—Original Article—

Impact of heat stress on germinal vesicle breakdown and lipolytic changes during *in vitro* maturation of bovine oocytes

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Abstract. Two studies were conducted with the overarching goal of determining the extent to which lipolytic changes relate to germinal vesicle breakdown (GVBD) in bovine oocytes matured under thermoneutral or hyperthermic conditions. To this end, cumulus-oocyte complexes underwent *in vitro* maturation for 0, 2, 4, 6 or 24 h at 38.5 (first study) or 38.5 and 41.0 C (second study; heat stress applied up through first 12 h only, then shifted to 38.5 C). Independent of maturation temperature, triglyceride and phospholipid content decreased markedly by 2 h of *in vitro* maturation (hIVM; $P < 0.0005$). Content was lowest at 24 hIVM with no detectable impact of heat stress when exposure occurred during first 12 hIVM. Germinal vesicle breakdown occurred earlier in oocytes experiencing heat stress with effects observed as soon as 4 hIVM ($P < 0.0001$). Germinal vesicle breakdown was associated with lipolytic changes ($R^2 = 0.2123$ and $P = 0.0030$ for triglyceride content; $R^2 = 0.2243$ and $P = 0.0026$ for phospholipid content). ATP content at 24 hIVM was higher in oocytes experiencing heat stress ($P = 0.0082$). In summary, GVBD occurs sooner in heat-stressed oocytes. Although marked decreases in triglyceride and phospholipid content were noted as early as 2 hIVM and preceded GVBD, lipolytic changes such as these are not likely serving as an initial driver of GVBD in heat-stressed oocytes because changes occurred similarly in oocytes matured at thermoneutral conditions.
Key words: Heat stress, Lipolysis, Oocyte, Oocyte Maturation, Triglyceride-Phospholipid Content

(**J. Reprod. Dev. 61:** 459–464, 2015)

Findings in two different species document the potential for heat stress exposure during meiotic maturation to hasten developmentally-important processes in the oocyte as it matures in preparation for fertilization. Baumgartner and Chrisman [1] 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.* [2], suggesting that short term heat shock exposure of murine oocytes during meiotic maturation accelerated germinal vesicle breakdown (GVBD). After direct application of heat stress to bovine oocytes, Edwards *et al.* [3] reported that a greater proportion had 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 to non-stressed oocytes. The potential for heat stress to hasten developmentally-important processes is not without consequence, because fertilization within the timeframe expected to yield optimal development from oocytes effectively results in the fertilization of an "aged" oocyte. In support of this, insemination of heat-stressed oocytes 4 to 6 h earlier than the physiologically relevant 24 h timeframe improved blastocyst development [3, 4]. Furthermore, Rispoli *et al.* [5] observed similar blastocyst development in heat-stressed oocytes chemically activated at 24 hIVM and aged oocytes chemically activated at 30 hIVM.

Although the underlying mechanism(s) responsible for hastening the onset of meiotic maturation in heat-stressed oocytes remain unclear, increased mitogen activated protein kinase (MAPK) activity has been noted in other cell types experiencing a mild heat stress [reviewed by 6]. MAPK is an important driver of GVBD in bovine oocytes [7, 8] and acts to phosphorylate hormone sensitive lipase in other cell types resulting in lipolysis of stored lipid [9, 10]. Bovine oocytes contain an abundance of lipid, comprised mostly of triglycerides and phospholipids [11], which may influence developmental competence after *in vitro* fertilization [12]. Although very little is known about the regulation of lipid metabolism during maturation, triglyceride content of mature bovine oocytes is significantly less than that contained within germinal vesicle stage oocytes [13, 14]. Decreases in triglyceride content during oocyte maturation have been coincident with increased lipase activity [15, 16].

Depending on the extent to which exposure of bovine oocytes to a physiologically-relevant heat stress alters MAPK activity, it was hypothesized that lipid metabolism during maturation may be altered in heat-stressed oocytes. In support of this notion, heat stress altered lipolytic activity in other cell types. For instance, fatty acid release in skeletal muscle cells was increased when human patients with Malignant Hyperthermia (a genetic disorder causing fever after administration of general anesthetics) became feverish [17], possibly as a result of increased triglyceride catabolism [18].

