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Susceptibility of Bovine Germinal Vesicle-Stage Oocytes from Antral Follicles to Direct Effects of Heat Stress In Vitro[1]

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ABSTRACT

Delineation of maternal versus direct effects of heat stress in reducing development at the germinal vesicle (GV) stage is challenging, because oocytes spontaneously resume meiosis after removal from antral follicles. The use of *S*-roscovitine (inhibitor of p34^{cdc2}/cyclin B kinase) to hold bovine oocytes at the GV stage without compromising early embryo development was previously validated in our laboratory. The objective of the present study was to assess the direct effects of an elevated temperature commonly seen in heat-stressed dairy cows on cumulus-oocyte complexes (COCs) held at the GV stage using 50 μM *S*-roscovitine. During roscovitine culture, GV-stage COCs (antral follicle diameter, 3–8 mm) were cultured at 38.5 or 41°C. Thereafter, oocytes were removed from roscovitine medium and allowed to undergo in vitro maturation, fertilization, and culture. Zona pellucida hardening (solubility to 0.5% pronase), nuclear stage (Hoechst 33342), cortical granule type (*lens culinaris* agglutinin-fluorescein isothiocyanate [FITC]), and early embryo development were evaluated. Culture of GV-stage COCs at 41°C increased the proportion that had type III cortical granules and reduced the proportion that progressed to metaphase II after in vitro maturation. Effects of 41°C on zona pellucida hardening, fertilization (penetration, sperm per oocyte, pronuclear formation, and monospermic and putative embryos), and cleavage of putative zygotes were not noted. However, culture of GV-stage COCs at 41°C for 6 h decreased the proportion of 8- to 16-cell embryos, whereas 41°C for 12 h reduced blastocyst development. In summary, antral follicle COCs are susceptible to direct effects of elevated body temperature, which may account in part for reduced fertility in heat-stressed cows.

environment, gamete biology, meiosis, oocyte development, stress

INTRODUCTION

In the bovine, germinal vesicle (GV)-stage cumulus-oocyte complexes (COCs) reside in the antral follicles for as long as 42 days [1] and, thus, may be exposed to daily fluctuations of elevated body temperatures exceeding 41°C [2–9] during environmental heat stress. Although intuitive, the direct effect of elevated body temperature in compromising GV-stage COCs remains unclear. Rocha et al. [10] reported reduced blastocyst development of GV-stage COCs derived from ovaries removed from heat-stressed cows. Similar observations have been noted in temperate [11] and subtropical [12] regions of the United States. Whether seasonal reductions in embryonic development are a consequence of altered maternal/follicle environment, a result of direct effects of elevated body temperature on the GV-stage COCs, or both has yet to be delineated.

Efforts to isolate GV-stage COCs from the maternal/follicle environment to assess the direct effects of a physiologically relevant elevated temperature in vitro have been hampered, because the oocyte spontaneously resumes meiosis after removal from an antral follicle (i.e., the germinal vesicle breaks down, and nuclear and cytoplasmic maturation ensue [13]), thereby precluding in vitro studies. Mermillod et al. [14], however, reported the effectiveness of the *R*-enantiomer of roscovitine (inhibitor of p34^{cdc2}/cyclin B kinase) to hold bovine COCs at the GV stage for 24 h after removal from antral follicles without compromising early embryo development. We extended these findings by showing that 50 μM of the *S*-enantiomer of roscovitine inhibits gonadotropin-induced cumulus expansion and maintains more than 90% of bovine COCs at the GV stage for as long as 48 h without compromising nuclear maturation, fertilization, or early embryo development [15–17].

In an attempt to determine the extent to which an elevated temperature commonly seen in heat-stressed dairy cows [2–9] has a direct impact on the physiology of GV-stage COCs, we conducted a series of studies. The approach was to isolate COCs from antral follicles (diameter, 3–8 mm), use roscovitine to maintain COCs at the GV stage, culture oocytes at a control or physiologically relevant elevated temperature, and then assess the direct effects of heat stress on various aspects of continued development. Developmental end points included nuclear and cytoplasmic maturation, fertilization, and early embryo development. Putative mechanisms through which heat stress may alter oocyte development were investigated by examining zona pellucida (ZP) hardening, translocation of cortical granules to the oolemma (cytoplasmic maturation), and functional competence of the ooplasm to remodel sperm chromatin (pronuclear formation).

