Disparate consequences of heat stress exposure during meiotic maturation: embryo development after chemical activation vs fertilization of bovine oocytes

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Abstract

Consequences of heat stress exposure during the first 12 h of meiotic maturation differed depending on how and when bovine oocytes were activated. If heat-stressed oocytes underwent IVF at ~24 h, blastocyst development was less than for respective controls and similar to that obtained for nonheat-stressed oocytes undergoing IVF at 30 h (i.e. slightly aged). In contrast, if heat-stressed oocytes underwent chemical activation with ionomycin/6-dimethylaminopurine at 24 h, blastocyst development was not only higher than respective controls, but also equivalent to development obtained after activation of nonheat-stressed oocytes at 30 h. Developmental differences in chemically activated vs IVF-derived embryos were not related to fertilization failure or gross alterations in cytoskeletal components. Rather, ionomycin-induced calcium release and MAP kinase activity were less in heat-stressed oocytes. While underlying mechanisms are multifactorial, ability to obtain equivalent or higher development after parthenogenetic activation demonstrates that oocytes experiencing heat stress during the first 12 h of meiotic maturation have the necessary components to develop to the blastocyst stage, but fail to do so after fertilization.

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Introduction

Heat stress is an important economic problem in agricultural animals in large part because of heat-induced reductions in fertility. Reduced fertility is problematic when females experience hyperthermia near the time period of breeding (bovine, Stott & Williams 1962; ovine, Dutt 1963; porcine, Tompkins *et al.* 1967). Extensive research in the bovine has demonstrated direct consequences of elevated temperature on the maturing oocyte. For instance, Putney *et al.* (1989) reported reductions in embryonic viability when rectal temperatures were elevated during estrus. Similar consequences were noted after direct application of physiologically relevant elevated temperatures to oocytes undergoing maturation *in vitro* (Edwards & Hansen 1996, Edwards *et al.* 2005, Schrock *et al.* 2007).

Marked changes occurring in the nucleus and ooplasm may explain why maturing oocytes are susceptible to direct effects of elevated body temperatures. Although the magnitude of response in bovine oocytes depends on severity and duration (Edwards &

Hansen 1996, Payton *et al.* 2004), consequences after direct application include heat-induced reductions in embryonic development by as much as 42–65% (Edwards & Hansen 1996, Roth & Hansen 2004a, Edwards *et al.* 2005, Schrock *et al.* 2007) and yet unidentified effects that carry over to alter competence of otherwise morphologically normal embryos (Edwards *et al.* 2009).

Although underlying mechanism(s) remain unclear, collective findings in two different species document effects of physiologically relevant heat stress exposures during meiotic maturation to hasten developmentally important processes. For instance, Baumgartner & Chrisman (1981) reported a higher incidence of murine oocytes having a bicellular classification (i.e. oocytes contained two cells, with one presumed to be the first polar body) after *in vivo* heat stress exposure. This is consistent with results reported by Kim *et al.* (2002) suggesting that a short-term heat shock permissive of meiotic maturation in murine oocytes accelerated germinal vesicle breakdown. After direct application of heat stress, Edwards *et al.* (2005) demonstrated that more

bovine oocytes progressed to metaphase I by 8 h of *in vitro* maturation (hIVM), metaphase II by 18 hIVM, and completed cortical granule translocation to the oolemma by 24 hIVM compared with those not exposed to heat stress. Also, heat stress exposure between 18 and 21 hIVM increased the percentage of bovine oocytes exhibiting a type IV cortical granule distribution suggesting earlier exocytosis (Andreu-Vazquez *et al.* 2010).

Regardless of the specific process or component affected, Schrock *et al.* (2007) showed that earlier fertilization increased developmental competence of heat-stressed oocytes. Using multi-source regression, optimal development for heat-stressed oocytes depended on performing IVF at 19.5 hIVM compared with 26.7 hIVM for nonheat-stressed controls. Collectively, these findings provide compelling evidence for heat-stressed oocytes to mature faster than nonheat-stressed counterparts. To this end, fertilization of heat-stressed oocytes during the 'fertile window of time' ascribed for nonheat-stressed oocytes would effectively result in the fertilization of an 'aged' oocyte.

To further test the heat-induced 'aging hypothesis' initially postulated but not investigated by Baumgartner & Chrisman (1981), experiments were conducted to examine the development of heat-stressed oocytes after chemical activation with ionomycin followed by 6-dimethylaminopurine exposure as per Susko-Parrish *et al.* (1994). Preference for use of this parthenogenetic model was based on previous efforts showing responsiveness of bovine oocytes to be age-dependent (i.e. aged oocytes activate more readily than younger oocytes; Susko-Parrish *et al.* 1994). Also, this approach has been demonstrated to support embryo and fetal development of somatic cell nuclear transfer clones (Davies *et al.* 2004, Lawrence *et al.* 2004).

In the first study, developmental competence of heat-stressed oocytes was assessed after chemical activation

at 19 vs 24 hIVM. In a second study, developmental competence of heat-stressed oocytes was assessed after chemical activation or IVF at 24 or 30 hIVM. Results from those two studies prompted a closer examination of fertilization rates, intracellular calcium release and activity related to maturation-promoting factor (MPF) and MAP kinase in control, heat-stressed, and aged oocytes. Since consequences of heat stress and aging may include perturbations in cytoskeletal components (Eichenlaub-Ritter *et al.* 1986, Tarin 1996, Ju & Tseng 2004, Roth & Hansen 2005), F-actin in maturing oocytes was also examined.

Results

Development of heat-stressed oocytes after chemical activation at 19 or 24 hIVM

Cumulus–oocyte complexes were matured at 38.5 (control) or 41.0 °C (heat stress was applied during first 12 hIVM only) and chemically activated with ionomycin/6-dimethylaminopurine at 19 or 24 hIVM. Heat stress exposure did not alter the proportion of oocytes recovered or those that had visibly lysed after denudement of cumulus cells before chemical activation at 19 or 24 hIVM (Table 1). The proportion of heat-stressed oocytes that underwent cleavage and blastocyst development after chemical activation at 19 and 24 hIVM was similar to those matured at 38.5 °C (Table 1). Blastocyst stage scores were slightly lower in embryos from heat-stressed oocytes activated at 19 and 24 hIVM (Table 1). Independent of heat stress exposure, proportion of oocytes that were visibly lysed was slightly higher when denudement occurred at 24 vs 19 hIVM (Table 1). Ability of oocytes to undergo cleavage and progress to the 8- to 16-cell and blastocyst stages was higher when chemical activation was performed at 24 vs 19 hIVM (Table 1).