Furthermore, application of radiant energy to fatty areas of the human body sufficient to elevate the temperature of adipocytes to approximately 41 C increases the rate of lipolysis [Laser Lipolysis; 19]. Because the body temperature of heat stressed-dairy cows may reach or exceed 41 C [20–22] and bovine oocytes have a relatively high lipid content, an overarching goal of the two studies described herein was to characterize lipolytic changes in bovine oocytes matured at thermoneutral and hyperthermic conditions. Because of the importance of relating changes, if any, to the incidence of GVBD, we first examined lipolytic changes in triglyceride and phospholipid content at thermoneutral conditions during the time period of maturation when GVBD first becomes evident. A second study was then performed examining lipolytic changes in triglyceride and phospholipid content in relation to the timing of GVBD in oocytes matured at thermoneutral and hyperthermic conditions.

Materials and Methods

Unless otherwise stipulated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Collection and in vitro maturation of oocytes

In general, the methods used to collect and mature bovine oocytes *in vitro* were as described previously by Rispoli *et al.* [5]. Depending on study, approximately 35 to 50 cumulus-oocyte complexes (COCs) were randomly grouped for maturation at 38.5 C and/or 41.0 C (heat-stressed COCs were transferred to 38.5 C after the first 12 hIVM) for up to 24 h. Immediately before placement into maturation medium (0 h), or at designated times thereafter, a subset of COCs was removed from culture and denuded completely of cumulus cells. Between 0 to 12 hIVM, oocytes were denuded by vortexing in HEPES-TALP; whereas COCs from the 24 h groups were vortexed in HEPES-TALP containing 0.3 mg/ml of hyaluronidase. Denuded oocytes determined free of cumulus cells were fixed in 3% paraformaldehyde-Dulbecco's Phosphate Buffered Saline (DPBS, without $CaCl_2$ or $MgCl_2$) for 1 h at room temperature, protected from light.

Study One: timing of lipolytic changes and GVBD in bovine oocytes undergoing in vitro maturation at 38.5 C

Lipolysis was evaluated by examining triglyceride and phospholipid content in COCs cultured for 0, 2, 4, 6 or 24 hIVM as modified from Genicot *et al.* [23] and Auclair *et al.* [15]. Fixed oocytes were incubated in 0.2 µg/ml Nile Red fluorescent lipophilic stain in 1% PVP-DPBS for 2 h at room temperature, protected from light. Stained oocytes were washed in 1% PVP-DPBS and then transferred in groups of ten per 100 µl 1% PVP-DPBS into separate wells of a 96-well black microplate with a transparent bottom (Thermo Scientific Nunc – Thermo Fisher Scientific; Rochester, NY, USA). Fluorescent readings were obtained using a Synergy H1 microplate reader (BioTek Instruments, VT, USA) at two fluorescent settings: excitation 485/emission 588 (triglyceride) and excitation 549/emission 628 [phospholipid; 24, 25]. Once measurements were obtained, background fluorescence was subtracted and the corrected value from each well was divided by the number of oocytes in said well to determine the arbitrary fluorescent units (A.F.U.) per oocyte. After fluorescence was recorded, oocytes were removed from the 96-well microplate and stained with Hoechst 33342 (0.5 µg/ml) before mounting to a slide under a coverslip. Nuclear stage of individual oocytes was determined using fluorescence (excitation 330 – 380/emission ≥ 420) on a Nikon TE300 Inverted Fluorescent microscope. Oocytes were determined to have undergone GVBD if the germinal vesicle (GV) was no longer detectable and the nuclear material was in a condensed chromatin (CC) configuration or at MI. This study was replicated on six different occasions using 1,237 oocytes in total (the total number of pools per each of the five treatment groups ranged from 17 to 25).

Study Two: lipolytic changes, GVBD and ATP content during in vitro maturation of bovine oocytes at 38.5 and 41.0 C

Triglyceride and phospholipid content of control and heat-stressed oocytes was assessed at 0, 2, 4, 6 and 24 hIVM as previously described. To better control for variability, each plate was read ten times instead of once and values were averaged separately for each well. After the average fluorescence was recorded, oocytes were transferred from the 96-well microplate and prepared for nuclear stage assessment using Hoechst 33342.

