Exposure to a Physiologically Relevant Elevated Temperature Hastens In Vitro Maturation in Bovine Oocytes

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ABSTRACT

The objective of this study was to evaluate nuclear (progression to metaphase II) and cytoplasmic (translocation of cortical granules to the oolemma) maturation in control (38.5°C) and heat-stressed (41.0°C) oocytes. Hoechst staining indicated that a similar proportion of control and heat-stressed oocytes progressed to metaphase II. More heat-stressed oocytes had type III cortical granule distribution suggesting that heat stress accelerated cytoplasmic maturation. The kinetics of nuclear maturation was examined in a second experiment in which a higher proportion of heat-stressed oocytes progressed to metaphase I by 8 h and arrested at metaphase II at 16 and 18 h after placement into maturation medium. However, differences related to maturation temperature were no longer apparent by 21 h. Heat-induced alterations in kinetics of nuclear and cytoplasmic maturation prompted a third experiment to evaluate if earlier insemination of heat-stressed oocytes ameliorates heat-induced reductions in development. A significant temperature × insemination time interaction was noted when evaluating blastocyst development. Blastocyst development was reduced when heat-stressed oocytes were inseminated with sperm 24 h after placement into maturation medium compared with controls. In contrast, blastocyst development was similar to controls when heat-stressed oocytes were inseminated at 19 h. Based on this interaction, earlier insemination in vitro prevented heat-induced reductions in oocyte development. Collectively, these studies suggest a cumulative effect of heat stress to hasten in vitro maturation in bovine oocytes.

(**Key words:** heat stress, oocyte, maturation)

Abbreviation key: COC = cumulus-oocyte complexes, GV = germinal vesicle, HS = heat stress, IVF = in vitro fertilization, IVM = in vitro maturation, M199 = tissue

culture medium-199, MI = metaphase I, MII = metaphase II, OMM = oocyte maturation medium, PZ = putative zygotes.

INTRODUCTION

Infertility caused by environmental heat stress is one of the most important economic problems facing the dairy industry and may be due to direct effects of elevated maternal temperatures to compromise reproduction. Rectal temperatures in heat-stressed dairy cows may reach or exceed 41°C (Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993). Elevations in temperature of this magnitude are not without consequence as pregnancy rates have been shown to decrease with each degree increase in rectal temperature (Ulberg and Burfening, 1967).

Effects of heat stress to reduce fertility are prominent when occurring at or near the time of estrus. Specifically, exposure of superovulated heifers to heat stress sufficient to elevate body temperature ≥41.0°C during estrus increased the proportion of degenerate/retarded embryos recovered from the uterus on d 7 (Putney et al., 1989). Direct effects of elevated maternal temperature to compromise the oocyte are likely real as in vitro exposure to 41.0°C reduces embryonic development (Edwards and Hansen, 1996, 1997; Lawrence et al., 2004) in a manner similar to that reported by Putney et al. (1989).

Oocyte maturation coincides with expression of estrus in the cow and is important to prepare the oocyte for fertilization. Marked changes in the nucleus are collectively referred to as nuclear maturation and are important in reducing number of maternal copies of chromosomes from 4 to 2; that is, the germinal vesicle breaks down, the oocyte becomes transcriptionally quiescent (Hyttel et al., 1997), and resumption of meiosis ensues culminating in extrusion of the first polar body and arrest of maternal chromatin at metaphase II (MII; Picton and Gosden, 1999). Marked changes occurring in the ooplasm are collectively referred to as cytoplasmic maturation and include alterations in the cytoskeleton, redistribution of organelles (Hosoe and Shioya, 1997),

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altered metabolism (Cetica et al., 2001), reductions in mRNA levels (Lequarre et al., 2004), and changes in protein synthetic profiles (Coenen et al., 2004).

