REPRODUCTION

Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation

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Abstract

Meiotic maturation in mammalian oocytes is a complex process which involves extensive rearrangement of microtubules, actin filaments and chromosomes. Since cytoskeletal elements are sensitive to disruption by heat shock, a series of experiments were performed to determine whether physiologically relevant heat shock disrupts the progression of the oocyte through meiosis, fertilization and zygote formation. Cumulus–oocyte complexes were cultured at 38.5, 40.0 or 41.0 °C for the first 12 h of maturation. Incubation during the last 10 h of maturation and 18 h after fertilization was at 38.5 °C and in 5% (v/v) CO_2 for both treatments. Examination of the cytoskeleton and the chromosome organization in matured oocytes revealed that oocytes matured at 38.5°C were mostly at metaphase II (MII) stage, while the majority of heat-shocked oocytes were blocked at the first metaphase (MI), first anaphase or first telophase stages. A subset of heat-shocked oocytes possessed misshapen MI spindles with disorganized microtubules and unaligned chromosomes. A higher percentage of TUNEL-positive oocytes was noted for oocytes matured at 41.0 °C. Addition of 50 nmol/l sphingosine 1-phosphate to maturation medium blocked the effect of heat shock on progression through meiosis and apoptosis and increased the proportion of oocytes matured at 41.0 °C that were at MII. Following insemination, a high percentage of heat-shocked oocytes were unfertilized, while the majority of the control zygotes were fertilized and had two visible pronuclei. In conclusion, heat shock disrupts nuclear maturation and induces apoptosis. These alterations are likely to be involved in the mechanism underlying heat-shock-induced disruption of oocyte capacity for fertilization and subsequent development.
Reproduction (2005) **129** 235–244

Introduction

Oocyte maturation involves a series of nuclear and cytoplasmic maturational events that result in acquisition of the capacity for fertilization and subsequent development. Nuclear reorganization involves the breakdown of the germinal vesicle, chromosome condensation and segregation, completion of meiosis I, extrusion of the first polar body, and arrest at metaphase of the second meiotic division (MII). Exposure of cultured oocytes to elevated temperature (i.e. heat shock) at the germinal vesicle stage (Payton *et al.* 2004) or during early stages of maturation (Edwards & Hansen 1997, Roth & Hansen 2004*a,b*) interferes with the processes of oocyte maturation so that the capacity for embryonic development after insemination is reduced.

The mechanisms for the disruption in oocyte maturational processes by heat shock are likely to be complex and to involve the cytoskeleton. This is so because nuclear maturation is dependent upon extensive rearrangement of the cytoskeleton (Gallicano 2001, Shin & Kim 2003). Indeed, nuclear maturation can be disrupted by addition of drugs that inhibit microtubule formation (Ruder & Murry 1996, Kim *et al.* 2000, Brunet *et al.* 2003) and microfilament assembly (Kim *et al.* 2000). Additionally, cytoskeletal elements are sensitive to disruption by heat shock (Coss & Linnemans 1996). Exposure of somatic cells to heat shock during the S phase can result in spontaneous premature chromosome condensation and formation of micronuclei (Swanson *et al.* 1995). Heat shock during the M phase can result in disassembly of the mitotic spindle, polyploidy and failure of cytokinesis (Coss *et al.* 1982, Vidair *et al.* 1993). Many studies evaluating effects of heat shock on the cytoskeleton involve temperatures higher than would be seen in mammals exposed to heat stress. Recently, however, more physiologically

relevant temperatures of 41–41.5 °C have been shown to cause alterations in microtubule and microfilament structure in matured bovine oocytes (Tseng *et al.* 2004), porcine oocytes subjected to heat shock during maturation (Ju & Tseng 2004) and two-cell bovine embryos (Rivera *et al.* 2004*a*).

The fact that nuclear maturation is dependent upon the cytoskeleton and that heat shock can disrupt cytoskeletal elements leads to the hypothesis tested in these studies that effects of heat shock on oocyte maturation involve alterations in nuclear maturation. A second objective was to evaluate the relationship between oocyte apoptosis (Roth & Hansen 2004*a*) and nuclear changes in the heat-shocked oocyte. Recently, Roth & Hansen (2004*b*) demonstrated that maturation in the presence of sphingosine 1-phosphate (S1P), a sphingomyelin metabolite, reduced the deleterious effect of heat shock during maturation on the proportion of bovine oocytes that could cleave and progress in development following insemination. Therefore, a third objective was to determine whether S1P acts to improve maintenance of oocyte function during heat shock by allowing nuclear maturation to take place.

Materials and Methods

Materials

Alexa Flour 594 phalloidin, Zenon Alexa Fluor 488 IgG labeling kit and mouse anti-bovine α-tubulin were from Molecular Probes (Eugene, OR, USA). Mouse $IgG_1\kappa$, S1P and Hoechst 33342 dye were from Sigma-Aldrich. The *in situ* cell death detection kit (fluorescein) was obtained from Roche. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY, USA), the Prolong Antifade Kit was from Molecular Probes, and RQ1 RNA-free DNase was from Promega. Oocyte collection medium (OCM) was Tissue Culture Medium 199 (TCM-199) with Hanks' salts and without phenol red (Hyclone, Logan, UT, USA) supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin; Pel-Freez, Rogers, AR, USA), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin and 1 mmol/l glutamine. Oocyte maturation medium (OMM) was TCM-199 with Earle's salts (Invitrogen) supplemented with 10% (v/v) steer serum (Pel-Freez), 22 μg/ml sodium pyruvate, 20 μg/ml follicle stimulating hormone (Follotropin–V; Vetrepharm Canada, London, ON, Canada), 2 μg/ml estradiol 17-β, 50 μg/ml gentamicin and 1 mmol/l glutamine. Hepes–Tyrode's Lactate (Hepes-TL), *in vitro* fertilization Tyrode's Lactate (IVF-TL) and Sperm-TL were obtained from Cell and Molecular Technologies (Lavallette, NJ, USA) and used to prepare Hepes-Tyrode's Albumin Lactate Pyruvate (TALP), IVF-TALP and Sperm-TALP (Parrish *et al.* 1986). BSA Fraction V and essentially fatty-acid-free BSA were purchased from Sigma-Aldrich. Percoll was from Amersham Pharmacia Biotech. Frozen semen from various bulls was donated by Southeastern Breeders Service (Wellborn, FL, USA).