Concurrent with efforts described above, ATP content was measured in a subset of oocytes taken before fixation from each treatment group at five different time points (0, 2, 4, 6 and 24 and cultured at 38.5 C and 41.0 C). Levels of ATP were evaluated in oocytes to serve as an indirect measure of mitochondrial β-oxidation released during lipolysis. To this end, oocytes were denuded of surrounding cumulus cells and the zona pellucida was removed using 0.5% pronase. Oocytes were then transferred individually to microcentrifuge tubes, lysed in sterile water, and stored at –80 C. Oocyte lysates were assessed for ATP content using the ATP determination kit from Invitrogen (Division of Life Technologies; Carlsbad, CA, USA) and a tube-based luminometer (Berthold, Huntsville, AL, USA) set to read the sample for ten seconds after a three second hold-time. The total amount of ATP in each oocyte lysate was determined using a standard curve ranging from 0 to 10 pmol. This study was replicated on six different occasions using 2,680 oocytes for lipid analysis (14 to 16 pools per each of nine treatment groups) and 270 for ATP analysis (21 to 39 oocytes per treatment group).

Statistical analyses

Data from Studies One and Two were analyzed as a randomized block design, blocking on date of collection with fixed effects of maturation time and temperature where appropriate, using generalized linear mixed models (PROC GLIMMIX, SAS 9.4, SAS Institute, Cary, NC, USA). The experimental unit for all data analyses was the 4-well Nunc plate in which the oocytes were incubated during IVM, as treatments were applied to a plate rather than individual oocytes. Treatment differences from all analyses were determined using F-protected least significant differences and reported as least squares means ± standard error of the mean (SEM). The association of nuclear maturation to lipolytic changes during IVM was assessed for each study using regression with GVBD serving as the dependent variable and blocking on collection date. The fixed effect of maturation temperature was included when appropriate. R-square was calculated from likelihoods (PROC GLIMMIX) as per Nagelkerke [26].

Results

Study One: timing of lipolytic changes and GVBD in bovine oocytes undergoing in vitro maturation at 38.5 C

Triglyceride content per oocyte was greatest in GV-stage oocytes at 0 hIVM (P < 0.0001; Fig. 1, panel A). By 2 hIVM triglyceride content decreased, with content being lower at 24 hIVM (P < 0.0001; Fig. 1, panel A). Similar to the triglyceride content, phospholipid content per oocyte was greatest at 0 hIVM, decreased by approximately half after 2 hIVM, and then decreased further by 6 hIVM (P < 0.0001; Fig. 1, panel B). Independent of IVM time, triglyceride content was almost double that of phospholipid content (283.14 *vs.* 156.56; SEM = 21.35). The resultant ratio of 1.81 to 1 did not change during *in vitro* oocyte maturation (P = 0.4201).

At 0 hIVM, the GV was intact in all oocytes that were evaluated. Percentage of oocytes undergoing GVBD increased at 4 hIVM (P < 0.0001; Fig. 1, panel C). Condensed chromatin was evident in almost all of the oocytes that had undergone GVBD by 4 hIVM. Incidence of GVBD was more pronounced at 6 hIVM (P < 0.0001; Fig. 1, panel C). Less than 1% of oocytes had reached the MI stage by 6 hIVM. After 24 hIVM the majority of oocytes were at the MII stage (83.97 ± 0.02%).

Lipolytic changes in triglyceride (R^2 = 0.2477; P = 0.0095) and phospholipid (R^2 = 0.2335; P = 0.0121) content were associated with GVBD in bovine oocytes undergoing meiotic maturation. For example, when lipid content was typically high at 0 hIVM incidence of GVBD was low; however, when lipid content was low at 24 hIVM the incidence of GVBD was high.