Table 1 Embryo development of control^a and heat-stressed^b bovine oocytes after chemical activation at 19 or 24 hIVM.

Oocyte treatment	Activation time (hIVM)	No. of oocytes	Cleavage (71.5–74.5 hpa)					Blastocyst (186–194.5 hpa)		
			Rcd (%) ^c	Lysed (%) ^d	Cleaved (%)	Four-cell (%) ^e	8- to 16-cell (%) ^e	Percent of oocytes	Percent of cleaved	Stage
Control	19	539	95.6	0.4	50.5	10.0	76.1	19.1	37.9	6.3
Heat stress	19	522	95.4	0.8	42.3	12.1	73.3	21.3	50.2	6.1
Control	24	516	97.1	1.8	77.7	5.2	85.5	45.0	57.9	6.3
Heat stress	24	470	96.2	1.6	74.0	7.3	83.1	44.7	60.3	6.2
		S.E.M.	1.4	0.5	4.3	2.6	3.7	3.0	4.1	0.06
		Oocyte treatment <i>P</i> value	0.661	0.610	0.207	0.157	0.469	0.697	0.090	0.020
		Activation time <i>P</i> value	0.425	0.019	<0.0001	0.005	0.014	<0.0001	0.002	0.038
		Oocyte treatment × activation time <i>P</i> value	0.777	0.448	0.759	0.713	0.931	0.644	0.256	0.587

hIVM, hours of *in vitro* maturation; hpa, hours post activation.

^aControl, oocytes were matured at 38.5 °C. ^bHeat stress, 41 °C was applied during first 12 hIVM only; thereafter oocytes were matured at 38.5 °C.

^cRcd, oocytes recovered after denuding as a proportion of total number of oocytes matured. ^dLysed, oocytes without an intact plasma membrane after denuding as a proportion of recovered. ^eRelative to proportion of embryos cleaved. Significant *P* values are shown in bold.

Figure 1 Schematic of experimental design for evaluating blastocyst development of bovine cumulus-oocyte complexes matured at 38.5 °C (control) or 41.0 °C (heat stress applied during first 12 h only) and undergoing chemical activation or IVF at 24 or 30 h of *in vitro* maturation (hIVM). This experiment was replicated on eight different occasions using a total of 164–282 cumulus-oocyte complexes per treatment group.

Development of control, heat-stressed, and aged oocytes: different activation approaches and times

Absence of heat stress effects to reduce blastocyst development in the first study prompted evaluation of development after cumulus-oocyte complexes, matured at 38.5 °C or 41.0 °C (heat stress applied during first 12 hIVM only), underwent chemical activation with ionomycin/6-dimethylaminopurine or IVF after 24 or 30 hIVM (experimental schematic provided in Fig. 1). Heat stress exposure did not alter the proportion of oocytes

recovered or those that had visibly lysed after denudation of cumulus cells before chemical activation or after IVF at 24 or 30 hIVM (Table 2). When evaluating the proportion of oocytes that underwent cleavage and progressed to the 8- to 16-cell stages, no prominent heat stress effects were noted (Table 2). Rather, cleavage was most influenced by activation method and time (Table 2). For instance, performing IVF at 30 vs 24 hIVM decreased the proportion of 8- to 16-cell embryos and increased the proportion of two- and four-cell embryos, whereas

Table 2 Cleavage development of control^a and heat-stressed^b bovine oocytes after undergoing chemical activation or IVF at 24 or 30 hIVM.

Oocyte treatment	Activation method	Activation time (hIVM)	No. of oocytes/PZ	Rcd (%) ^c	Lysed (%) ^d	Cleavage (69–74 hpa)			
						Cleaved (%)	Two-cell (%) ^e	Four-cell (%) ^e	8- to 16-cell (%) ^e
Control	CA	24	262	96.1	0.6	77.1	6.9	3.5	89.6
Heat stress	CA	24	281	96.1	0.2	82.6	5.6	6.0	88.4
Control	IVF	24	211	92.4	1.2	76.8	8.6	3.1	88.3
Heat stress	IVF	24	203	94.3	0.4	64.0	16.9	6.2	76.9
Control	CA	30	225	96.0	0.8	88.0	6.6	2.0	91.4
Heat stress	CA	30	209	96.0	1.4	87.6	6.0	6.0	88.0
Control	IVF	30	153	92.5	1.6	60.8	20.4	14.0	65.6
Heat stress	IVF	30	137	85.4	1.2	56.2	35.1	9.1	55.8
S.E.M.				2.3	1.3	4.1	3.6	2.5	4.9
Oocyte treatment <i>P</i> value				0.707	0.336	0.457	0.242	0.148	0.088
Activation method <i>P</i> value				0.009	0.320	<0.0001	<0.0001	0.075	<0.0001
Oocyte treatment×activation method <i>P</i> value				0.703	0.656	0.109	0.075	0.270	0.469
Activation time <i>P</i> value				0.362	0.080	0.895	0.054	0.265	0.034
Oocyte treatment×activation time <i>P</i> value				0.356	0.227	0.944	0.914	0.622	0.875
Activation method×activation time <i>P</i> value				0.389	0.604	0.002	0.058	0.055	0.015
Oocyte treatment×activation method×activation time <i>P</i> value				0.377	0.641	0.237	0.877	0.191	0.513

hIVM, hours of *in vitro* maturation; hpa, hours post activation; CA, chemical activation; PZ, presumptive zygotes.

^aControl, oocytes were matured at 38.5 °C. ^bHeat stress, 41 °C was applied during first 12 hIVM only; thereafter oocytes were matured at 38.5 °C.

^cRcd, oocytes/PZ recovered after denuding as a proportion of total number of oocytes matured. ^dLysed, oocytes/PZ without an intact plasma membrane after denuding as a proportion of recovered. ^eRelative to proportion of embryos cleaved. Significant *P* values are shown in bold.

Figure 2 Least squares means for blastocyst development after bovine cumulus–oocyte complexes were matured at 38.5 °C (control) or 41.0 °C (heat stress applied during first 12 h only) and underwent chemical activation (CA) or IVF at 24 or 30 h of *in vitro* maturation (hIVM). There was an oocyte treatment × activation time × activation method interaction ($P=0.025$; S.E.M. = 3.5). ^{ABCD} Bars with different letters differ. Numeric scores for blastocyst stage (S) and quality (Q) are indicated for each treatment combination within individual bars.

chemical activation at 30 vs 24 hIVM did not alter the proportion of 2-, 4-, or 8- to 16-cell stage embryos.