During maturation, reductions in protein synthesis by 30 to 50% in heat-stressed oocytes have been reported (Edwards and Hansen, 1996, 1997). Moreover, prolonged exposure of bovine oocytes to 41.0°C resulted in fewer oocytes with a discernible first polar body and may have altered cumulus cell function (Lenz et al., 1983). Taken together, these results suggest that heat-induced perturbations occurring in the ooplasm, nucleus, or cumulus cells may compromise continued development of the bovine oocyte.

Effects of heat stress to compromise the oocyte are temperature (Edwards and Hansen, 1996) and duration dependent (Payton et al., 2004) suggesting that regardless of the specific component altered, effects are not necessarily irreparable. Lawrence et al. (2004) showed that addition of all-*trans* retinol to in vitro maturation (IVM) medium prevented heat-induced reductions in development of oocytes. These results are not likely an in vitro phenomenon as short-term cooling (Ealy et al., 1994) and administration of antioxidants (Aréchiga et al., 1998) have been shown to provide slight improvements in pregnancy rates in heat-stressed cows.

To examine the extent to which an elevated temperature commonly seen in heat-stressed dairy cows (Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993) influences the oocyte as it matures, a series of experiments was conducted to evaluate ability of heat-stressed oocytes to undergo nuclear (i.e., progression to MII) and cytoplasmic (i.e., translocation of cortical granules to the oolemma) maturation. A putative mechanism through which heat stress may reduce oocyte development was also investigated by examining kinetics of nuclear maturation. Results from this study prompted a final experiment to evaluate if earlier insemination in vitro ameliorates heat-induced reductions in oocyte development.

MATERIALS AND METHODS

Materials

All reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Tissue culture medium-199 (**M199**), gentamicin, L-glutamine, nonessential amino acids, and penicillin-streptomycin were purchased from Specialty Media (Phillipsburg, NJ). Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD). Luteinizing hormone was obtained from the USDA (Beltsville, MD). Follicle stimulating hormone was obtained from Vetrepharm Canada Inc. (London, Ontario, Canada). Bovine ovaries were purchased from a commercial abattoir

(*Bos indicus* cattle represented <1% of the females processed) between the months of October and May. Harrogate Genetics International (Harrogate, TN) donated frozen semen. Media for in vitro production of embryos were prepared as previously described (HEPES-TALP, in vitro fertilization (**IVF**)-TALP, and Sperm-TALP (Parrish et al., 1988); potassium simplex optimized medium containing 0.5% BSA, 10 mM glycine, 1 mM L-glutamine, 1× nonessential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin (Biggers et al., 2000).

In Vitro Production of Embryos

In vitro maturation, IVF, and embryo culture were performed as previously described (Lawrence et al., 2004). Cumulus-oocyte complexes (**COC**) were collected from antral follicles (3 to 8 mm) and cultured for 24 h in 500 µL of oocyte maturation medium (**OMM**; M199 with Earle's salts, 10% fetal bovine serum, 50 µg/mL gentamicin, 5.0 µg/mL FSH, 0.3 µg/mL LH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine) in Nunclon 4-well plates (Fisher Scientific, Pittsburgh, PA) at either 38.5 or 41.0°C in 5.5% CO₂ and humidified air as described in the following experiments. After 24 h in culture, COC were fertilized with Percoll-prepared frozen-thawed bovine sperm (400,000 to 600,000 motile/mL). Pooled semen from the same 2 bulls was used for each experimental replicate of IVF. Putative zygotes (**PZ**) were denuded of cumulus and associated spermatozoa (8 to 18 h after IVF) by vortexing for 4 min in HEPES-TALP and then cultured in potassium simplex optimized medium at 38.5°C in 5.5% CO₂, 7.0% O₂, and 87.5% N₂ in humidified air.