In vitro *maturation and fertilization of oocytes*

Oocyte maturation and fertilization were performed using procedures described earlier (Roth & Hansen 2004*a*). Briefly, ovaries were sliced and cumulus–oocyte complexes (COCs) were collected into a beaker containing OCM. COCs were washed and matured in groups of ten in 50 μl drops of OMM overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 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 ($\sim 1 \times 10^6$) Percoll-purified spermatozoa supplemented with 25 μl 0.5 mmol/l penicillamine, 0.25 mmol/l hypotaurine and 25 μmol/l 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 hyalurinidase (1000 U/ml in 1 ml Hepes-TALP medium for 5 min), and washed two or three times in Hepes-TALP. Matured oocytes were harvested after 22 h of maturation and putative zygotes were collected 18 h after fertilization for further examination.

Effect of heat shock during maturation on nuclear maturation and apoptosis

Freshly collected COCs were placed in maturation drops as described earlier and cultured for 22 h at either 38.5°C for 22 h or 40 or 41°C for 12 h followed by 38.5°C for 10 h. The period of 12 h was chosen because this duration of heat shock caused changes in oocyte competence for embryonic development in an earlier study (Edwards & Hansen 1997) and because the period of elevated body temperatures during heat stress can be that long (Rivera & Hansen 2001). The gaseous atmosphere was CO_2 in humidified air. The percent CO_2 was adjusted to ensure that the concentration of dissolved CO_2 was similar between treatments and to maintain pH at ~ 7.4 (5, 6 and 7% for 38.5, 40 and 41°C respectively). Matured oocytes were harvested after 22 h of maturation and denuded of cumulus cells by vortexing COCs with 100 μl hyaluronidase (1000 U/ml in 1 ml Hepes-TALP medium for 5 min) and processed for TUNEL and Hoechst labeling. In particular, oocytes were removed from culture medium, washed three times in 100 μl drops of 10 mmol/l KPO_4, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 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 weeks before assay. The experiment was replicated six times using 163–212 oocytes per treatment.

The procedure for dual-labeling of DNA using Hoechst 33342 and DNA fragmentation using the TUNEL procedure was as described by Roth & Hansen (2004*a*). Briefly, oocytes were permeabilized 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. Specimens were then incubated in 50 μl drops of TUNEL reaction

mixture for 1 h at 37 °C in the dark, washed in PBS-PVP, and transferred to 50 µl drops of 1 µg/ml Hoechst 33342 in PBS-PVP for 30 min at room temperature. Each TUNEL procedure contained samples 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. 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 oocyte was evaluated for the stage of meiosis and for the presence of TUNEL labeling of chromatin.

Effect of S1P on nuclear maturation

For three replicates of the above experiment, the ability of S1P to prevent effects of a 41 °C heat shock on nuclear maturation was tested by performing maturation in medium with either 50 nmol/l S1P or an equivalent amount of vehicle (0.1% (v/v) methanol). A total of 56–80 oocytes per treatment were examined.

Effects of heat shock during maturation on cytoskeletal organization

COCs were placed in maturation drops and cultured for 22 h at either 38.5°C for 22 h or 41°C for 12 h followed by 38.5°C for 10 h as described above. Oocytes were then harvested, denuded of cumulus cells, and processed for localization of actin, tubulin and DNA. A total of 33–54 oocytes per treatment were analyzed.

For localization, oocytes were fixed in 3.7% (v/v) formaldehyde in PBS for 10 min at 39°C, washed three times in PBS-PVP and stored in PBS-PVP at 4 °C for up to 2 weeks. Samples were then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min followed by three washes with PBS-PVP. To block nonspecific binding, samples were preincubated in 10% (v/v) normal goat serum in PBS (PBS + S) at room temperature for 30 min. Samples were incubated for 30 min in a labeling solution that consisted of 120 µl phalloidin conjugated to Alexa Flour 594, 40 µl mouse anti-bovine α-tubulin labeled with Zenon Alexa Fluor 488 and 40 µl 5 µg/ml Hoechst 33342 in PBS + S. The phalloidin conjugated to Alexa Flour 594 was prepared by dilution of 5 µl 200 U/ml methanolic phalloidin in a final volume of 200 µl PBS + S. The anti-tubulin was labeled with Zenon Alexa Flour 488 by incubation of 1 µg anti-α-tubulin (200 µg/ml stock solution in PBS containing 1% (w/v) BSA) with 5 µl Zenon IgG labeling reagent for 5 min at room temperature followed by incubation of the reaction mixture with 5 µl of blocking reagent (from the labeling kit). Following incubation of specimens with the labeling mixture, slides were washed three times in PBS + S, fixed again in 3.7% (v/v) formaldehyde for 15 min at room temperature, washed three times in PBS-PVP and placed on a glass slide with a cover slip mounted with 5 µl ProLong Antifade solution. Negative controls were stained similarly except that phalloidin was omitted from the reaction mixture and 1 µg $IgG_1\kappa$ was used instead of 1 µg anti-α-tubulin for preparation of the antibody-Zenon complex. Specimens were viewed using a Zeiss Axioplan 2 epifluorescent microscope connected to an AxioCam MRm digital camera and images were acquired using AxioVision software (Carl Zeiss).