The impact of heat stress exposure to affect blastocyst development differed depending on activation method and time (oocyte treatment × activation method × activation time interaction $P=0.025$; S.E.M. = 3.5; Fig. 2). Specifically, when heat stress exposure occurred during the first 12 h of meiotic maturation and oocytes underwent IVF at 24 hIVM, blastocyst development was lower than for respective nonheat-stressed controls. However, when heat-stressed oocytes underwent chemical activation at 24 hIVM, blastocyst development was higher than for respective nonheat-stressed controls and equivalent to development of nonheat-stressed oocytes chemically activated at 30 hIVM (i.e. slightly aged oocytes). At 30 hIVM, blastocyst development of heat-stressed oocytes after chemical activation was similar to respective nonheat-stressed controls. Blastocyst development of nonheat-stressed oocytes undergoing IVF at 30 hIVM was similar to that obtained for heat-stressed oocytes undergoing IVF at 24 hIVM. Performing IVF at 30 hIVM did not result in further reductions in the development of heat-stressed oocytes. Stage and quality scores of resultant blastocysts were similar regardless of maturation temperature or activation method.

Fertilization and pronuclear formation in control, heat-stressed, and aged oocytes

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM only) for 24 h or 38.5 °C for 30 h (aged) before undergoing IVF. The proportion of oocytes that were penetrated and underwent pronuclear formation thereafter was evaluated at 6, 10, 13, and 16 h (3 × 4 factorial treatment arrangement). Subsets of control and heat-stressed oocytes (five out of eight total replicates) were allowed to develop to the blastocyst stage to document the extent to which heat stress reduced development. Using experimental conditions whereby heat stress reduced blastocyst development by ~37% without impacting cleavage rates (see Table 3), neither heat stress nor slight aging altered the ability of oocytes to undergo penetration or pronuclear formation (Table 4).

Actin filaments in heat-stressed oocytes

Cumulus–oocyte complexes were assessed at 38.5 (control), 41.0 °C or 43.0 °C (heat stress temperatures applied during first 12 hIVM only). Heat stress exposure did not alter the proportion of oocytes recovered or those that had visibly lysed after denudement of cumulus cells.

Table 3 Embryo development of control^a and heat-stressed^b bovine oocytes after IVF at 24 hIVM.

Oocyte treatment	No. of oocytes	Rcd (%) ^c	Lysed (%) ^d	Cleavage (67–73 hpi)		Blastocyst (188–191 hpi)		
				Cleaved (%)	8- to 16-cell (%) ^e	Percent of PZ	Stage	Quality
Control	250	93.2	4.7	75.3	82.5	35.3*	6.3	1.5
Heat stress	250	93.2	4.3	66.6	80.2	22.2 [†]	6.3	1.9
	S.E.M.	1.6	1.4	5.0	6.8	1.1	0.2	0.14
	P value	1.0	0.832	0.133	0.645	0.008	0.794	0.107

hIVM, hours of *in vitro* maturation; hpi, hours post IVF; PZ, presumptive zygotes.

^aControl: oocytes were matured at 38.5 °C. ^bHeat stress, 41 °C was applied during first 12 hIVM only; thereafter oocytes were matured at 38.5 °C.

^cRcd, oocytes/PZ recovered after denuding as a proportion of total number of oocytes matured. ^dLysed, oocytes/PZ without an intact plasma membrane after denuding as a proportion of recovered. ^eRelative to proportion of embryos cleaved. *,[†]Means within a column differ. Significant P values are shown in bold.

Table 4 Fertilization and pronuclear formation in control^a, heat-stressed^b, and aged^c bovine oocytes.

Oocyte treatment	Hours after IVF	No. evaluated	Fertilized ^d (%)	Monospermic ^e (%)	PNF ^f (%)	2PN ^g (%)
Control	6	173	29.8	96.2	–	–
Heat stress	6	180	34.4	95.6	–	–
Aged	6	105	34.4	87.5	–	–
Control	10	165	67.4	77.1	26.8	66.7
Heat stress	10	176	55.1	85.9	29.0	62.5
Aged	10	108	57.1	79.2	52.8	68.0
Control	13	172	68.7	82.1	67.8	90.9
Heat stress	13	143	71.5	71.4	64.4	89.6
Aged	13	103	61.0	62.2	67.2	92.0
Control	16	172	75.0	67.9	86.7	92.1
Heat stress	16	168	77.5	72.2	83.5	94.9
Aged	16	92	72.6	72.5	79.1	88.9
		S.E.M.	7.2	5.5	7.9	6.8
		Oocyte treatment <i>P</i> value	0.723	0.094	0.740	0.979
		Hours after IVF <i>P</i> value	<0.0001	<0.0001	<0.0001	0.0003
		Oocyte treatment×hours after IVF <i>P</i> value	0.516	0.096	0.403	0.889

hIVM, hours of *in vitro* maturation; PNF, pronuclear formation; PN, pronuclei.

^aControl, oocytes were matured at 38.5 °C for 24 h. ^bHeat stress, 41 °C was applied during first 12 hIVM only; thereafter oocytes were matured at 38.5 °C. ^cAged, oocytes were matured at 38.5 °C for 30 h. ^dFertilized, proportion of oocytes penetrated by at least one spermatozoon.

^eMonospermic, proportion of fertilized oocytes at different stages of meiosis (e.g. MII, AII, TII, pronucleus) that were penetrated by one spermatozoon as indicated by the presence of a condensed or swollen sperm head, or as a pronucleus. ^fPNF, proportion of monospermic oocytes having at least one pronuclear structure. ^g2PN, proportion of PNF that had two pronuclei at time of evaluation. Significant *P* values are shown in bold.

However, it was noted during staining procedure manipulations that more oocytes exposed to 43 °C underwent lysis (59.1% vs 13.5 and 22.2% for 43.0 °C, 38.5 °C, and 41.0 °C, respectively; *P*<0.01; S.E.M. = 6.2).

When matured at 38.5 °C or 41.0 °C for 12 or 24 hIVM, F-actin staining as assessed using fluorescent-labeled phalloidin was localized to the cortex in majority of oocytes examined (Fig. 3A–D and G–J). In a few oocytes, light but diffuse staining was noted in ooplasm (Fig. 3B). In some oocytes matured at 41 °C for 12 hIVM (Fig. 3D), intense staining as spherical shapes was also noted. Although proportionately fewer, F-actin staining in majority of membrane-intact oocytes after 43.0 °C exposure was similar to those undergoing maturation at 38.5 °C or 41.0 °C. However, in some of the oocytes examined at 12 hIVM at 43.0 °C, microfilament breaks were noted (Fig. 3E and F). These were not detected when oocytes were examined at 24 hIVM; rather, dense staining was noted in ooplasm and was also localized to cortex (Fig. 3K and L). No fluorescence was observed in oocytes when the Alexa Fluor 594-conjugated phalloidin was omitted from the staining procedure.