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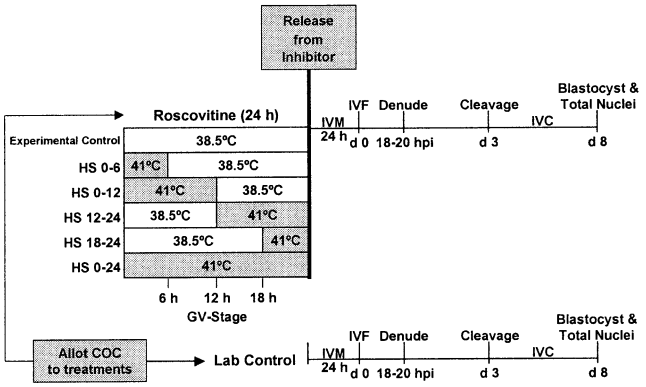

FIG. 1. Schematic depicting design of experiment 1 (embryo development of GV-stage bovine oocytes after culture at 41°C).

Use of a pharmacological inhibitor to hold COCs at the GV stage in vitro after removal from antral follicles eliminates the effects of heat stress on the maternal/follicle environment, thereby providing a novel approach to assess direct effects of an elevated temperature. Determining the extent to which GV-stage COCs are susceptible to direct effects of elevated body temperatures is a necessary first step toward the development of management strategies to alleviate the effects of heat stress on reproductive performance.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture medium 199 (TCM-199), gentamicin, L-glutamine, and penicillin-streptomycin were purchased from Specialty Media (Phillipsburg, NJ). Bovine ovaries were purchased from a commercial abattoir (Gaffney, SC). Roscovitine [2-(S)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] was purchased from Calbiochem (La Jolla, CA) and solubilized in dimethyl sulfoxide (DMSO) before freezing at −20°C as a 10 mM stock. Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). Luteinizing hormone was obtained from the U.S. Department of Agriculture (Beltsville, MD). Follicle-stimulating hormone was obtained from Vetrepharm Canada, Inc. (London, ON, Canada). Media for in vitro production of embryos were prepared as previously described: Hepes-Tyrode albumin lactate pyruvate (TALP), in vitro fertilization (IVF)-TALP, and sperm-TALP [18] as well as potassium simplex optimized medium (KSOM) containing 0.5% (w/v) BSA, 10 mM glycine, 1 mM L-glutamine, 1× nonessential amino acids, 50 U/ml of penicillin, and 50 μg/ml of streptomycin [19].

In Vitro Maturation, IVF, and In Vitro Culture of Embryos

In vitro maturation (IVM), IVF, and in vitro culture (IVC) of embryos were performed as previously described [20] with a few modifications. Cumulus-oocyte complexes were collected from antral follicles (diameter, 3–8 mm) and cultured for 24 h (30–40 COCs per 500 μl of oocyte maturation medium [OMM; TCM-199 with Earle salts, 10% [v/v] FBS, 50 μg/ml of gentamicin, 5.0 μg/ml of FSH, 0.3 μg/ml of LH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine) in Nunclon four-well plates (Fisher Scientific, Pittsburgh, PA) at 38.5°C in 5.5% CO_2 and humidified air. After 22.5–24 h, COCs were fertilized (750 000 total sperm/ml) with Percoll-prepared, frozen-thawed semen pooled from two different bulls. The same two bulls were used for each experimental replicate. Putative zygotes were denuded of cumulus and associated spermatozoa at 18- to 20-h postinsemination (hpi) by vortexing (4 min) and then cultured in KSOM pre-equilibrated in 5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C in humidified air. Ability of putative zygotes to cleave (assessed by recording the number of 1-, 2-, 4-, and 8- to 16-cell embryos present at 72–75 hpi) and to develop to the blastocyst stage was recorded on Day 3 and Day 8 post-IVF, respectively. The total number of nuclei within blastocysts (Day 8, 186–193 hpi) was determined by staining with Hoechst 33342 (10 μg/ml).

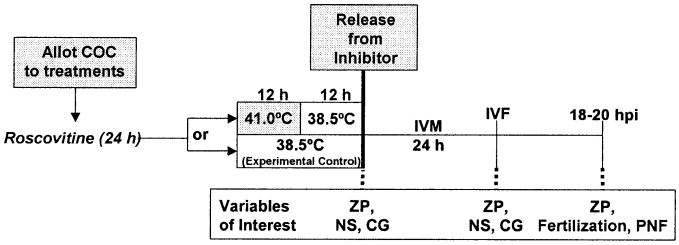

FIG. 2. Schematic depicting the design of experiment 2 along with variables of interest.