Study Two: lipolytic changes, GVBD and ATP content during in vitro maturation of bovine oocytes at 38.5 and 41.0 C

Triglyceride content decreased by 2 hIVM similarly in control and heat-stressed oocytes (P = 0.0009; SEM = 40.23). Independent of maturation temperature, triglyceride content remained similar from 2 and 6 hIVM, yet was further decreased at 24 hIVM (P = 0.0003; Fig. 2, panel A). Similar to triglyceride content, phospholipid content decreased by 2 hIVM similarly in control and heat-stressed oocytes (P = 0.0005; SEM = 21.54). Independent of maturation temperature, phospholipid content was lower at 6 hIVM and decreased even further by 24 hIVM (P < 0.0001; Fig. 2, panel B). Triglyceride content was almost double that of phospholipid (184.82 *vs.* 93.33; SEM = 17.42). The resultant ratio of 1.98 to 1 was not affected by maturation time (P = 0.1841) or temperature (P = 0.3927).

The effect of maturation temperature on the ability of oocytes to undergo GVBD differed depending upon the length of IVM (IVM temperature × hIVM interaction; P < 0.0001; Table 1). Specifically, the proportion of control and heat-stressed oocytes undergoing GVBD was similar at 2 hIVM. At 4 hIVM, however more heat-stressed oocytes had undergone GVBD; the effect of heat stress to increase the incidence of GVBD was more pronounced at 6 hIVM (Table 1). At 24 hIVM, a similar proportion of control and heat-stressed oocytes had undergone GVBD and progressed to MII (Table 1).

Independent of maturation temperature, lipolytic changes in triglyceride (R^2 = 0.2123; P = 0.0030) and phospholipid (R^2 = 0.2243; P = 0.0026) content were associated with incidence of GVBD in bovine oocytes undergoing meiotic maturation. Differences in oocyte
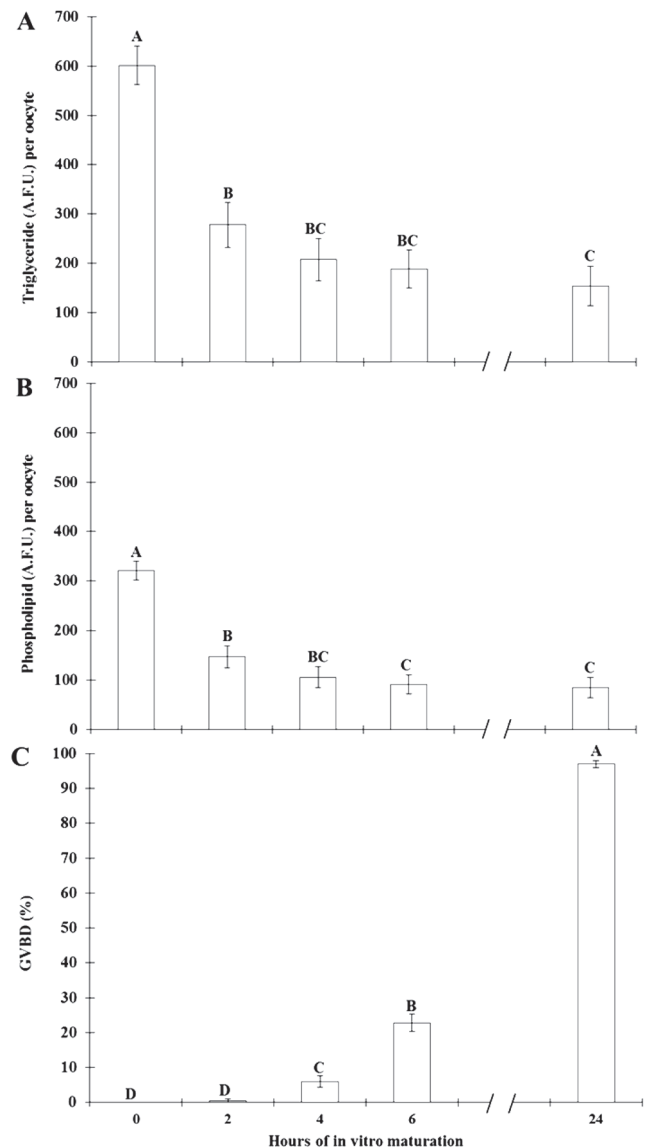

Fig. 1. Triglyceride (Panel A) and phospholipid (Panel B) content (average fluorescence units (A.F.U.) ± SEM) in bovine oocytes matured *in vitro* for 0, 2, 4, 6 or 24 h. Panel C: Percentage of bovine oocytes at 0, 2, 4, 6 or 24 hIVM that had undergone germinal vesicle breakdown (GVBD). [A-D] Letters within a panel differ, P < 0.05.