Ionomycin-induced calcium release in control, heat-stressed, and aged oocytes

Since loss of heat stress effects to reduce blastocyst development was coincident with calcium ionophore exposure, basal levels of free cytosolic calcium and its release after ionomycin exposure in control, heat-stressed, and aged Fura-2-loaded oocytes were examined (representative image and variables of interest depicted in Fig. 4). If the external medium contained calcium, basal (i.e. resting) levels of intracellular

calcium did not differ between control (24 hIVM at 38.5 °C), heat-stressed (12 of the 24 hIVM at 41.0 °C), and aged (30–32 hIVM at 38.5 °C) oocytes (Table 5). Ionomycin exposure stimulated immediate calcium release; time of rise (i.e. time from initiation of release until maximum peak), amplitude of release (i.e. difference between basal and maximum peak), area under the curve and post-release plateau of intracellular calcium concentrations were similar in control, heat-stressed, and aged oocytes (Table 5). Interestingly, the time of decline in intracellular calcium levels (i.e. time from maximum peak until plateau level was reached) was longer in heat-stressed oocytes compared with control and aged oocytes. Consequently, the duration of release (time from initiation to termination of intracellular calcium release in response to ionomycin) was longer in heat-stressed oocytes compared with controls and aged oocytes (Table 5).

Evaluating ionomycin-induced intracellular calcium release using a calcium-deficient medium allowed for assessing calcium release from intracellular stores. When compared with nonheat-stressed controls, response of heat-stressed oocytes to ionomycin was similar (i.e. comparable times in the rise and decline of intracellular calcium concentrations and duration of response). Differences included lower intracellular basal levels of calcium, amplitude of its release, total area under the curve, and post-release plateau indicating heat-induced perturbations in calcium stores and/or components important for release (Table 5). Distinguishing features of aged oocytes included higher basal levels of intracellular calcium and quicker response to ionomycin than observed in control and heat-stressed oocytes (Table 5). While the amplitude of response was

Figure 3 Representative confocal images of F-actin localization in bovine oocytes matured at 38.5 °C (control), 41.0 °C or 43.0 °C (heat stress temperatures applied during first 12 h only). Regardless of temperature, most oocytes matured for 12 h of *in vitro* maturation (hIVM), had the majority of staining at the oolemma, forming a cortical ring (A–C, bar=20 µm). Some oocytes matured at 41.0 °C for the first 12 hIVM had round areas of intense staining underlying the oolemma (D, bar=20 µm). More extreme temperature of 43.0 °C during the first 12 hIVM induced some oocytes to display more prominent staining throughout the ooplasm (E, bar=20 µm) with distinct breaks in some microfilaments visualized (F, bar=4 µm, breakage indicated by arrows). At 24 hIVM, most oocytes had majority of staining at the cortex with diffuse staining throughout the ooplasm (G and I, bar=20 µm) and prominent staining at the point of polar body extrusion (H and J, bar=20 µm). At 24 hIVM, oocytes exposed to 43.0 °C during the first 12 hIVM displayed a cortical ring of F-actin but also had a distinct network of F-actin throughout the ooplasm (K, bar=20 µm; L, bar=4 µm). Each image was taken near the equatorial region of the oocyte. Panels (F and L) were generated using optical zoom on oocyte in preceding panel. The number of oocytes displaying each phenotype is indicated in the lower left corner of each panel.

similar to controls, duration of response was less and more comparable to that observed in heat-stressed oocytes. Post-release plateau values were highest in aged oocytes.

MPF and MAP kinase activities in control, heat-stressed, and aged oocytes

MPF activity was similar in control (24 hIVM at 38.5 °C), heat-stressed (41.0 °C during first 12 hIVM only, thereafter 38.5 °C), and aged (32 hIVM at 38.5 °C) oocytes ($P=0.173$; Fig. 5A). In contrast, MAP kinase activity differed depending on treatment ($P=0.083$). When compared with nonheat-stressed controls, heat stress exposure reduced MAP kinase activity ($P=0.047$) to levels similar to those observed in aged oocytes ($P=0.886$; Fig. 5B).

Discussion

A parthenogenetic approach was utilized to further test the heat-induced 'aging' hypothesis, as age dependence of activation protocols sufficient to support pronuclear formation, cleavage, and blastocyst development has been well documented (i.e. older MII oocytes activate more readily than younger MII oocytes; Susko-Parrish *et al.* 1994, Suzuki *et al.* 1999, Tian *et al.* 2002). Results

clearly showed that consequences of heat stress exposure during the first 12 h of meiotic maturation differed depending on how and when bovine oocytes were activated. For instance, if heat-stressed oocytes underwent IVF at ~24 hIVM, blastocyst development was less than respective control which is in agreement with numerous other studies (Edwards & Hansen 1996, Lawrence *et al.* 2004, Roth & Hansen 2004a, 2004b, Edwards *et al.* 2005, de Castro e Paula & Hansen 2007, Schrock *et al.* 2007, Sugiyama *et al.* 2007, Soto & Smith 2008, Zhandi *et al.* 2009). Furthermore, blastocyst development obtained after heat-stressed oocytes underwent IVF at 24 hIVM was similar to that obtained for nonheat-stressed oocytes undergoing IVF at 30 hIVM (i.e. slightly aged). In contrast, when heat-stressed oocytes underwent chemical activation at 24 hIVM, blastocyst development was not only higher than respective controls but also equivalent to development obtained after activation at 30 hIVM of slightly aged nonheat-stressed oocytes. Collectively, these findings along with previous results (Kim *et al.* 2002, Edwards *et al.* 2005, Schrock *et al.* 2007) provide compelling evidence for a major effect of heat stress exposure during the first part of meiotic maturation to hasten developmentally important processes.