Effect of heat shock during maturation on fertilization

COCs were matured at either 38.5°C for 22 h or 41°C for 12 h followed by 38.5°C for 10 h as described earlier. Thereafter, oocytes were fertilized at 38.5°C in an atmosphere of 5% CO_2 in humidified air. After 18 h, putative zygotes were removed from fertilization wells, denuded of cumulus cells with hyaluronidase, and processed for Hoechst staining of DNA as described above. A total of 46–61 oocytes per treatment were analyzed.

Statistical analysis

The CATMOD procedure of SAS (SAS System for Windows, Release 8.20; Cary, NC, USA) was used to determine whether treatments affected the distribution of oocytes into various classes of nuclear maturation as well as the proportion of oocytes that were apoptotic.

Results

Heat shock during maturation affects nuclear maturation

Oocytes were classified by the stage of meiotic progression as being either condensed, or at metaphase I (MI), anaphase I (AI), telophase I (TI) or MII (for representative pictures, see Fig. 1). Heat shock affected ($P < 0.001$) the distribution of oocytes into these different classes (Fig. 2). In particular, a higher proportion of oocytes matured at 38.5°C reached MII (characterized by a metaphase plate and formation of the first polar body; see Fig. 1e) than oocytes matured at 40 and 41°C. Since heat shock reduced the proportion of oocytes that reached MII, there was a corresponding increase in the proportion of heat-shocked oocytes at other stages. For oocytes at 40°C, oocytes that were arrested were distributed in approximately equal numbers at the various stages of meiosis (Fig. 2). For oocytes at 41°C, however, more arrested oocytes were at MI than at later stages (Fig. 2).

There was an effect of treatment ($P < 0.001$) on the percentage of oocytes with TUNEL-positive chromatin because both heat shocks (40 and 41°C) increased the percentage of oocytes having TUNEL-positive chromatin (Fig. 3). As illustrated in Fig. 1f–j, TUNEL-positive chromatin was observed at all stages of nuclear maturation.
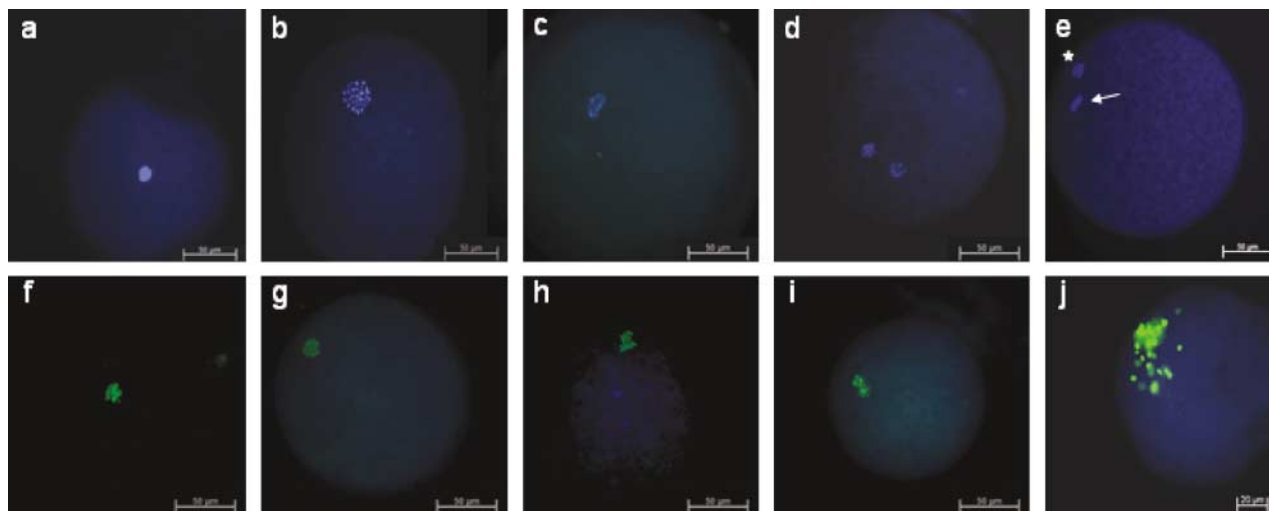
Figure 1 Representative images of nuclear staining (a–e) and TUNEL labeling (f–j) of bovine oocytes after maturation. Shown are condensed nucleus (a, f), MI (b, g), AI (c, h), TI (d, i) and MII (e, j) stages. Note that DNA was visualized with blue fluorescence after staining with Hoechst 33342, while TUNEL-positive chromatin is labeled green. The asterisk identifies polar bodies and the arrow identifies the MII plate (e).

S1P affects nuclear maturation

In a subset of replicates from the above-described experiments, additional groups of oocytes at each temperature were cultured with 50 nmol/l S1P. In this subset of replicates, heat shock also affected ($P < 0.001$) the distribution of oocytes in stages of nuclear maturation (Fig. 4). There was a temperature $\times$ S1P interaction ($P < 0.001$) on distribution of oocytes into nuclear classes because S1P blocked the effect of heat shock on progression to MII. As illustrated in Fig. 5, S1P also blocked the increase in percentage of oocytes that were classified as apoptotic based on TUNEL assay (temperature $\times$ treatment, $P < 0.005$).