Nuclear and Cytoplasmic Maturation in Bovine Oocytes

Nuclear maturation was defined as proportion of oocytes that progressed to MII after culture in OMM for 24 h. Translocation of cortical granules to the oolemma has been used previously as indicator of cytoplasmic maturation (Hosoe and Shioya, 1997). After 24 h in OMM, oocytes were denuded of cumulus. The number of oocytes recovered, lysed, and those having a discernible polar body were recorded. The zona pellucida was removed using 0.5% pronase before fixation (3% paraformaldehyde) and staining (10 µg/mL lens culinaris agglutinin conjugated to fluorescein isothiocyanate and 0.5 µg/mL Hoechst 33342) to evaluate nuclear stage and cortical granule type (type I, large aggregates; type II, aggregates with some dispersion, and type III, complete dispersion of granules) in each oocyte using epifluorescence microscopy (Payton et al., 2004). To validate that cortical granule types (I, II, and III) were

indicative of the extent to which translocation to the oolemma had occurred, a subset of the oocytes previously examined with epifluorescence was also evaluated by confocal microscopy (Wang et al., 1997). In every oocyte examined ($n = 25$ per treatment), confocal was concordant with epifluorescence analysis. Two independent evaluators, blind to treatments, assessed variables of interest.

Experiment 1: Nuclear and Cytoplasmic Maturation of Heat-Stressed Oocytes

On a given day, COC were randomly allocated to treatment in groups of 50 and then cultured at 38.5°C for 24 h (control), 41.0°C (heat stress; **HS**) for the first or last 6 h of IVM; HS 0–6 and HS 18–24, respectively, 41.0°C for the first or last 12 h of IVM (HS 0–12 and HS 12–24, respectively), or 41.0°C for the entire 24 h period of IVM (HS 0–24). After a total of 24 h in OMM, oocytes were kept separate according to treatment, and denuded of cumulus. Membrane-intact oocytes were evaluated for nuclear and cytoplasmic maturation. This experiment was replicated on 5 different days (i.e., one experimental unit (group of oocytes) per treatment per day). Disparity in the total number of oocytes for which data were derived ($n = 205$ to 239 per treatment) vs. total number of COC placed in culture ($n = 250$ per treatment) was due to lysis after denudement and technical issues related to mounting oocytes on glass slides.

Experiment 2: Kinetics of Nuclear Maturation in Heat-Stressed Oocytes

Cumulus-oocyte complexes were cultured in OMM at 38.5 or 41.0°C during the first 12 h of IVM; thereafter, COC were cultured at 38.5°C. At 0 ($n = 41$), 4 ($n = 149$), 8 ($n = 157$), 12 ($n = 146$), 16 ($n = 201$), 18 ($n = 154$), or 21 ($n = 148$) h after placement in OMM, subsets of COC were removed from culture (12 to 16 COC per treatment group except for 0 h), kept separate according to treatment, and then denuded of cumulus. Number of oocytes that had visibly lysed and those with a discernible polar body were recorded. Nuclear stage of oocytes with an intact membrane was determined with Hoechst staining (0.5 $\mu\text{g/mL}$). This experiment was performed on 3 different days with 2 experimental units per treatment per day yielding 6 total experimental units per treatment.

Experiment 3: Early Insemination of Heat-Stressed Oocytes

Results from the first 2 experiments prompted a third to evaluate if earlier insemination in vitro ameliorates

heat-induced reductions in oocyte development. For this study, COC were cultured at 38.5 or 41.0°C (first 12 h) in OMM (30 to 40 per 500 μL of OMM). Sperm were added to control and heat-stressed COC at 19 or 24 h after placement in OMM (2 $\times$ 2 factorial treatment arrangement). Number of PZ recovered and the proportion that had visibly lysed after denudement was recorded. Ability of PZ to cleave (assessed by recording the number of 1-, 2-, 4-, and 8- to 16-cell embryos 72 to 74 h after IVF) and develop to the blastocyst stage (192 to 194 h after IVF) was evaluated. Blastocyst embryos were stained with Hoechst 33342 to enumerate total number of nuclei according to Lawrence et al. (2004). Two independent evaluators blind to treatments assessed variables of interest. This experiment was performed on 4 different days with 1 to 2 experimental units per treatment per day yielding a total of 8 experimental units per treatment (340 to 360 total COC were cultured per treatment group).

