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Does heat stress provoke the loss of a continuous layer of cortical granules beneath the plasma membrane during oocyte maturation?

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Summary

The objective of the present study was to evaluate the influence of heat stress on bovine oocyte maturation. Both nuclear stage and distribution of cortical granules (CG) were simultaneously evaluated in each oocyte. Oocyte overmaturation under standard conditions of culture was also evaluated. For this purpose, logistic regression procedures were used to evaluate possible effects of factors such as heat stress, overmaturation, replicate, CG distribution and metaphase II (MII) morphology on oocyte maturation. Based on the odds ratio, oocytes on heat stressed (HSO) and overmaturated (OMO) oocyte group were, respectively, 14.5 and 5.4 times more likely to show anomalous MII morphology than those matured under control conditions (CO). The likelihood for an oocyte of showing the CG distribution pattern IV (aging oocyte) was 6.3 and 9.3 times higher for HSO and OMO groups, respectively, than for the CO group. The risk of undergoing anomalous oocyte maturation, considering both nuclear stage and distribution of CG was 17.1 and 18 times greater in oocytes cultured in HSO and OMO groups, respectively, than those in the CO group. In conclusion, heat stress proved to be valuable in aging oocytes. Heat stress advanced age for nuclear and cytoplasmic processes in a similar form to that of oocyte overmaturation.

Keywords: Aging, Cortical granules, Heat stress, Oocyte maturation, Overmaturation

Introduction

Although molecular studies are now considered especially important (Bhojwani *et al.*, 2006; Fair *et al.*, 2007; Evsikov & Marin de Evsikova, 2009; Siemer *et al.*, 2009) classical concepts concerning the maturation of mammalian oocytes (Szollosi, 1975b; Thibault *et al.*, 1987) still focus on two aspects: nuclear maturation and cytoplasmic maturation (Sirard, 2001; Ferreira *et al.*, 2009) that are usually highly coordinated (Eppig, 1996). Nuclear maturation involves the transition from a germinal vesicle nucleus to a second metaphase arrangement of the chromosomes and formation of a first polar body by the time of ovulation in most species so far studied. Cytoplasmic maturation is expressed as changes in protein composition, but most conspicuously in the redistribution of organelles that are termed the cortical granules (CG) (Szollosi, 1962, 1967). Such granules are, in fact, small vesicles that contain enzymes. During resumption of meiosis, the CG migrate from the Golgi apparatus to close to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane (Ducibella & Buetow, 1994). Only when situated just beneath the plasma membrane can they undergo exocytosis by fusing with the egg membrane. This fusion enables release of the CG contents into the perivitelline space, an important step in membranous maturation and in instigating a block to polyspermy (Szollosi, 1967; Hosoe & Shioya, 1997; Wang *et al.*, 1997).

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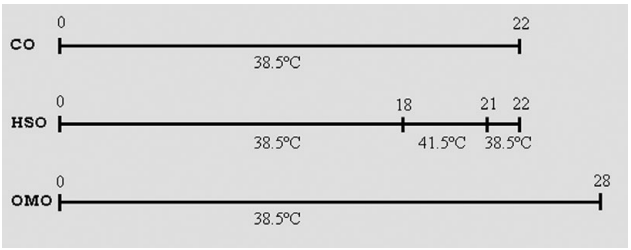

Figure 1 A time-line figure illustrating the experimental setup for cumulus–oocyte complexes (COCs) culture and groups of study: CO (control oocytes; $n = 75$), HSO (heat stress oocytes; $n = 80$) and OMO (overmaturation oocytes; $n = 20$).

There have been diverse studies that focussed on the formation and distribution of the CG in mammalian oocytes (Szollosi, 1967, 1978; Flechon, 1970; Thibault *et al.*, 1987; Hosoe & Shioya, 1997; Wang *et al.*, 1997; Wessel *et al.*, 2001, 2002) but none in the present context. In this study on maturation of bovine oocytes, the influence of heat stress on both nuclear stage and distribution of cortical granules has been evaluated in each oocyte. As heat stress seems to induce premature aging of oocytes (Lawrence *et al.*, 2004; Edwards *et al.*, 2005; Schrock *et al.*, 2007) the effect of overmaturation on nuclear and cortical granules distribution features was also evaluated. This research has relevance to bovine reproduction as heat stress has been associated with reduced fertility in many countries (de Rensis & Scaramuzzi, 2003; López-Gatius, 2003) especially when it coincides with the insemination time (Putney *et al.*, 1989; García-Ispierto *et al.*, 2007).