Microfilament and microtubule rearrangement associated with effects of heat shock on nuclear maturation

For oocytes used in this study, 81% of those matured at 38.5°C were at the MII stage, while only 30% of heat-shocked oocytes were at MII, with the remainder mostly at AI or TI (temperature; $P < 0.05$).
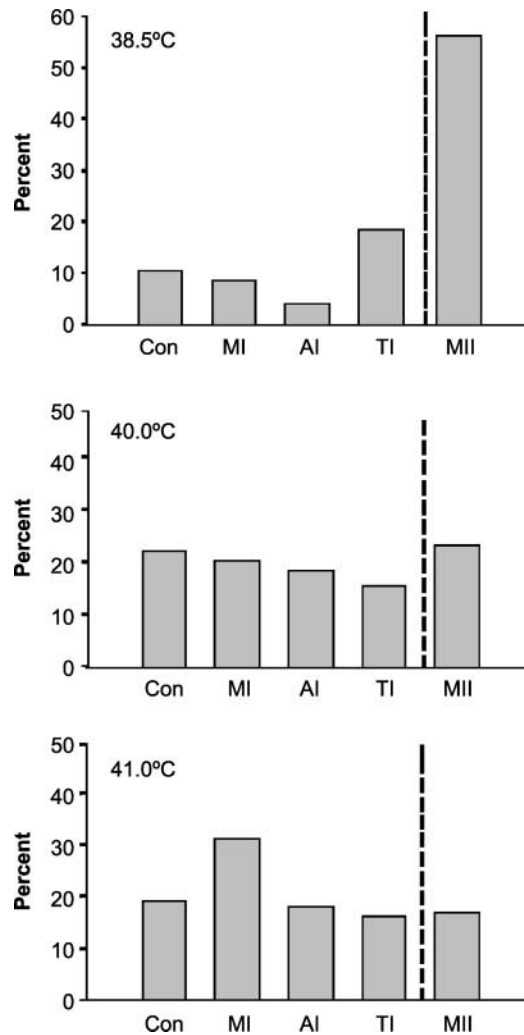
The pattern of microfilament localization was affected by stage of nuclear maturation and by heat shock. Oocytes at the MI stage were characterized by condensed chromatin, a large metaphase plate and lack of a polar body (for representative picture see Fig. 1b and g). At this stage, microfilaments were observed within the oocyte cytoplasm but did not surround the pronucleus (Fig. 6a and 6a). At MII stage, the polar body was present and microfilaments were associated with this structure (Fig. 6e and 6e). Actin microfilaments were more distinct in the cytoplasm of heat-shocked oocytes than in oocytes cultured at 38.5°C (compare Fig. 6a and Fig. 6a). An intense ring of actin was present under the plasma membrane in oocytes matured at 38.5°C (Fig. 6a, c and e). Microfilaments were also observed to transverse the zona pellucida of this group of oocytes. In contrast the peri-cellular actin ring and trans-zonal actin processes were generally absent for oocytes matured at 41°C (Fig. 6a and 6e). Microtubules were identified within the meiotic spindle of pronuclei at MI, AI, TI and MII stages (Fig. 6, all images) and, for MII oocytes, in the first polar body (Fig. 6f and 6f).

For oocytes matured at 38.5°C, the meiotic spindles of both MI and MII pronuclei were well organized with sharply defined structures and with chromosomes aligned along the midline. This pattern also predominated for heat-shocked oocytes that developed to MII. In contrast, the heat-shocked oocytes that remained at MI had normal spindles in only 24% of the cases (see Fig. 7a for examples). Rather, 76% of the heat-shocked oocytes at MI had abnormal spindles. For some oocytes, spindles had a rounded shape and some unaligned chromosomes (Fig. 7b), while for others spindles were of amorphous shape and with a large degree of disorganization of chromosomal alignment (Fig. 7c).

Heat shock during maturation reduces the proportion of oocytes fertilized

Examination of the chromosomal organization for putative zygotes (18 h after fertilization) revealed that shock during oocyte maturation affected ($P < 0.001$) the distribution of putative zygotes into nuclear classes (Table 1). The percentage of putative zygotes with a normal pattern of two pronuclei was higher for oocytes that matured at 38.5°C than for oocytes matured at 41°C (for representative picture see Fig. 8a–c). Conversely, the proportion of oocytes that were unfertilized was higher for oocytes at 41°C. These unfertilized oocytes were observed to be at all stages of nuclear maturation from MI through MII (for
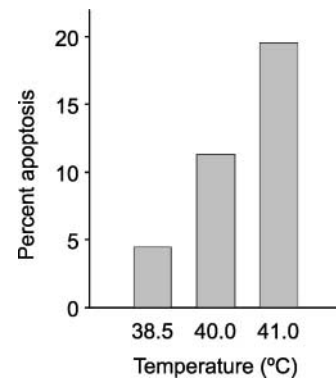
Figure 3 Effect of heat shock on oocyte apoptosis. Oocytes were matured for either 22 h at 38.5 °C or for 12 h at either 40 or 41 °C followed by 10 h at 38.5 °C. Apoptosis was then determined by TUNEL assay. Results represent analysis of 163–212 oocytes per treatment. The proportion of oocytes that were apoptotic was affected ($P < 0.001$) by temperature.

Figure 2 Effect of heat shock on nuclear maturation. Oocytes were matured for either 22 h at 38.5 °C or for 12 h at either 40 or 41 °C followed by 10 h at 38.5 °C. Status of nuclear maturation was then determined. Results represent analysis of 161–210 oocytes per treatment. Distribution of oocytes into nuclear classes was affected ($P < 0.001$) by temperature. Con, condensed.

examples, see Fig. 8d–f). The small subset of oocytes that had more than two pronuclei or were polyspermic was of similar abundance for oocytes at 38.5 and 41 °C (see Fig. 8g–i for example).