Assessment of ZP Hardening, Nuclear Stage, Cortical Granule Type, and Fertilization

Hardening of the ZP was estimated as previously described [21], with the exception that 0.5% (w/v) pronase was used. Nuclear stage and cortical granule types (type I, aggregates; type II, aggregates with some dispersion; and type III, dispersion of granules) were evaluated as previously described [22]. In brief, the ZP was removed using 0.5% (w/v) pronase, and oocytes were fixed in 3% (w/v) paraformaldehyde, washed with blocking solution (Dulbecco PBS containing 1% [w/v] BSA, 10 mM glycine, and 0.05% [v/v] Triton X-100), and then stained with 10 μg/ml *lens culinaris* agglutinin conjugated to fluorescein isothiocyanate (FITC) and 0.5 μg/ml of Hoechst 33342 [22, 23]. Fertilization of oocytes was assessed at 18–20 hpi by removing the ZP (0.5% [w/v] pronase) and staining putative zygotes with Hoechst 33342 (0.5 μg/ml). Oocytes and presumptive zygotes were evaluated with a Nikon Eclipse TE300 (4′,6′-diamidino-2-phenylindole filter: excites at 330–380 nm; FITC filter: excites at 450–490 nm) by at least two independent evaluators who were uninformed regarding the treatment used. Variables of interest included penetration (oocytes penetrated by at least one sperm), sperm per oocyte, pronuclear formation (penetrated oocytes having one or more pronuclei), and monospermic (oocytes with one pronucleus and one sperm head either condensed, swollen, or as a pronucleus) and putative embryos (monospermic embryos having two pronuclei).

Experiment 1: Embryo Development after Heat Stress of GV-Stage Oocytes

Cumulus-oocyte complexes were cultured in 50 μM roscovitine medium (TCM-199 containing 10% [v/v] FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml of penicillin, and 50 μg/ml of streptomycin) at 38.5°C for 24 h (experimental control; n = 202) or 41°C for the first or last 6 h of a 24-h culture period (HS 0–6 [n = 310] or HS 18–24 [n = 265]), at 41°C for the first or last 12 h of culture (HS 0–12 [n = 315] or HS 12–24 [n = 315]), or at 41°C for 24 h (HS 0–24 [n = 350]) in 5.5% CO_2 and humidified air (Fig. 1). In addition, a group of COCs was placed in OMM to undergo IVM (lab control [n = 204]) to assess the effects of 50 μM roscovitine solubilized in DMSO (0.5% final concentration in roscovitine medium). After a total of 24 h, COCs were removed from the roscovitine medium and allowed to undergo IVM, IVF, and IVC similar to the lab control group. Ability of putative zygotes to cleave and develop to the blastocyst stage was recorded. In addition, the total number of nuclei within blastocysts was determined. This experiment was replicated on seven different days.

Experiment 2: Effects of Heat Stress on GV-Stage Oocytes to Alter ZP Hardening, Nuclear Stage, Cortical Granule Type, and Fertilization

Cumulus-oocyte complexes were cultured in 50 μM roscovitine for 24 h at 38.5°C (experimental control) or 41.0°C (heat stress; 12 h followed by 38.5°C for the remaining 12 h) (Fig. 2). After 24 h, COCs were removed from the roscovitine medium and allowed to undergo IVM and IVF. The number of oocytes recovered and lysed after denuding, ZP hardening, nuclear stage, cortical granule type, and fertilization was recorded. Hardening of the ZP was assessed before IVM (immediately after removal of the COCs from antral follicles or after culture in roscovitine medium), after IVM, and after IVF (18–20 hpi) (Fig. 2). Nuclear stage and cortical granule type of the oocytes were assessed before and after IVM. Fertilization of COCs was assessed 18–20 hpi by examining penetration, number of sperm per oocyte, pronuclear formation (indirect assessment of the functional competence of the heat-stressed ooplasm to remodel sperm

chromatin), and monospermic and putative embryos. This experiment was conducted concurrently with an experiment described by Edwards et al. [17], which showed that culture of COCs in 50 μM roscovitine for 24 h did not alter ZP hardening, maintained greater than 90% of oocytes at the GV stage, and did not compromise nuclear or cytoplasmic maturation or fertilization (penetration, number of sperm/oocyte, median number of sperm, and proportion monospermic or putative embryos), because values were at least comparable to those of a lab control (oocytes were not cultured in roscovitine but were allowed to undergo IVM immediately after removal from antral follicles). This experiment was replicated on three separate days. Data generated from culture of oocytes in roscovitine for 24 h at 38.5°C [17] were utilized as the experimental control for this experiment. Use of an identical control group in the two separate studies maximized the use of available oocytes and ensured that necessary controls were included to draw sound conclusions.