ATP content were dependent upon both maturation temperature and time (IVM temperature × time interaction; P = 0.0082; Fig. 2, panel C). Oocyte ATP content was not different in control and heat-stressed oocytes matured for 6 h after placement in maturation medium. However, at 24 hIVM ATP content was higher in heat-stressed oocytes (Fig. 2, panel C).

Discussion

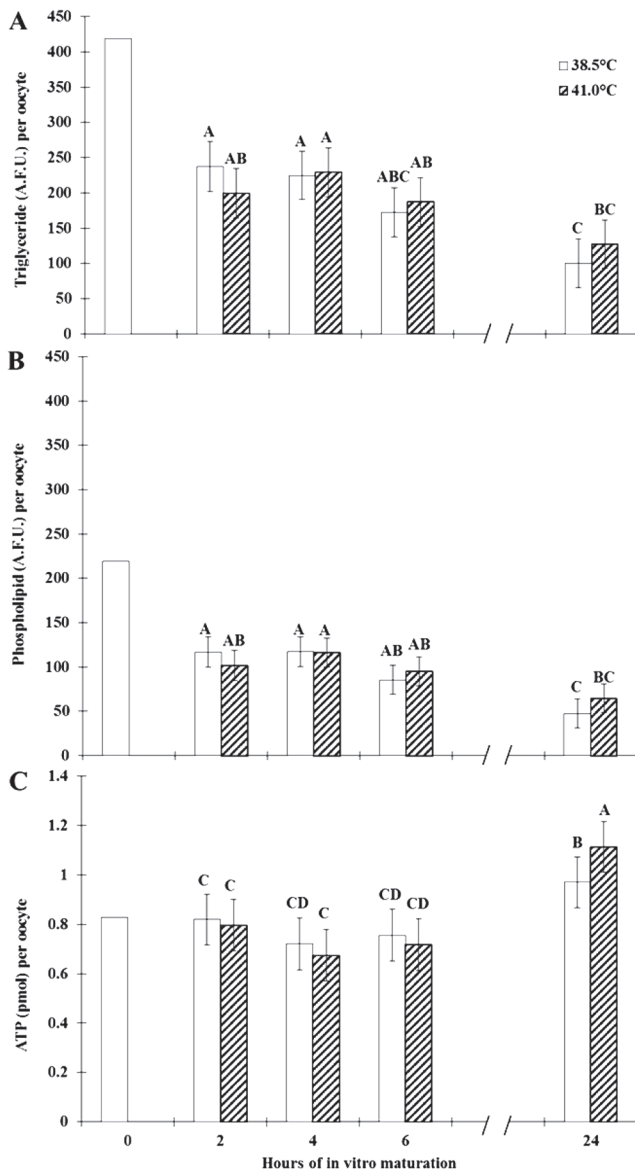Novel findings described herein provide additional evidence that

Fig. 2. Triglyceride (Panel A) and phospholipid (Panel B) content (average fluorescence units (A.F.U.) ± SEM) and ATP content (Panel C; pmol per oocyte ± SEM) in bovine oocytes matured *in vitro* for 0, 2, 4, 6 or 24 h at 38.5 or 41.0 C (first 12 h only). [A–D] Letters within a panel differ, P < 0.05.

exposure of bovine oocytes to a physiologically-relevant heat stress hastens the incidence of GVBD. Although underlying mechanisms remain unclear, changes in lipolytic activity are not likely problematic because triglyceride and phospholipid content changed similarly in oocytes matured at thermoneutral and heat-stressed conditions. Interestingly, ATP content at 24 hIVM was higher in bovine oocytes heat stressed during the first 12 h of maturation. Whether this novel finding serves as an indicator of downstream lipolytic changes involving heightened fatty acid β-oxidation, or representative of a surplus of ATP from mitochondrial dysfunction, changes in protein

synthetic capabilities, or other alterations yet to be identified, remains unclear. Nonetheless, in two different studies, marked reductions in triglyceride and phospholipid content were noted after 2 hIVM in bovine oocytes. Regardless of maturation temperature, lipolytic changes in the oocyte preceded incidence of GVBD.