Statistical Analyses

Data obtained were analyzed as a randomized block design, blocking on replicate, using generalized linear mixed models (Proc Glimmix) in SAS (2005). This permits random and fixed effect models for normally distributed (number of nuclei) or binomial response data (all other variables). Fixed effects were either 6 timings of heat stress (experiment 1), factorial combinations of 6 times and 2 heat stress treatments (experiment 2), or factorial combinations of 2 fertilization times and 2 heat stress treatments (experiment 3). The 6 times in experiment 2 were split between 2 analyses, 4 to 12 h for metaphase I and 16 to 21 h for metaphase II, because each time only had meaning for one outcome. Because the experimental unit was a group of COC receiving the same treatment within replicate, the replicate by treatment interaction was included in the model as needed to create the correct error term. Data are expressed as least squares means $\pm$ SEM using the inverse link option, and as a proportion of total COC for ease of comparison with results in the literature. Orthogonal contrasts were performed to partition treatment effects in experiment 1, and protected LSD was used to identify individual treatment differences in all experiments.

RESULTS

Experiment 1: Nuclear and Cytoplasmic Maturation of Heat-Stressed Oocytes

Culture at 41.0°C for up to 24 h did not alter number of oocytes that were recovered after cumulus denudement (91.1 to 97.87%; SEM = 1.6; $P > 0.07$), nor did it increase lysis of the oolemma (2.4 to 4.9%; SEM =

Table 1. Nuclear and cytoplasmic maturation of bovine oocytes heat-stressed during in vitro maturation.

Treatment ¹	n	PB ²	MII ³	CG Type I (%)	CG Type II (%)	CG Type III (%)
Control	208	66.2 ^a	83.9	2.7	31.7 ^a	65.7 ^a
HS 0–6	221	55.3 ^b	83.0	1.2	30.7 ^a	68.1 ^a
HS 0–12	205	55.4 ^b	87.1	0.7	19.9 ^b	79.5 ^b
HS 12–24	207	61.4 ^{ab}	89.5	0.6	20.0 ^b	79.4 ^b
HS 18–24	210	65.2 ^a	88.9	0.6	32.2 ^a	67.2 ^a
HS 0–24	239	38.8 ^c	84.5	0.0	9.3 ^c	90.8 ^c
SEM		4.5	2.9	0.6	4.5	4.7
P-value		0.0001	0.54	0.5	0.002	0.002

^{a,b,c}Means with different superscripts differ. The following orthogonal contrasts were performed for cortical granule (CG) Type II: 1) Control vs. HS, $P < 0.02$, 2) HS 0–24 vs. HS 0–6 + HS 0–12, $P < 0.001$, 3) HS 0–6 vs. HS 0–12, $P < 0.01$, 4) HS 0–6 (early) vs. HS 18–24 (late), $P > 0.7$, and 5) HS 0–12 (early) vs. HS 12–24 (late), $P > 0.9$ and CG Type III: 1) Control vs. HS, $P < 0.01$, 2) HS 0–24 vs. HS 0–6 + HS 0–12, $P < 0.001$, 3) HS 0–6 vs. HS 0–12, $P < 0.005$, 4) HS 0–6 (early) vs. HS 18–24 (late), $P > 0.8$, and 5) HS 0–12 (early) vs. HS 12–24 (late), $P > 0.9$.

¹Treatments: cumulus-oocyte complexes were randomly allocated to treatment in groups of 50 and then cultured at 38.5°C for 24 h (control); 41.0°C (heat stress; HS) for the first (HS 0–6) or last (HS 18–24) 6 h of in vitro maturation (IVM); 41.0°C for the first (HS 0–12) or last (HS 12–24) 12 h of IVM; or 41.0°C for the entire 24 h period of IVM (HS 0–24).

