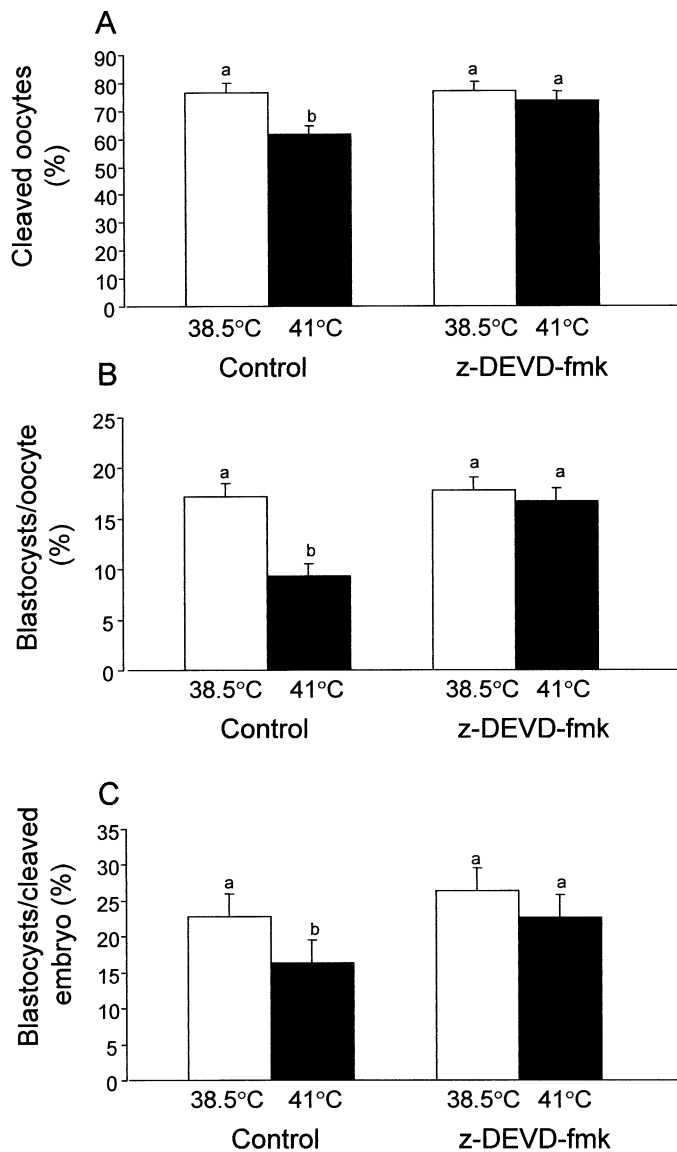


FIG. 5. Inhibition of the deleterious effect of heat shock during oocyte maturation by addition of z-DEVD-fmk, a group II caspase inhibitor. Shown are cleavage rate (A) and the percent of oocytes (B) and cleaved embryos (C) developing to the blastocyst stage at Day 8 after insemination. Data represent least-squares means  $\pm$  SEM. Note that the lower development in this experiment as compared with results shown in Figure 1 probably reflects the addition of the DMSO vehicle to cultures.

heat shock during maturation have been reported before [16, 32]. In addition, exposure of superovulated cows to heat stress during the time of oocyte maturation also disrupted subsequent development of oocytes after fertilization [7]. Thus, oocyte damage caused by heat shock *in vitro* is likely to be relevant to understanding the reduction in fertility caused by heat stress *in vivo*. The present study provides clear evidence that heat shock during maturation induces apoptotic events in bovine oocytes and that the activation of these processes is a crucial event for the loss of developmental competence of the oocyte following heat shock. The evidence for this conclusion is twofold. First, heat shock increased caspase activity and the proportion of oocytes with a TUNEL-positive pronucleus. Second, administration of z-DEVD-fmk, an inhibitor of group-II caspases (caspase-2, -3, and -7), completely blocked the del-

eterious effects of heat shock on cleavage rate and subsequent development to the blastocyst stage.