Discussion

Meiotic maturation in mammalian oocytes is a complex process that involves coordinated reorganization of the cytoskeleton and chromatin. As shown in the current studies, heat shock during oocyte maturation can impair resumption of meiosis and thereby attenuate ability of oocytes to be fertilized. In particular, heat shock blocked the progression from MI to MII and increased the proportion of oocytes that were apoptotic as indicated by TUNEL labeling. Moreover, results imply that the induction

Figure 4 Alteration in the effect of heat shock on nuclear maturation caused by S1P. Oocytes were matured in the presence (black bars) or absence (open bars) of S1P for either 22 h at 38.5 °C or for 12 h at 41 °C followed by 10 h at 38.5 °C. Status of nuclear maturation was then determined. Results represent analysis of 54–79 oocytes per treatment. Distribution of oocytes into nuclear classes was affected ($P < 0.001$) by temperature, treatment and temperature × treatment. Con, condensed.

of apoptosis by heat shock is functionally related to the inhibition of meiotic progression. This conclusion is based on the present observation that the anti-apoptotic molecule, S1P, blocked the effect of heat shock on
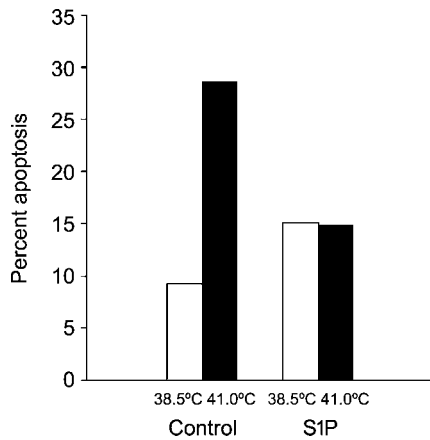
Figure 5 Inhibition of heat-shock induction of apoptosis by S1P. Oocytes were matured in the presence or absence of S1P for either 22 h at 38.5 °C (open bars) or for 12 h at 41 °C (black bars) followed by 10 h at 38.5 °C. Apoptosis was then determined by TUNEL assay. Results represent analysis of 56–80 oocytes per treatment. The proportion of oocytes that were apoptotic was affected *(P < 0.005)* by temperature × treatment.

progression through meiosis. The ability of S1P to block effects of heat shock suggests that one approach to overcome effects of heat stress on oocyte function in heat-stressed females is to manipulate sphingomyelin metabolism.