²PB = first polar body (%).

³MII = Metaphase II (%).

1.6; $P > 0.6$). Application of heat stress reduced the proportion of oocytes having a discernible polar body. However, this effect was without consequence on nuclear maturation as a similar proportion of heat-stressed oocytes progressed to MII (Table 1). Greater than 70% of oocytes without a discernible polar body were determined to have a nuclear stage of MII (70.6 for control vs. 72.6, 74.4, 82.1, 82.1, and 80.9% for HS 0–6, HS 0–12, HS 12–24, HS 18–24, and HS 0–24, respectively; SEM = 5.2; $P > 0.4$). The nuclear stage of oocytes not progressing to MII was predominantly metaphase I (10.7 vs. 8.1, 10.3, 7.3, 7.1, and 10.8% for control, HS 0–6, HS 0–12, HS 12–24, HS 18–24, and HS 0–24, respectively; SEM = 2.0; $P > 0.50$).

Analysis of a small subset of oocytes before placement in OMM (soon after removal from antral follicles) showed that the majority contained type I (51.4%; SEM = 6.1) or type II (42.9%; SEM = 7.2) cortical granules. Effects of heat stress on cortical granule types were not dependent on when maturing oocytes were cultured at 41.0°C in relation to when resumption of meiosis occurred (i.e., the first or last 6 h of maturation, HS 0–6 (early) vs. HS 18–24 (late), $P > 0.7$ for cortical granule type II and $P > 0.8$ for cortical granule type III, or the first 12 or last 12 h of culture, HS 0–12 (early) vs. HS 12–24 (late), $P > 0.9$ for cortical granule type II and $P > 0.9$ for cortical granule type III; Table 1). Data

Figure 1. A) Micrographs showing typical cortical granules (CG). Panels 1 and 2 are type I (large aggregates); panels 3 and 4 are type II (smaller aggregates with dispersion); and panels 5 and 6 are type III (dispersion). The lower set of panels (2, 4, and 6) show confocal images taken at the equatorial region to assess translocation to the oolemma. B) and C) Proportion of matured oocytes for which CG were of type II and type III, respectively. Cortical granule type was assessed in individual oocytes 24 h after placement into oocyte maturation medium and culture at 38.5°C (control) or 41.0°C (heat stress) for 6 (HS6), 12 (HS12), or 24 (HS24) h. ^{a,b,c}Means with different superscripts differ ($P < 0.05$).

within individual periods were then pooled to examine duration-dependent effects of heat stress on cortical granule types II and III. Culture of oocytes at 41.0°C for 6 h did not alter cortical granule types compared with controls ($P > 0.6$; Figure 1). However, culture at

41.0°C for 12 h reduced the proportion of oocytes having type II ($P < 0.02$), which was coincident with heat-induced increases in type III ($P < 0.01$; Figure 1). Effects of heat stress on cortical granules types II and III were more prominent when oocytes were cultured at 41.0°C for 24 h ($P < 0.01$).

Experiment 2: Kinetics of Nuclear Maturation in Heat-Stressed Oocytes

Culture at 41.0°C did not alter number of oocytes that were recovered after cumulus denudement (99.4 vs. 99.1%; SEM = 2.8; $P > 0.9$; for control and heat stress, respectively), nor did it increase lysis of the oolemma (1.0 vs. 1.6%; SEM = 0.9; $P > 0.9$; for control and heat stress, respectively). Presence of a discernible polar body was first noted in the perivitelline space at 16 h. Culture at 41.0°C reduced number of oocytes that had a discernible polar body 21 h after placement in OMM (64.1 and 41.7% for control and heat stress, respectively; SEM = 5.6; $P < 0.05$).