Thus, fertilization of heat-stressed oocytes during the 'fertile window of time' ascribed for nonheat-stressed

Figure 4 Representative image depicting calcium concentration (nM) in a bovine oocyte before and after ionomycin exposure. Analysis variables included basal levels (average intracellular levels before ionomycin addition), time of rise (time from addition of ionomycin until maximum peak), amplitude of release (difference between basal and maximum peak), area under the curve, time of decline (time from maximum peak until plateau level was reached), duration of release (time from initiation to termination of intracellular calcium release), and post-release plateau (average intracellular levels post termination of release).

oocytes likely results in the fertilization of an 'aged' oocyte. Notably, numerous similarities exist between heat-stressed oocytes and those that have been aged after reaching MII. Ward *et al.* (2002) and Agung *et al.* (2006) reported a 35–50% reduction in blastocyst development after oocytes were aged 8–10 h before IVF. Typically heat-stressed oocytes experience a 42–65% reduction in blastocyst development when IVF is performed at ~24 hIVM (Edwards & Hansen 1996, Roth & Hansen 2004a, Edwards *et al.* 2005, Schrock *et al.* 2007). Other pertinent similarities include reduced *de novo* synthesis of intracellular proteins and MAP kinase activity

(Edwards & Hansen 1996, 1997, Xu *et al.* 1997, Ma *et al.* 2005, Lee & Campbell 2006, Tatone *et al.* 2006, this study). Similar to aged oocytes, MAP kinase activity was lower in heat-stressed oocytes examined at 24 hIVM. Xu *et al.* (1997) associated partial decreases in MPF and MAP kinase activities to increased sensitivity of aged oocytes to calcium increasing agents, possibly providing some explanation for improved development of heat-stressed oocytes after chemical activation.

Disparate consequences of heat stress exposure during the first half of meiotic maturation to impact blastocyst development after activation with a chemical or a spermatozoon were not related to fertilization failure or obvious alterations in cytoskeleton. Examination of >1700 oocytes showed similar fertilization rates and pronuclear formation in control, heat-stressed, and aged oocytes, despite heat stress effects to reduce blastocyst development by ~37%. Additional efforts showed that the predominance of F-actin in the cortical region underlying the oolemma was similar to that reported by others in bovine (Li *et al.* 2005), porcine (Wang *et al.* 2000), and rat (Eliyahu *et al.* 2005, Meng *et al.* 2006) oocytes; 41.0 °C exposure had minimal impact. Other studies reporting minimal to no heat stress effects on cleavage rates support fertilization and cytoskeletal data described herein (*in vivo*, Putney *et al.* 1989, Matsuzuka *et al.* 2004 and *in vitro*, Edwards & Hansen 1996, Lawrence *et al.* 2004, Edwards *et al.* 2005, de Castro e Paula & Hansen 2007, Schrock *et al.* 2007, Sugiyama *et al.* 2007, Edwards *et al.* 2009, Zhandi *et al.* 2009).

Notably, results document that oocytes experiencing heat stress during the first 12 h of meiotic maturation, like aged-MII oocytes (Susko-Parrish *et al.* 1994), are capable of developing to the blastocyst stage (i.e. they have the necessary components) but fail to do so after fertilization. While the underlying mechanism(s) remain

Table 5 Characteristics of intracellular calcium levels before and after 5 µM ionomycin exposure of control^a, heat-stressed^b, and aged^c bovine oocytes.

Oocyte treatment	No. of evaluated	Basal levels (Ca ²⁺ nM)	Time of rise ^d (s)	Amplitude of release ^e (Ca ²⁺ nM)	Time of decline ^f (s)	Release duration ^g (s)	Area under the curve	Post-release plateau (Ca ²⁺ nM)
Calcium-containing medium								
Control	49	121.3	16.6	1198.3	52.6 [†]	69.2 [†]	52 357	556.3
Heat stress	44	116.8	15.4	1039.1	61.3*	79.6*	49 629	448.7
Aged	100	134.7	15.8	1153.2	53.8 [†]	69.5 [†]	47 951	504.8
	S.E.M.	6.7	0.6	113.2	2.8	3.0	4833	41.5
	P value	0.112	0.373	0.613	0.009	0.048	0.709	0.141
Calcium-free medium								
Control	59	90.0 [†]	18.1*	685.7*	59.1	77.3*	28 963*	213.1 [†]
Heat stress	33	55.2 [†]	18.2*	505.9 [†]	55.5	73.8* [†]	20 894 [†]	148.3 [†]
Aged	65	146.2*	14.7 [†]	787.9*	55.4	70.1 [†]	29 935*	280.6*
	S.E.M.	18.9	1.3	114.3	3.5	4.6	5785	37.3
	P value	<0.0001	0.0001	0.0004	0.250	0.042	<0.0001	<0.0001

hIVM, hours of *in vitro* maturation.

^aControl, oocytes were matured at 38.5 °C for 24 h. ^bHeat stress, 41 °C was applied during first 12 hIVM only; thereafter oocytes were matured at 38.5 °C. ^cAged, oocytes were matured at 38.5 °C for 30–32 h. ^dTime in seconds from initiation of release until maximum peak reached. ^eDifference between initial basal and maximum peak intracellular calcium ([Ca²⁺]_i) levels. ^fTime in seconds from maximal peak until termination of release.

^gTime from initiation to termination of [Ca²⁺]_i elevation. *,[†] Means within a column and medium differ. Significant P values are shown in bold.

Figure 5 Relative kinase activity (densitometric units) for maturation-promoting factor (MPF) using histone H1 (panel A) and MAP kinase (MAP kinase; panel B) using myelin basic protein (MBP) in bovine oocytes after maturation at 38.5 °C (control (C)) or 41 °C (heat stress, HS applied during first 12 hIVM only) for 24 h or at 38.5 °C for 32 h (aged (A)). Insets within each panel depict a representative autoradiograph from phosphorylation of histone H1 and myelin basic protein respectively. ^{AB}Bars with different letters differ ($P < 0.05$).

unclear, ability to obtain equivalent or higher blastocyst development after exposure to chemicals targeting cytoplasmic components support the view for much of the negative heat stress effects to be occurring at the level of the ooplasm rather than nuclear components. Wang *et al.* (2009) came to a similar conclusion after performing chromosome spindle exchanges between murine oocytes matured *in vivo* and *in vitro* at different temperatures.

Loss of heat stress effects to reduce blastocyst development after parthenogenetic activation was functionally related to the induction of single calcium transient using ionomycin followed by inhibition of serine–threonine kinases with 6-dimethylaminopurine after cumulus denudement. Ionomycin exposure induces a single large calcium transient originating from internal stores and the external medium through effects on store-regulated Ca^{2+} channels (Morgan & Jacob 1994, Dedkova *et al.* 2000). Additional effort showed that when calcium was present in the external medium, ionomycin-induced calcium release in heat-stressed oocytes was comparable to that observed in nonheat-stressed and aged oocytes, with one notable

exception. Decline in calcium levels was slower in heat-stressed oocytes, suggesting possible heat-induced perturbations in components important for reducing cytosolic calcium levels (reviewed by Berridge *et al.* (2003)). Use of specific Ca^{2+} -ATPase inhibitors targeting ATP-dependent Ca^{2+} pumps would allow for differentiating between possible effects occurring at the level of the endoplasmic reticulum or plasma membrane (Igarashi *et al.* 1997).