Thermal stress *in vivo* during oocyte maturation has been previously shown in mice to compromise the ability of oocytes to progress through meiosis and form embryos capable of developing to the blastocyst stage (Baumgartner & Chrisman 1981, Matsuzuka *et al.* 2004). Exposure of oocytes maturing *in vitro* to 43°C also blocks meiotic progression in mice (Kim *et al.* 2002). The temperatures used here to test effects of heat shock on maturation *in vitro* are much more physiologically relevant than the 43°C used by Kim *et al.* (2002); average maximal rectal temperatures in heat-stressed lactating cows experiencing infertility ranged from 40.1 to 40.5°C (Roth *et al.* 2000). Because a temperature as low as 40°C was sufficient to reduce meiotic progression in this study, effects of heat shock seen *in vitro* are likely to also occur in heat-stressed
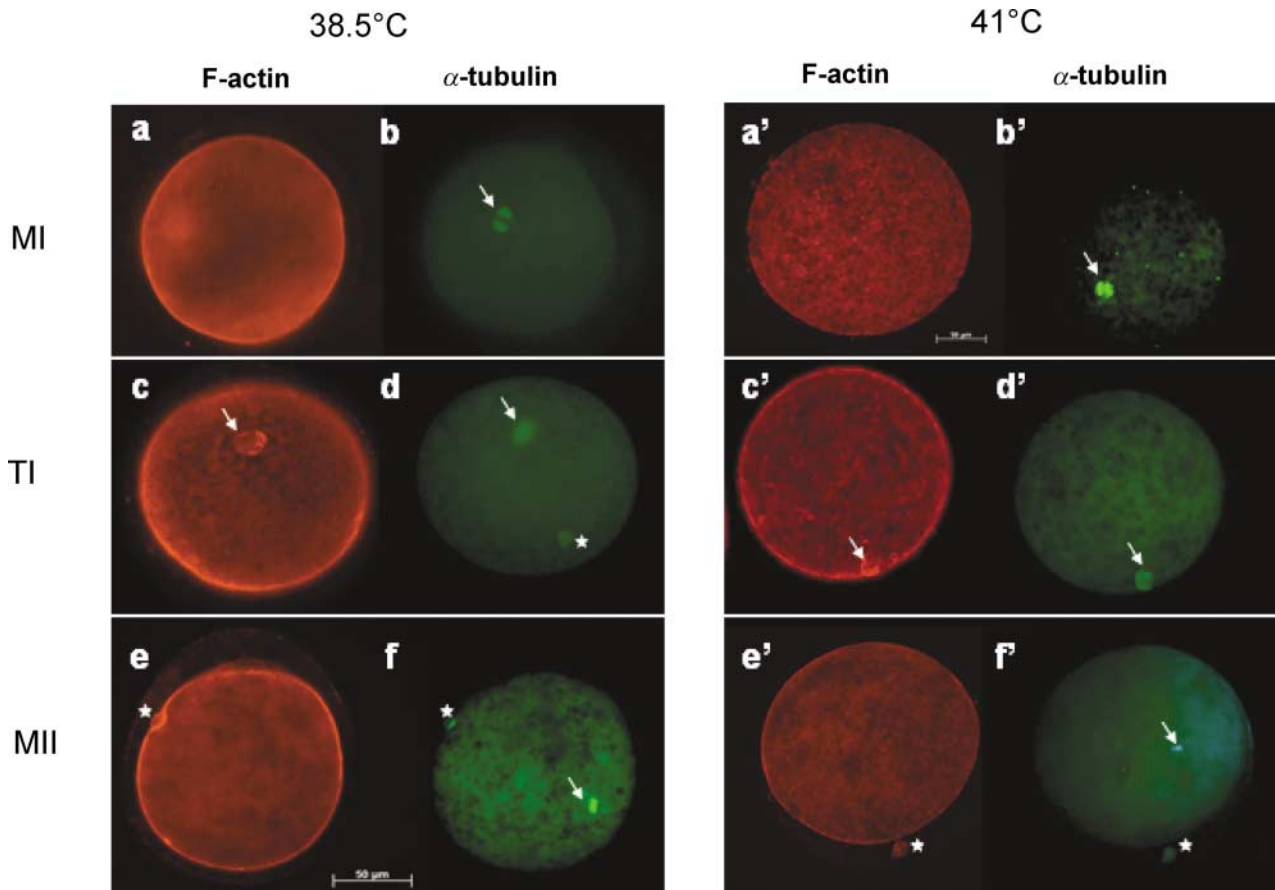
Figure 6 Representative patterns of labeling of bovine matured oocytes for microfilaments (F-actin) and microtubules (α-tubulin). Microfilaments were visualized by reaction with phalloidin conjugated to Alexa Flour 594. Microtubules were localized by immunofluorescence. The oocyte in f was also stained with Hoechst 33342. Each pair of images (for example a and b) are from the same oocyte. Arrows identify meiotic spindles of pronuclei at different stages of meiosis and asterisks identify polar bodies.
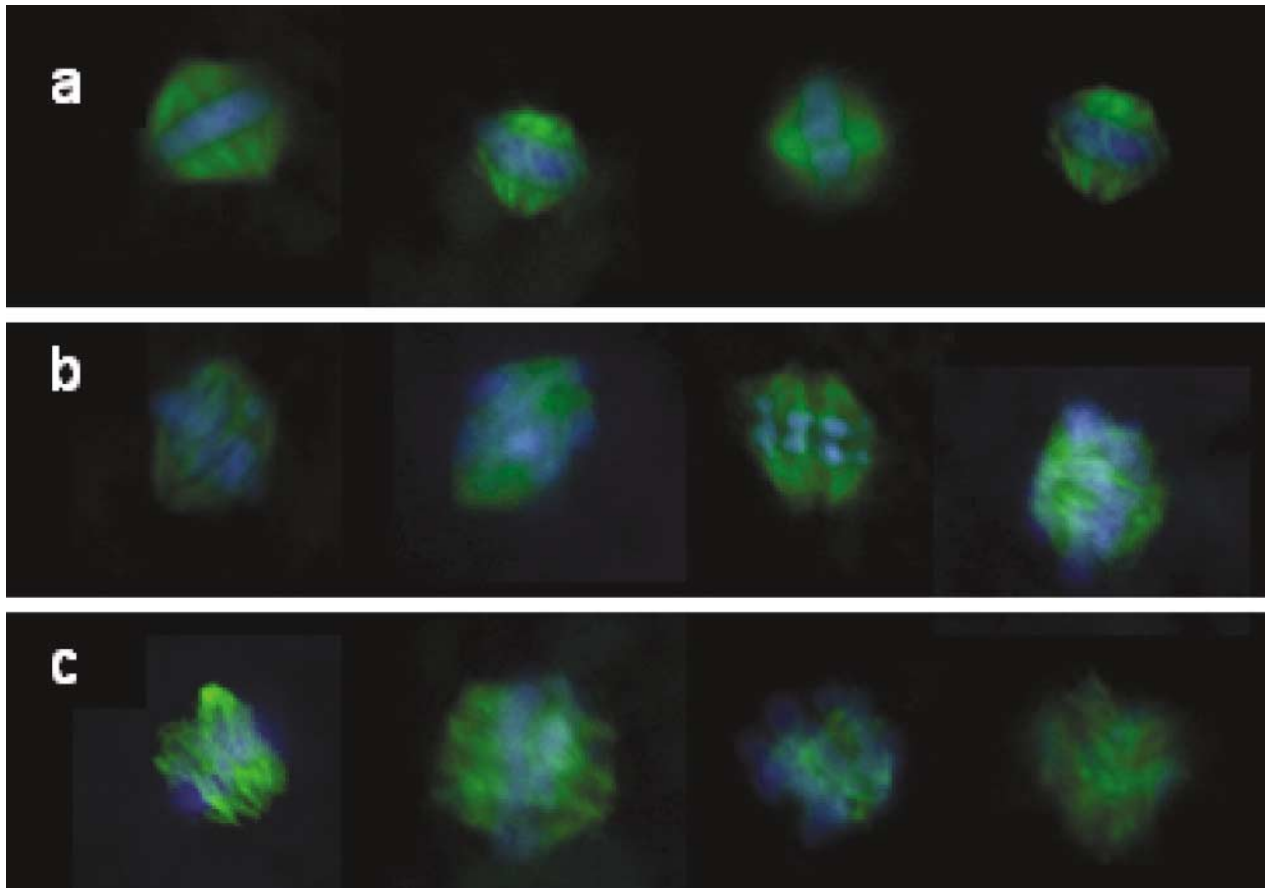
Figure 7 Representative images of MI spindles of oocytes exposed to heat shock (41 °C) during the first 12 h of maturation. Shown are spindles with sharply defined structures and with chromosomes aligned along their midline (a), spindles with more rounded shape and unaligned chromosomes (b) and spindles with amorphous structures and disorganized chromosomes (c).

cows. Nonetheless, further experiments are necessary to determine whether this is the case. Exposure of oocytes to 41°C had more severe effects on meiosis since oocytes were less likely to pass MI after exposure to 41°C than oocytes exposed to 38.5 or 40°C.