Statistical Analyses

To determine the effects of heat stress on GV-stage oocytes, data were analyzed comparing heat-stress treatments to the experimental control (COCs cultured in 50 μM roscovitine for 24 h at 38.5°C). In experiment 1, embryo development data were analyzed as a randomized, incomplete block design (in three of the seven experimental replicates, one treatment was inadvertently omitted) using mixed models of SAS [24]. An additional analysis was conducted comparing the lab and the experimental control to assess the effects of 50 μM roscovitine solubilized in DMSO on oocyte development. Least-square means ± SEM were expressed as a proportion of the total oocytes or putative zygotes for ease of comparison to data in the literature. A linear contrast was conducted to compare the average of the experimental control and HS 0–6 to HS 0–12 groups. Polynomial contrasts were conducted to examine linear and quadratic relationships of the experimental control, HS 0–12, and HS 0–24 groups.

In experiment 2, continuous data, including ZP hardening and sperm per oocyte, were analyzed as a randomized block using mixed models of SAS [24] after testing for normality (Shapiro-Wilk, >0.90). Nonnormal data were log (ZP hardening) or rank (sperm per oocyte) transformed. Number of sperm per oocyte was skewed in one experimental replicate, so the median number of sperm per oocyte was also examined. Proportional data, including sperm penetration, pronuclear formation, and monospermic and putative embryos, were analyzed using the Fisher exact test.

RESULTS

Experiment 1: Embryo Development after Heat Stress of GV-Stage Oocytes

Heat stress did not increase oocyte lysis (8.0–11.1%, $P > 0.95$, SEM = 2.7). Ability of GV-stage COCs to cleave after IVM and IVF was not compromised by heat stress (Fig. 3). However, heat stress for as few as 6 h increased the number of 4-cell ($P < 0.006$) (Fig. 3) and decreased the number of 8- to 16-cell ($P < 0.0008$) embryos. The proportion of 1- and 2-cell embryos was similar regardless of treatment (data not shown). A linear contrast comparing the average of the experimental control and the HS 0–6 groups to the HS 0–12 group showed that heat stress for 12 h reduced development to the blastocyst stage ($P < 0.006$). Effects of heat stress to reduce oocyte development increased as the duration of the 41°C treatment increased ($P < 0.04$). Reductions in oocyte development to the 8- to 16-cell ($P < 0.0008$) and blastocyst ($P < 0.008$) stages were similar regardless of when the heat stress was applied (i.e., the first or last part of the 24-h culture period) (Fig. 3). The number of nuclei contained within blastocyst-stage embryos varied (67.8, 65.6, 82.4, 69.0, 79.0, and 59.5 for the experimental control, HS 0–6, HS 0–12, HS 12–24, HS 18–24, and HS 0–24 groups, respectively; $P < 0.04$, SEM = 6.6). No treatment group differed from the experimental control.

For the lab and experimental controls, cleavage (80.5% and 73.4%, respectively; $P > 0.40$, SEM = 5.8), development to 8- to 16-cell stage (50.4% and 52.6%, respectively; $P > 0.75$, SEM = 4.6), and development to the blastocyst stage (29.7% and 24.8%, respectively; $P > 0.35$, SEM = 3.2) were similar, indicating minimal effects of the roscovitine medium containing 0.5% (v/v) DMSO on oocyte development.