These findings are the first evidence that elevated temperature within the physiological range (40 and 41°C) is a stimulus for programmed cell death in mammalian oocytes. A variety of other adverse conditions can also induce oocyte apoptosis, including exposure to the chemotherapeutic drug doxorubicin [19], environmental toxicants [20, 21], ionizing radiation [19, 33], oxidative stress [34], and cryopreservation [35]. It has been documented that programmed cell death is the mechanism underlying the depletion of oocytes from the ovarian pool [17, 18] and that the capacity of an oocyte to undergo apoptosis exists at early stages (primordial, primary, and preantral) of follicular growth [36]. Therefore, it is possible that heat stress could lead to oocyte apoptosis not only during maturation but also at earlier stages in follicular development. Consistent with this idea are observations that oocyte competence does not become restored following thermal stress until a period of 2–3 estrous cycles [15].

Spontaneous apoptosis of unfertilized murine oocytes was characterized by cellular budding, fragmentation, caspase activation, and DNA cleavage [37]. Apoptosis in cryopreserved bovine oocytes was also characterized by cytoplasmic condensation, fragmentation of cytoplasm, and formation of apoptotic bodies with or without DNA fragmentation [35]. In the present study, in contrast, these gross morphological changes were not noted in association with increased caspase activity. Rather, oocytes with high caspase activity had an expanded cytoplasm. Cellular fragmentation in oocytes [38] and embryos [39] is not always a result of caspase activation and, therefore, it is possible that heat shock induced caspase activity and nuclear fragmentation without inducing the full complement of cellular changes associated with apoptosis. Alternatively, such changes do occur but beyond the 24 h post-heat-shock time frame of our studies.

That only a fraction of oocytes exposed to heat shock experienced apoptotic changes points out that some oocytes are better equipped to survive cellular stress. The inter- and intra-cellular components that define whether an oocyte responds to heat shock by undergoing apoptosis or by surviving are not known. Cumulus cells were found to have a critical role in protecting porcine oocytes against oxidative stress-induced apoptosis [34] and provide thermoprotection to bovine oocytes [32], and perhaps the integrity and function of the COC might affect the ability of a matured oocyte to survive heat shock. Another possibility is that oocytes differ in amounts of heat shock protein 70 (HSP70). While oocytes cannot synthesize increased amounts of HSP70 in response to heat shock [16, 32], HSP70 produced constitutively might play a role in thermal resistance. Indeed, HSP70 blocks heat-shock-induced apoptosis at many points along the apoptosis pathway, including caspase-3 activation and the c-Jun N-terminal kinase pathway for apoptosis [40]. Injection of HSP70 mRNA into mouse oocytes increased resistance to heat shock [41]. The number and the status of mitochondria found in the oocyte might also have an impact on oocyte resistance to stress. Mitochondrial distribution and ATP levels during maturation differed between morphologically good and poor oocytes and were associated with developmental competence in the bovine [42], pig [43], and human [44]. Infusion of mitochondria-enriched cytoplasts to mouse oocytes resulted in an increase in ATP production [45] and improved embryonic development in humans [46]. Mitochondria are not only involved



in energy metabolism but also play a crucial role in regulation of cell death pathways [47]. Microinjection of mitochondria purified from nonapoptotic granulosa cells into oocytes decreases the occurrence of apoptosis in these oocytes [48].

While not addressed experimentally in these studies, it is possible that effects of heat shock on oocyte apoptosis and competence to undergo cleavage and subsequent development depend on interactions with the cumulus cells that formed part of the cumulus-oocyte complex that was cultured in these studies. Studies in mice indicate that completion of oocyte maturation is dependent on communication between the oocyte and cumulus cells, whereby a paracrine signal from the oocyte enables the cumulus to undergo activation of mitogen-activated protein kinase in response to gonadotropin, which in turn leads to a signal from the cumulus necessary for oocyte maturation [49]. In cattle, too, interactions between cumulus and oocyte are important for regulation of oocyte function. Maintenance of gap junctional complexes between cumulus cells and the oocyte are involved in the inhibition of maturation [50], and removal of cumulus cells increased oocyte sensitivity to the inhibitory effects of heat shock on oocyte protein synthesis [10]. Disruption of one or more of the interactions between the oocyte and its cumulus investments by heat shock could be responsible for the induction of oocyte apoptosis and the reduction in oocyte competence to undergo cleavage and subsequent development.