Actions of heat shock on progression of the maturing oocyte through meiosis are the proximal cause for the reduction in fertilization rate for oocytes matured at 40 and 41°C as compared with oocytes at 38.5°C (present results and Roth & Hansen 2004a,b). Heat shock causes several changes in oocyte function that could contribute to the inhibition in progression through meiosis. Protein

Table 1 Effect of heat shock during maturation on fertilization: effect of temperature on the distribution of oocytes into classes ($P < 0.001$).

Treatment	Oocytes (n)	Unfertilized[a] (n (%))	2PN[b] (n (%))	>2PN[c] (n (%))
38.5°C	46	13 (28.2)	26 (56.6)	7 (15.2)
41°C	61	41 (67.2)	12 (19.7)	8 (13.1)

[a] Oocytes that blocked at MI, AI, TI or MII stages.
[b] Two pronuclei.
[c] More than two pronuclei.

synthesis is required for nuclear maturation (Tatemoto & Horiuchi 1995) and heat shock can reduce protein synthesis by the oocyte (Edwards & Hansen 1996, 1997). Microtubules are critical for meiotic division (Brunet *et al.* 2003) as are microfilaments, which play a pivotal role in the migration of the MI spindle to the plasma membrane and extrusion of the first polar body and rotation of the MII plate before activation (Gallicano 2001). Effects of heat shock on microfilament organization in the present study were apparent as a decrease in membrane-associated microfilaments and a corresponding increase in cytoplasmic actin. Disruption of microtubules by heat shock was indicated by the increase in the frequency of deformed spindle apparatuses. A similar effect of heat shock on membrane-associated actin was reported for bovine two-cell embryos (Rivera *et al.* 2004a) and heat-shock-induced alterations in the spindle apparatus have been reported in matured porcine oocytes (Ju & Tseng 2004) and parthenogenetically activated bovine oocytes (Tseng *et al.* 2004).

Progression of meiosis is controlled by the spindle checkpoint mechanism that ensures proper chromosome segregation (Brunet *et al.* 2003). Incorrect chromosome
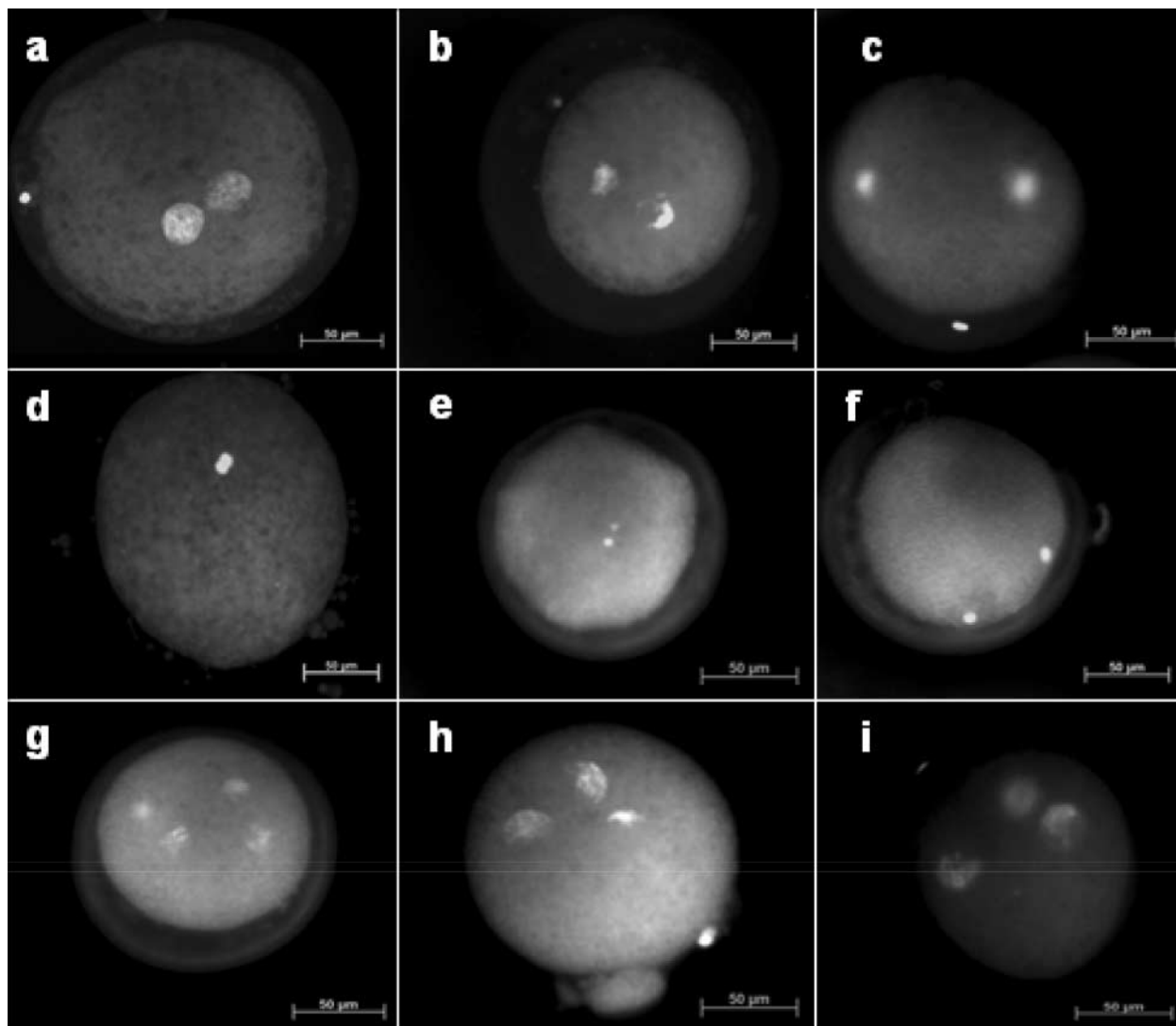
Figure 8 Representative images of oocytes at 18 h after fertilization. Shown are one-cell stage embryos with two pronuclei (a–c), unfertilized oocytes (d–f) and one-cell stage embryos with three or four pronuclei (g–i).