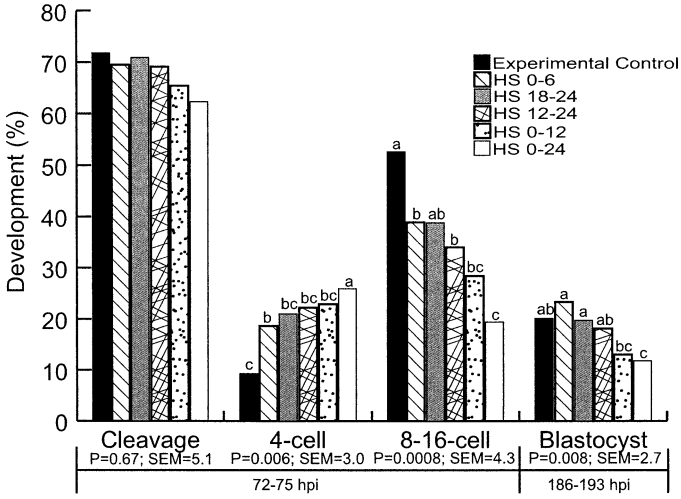

FIG. 3. Embryo development (%) after bovine oocytes at the GV stage were exposed to heat stress. The proportion of 1- and 2-cell embryos is not shown. Superscripts indicate differences among bars within developmental stage.

Experiment 2: Effects of Heat Stress on GV-Stage Oocytes to Alter ZP Hardening, Nuclear Stage, Cortical Granule Type, and Fertilization

Culture of GV-stage COCs at 41°C did not alter ZP hardening (202.9–237.0 sec, $P > 0.90$, SEM = 25.3) before or after IVM or after IVF. Heat stress increased the proportion of GV-stage oocytes that had type III cortical granules ($P < 0.002$) (Table 1). Culture of GV-stage COCs at 41°C for 12 h reduced the proportion that progressed to metaphase II after IVM ($P < 0.053$) (Table 2). Exposure of GV-stage COCs to 41°C had few, if any, effects on fertilization or functional competence of the ooplasm to remodel sperm nuclei, because penetration and pronuclear formation were similar in control and heat-stressed oocytes (Table 3). Heat stress may have increased the number of sperm per oocyte (1.60 and 1.78 for experimental control and heat stress, respectively; $P = 0.05$); however, the median number of sperm per oocyte was 1.00 regardless of treatment ($P > 0.15$).

DISCUSSION

The two experiments of the present study are significant, because they show that a physiologically relevant elevated temperature compromises continued development of antral follicle, GV-stage COCs in a direct manner. The consequences of direct exposure of GV-stage COCs to heat stress included reduced ability to undergo nuclear maturation and embryo development. Premature translocation of the cortical granules to the oolemma indicates that heat stress alters oocyte physiology through induction and/or acceleration of processes occurring within the ooplasm.

Roscovitine treatment may increase the sensitivity of GV-stage oocytes to an elevated temperature in vitro. However, reductions in the development of GV-stage COCs after heat stress were comparable to those reported previously (i.e., when heat stress was applied to GV-stage COCs while contained within the ovarian follicles of heat-stressed cows

TABLE 1. Cortical granule (CG) types before and after IVM in bovine oocytes heat stressed at the GV stage.

	Before IVM				After IVM			
		CG Types (%)			Metaphase II	CG Types (%)		
Treatments	GV (n)	I	II	III	(n)	I	II	III
Experimental control	120	30.0	65.0	5.0[a]	113	3.5	24.8	71.7
Heat stress	121	18.2	63.6	18.2[b]	102	1.0	32.4	66.7
P				0.0018				0.69

[a,b] Means differ within column.

[10–12, 25]). Moreover, duration-dependent effects of heat stress to reduce development of GV-stage COCs were comparable to heat-induced reductions in the development of maturing oocytes [20, 26, 27]. Taken together, these results suggest that effects of 41°C to reduce oocyte development are real and not an artifact of in vitro conditions.

Effects of heat stress to reduce the development of GV-stage COCs were not dependent on when the elevated temperature was administered in relation to when the resumption of meiosis occurred (i.e., the first or last 6 or 12 h of culture). This is not the case for maturing oocytes, because reductions in development have been noted only when heat stress was applied during the first 12 h of maturation [20]. This disparity of results may reflect inherent differences in GV-stage versus maturing COCs (i.e., GV intact vs. broken down [28], cortical granules in aggregates vs. dispersal during translocation to the oolemma [22, 26], and differences in mRNA levels [29], protein synthetic profiles [20, 30], M-phase promoting factor and mitogen-activated protein kinase activity [28], and metabolism [31] in GV-stage COCs vs. maturing COCs, respectively).