When the external medium was calcium deficient, several distinguishing features were noted in heat-stressed oocytes. Basal levels were lower than nonheat-stressed oocytes that represent the steady state achieved by balancing calcium leakage in and constant removal out of the cytosol (reviewed by Berridge *et al.* (2003)). Obtaining lower amplitude of response, area under the curve and post-release plateau after ionomycin exposure suggest possible heat-induced reductions of internal calcium stores or perturbations in components important for ionomycin-induced transients that may involve IP_3 -sensitive mechanisms (*Xenopus laevis* oocytes; Yoshida & Plant 1992). Use of IP_3 -receptor agonists and antagonists would be helpful for discerning underlying mechanism(s). Depending on the extent to which alterations occur, developmentally important calcium oscillations induced by the fertilizing spermatozoon may be perturbed in heat-stressed oocytes. This seems plausible since developmental consequences of reduced amplitude or frequency of calcium oscillations in rabbit oocytes often parallel those observed after heat stress exposure (i.e. reduced compaction rates and blastocyst development (Ozil 1990, Edwards *et al.* 2009) and increased incidence of post-implantation/attachment losses (Ozil & Huneau 2001, Ozil *et al.* 2006, Rutigliano *et al.* 2008) without consequences on cleavage rates (Ozil & Huneau 2001, Schrock *et al.* 2007, Edwards *et al.* 2009)).

Although oocytes undergo numerous changes such as reorganization of the endoplasmic reticulum (Kruip *et al.* 1983) and increased number of IP_3 receptors (He *et al.* 1997, Wang *et al.* 2005) for optimizing intracellular calcium responsiveness (reviewed by Ajduk *et al.* (2008)) during the first 12 h of maturation, heat-related consequences described herein may not be stage or species specific. For instance, Tseng *et al.* (2009) showed that a 2 h heat stress exposure after porcine oocytes had reached maturity (i.e. MII stage) reduced thimerosal-induced calcium release.

Bovine oocytes that had been slightly aged had higher basal levels of intracellular calcium that is consistent with preliminary work by Susko-Parrish *et al.* (1994) and consequences noted in murine oocytes (Takahashi *et al.* 2000). After ionomycin exposure, amplitude of calcium release was highest in aged oocytes. While consistent with consequences reported after aging MII murine oocytes (Vincent *et al.* 1992), these findings were inconsistent with results obtained for heat-stressed

oocytes. This suggests heat-induced perturbations in specific ooplasmic component(s) important for calcium homeostasis or mobilization rather than merely a consequence of hastened maturation which may explain why performing earlier IVF is not entirely effective for eliminating negative heat stress effects on blastocyst development (Schrock *et al.* 2007). Since the calcium transient in heat-stressed oocytes was largely similar to that observed in nonheat-stressed oocytes, when calcium was present in the external medium, ionomycin exposure may have circumvented some of the potential problems related to perturbations in cytoplasmic component(s) important for calcium homeostasis or mobilization.

It is also possible for loss of heat stress effects to reduce blastocyst development after parthenogenetic activation to be functionally related to the absence of a sperm. In some instances glutathione content is lower in heat-stressed oocytes (Wang *et al.* 2009, Nabenishi *et al.* 2011). In our laboratory, heat-stressed oocytes may contain ~12% less glutathione than nonheat-stressed counterparts (J L Edwards & R R Payton 2011, unpublished observations). Important for reduction of disulfide bonds in the sperm nucleus, glutathione decreases after fertilization but remains unchanged after electrical-induced parthenogenetic activation (Funahashi *et al.* 1995). In the absence of a spermatozoon, activated oocytes would have an increased abundance of glutathione available to neutralize heat-induced increases in reactive oxygen species (Nabenishi *et al.* 2011).

Using a different stage of oocyte, others have noted a loss of heat stress effects after parthenogenetic activation. Tseng *et al.* (2009) performed chromosome spindle exchanges to isolate confounding effects of heat stress on the nucleus and ooplasm by applying 41.5 °C for 2 h to porcine oocytes at MII stage and noted higher blastocyst development after parthenogenetic activation. Admittedly, results were not expected as in two different studies heat stress exposure reduced rather than increased blastocyst development (Tseng *et al.* 2006, Tseng & Ju 2009). Because spindle exchanges took ~1–2 h, the importance of recovery time after heat stress exposure was speculated to be beneficial. Similarly, improved development herein was coincident with a 12 h recovery period after a heat stress exposure applied during the first 12 h of meiotic maturation. Disparate results of Tseng and coworkers were coincident with use of 1000-fold lower dose of 6-dimethylaminopurine, which may not be maximally effective to activate oocytes (Susko-Parrish *et al.* 1994). This may explain why Wang *et al.* (2009) did not observe a 'beneficial' effect of heat stress to improve blastocyst development when activating reconstructed murine oocytes with just SrCl₂. Studies documenting negative impacts on blastocyst development after parthenogenetic activation (Tseng *et al.* 2006, Tseng & Ju 2009, Wang *et al.* 2009) include cumulus denudement before activation procedure,

effectively eliminating possible influences on loss of heat stress effects observed in this or other studies.

Current studies are focused on identifying components of the maturing oocyte affected by heat stress and consequences thereof, as it is this oocyte that will contribute >99% of its cytoplasm and half of its genetic material to the resultant embryo after fertilization. Assessing the direct impact *in vitro* is a necessary first step toward identifying specific components in the *in vivo* matured oocyte altered by heat stress while resident within the Graafian follicle. Doing so is of utmost importance toward finding a solution to the problem of heat stress to alter oocyte competence and minimizing animal numbers necessary to conduct more focused *in vivo* studies.