Ability of control and heat-stressed oocytes to undergo nuclear maturation was assessed by recording the number of oocytes undergoing germinal vesicle breakdown/chromatin condensation and progression to metaphase I (MI) during the first 4, 8, and 12 h of maturation. Proportion of oocytes that progressed from MI to MII was recorded at 16, 18, and 21 h of maturation. Analysis of a small subset of oocytes before culture indicated that 100% had an intact germinal vesicle (GV). Four hours after placement into OMM, a similar proportion of control and heat-stressed oocytes ($P > 0.7$) had an intact GV (72.1 vs. 75.0%; SEM = 5.5) or had already undergone germinal vesicle breakdown/chromatin condensation as evidenced by the presence of condensed chromatin in the apparent absence of the GV (27.9 vs. 25.0%; SEM = 5.5). At 8 h, however, fewer heat-stressed oocytes had an intact GV compared with controls (7.9 vs. 27.7%; SEM = 9; $P < 0.02$). Coincident with this effect was a higher proportion of heat-stressed oocytes at MI ($P < 0.02$; Figure 2). Treatment differences were no longer apparent at 12 h (Figure 2) because this is a time when the majority of control oocytes are expected to be at MI (97.4 vs. 98.5% for control and heat-stressed oocytes, respectively; SEM = 1.7; Figure 2). Continued examination showed a strong tendency at 16 h ($P < 0.07$) and a significant effect at 18 h ($P < 0.01$) for heat stress to increase the proportion of oocytes arrested at MII compared with controls (Figure 2). When the majority of control oocytes had reached MII at 21 h (84.1 vs. 83.5% for control and heat-stressed oocytes, respectively; SEM = 5.2), differences related to maturation temperature were no longer apparent (Figure 2).

Figure 2. Proportion of oocytes at metaphase I and metaphase II at 0, 4, 8, 12, 16, 18, and 21 h after culture in oocyte maturation medium at 38.5 (white bar) and 41.0°C (black bar). Nuclear stage was determined with Hoechst staining (0.5 µg/mL).

Experiment 3: Early Insemination of Heat-Stressed Oocytes

Oocyte culture at 41.0°C did not alter proportion of PZ recovered after removal of associated cumulus and spermatozoa (93.8 and 93.1% for 38.5 and 41.0°C, respectively; SEM = 1.0; $P > 0.5$) or those that had visibly lysed (1.1 and 1.4% for 38.5 and 41.0°C, respectively; SEM = 0.4; $P > 0.6$). Ability of PZ to cleave (66.2 and 61.9% for 38.5 and 41.0°C, respectively; SEM = 16.9; $P > 0.3$) and develop to 8- to 16-cell stage (51.6 and 52.5% for 38.5 and 41.0°C, respectively; SEM = 19.1; $P > 0.8$) was similar regardless of maturation temperature. In addition, blastocyst-stage embryos derived from control or heat-stressed oocytes contained similar numbers of nuclei (75.5 and 78.0 for 38.5 and 41.0°C, respectively, SEM = 5.8; $P > 0.7$).

Addition of sperm at 19 vs. 24 h after placement of COC in OMM did not alter the proportion of PZ recovered after removal of associated cumulus and spermatozoa (94.6 and 92.1% for 19 and 24 h, respectively; SEM = 0.9; $P < 0.1$) or those that had visibly lysed (1.2 and 1.3% for 19 and 24 h, respectively; SEM = 0.4; $P > 0.8$). Ability of PZ to cleave (63.3 and 64.9% for 19 and 24 h, respectively; SEM = 16.9; $P > 0.6$) or develop to the 8- to 16-cell stage (53.0 and 51.0% for 19 and 24 h, respectively; SEM = 19.1; $P > 0.6$) was similar regardless of insemination time. Number of nuclei contained within blastocyst-stage embryos derived from oocytes fertilized at 19 vs. 24 h was similar (75.3 and 78.1 for 19 and 24 h, respectively; SEM = 5.8; $P > 0.7$).