Exposure of GV-stage COCs to 41°C for as few as 6 h retarded development of cleaved embryos. Only after exposure to 41°C for 12 h was reduced blastocyst development observed. Ability of heat stress to retard versus inhibit oocyte development suggests that elevated temperature may alter maternal pools of mRNA and/or efficiency of translation. In the bovine, transcriptional activity of GV-stage oocytes residing within late-tertiary follicles (diameter, >3 mm) is minimal [32] even when exposed to noxious environmental stimuli. Edwards and Hansen [20] showed that bovine oocytes that were heat-shocked immediately after removal from antral follicles (diameter, 3–8 mm) were incapable of mounting a heat shock response. Therefore, maternal pools of mRNA, previously accumulated during the growth phase [33–36], must be maintained to provide the necessary transcripts for protein synthesis before and after resumption of meiosis and up to the embryonic genome activation occurring at the late 4-cell to the 8- to 16-cell stages in the bovine [37]. Literature describing mechanisms involved in the maintenance of maternal pools of mRNA and how elevated temperature might affect those pools remains scant. In other cell types, however, elevated temperature does not necessarily induce degradation of RNA but, instead, alters translation such that heat shock protein (HSP) mRNA is preferentially translated [38].

TABLE 2. Nuclear stage of bovine oocytes before and after IVM when heat stressed at the GV stage.

	Before IVM		After IVM	
	Oocytes	GV	Oocytes	Metaphase II
Treatments	(n)	(%)	(n)	(%)
Experimental control	130	93.9	124	91.9[a]
Heat stress	128	96.1	122	83.6[b]
P		0.57		0.0525

[a,b] Means differ within column.

Reduced synthesis of intracellular proteins has been observed in heat-stressed oocytes. Curci et al. [39] showed that murine oocytes held at the GV-stage using dibutyryl cAMP exposed to 43°C showed reduced de novo synthesis of intracellular proteins. Moreover, heat-induced reductions in protein synthesis increased as the duration of the heat shock increased. Similar effects have been described in bovine oocytes cultured at a more physiologically relevant elevated temperature (2.5–3.5°C above normal body temperature) immediately before (intact GV) and after resumption of meiosis [20]. Specific proteins in the oocyte altered by heat stress are largely unknown. Cognate thermoprotective molecules, such as HSP70 molecules, may be decreased [40], whereas glutathione content may be increased [41], suggesting possible increases in free radical production. The possibility for heat stress to denature proteins and/or damage the maternal chromatin is unlikely given that exposure to supraphysiological temperatures may be required [42, 43].

Heat-induced alterations in cortical granule types in GV-stage (present study) and maturing COCs [26] are coincident with reduced development and may be a consequence of alterations in specific components comprising the cytoskeleton. Translocation of cortical granules to the oolemma after resumption of meiosis occurs via actin microfilaments [44]. Most recently, it has been reported that microfilaments are altered in porcine oocytes [45] and in bovine 2-cell embryos [46] after application of heat stress. To our knowledge, whether similar heat-induced alterations are occurring in antral GV-stage COCs remains to be determined.

Nonetheless, similar effects of heat stress to alter oocyte development [20, 27, 40], protein synthesis [20, 39, 40], and translocation of the cortical granules to the oolemma (present study) [26] in both GV-stage and maturing oocytes suggest common mechanisms. Moreover, altered kinetics within the ooplasm (premature or accelerated translocation of the cortical granules to the oolemma) and possible increases in free radical production [41] suggest that a major effect of heat stress may be to ''age'' the oocyte. Like heat stress, aging reduces developmental competence of the oocyte [47] and is associated with increased free radical production [48].

Some of the effects of heat stress to reduce oocyte development may be to affect the surrounding cumulus. Lenz et al. [49] showed that prolonged exposure to a physiologically relevant elevated temperature altered cumulus function, because culture at 41°C for 24 h reduced hyaluronic acid production. Heat-induced alterations in cumulus cells may not be without consequence, because they project through the ZP and oolemma to establish an intercellular, bidirectional form of communication [50] that provides

TABLE 3. Fertilization and pronuclear formation in bovine oocytes heat stressed at the GV stage.

Treatments	Oocytes (n)	Penetration (%)	Pronuclear formation (%)	Monospermy (%)	Putative embryos (%)
Experimental control	128	71.9	70.7	67.4	48.4
Heat stress	122	72.1	71.6	58.0	41.8
P		1.00	1.00	0.22	0.31

metabolic and protective roles before and during cytoplasmic maturation [51].

In conclusion, we have shown that antral follicle GV-stage COCs are susceptible to direct effects of a physiologically relevant elevated temperature. Perturbations in the ooplasm were coincident with reductions in oocyte development. Future efforts will focus on identifying the cellular and molecular components of the COC that are altered by heat stress to account for reduced development.

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