Materials and Methods

Materials

Majority of reagents and chemicals were purchased from Sigma Chemical Co. Medium-199, gentamicin, penicillin–streptomycin, Fura-2/acetoxymethylester (Fura-2/AM), Fura-2 pentapotassium salt (Fura-2 salt), pluronic acid F-127, Alexa Fluor 594 phalloidin, and ProLong Gold antifade solution were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD, USA), whereas FSH (Folltropin-V) was obtained from Vetrepur Canada, Inc. (London, ON, Canada). Ionomycin was purchased from Calbiochem (La Jolla, CA, USA) and Invitrogen. Oocyte collection medium was prepared in the laboratory containing M-199 with Hank's salts (Mediatech, Manassas, VA, USA), 1–2% (v/v) FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Oocyte maturation medium contained M-199 with Earle's salts (Invitrogen), 10% (v/v) FBS, 50 µg/ml gentamicin, 5 µg/ml Folltropin-V, 0.2 mM sodium pyruvate, and 2 mM L-glutamine. Potassium simplex optimized medium was prepared in the laboratory according to Biggers *et al.* (2000) but modified to include 1 mM glutamine, 10 mM glycine, 1× non-essential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin (mKSOM). Additional media including IVF Tyrode's albumin lactate pyruvate (IVF-TALP), Sperm-TALP, and HEPES-TALP were prepared as per Parrish *et al.* (1988). Frozen semen was donated by Harrogate Genetics International, Inc. (Harrogate, TN, USA). Bovine ovaries were purchased from a commercial abattoir (Brown Packing Company, Gaffney, SC, USA).

General methods for *in vitro* production of embryos

IVM, IVF and embryo culture were performed as described previously (Lawrence *et al.* 2004, Edwards *et al.* 2005, Schrock *et al.* 2007). Frozen–thawed Percoll-prepared semen from various bulls (500 000–600 000 motile sperm/ml; dairy and beef breeds; two of which were pooled on a given day) were used for each experimental replicate requiring IVF. Presumptive zygotes were cultured in 500 µl of mKSOM at 38.5 °C in 5.5% CO₂, 7.0% O₂, and 87.5% N₂. Cleavage was assessed ~3 days post IVF by recording number of 1-, 2-, 4-, and 8- to

16-cell embryos. Stage and/or quality scores of resultant blastocysts were recorded ~8 days post IVF as per Robertson & Nelson (1998).

Development of heat-stressed oocytes after chemical activation at 19 or 24 hIVM

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM). After a total of 19 or 24 hIVM, oocytes were denuded of cumulus (i.e. vortexed ~4 min in HEPES-TALP containing 0.3 mg/ml hyaluronidase) before chemical activation with 5 µM ionomycin (4 min) followed by exposure to 2 mM 6-dimethylaminopurine (3.5 h) as per Susko-Parrish *et al.* (1994), resulting in four treatment combinations per experimental replicate. Thereafter, culture was performed in 500 µl of mKSOM at 38.5 °C in 5.5% CO₂, 7.0% O₂, and 87.5% N₂. Ability to undergo cleavage and develop to the blastocyst stage was assessed between 71.5–74.5 and 186–194.5 h, after chemical activation respectively. Preference for chemical activation method was based on its effectiveness for supporting fetal development of embryos made via somatic cell nuclear transfer (Davies *et al.* 2004, Lawrence *et al.* 2004). This study was replicated on eight different occasions with a total of 136–280 oocytes per treatment combination.

Development of heat-stressed and aged oocytes: different activation approaches and times

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM). After a total of 24 or 30 hIVM, oocytes were chemically activated as per first study or underwent IVF resulting in eight treatment combinations per experimental replicate (Fig. 1). Ability to cleave was assessed 69–74 h after IVF or chemical activation; whereas, blastocyst development was assessed between 184 and 194 h. This experiment was replicated on eight different occasions with a total of 164–282 cumulus–oocyte complexes cultured per treatment combination.

Fertilization and pronuclear formation in control, heat-stressed, and aged oocytes

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM) for 24 h (control and heat stress respectively) or at 38.5 °C for 30 h (aged) before undergoing IVF. At 6, 10, 13, or 16 h after IVF, number of oocytes that had been penetrated by sperm (i.e. underwent fertilization) was assessed. To this end, the zona pellucida was removed (0.5% (w/v) pronase), oocytes were fixed (3% (w/v) paraformaldehyde) and then stained with Hoechst 33342 (0.5 µg/ml). Stage of maternal and paternal chromatin was recorded by an individual uninformed of treatment using a Nikon Eclipse TE300 (DAPI filter: excites at 330–380 nm). In total, there were 12 treatment combinations examined per experimental replicate (i.e. three oocyte treatments assessed four different times after IVF). This experiment was performed on six different occasions with 92–180 presumptive zygotes evaluated per treatment

combination. On five different occasions, subsets of control and heat-stressed oocytes were allowed to develop to the blastocyst stage to document the extent to which heat stress reduced subsequent development.

Actin filaments in heat-stressed oocytes

Cumulus–oocyte complexes were assessed at 12 and 24 hIVM after culture at 38.5 °C, 41.0 °C, or 43.0 °C (heat stress applied during first 12 hIVM). Oocytes were denuded of associated cumulus cells and the zona pellucida was removed (0.5% (w/v) pronase). Oocytes were fixed (3% (w/v) paraformaldehyde), permeabilized in 0.1% (v/v) Triton-X, and incubated in DPBS with 1% (w/v) BSA (DPBS+BSA) for 30 min to reduce nonspecific binding. Staining for F-actin was performed by incubating oocytes in 165 nM Alexa Fluor 594 phalloidin in DPBS+BSA for 30 min in the dark. Stained oocytes were washed twice in DPBS+BSA before mounting on glass slides in ProLong Gold antifade solution. Negative controls were processed similarly except the Alexa Fluor 594 phalloidin was omitted. Images of different planes throughout each oocyte were captured using confocal microscopy (Leica TCS SP2, Wetzlar, Germany). Experiment was replicated on three different days of oocyte collection with a total of 157 oocytes.

Ionomycin-induced calcium release in control, heat-stressed, and aged oocytes

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM) for 24 h (control and heat stress treatments respectively) or at 38.5 °C for 30–32 h (slightly aged). After maturation, oocytes were completely denuded of cumulus and placed in IVF-TALP (equilibrated at 38.5 °C in a humidified environment of 5.5% CO₂) containing 2 µM Fura-2/AM and pluronic acid F-127 (0.04% (w/v)) for minimum of 45 min at 38.5 °C. Thereafter, oocytes were extensively washed in HEPES-TALP (pre-warmed to 38.5 °C) and intracellular Ca²⁺ was monitored in each oocyte using a Nikon Ti-E microscope with Super Fluor 10×0.5 NA objective and heated stage adaptor (Delta T Open Dish System; Bioprotech, Butler, PA, USA). For each maturation group, pools of dye-loaded oocytes were split into two groups and washed in pre-warmed HEPES-TL (BSA-free) with or without Ca²⁺ before being positioned in a 40 µl droplet of appropriate HEPES-TL (overlaid with mineral oil pre-warmed to 38.5 °C) on Delta T dish (Bioprotech) pre-coated with poly-L-lysine. Individual oocyte measurements were made within 30 min of washing and placement on imaging dish. Using Nikon Elements Advanced Research Software, fluorescence values (340 and 380 nm excitations with 510 nm emission) were obtained at 1 s intervals; baseline levels were established (30 s) before addition of ionomycin diluted in appropriate HEPES-TL (5 µM final concentration; 300 s). Intracellular Ca²⁺ concentrations at each time point were calculated from ratio of 340 to 380 nm fluorescence (R) according to following formula: $[Ca^{2+}] = K_d \times B \times ((R - R_{min}) / (R_{max} - R))$ as described previously (Grynkiewicz *et al.* 1985, Fissore *et al.* 1992). Calibration solution of 2 µM Fura-2 salt in HEPES-TL supplemented with 60% sucrose (w/v) to correct for