Heat-induced hastening of GVBD was evident as early as 4 hIVM with effects being more pronounced at 6 hIVM. Similar findings were previously reported by Edwards *et al.* [3] showing that a higher proportion of heat-stressed oocytes undergo GVBD sooner than non-heat stressed counterparts. The fact that an equivalent number of control and heat-stressed oocytes progressed to MII after 24 hIVM provides further evidence that there are instances whereby heat stress exposure at the beginning of maturation hastens, rather than inhibits, meiotic maturation. The increased ATP content of heat-stressed oocytes at 24 hIVM is consistent with age-related changes occurring after oocyte maturation is completed. Koyama *et al.* [27] reported that bovine oocytes matured for 30 to 40 h had more ATP content than oocytes matured for 20 h. Depending upon the extent to which heat-induced hastening of maturation occurs, oocytes experiencing heat stress during the beginning of meiotic maturation are likely aged at the time of fertilization. This could explain some of the previously reported reductions in oocyte developmental competence after exposure to elevated temperatures during the onset of meiotic maturation [3–5].

Although GVBD was associated with lipolytic changes, increased incidence of GVBD in heat-stressed oocytes was not related to changes in triglyceride or phospholipid content. This may not preclude possible heat stress effects on triglyceride catabolism however, as the energy requirements of an oocyte could be met by the breakdown of a small amount of lipid (e.g., the breakdown of one mole of fatty acid typically results in the production of approximately 100 moles of ATP [28]). During the first 6 hIVM, ATP content remained unchanged and was similar in control and heat-stressed oocytes. Depending on the extent to which oocytes, like other cell types experiencing heat stress consume more ATP [reviewed by 29], any heat-related differences in fatty acid β-oxidation may have been masked. Although speculative, addition of L-Carnitine, an upregulator of β-oxidation, to porcine or murine oocytes during maturation increased the rate of meiotic progression [reviewed by 30, 31].

Heat-induced increases in ATP content at 24 hIVM could be attributable to changes in protein synthesizing capabilities. Protein synthesis is energetically taxing; thus, heat-induced reductions in de novo synthesis by as much as 30 to 50% in bovine oocytes [32] could create a surplus of unused ATP. Regardless of the underlying mechanism(s), increased ATP content due to the effect of heat stress is likely real. In other cell types, ATP content is increased after exposure to elevated temperatures, possibly due to a greater cellular requirement for energy (ATP) necessary for overcoming stress-related apoptosis [reviewed by 29]. Increased ATP content in heat-stressed oocytes may also be the result of alterations in metabolic pathways whereby ATP remains unused [reviewed by 33] or key components involved in ATP production are altered [ATP synthase; 34].

Nonetheless, immature oocytes have an abundance of intracellular lipid droplets, much of which are degraded during maturation [35]. Consistent with this finding, several studies [13–15], including ours, show that lipid content changes during maturation with triglyceride

Table 1. Meiotic progression of bovine oocytes undergoing IVM at 38.5 or 41.0 C

Treatment Combinations		Nuclear Stage (%)						
Hours of IVM	Temperature (C)	GV	CC	MI	GVBD[1]	AI[2]	TI[3]	MII
2	38.5	99.26^a	0.73^d	0^b	0.74^d	-	-	-
2	41.0	100.00^a	0^d	0^b	0^d	-	-	-
4	38.5	95.40^a	4.63^{cd}	0^b	4.59^d	-	-	-
4	41.0	88.76^b	11.27^b	0^b	11.24^c	-	-	-
6	38.5	88.76^b	9.94^{bc}	1.27^b	11.24^c	-	-	-
6	41.0	58.76^c	36.60^a	4.60^a	41.24^b	-	-	-
24	38.5	0.14^d	0^d	4.75^a	99.85^a	2.07^a	2.09^a	91.06^a
24	41.0	0.65^d	0^d	5.18^a	98.07^a	0.65^a	0.64^a	91.59^a