It is possible that some oocytes that experience caspase activation can survive the initiation of apoptosis and undergo development after fertilization, although with reduced capacity for sustained development. Such an idea is supported by the observation that the reduction in cleavage caused by heat shock (Table 1) was less than would be expected from the heat-shock-induced increase in oocytes with high caspase activity or positive TUNEL reaction (Table 3 and Fig. 3). Survival of cells that initiated apoptosis has been reported for *Caenorhabditis elegans* [51]. Further experiments to identify the pattern of apoptosis and development in cleaved embryos formed from heat-shock oocytes are warranted.

The observation that heat shock during maturation reduced the proportion of cleaved embryos becoming blastocysts means that embryos formed from heat-shocked oocytes were less competent to develop to the blastocyst stage. It seems that heat shock during maturation induces other cellular changes that are carried over to the embryonic stage and that impede subsequent development. Disruptions of these postcleavage events also involve caspase activation because treatment with z-DEVD-fmk blocked the effect of heat shock on the proportion of cleaved embryos that developed to the blastocyst stage.

The fact that maturing oocytes are capable of caspase activation and DNA fragmentation in response to heat shock is in direct contrast with the situation with the two-cell bovine embryo, which does not undergo apoptosis in response to heat shock [22], arsenic [23], or tumor necrosis factor- $\alpha$  [31]. In fact, heat-shock-induced apoptosis does not occur in the bovine preimplantation embryo until about the 16-cell stage [22]. Because the protein kinase inhibitor staurosporine can induce apoptosis in bovine embryos at the 1- to 16-cell stage [52], it is likely that the apoptosis pathway becomes inhibited at the two-cell stage through a kinase-dependent pathway and that inhibition lasts for several rounds of cell division. The role of apoptosis in embryonic resistance to stress is also likely to be much dif-

ferent for multicellular embryos than for oocytes and early cleavage-stage embryos. While inhibition of group II caspase activity reverses effects of heat shock in oocytes, addition of z-DEVD-fmk to embryos  $\geq 16$  cells at Day 5 after insemination exacerbates the deleterious effects of heat shock on development [24]. Under certain conditions, therefore, limited amounts of apoptosis can play a beneficial role in preimplantation embryos by eliminating damaged cells. This is clearly not the case for oocytes, however. Rather, apoptosis is detrimental to subsequent oocyte function, as is to be expected for a single cell. Moreover, it is possible that apoptosis represents a selection process in which only the healthiest and most viable oocytes survive stress.

The finding that z-DEVD-fmk blocked the deleterious effects of heat shock on oocyte maturation suggests that controlling the activity of caspase-3 or other upstream intracellular events might lead to practical treatments for blocking heat-shock-induced apoptosis. This idea is strengthened by the present observations that blastocysts derived from heat-shocked oocytes were similar to blastocysts from oocytes matured at 38.5°C with respect to total cell number, percentage of TUNEL-positive cells, and activity of group II caspases. Further studies using embryo transfer will lead to a conclusive determination of whether these blastocysts are actually of normal developmental potential. One possible point for therapeutic intervention is at the level of the bcl-2 gene family. Indeed, Bax-deficient oocytes were resistance to apoptosis induced by doxorubicin [19]. Additionally, targeted expression of the antiapoptotic molecule, Bcl-2, [53] protected mouse oocytes from apoptosis induced by doxorubicin. Ceramide also plays a pivotal role in stress-induced apoptosis [54]. Oocytes from mice lacking the gene encoding for acid sphingomyelinase, the enzyme that hydrolyzes sphingomyelin to ceramide, were resistant to chemotherapy-induced apoptosis in vitro and to radiation-induced apoptosis in vivo [33]. Sphingosine-1-phosphate, a metabolite of ceramide that functions as an antiapoptotic factor, protected mouse oocytes from apoptosis induced by chemotherapy and radiation [33]. Furthermore, addition of sphingosine-1-phosphate was recently shown to improve oocyte maturation in pigs [55].

In conclusion, activation of apoptotic processes mediated by group II caspases, as defined by both TUNEL and caspase activity assays, is a critical mechanism responsible for the disruption of oocyte capacity for cleavage and subsequent development caused by heat shock during the first 12 h of maturation. Controlling the activity of group II caspases or intracellular events upstream from activation of execution caspases might be a useful strategy for blocking heat-shock-induced apoptosis in oocytes and thereby improving fertility of heat-stressed females.

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