intracellular viscosity (Poenie 1990) was used to determine 340–380 nm fluorescence ratio at zero ($R_{\min}$; containing 2 mM EDTA) and saturating calcium ($R_{\max}$; containing 2 mM CaCl_2) concentrations. Ratio of fluorescence readings at 380 nm wavelength for zero Ca^{2+} to saturating Ca^{2+} solutions determined value of B . Background values for subtraction were obtained by measuring calibration solution without Fura-2 salt. Ratios of 0.07 and 0.48 correspond to 50 nM and 2 μM Ca^{2+} respectively. This experiment was replicated on three different occasions with a total of 350 oocytes.

Activity of MPF and MAP kinase in control, heat-stressed, and aged oocytes

Cumulus–oocyte complexes were matured at 38.5 °C or 41.0 °C (heat stress was applied during first 12 hIVM) for 24 h (control and heat stress respectively) or at 38.5 °C for 30–32 h (slightly aged). MPF and MAP kinase activities were assessed as per Fissore *et al.* (1996) and Lee & Campbell (2008) with some modifications. In brief, oocytes were denuded completely of cumulus and zona pellucidae removed before washing twice in cold (4 °C) DPBS containing 0.1% polyvinylpyrrolidone (DPBS–PVP). Groups of ten oocytes in 1 μl of DPBS–PVP were lysed in 5 μl cold kinase buffer (45 mM β -glycerophosphate, 12 mM EGTA, 12 mM MgCl_2 , 0.1 mM EDTA, 20 mM MOPS, 0.8 mM dithiothreitol, 20 $\mu\text{g/ml}$ leupeptin, 20 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ pepstatin A, 10 mM sodium fluoride, and 1.2 mM sodium orthovanadate) and stored at –80 °C until further analysis. At time of assay, lysates were repeatedly thawed/frozen to facilitate complete lysis before mixing with 5 μl of kinase buffer containing 2 mg/ml histone H1 (useful substrate for assessing MPF activity; EMD Biochemicals, Gibbstown, NJ, USA) or 1 mg/ml myelin basic protein (useful substrate for assessing MAP kinase activity; Active Motif, Carlsbad, CA, USA), 500 nM H-89 dihydrochloride hydrate (cAMP-dependent protein kinase inhibitor), 2.2 μM PKA inhibitor peptide, 0.6 mM ATP, and 250 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP (Perkin Elmer, Waltham, MA, USA). Reactions were incubated for 30 min at 37 °C and were terminated by adding 4 $\times$ LDS sample buffer with dithiothreitol (Expedeon, San Diego, CA, USA). Samples were denatured (70 °C for 10 min) before electrophoresis on 10–20% linear gradient SDS–polyacrylamide gels (Expedeon). Dried gels were exposed to film, and autoradiographs were scanned and quantified utilizing ImageJ Software (ver 1.43u; <http://imagej.nih.gov/ij/>). Multiple exposures were performed to avoid saturation of exposed film to ensure proper quantification. This study was replicated on three different days with a total of three pools per treatment for MPF and 4–8 pools per treatment for MAP kinase examined for kinase activity.

Statistical analysis

In general, data were analyzed as a randomized block design, blocking on replicate, by generalized linear mixed models (PROC GLIMMIX; SAS 9.2, SAS Institute, Inc., Cary, NC, USA) with binomial or normal distributions as appropriate. When required, correction for overdispersion and/or unequal variance was implemented. Treatment differences were

determined using protected least significant differences and reported as least squares means $\pm$ S.E.M. using the inverse link option. Development of heat-stressed oocytes after chemical activation was evaluated by including the fixed effects of IVM temperature (38.5 °C or 41.0 °C) and activation time (19 and 24 hIVM) in the statistical model. To examine development of heat-stressed oocytes after chemical activation or IVF at 24 or 30 hIVM, main effects included IVM temperature (38.5 °C or 41.0 °C), activation time (24 or 30 hIVM), activation method, and all interactions. Fertilization and pronuclear formation in control, heat-stressed, and aged oocytes were evaluated by including main effects of treatment (control, heat stress, or aged), evaluation time (6, 10, 13, and 16 h after IVF), and the interaction. Activity of MPF and MAP kinase was evaluated by including the main effect of treatment (control, heat stress, and aged) in statistical model.

Differences in control, heat-stressed, and aged oocytes response to ionomycin were determined by examining various parameters of the induced intracellular increase in calcium. Initiation and termination of $[\text{Ca}^{2+}]_i$ release was determined by fitting a four straight line segmented nonlinear model (PROC NLIN, SAS 9.2), with segments consisting of an initial flat baseline, then a rise to the peak, followed by a fall to the termination of release, and then a final plateau which generally was not flat. Join points provided estimates for initiation and termination, except in a few cases of unusual response where estimates were manually adjusted by a few seconds to better match visually obvious breaks in the $[\text{Ca}^{2+}]_i$ pattern. Once initiation and termination times were estimated, variables of interest were calculated from the data. Variables included initial basal level (average intracellular concentration before ionomycin addition), time of rise (duration from initiation of release until maximum concentration was observed), amplitude of response (difference between initial basal and maximum concentration), time of decline (duration from time of maximal concentration until termination of release), response duration (total time from initiation to termination of $[\text{Ca}^{2+}]_i$ release), area under the curve (representation of total change in intracellular calcium), and post-release plateau (average intracellular concentration post termination of release until end of experiment). Resulting data were tested for normality (Shapiro–Wilk $W \geq 0.90$) and subjected to logarithmic transformation before analysis with main effect of maturation condition (control, heat stress, or aged) and blocking on replicate by mixed model procedure of SAS. Values were back transformed to original scale for reporting.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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