Figure 3. The proportion of oocytes that developed to the blastocyst stage after in vitro maturation at 38.5 or 41.0°C; sperm were added at 19 or 24 h after placement into oocyte maturation medium. Blastocyst development was assessed at 192 to 194 h after in vitro fertilization (IVF). Asterisk (*) indicates a temperature $\times$ time of IVF interaction; SEM = 5.0; $P < 0.05$.

A significant interaction of temperature $\times$ insemination time was noted when evaluating ability of oocytes to develop to blastocyst (SEM = 5.0; $P < 0.05$; Figure 3). Specifically, when sperm were added at 19 vs. 24 h after placement of COC in OMM, heat stress did not compromise ability of oocytes to develop to blastocyst compared with controls.

DISCUSSION

The described studies provide new insight into specific processes within the oocyte that are altered by direct application of heat stress as the oocyte undergoes in vitro maturation. Culture of oocytes at 41.0°C did not compromise ability of the oocyte to undergo nuclear (i.e., progression to MII) or cytoplasmic maturation (i.e., cortical granule translocation to the oolemma). However, heat stress did reduce the time required for these critical processes (i.e., heat-stressed oocytes matured faster than controls). Moreover, IVF of heat-stressed oocytes 5 h earlier than usual ameliorated negative effects of heat stress to reduce embryo development after fertilization. Taken together, these data provide strong supportive evidence that a major effect of a physiologically relevant elevated temperature (Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993) may be to induce premature aging of the maturing oocyte.

In support of this, general effects of aging vs. heat stress of oocytes are comparable. Oocyte aging reduces developmental competence (reviewed by Fissore et al., 2002) as does heat stress (Edwards and Hansen, 1996, 1997; Lawrence et al., 2004). Changes in maternal transcripts and proteins have been described in aged (Xu et al., 1997) as well as heat-stressed oocytes (Edwards and Hansen, 1996; Payton and Edwards, 2005). In extreme cases, aged (reviewed by Fissore et al., 2002) and heat-stressed (Roth and Hansen, 2004) oocytes may undergo apoptosis.

The cumulative effect of heat stress to induce premature aging in the bovine oocyte is concerning because the fertile life span of in vivo "controls" is 8 to 10 h (Hunter, 1985). Fertile life span in vitro may be shorter because fertilization at 30 to 32 h after placement of oocytes into maturation medium reduces development to blastocyst (our unpublished data; Susko-Parrish et al., 1991). In this study, general effects of heat stress were to hasten oocyte maturation by 4 to 6 h. In this case, fertilization of heat-stressed oocytes at the same time as controls (24 h after placement into OMM) would result in the fertilization of an "aged" egg. Subsequent efforts of our laboratory have shown that development of heat-stressed oocytes is comparable to the development of nonheat-stressed oocytes fertilized at 30 h (our unpublished data).

Although it is possible that in vitro maturation might increase the sensitivity of maturing oocytes to heat stress, comparable results among in vitro (41.0°C culture temperature; Edwards and Hansen, 1996, 1997; Lawrence et al., 2004) and in vivo (41.0°C rectal temperatures; Putney et al., 1989) studies showing similar reductions in embryo development after application of heat stress during oocyte maturation suggest the relevance of our in vitro model. Similarity of in vivo vs. in vitro heat stress to reduce developmental competence of maturing oocytes may be attributable in part to the interconnectedness of cumulus cells surrounding the oocyte such that a similar microenvironment may be maintained after removal from the follicle. Because surrounding cumulus cells are intimately connected with the oocyte as they project through the zona pellucida and oolemma to establish an intercellular bidirectional form of communication (Kruip et al., 1983), some of the effects of heat stress to reduce oocyte development may be mediated through the cumulus. Lenz et al. (1983) showed that prolonged exposure to a physiologically relevant elevated temperature altered cumulus function because culture at 41.0°C for 24 h reduced hyaluronic acid production.