A = Representative image of germinal vesicle (GV)-stage oocyte, scale bar = 20 μm. B = Representative image of oocyte with condensed chromatin (CC). C = Representative image of oocyte with metaphase I (MI) chromatin configuration. D = Representative image of oocyte with metaphase II (MII) chromatin configuration. $^{a–d}$ means differ within a column (P < 0.05). [1]GVBD = Germinal Vesicle Breakdown. [2]AI = Anaphase I stage. [3]TI = Telophase I stage. – = nuclear stage not observed at these time periods.

levels being lower in mature oocytes compared to GV-stage oocytes at 0 hIVM. Previous efforts examining only two time points (i.e., at the beginning and end of maturation; 0 and 22–24 hIVM) speculated that maturation-related changes were most likely gradual [13, 14]. However, findings from our study examining changes during the first 6 hIVM document marked changes (i.e., reductions) in triglyceride and phospholipid content of bovine oocytes within the first 2 hIVM. Though the physiological significance of the marked decrease within 2 hIVM remains unclear, activation of mechanisms important for reducing much of the lipid content in an oocyte is likely critical in the continuum of remodeling/transforming cytoplasmic components in the oocyte to be "inherited" by the resultant zygote after fertilization. Interestingly, decreases in triglyceride content continue as the zygote begins to undergo cell cleavage divisions [13].

Another potential role for the rapid breakdown of lipids in the oocyte during the first 2 hIVM may be to release important proteins or histones that are packaged within lipid droplets. In a variety of different cell types, lipid droplets serve as a protein storage depot [reviewed by 36]. In Drosophila, approximately 50% of certain embryonic histones are physically attached to lipid droplets [37]. Because a fraction of H2Av was colocalized with lipid droplets in both nurse cells and in oocytes, Cermelli et al. [37] proposed that the lipid droplet may play an important role towards the storage of maternally-provided histones for early embryo development. Further evaluation showed that the associated histones were capable of translocation to the nucleus, where they could then take part in regulating DNA transcription. However, histones became unavailable for use in transcriptional regulation if lipid droplets did not properly redistribute during oocyte and early embryonic development [37].

Although it is not clear if lipid droplets play a similar role in the bovine oocyte, it is interesting to note that bovine cumulus oocyte-complexes require a 1 to 2 h transcriptional phase towards synthesis of necessary proteins for driving meiosis [reviewed by 38].

Various fatty acids are released from the breakdown of lipid droplet-triglycerides. However, it remains unclear where or how released fatty acids are utilized within the oocyte. When lipolytic changes are most pronounced, bovine oocytes are intimately associated with surrounding cumulus cells [39]. These intimate associations via gap junctional-complexes allow for a bidirectional flow of signals and metabolites between the oocyte and the cumulus [reviewed by 40]. Although cumulus cells were not evaluated as a part of this study, one cannot preclude the potential for these cells to receive, and possibly utilize, the by-products released from lipolytic activity occurring in the oocyte. Auclair et al. [15] reported that lipid droplet breakdown was greater in bovine oocytes matured with intact cumulus cells compared to those matured without surrounding cumulus. Furthermore, fatty acids are commonly packaged into vesicles to allow for transportation to membrane surfaces in other cell types [41]. Kruip et al. [35] observed small vesicles surrounding many of the cumulus cell processes in bovine oocytes during early maturation.

In conclusion, findings described herein provide additional evidence that exposure of bovine oocytes to a physiologically-relevant heat stress hastens the incidence of GVBD. Although underlying mechanisms remain unclear, changes in lipolytic activity reported herein are not likely problematic because triglyceride and phospholipid content decreased similarly in oocytes matured at thermoneutral and heat-stressed conditions. Whether higher ATP content at 24 hIVM in oocytes experiencing heat stress during the first 12 h of

maturation serves as an indicator of downstream lipolytic changes involving heightened fatty acid β-oxidation, or are representative of 1) a surplus of ATP from mitochondrial dysfunction, 2) changes in protein synthetic capabilities, or 3) other alterations yet to be identified, remains to be determined.

Acknowledgments

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