Materials and methods

Experiment design

Three groups were established: (1) control oocytes (CO; $n = 75$), maintained under culture conditions (38.5°C for 22 h); (2) heat stressed oocytes (HSO; $n = 80$) submitted to the heat treatment; and (3) overmaturated oocytes (OMO; $n = 20$) cultured for 28 h (Fig. 1).

As a 3 h heat treatment mimics hyperthermia real conditions (Tseng *et al.*, 2004) of the 22 h of the *in vitro* maturation (IVM) process, the HSO group was exposed to 41.5°C during the period of 18 to 21 h of maturation.

Chemicals and reagents

All chemicals were purchased from Sigma unless otherwise indicated.

Collection of oocytes

Ovaries recovered from slaughterhoused young heifers and placed into Dulbecco's phosphate buffered saline solution (PBS; DPBS 10×; GIBCO-Invitrogen) including 1% (v/v) antibiotic antimycotic solution (AA; 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml amphotericin B) and carried to the laboratory at room temperature. Ovaries were then washed twice in warm sterile PBS and were kept at 37.5°C until ovarian puncture that took place within 2 h from their recovery. Next, 2 to 8 mm-sized follicles were aspirated using an 18-gauge needle joined to a 5ml syringe. Cumulus–oocyte complexes (COCs) were obtained and placed into working medium (WM; Medium 199 with Earle's salts 25 mM HEPES and $NaHCO_3$, 1% v/v AA solution). Only oocytes enclosed in three or more layers of compact cumulus cells and presenting a homogeneous and translucent ooplasm were selected.

In vitro maturation

Selected COCs were washed twice in WM and once in maturation medium (MM; Medium 199 with Earle's salts, L-glutamine and $NaHCO_3$, supplemented with 20 µg/ml epidermal growth factor, 2 mM sodium pyruvate and 1% v/v AA solution) that had been pre-equilibrated for 3 h at 38.5°C in 5% (v/v) CO_2 in humidified air.

After washing, COCs were randomly allocated to groups of 20–25 and placed into 4-well dishes (Nunc) containing 500 µl of MM. All the processes were performed in a laminar flux booth during approximately 2 h from follicular aspiration to the entrance of COCs to IVM.

COCs were cultured at different temperature conditions for 22 h (CO and HSO groups) or 28 h (OMO group) according to the experimental design in an atmosphere of 5% (v/v) CO_2 in humidified air. To reduce variation of temperature during culture, incubation of control and overmaturation groups and heat stress group COCs were performed in two different CO_2 incubators. While one incubator was set at 38.5°C, the other was used for the heat treatment. Both incubators were kept closed during the whole maturation period and their temperature was verified by checking of clinical thermometers that were placed inside of the incubators.

Zona pellucida digestion and oocyte fixation

After 22 h (CO and HSO groups) or 28 h (OMO group) of IVM, COCs were removed from maturation wells and denuded of cumulus cells by pipetting and washed twice in PBS with 0.05% (w/v) of bovine serum

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albumin (BSA; fraction V BSA). Oocytes were then immersed in PBS containing 0.4% (w/v) pronase for 3 min to dissolve zona pellucida and washed five times in PBS with 0.05% (w/v) BSA. Oocytes were fixed in a PBS solution containing 4% (w/v) of paraformaldehyde for 45 min at room temperature and washed five times in PBS with 0.05% (w/v) BSA.

Oocyte permeabilization and cortical granules staining

Oocytes were immersed in a permeabilizing solution of PBS containing 0.3% Triton X100 and 0.05% (w/v) BSA for 5 min at room temperature and washed five times in PBS with 0.05% BSA. Oocytes were then incubated in the dark for 30 min at room temperature into a staining solution of PBS containing 100 µg/ml of fluorescein isothiocyanate-labelled *Lens culinaris* agglutinin (FITC–LCA) and 0.05% BSA.