The time required for the bovine oocyte to undergo maturation in vivo is finite (24 to 26 h; Kruip et al., 1983) and is coincident with estrus in the cow. The

preovulatory surge of LH stimulates the dictyate oocyte (arrested in prophase I) to resume meiosis. In most cases, the oocyte has completed maturation before ovulation thereby precluding the use of earlier insemination *in vivo* to obviate heat-induced reductions in oocyte development. If the cumulative effect of elevations in maternal temperature is to hasten maturation of the oocyte *in vivo*, fertilization during fertile life span will be dependent on the development of therapeutic strategies designed to hasten ovulation in heat-stressed lactating dairy cows.

One possible strategy may be through the use of GnRH. Kaim et al. (2003) reported that the interval from the onset of estrus to ovulation was reduced by 3 to 5 h when GnRH was administered immediately after the onset of estrus. In the same study, GnRH administered (10 µg of Buserelin) within 3 h of detected estrus increased conception rates of primiparous cows experiencing summer heat stress (Kaim et al., 2003). Another study reported by Ullah et al. (1996) showed that GnRH (100 µg of Factrel) administration at observed estrus improved 45 pregnancy rates in lactating Holsteins experiencing summer heat stress. Researchers speculated that improved fertility after GnRH administration may have been through enhancement of the magnitude of the LH surge (Kaim et al., 2003) and subsequent progesterone concentrations (Ullah et al., 1996) because the magnitude of the preovulatory surge of LH (Madan and Johnson, 1973) and progesterone concentrations (Howell et al., 1994) are often reduced in dairy cows experiencing chronic heat stress. However, it is interesting to speculate that improved pregnancy rates may have also been due to GnRH-induced reductions in the time interval from estrus to ovulation thereby maximizing fertilization of the oocyte during its fertile life span. In support of this, our data indicate effects of heat stress hasten *in vitro* oocyte maturation by 4 to 6 h. Moreover, *in vitro* fertilization of heat-stressed oocytes 5 h earlier than usual resulted in development comparable to controls. However, ineffectiveness of GnRH administration to improve fertility of multiparous cows (Kaim et al., 2003) along with limited animal numbers in both studies (Ullah et al., 1996; Kaim et al., 2003) precludes further inference.

It is important to note that heat-induced alterations in the maturing oocyte are likely one of many factors that contribute to infertility in heat-stressed dairy cows. This may explain in part why only slight improvements in fertility have been obtained so far. Clearly, oocytes at other stages of development (i.e., while contained within growing antral follicles; Rocha et al., 1998), and early embryos (Ealy et al., 1993) as well as other reproductive processes are also susceptible to negative effects of heat stress.

The effect of heat stress in the current study to reduce number of oocytes with a discernible polar body was in agreement with Lenz et al. (1983). The significance of these findings, however, remains unclear because Hoechst staining indicated that a similar number of control and heat-stressed oocytes progressed to MII. In other species, the first polar body is usually lost within several hours presumably by cytolysis (Donahue, 1973). It is plausible that heat stress may have hastened polar body degeneration in a manner similar to that reported for aged oocytes (Donahue, 1973).

CONCLUSIONS

Continued efforts to identify components of the oocyte that are affected by heat stress and consequences thereof are important as the matured oocyte contributes more than 99% of the cytoplasm and half of the genetic material to the embryo. Results showing that earlier insemination *in vitro* prevented heat-induced reductions in embryo development suggest that effects of physiologically relevant elevated temperatures on the oocyte are likely not irreparable. However, because the bovine oocyte undergoes nuclear maturation and the majority of cytoplasmic maturation while contained within the Graafian follicle before ovulation, additional studies are needed to evaluate the extent to which alterations in the timing of *in vitro* fertilization of heat-stressed oocytes affects embryo development, and to determine the extent to which elevated maternal temperatures alter specific components in the oocyte matured *in vivo*.

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