positioning or improper attachment to the spindle micro-tubule activates the spindle checkpoint and delays onset of anaphase by inhibition of anaphase-promoting complex/cyclosome (Zhou *et al.* 2002, Cleveland *et al.* 2003). Checkpoint mechanisms could theoretically be engaged as a result of heat shock. Arrested oocytes at 41°C were more likely to be at MI than at later stages, which supports the idea that heat shock at this temperature activates the spindle checkpoint mechanism. However, the anaphase checkpoint is not likely to be the only mechanism affected at 40°C because heat-shocked oocytes at this temperature that failed to progress to MII were blocked at several stages. A checkpoint monitoring the anaphase-to-telo-phase transition has also been described in yeast (Muhua *et al.* 1998) and perhaps this mechanism is also activated by heat shock.

While not studied here, it is also possible that heat shock can inhibit the onset of meiosis by interfering with germinal vesicle breakdown. However, there was no effect of heat shock at the germinal vesicle stage on the pro-portion of roscovitine-treated bovine oocytes that com-pleted germinal vesicle breakdown (Payton *et al.* 2004).

The fact that resumption of meiosis was attenuated by heat shock in a parallel manner with induction of oocyte apoptosis suggests that more than one mechanism is involved in heat-shock-induced disruption of oocyte com-petence and confirms the earlier finding of Roth & Hansen (2004*a*) that heat shock during maturation induces apop-tosis mediated by caspase activation in bovine oocytes. Apoptosis appears to be the key event that leads to arrest of fertilization failure (and presumably nuclear arrest). This conclusion is based on findings that effects of heat shock on nuclear maturation can be blocked by inhibition

of apoptosis, either by addition of a z-DEVD-fmk, an inhibitor of group II caspases, as described by Roth & Hansen (2004*a*) or by addition of S1P, as reported here for meiotic progression and earlier for cleavage (Roth & Hansen 2004*b*).

It is possible that alterations in the cytoskeleton caused by heat shock serve as a trigger for apoptosis. Anti-microtubule agents are known to promote apoptosis of tumor cells (Wang *et al.* 1999, Masuda *et al.* 2003). Alternatively, alterations in cytoskeletal architecture may be the result of activation of execution caspases. If the former possibility is true (cytoskeletal changes induce apoptosis), these changes in cytoskeleton induced by heat shock are not capable of preventing fertilization in the absence of apoptosis (Roth & Hansen 2004*a*). Since one would presume that fertilization is unlikely in the absence of nuclear maturation, one must conclude that either heat-shocked oocytes treated with apoptosis inhibitor do not experience a disruption of the cytoskeletal architecture or that these changes become reversed and the oocyte eventually completes nuclear maturation. Interestingly, the bovine two-cell embryo is capable of development to the eight-cell stage after heat shock despite extensive changes in organellar structure (Rivera *et al.* 2004*b*).

That S1P prevents the effect of heat shock on nuclear maturation provides evidence that alterations in nuclear maturation and apoptosis are linked features of the oocyte response to heat shock. S1P is a sphingomyelin metabolite that regulates a wide variety of cellular processes including growth, survival and cytoskeleton rearrangement (Pyne & Pyne 2000) and which is involved in maturation of porcine (Lee *et al.* 2003) and bovine oocytes (Roth & Hansen 2004*b*). Involvement of S1P in cytoskeletal rearrangement is mediated by a family of specific G protein-coupled receptors called $S1P_{1-5}$ receptors. For example, binding of S1P to $S1P_1$ receptor mediated cortical actin assembly and Rac activation (Spiegel & Milstien 2002). Binding of S1P to $S1P_2$ and $S1P_3$ induces stress fiber formation and activation of Rho family proteins that are important for cytoskeleton rearrangement (Okamoto *et al.* 2000). Thus, S1P could improve nuclear maturation in the face of heat shock by affecting the cytoskeleton. In addition, the thermoprotective action of S1P may involve anti-apoptotic effects also because S1P counteracts the pro-apoptotic effects of ceramide (Cuvillier *et al.* 1998).

Segregation errors during either the first or the second meiotic division can result in a chromosomally abnormal embryo. For example, oocytes of mice lacking the Formin homology gene ($Fmn2^{-/-}$), which is required for progression through metaphase, cannot correctly position the metaphase spindle during MI and cannot form the first polar body. Fertilization of $Fmn2^{-/-}$ oocytes resulted in formation of a polyploid embryo and pregnancy loss (Leader *et al.* 2002). Moreover, postimplantation embryos produced by mice exposed to thermal stress during the periovulatory period had a higher incidence of chromosomal abnormalities (Baumgartner & Chrisman 1988).

To verify whether this is the situation in heat-shocked bovine oocytes, the effect of heat shock on nuclear status in one-cell stage embryos was determined at 18 h after fertilization. Only a few oocytes matured at 41°C had more than two pronuclei or were polyspermic. The majority of heat-shocked oocytes failed to undergo fertilization and were blocked at the MI or through MII stages.

Taken together, these findings indicate that disruption in resumption of meiosis caused by heat shock is associated with oocyte apoptosis and cytoskeletal modifications. These alterations are likely to be involved in the mechanism underling heat-shock-induced disruption of oocyte competence and the reduction in fertility of heat-stressed females.

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