Nuclear staining and mounting

Stained oocytes were thoroughly washed in PBS with 0.05% (w/v) BSA to remove excess of FITC–LCA before mounting, and mounted between a pretreated with poly-L-lysine coverslip and a glass slide supported by a washer. The antifade mounting medium contained 4,6-diamidino-2-phenilidole (DAPI; Vectashield; Vector laboratories, Inc.) for counterstaining DNA. The coverslip was sealed with nail polish and preparations were kept at 4°C and protected from light until examination by fluorescence and laser confocal microscopy.

Nuclear and cytoplasmic maturation evaluation

Nuclear and cytoplasmic maturation status was evaluated for each oocyte. Nuclear content and apical and equatorial section of each oocyte were photographed under UV epifluorescent microscope (Nikon Eclipse TE 2000S) and laser confocal microscope (Leica TCS SP2) respectively.

Cultured oocytes were checked to have reached metaphase II (MII) or not. Normality in MII morphology was furthermore registered according to the modified classification of Tseng (2004) (normal MII: uniform alignment of the chromosomes on the spindle; anomalous MII: nuclear content changed into chromatin-like structure forming condensed aggregates or forming aberrantly distributed chromosomes).

Translocation of CG to the oolemma was used as an indicator of cytoplasmic maturation (Damiani *et al.*, 1996). The distribution of GC was classified into four patterns according the classification of Hosoe & Shioya (1997) (pattern I: GCs distributed in clusters or large aggregates; pattern II: GC individually dispersed and partially clustered or aggregated; pattern III: GC completely dispersed; pattern IV: no CG).

Statistical analysis

Only oocytes that reached MII nuclear stage were included in the statistical analysis. The following data were recorded for each oocyte: replicate (1–4), group (CO, HSO or OMO), MII morphology (normal MII versus anomalous MII) and CG distribution pattern (I, II, III or IV).

On data from each oocyte, a first logistic regression analysis was performed using MII anomalous morphology as the dependent variable (0 or 1) and replicate, group and CG distribution pattern as independent factors. A second logistic regression analysis was performed using CG distribution pattern IV as dependent variable (0 or 1) and replicate, group and MII morphology as independent factors. A further logistic regression analysis was performed using anomalous oocyte maturation, considering both MII anomalous morphology and GC distribution pattern IV as the dependent variable (0 or 1) and replicate and group as independent factors. All variables above were considered as class variables.

Logistic regressions analyses were performed using the SPSS package, version 17.0 (SPSS Inc.) according to the method of Hosmer & Lemeshow (1987). Basically, this method involves five steps as follows: (1) preliminary screening of all variables for univariate associations; (2) construction of a full model using all the variables found to be significant in the univariate analysis; (3) stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; (4) evaluation of plausible two ways interactions among variables; and (5) assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing p-values <0.25 were included in the initial model. We continued modelling until all the main effects or interaction terms were significant according to the Wald statistic at $p < 0.05$.

Results

Effects of heat stress on nuclear maturation

Of the 175 cultured and evaluated oocytes, four showed immature nuclear stages after IVM and were excluded from the study.

Logistic regression analysis indicated no significant effect of replicate or CG distribution pattern on MII morphology. Table 1 shows the adjusted odds ratios of the variable finally included in the logistic model.

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Table 1 Odds ratios of variables included in the final logistic regression model for metaphase II (MII) anomalous morphology.

		MII anomalous morphology				
Factor	Class	*n*	%	Odds ratio	95% CI[a]	*p*-value
Group	Control	8/73	10.9	Reference	–	–
	Heat stress	28/78	35.9	14.5	6.1–34.6	<0.001
	Overmaturation	8/20	40.0	5.4	1.7–17.2	0.004

Likelihood ratio test 48.85; 2 d.f., $p < 0.0001$. Nagelkerke $r^2 = 0.337$.
[a]Confidence interval for the odds ratio.

Table 2 Odds ratios of variables included in the final logistic regression model for cortical granules (CG) distribution pattern IV.

		CG distribution pattern IV				
Factor	Class	*n*	%	Odds Ratio	95% CI[a]	*p*-value
Group	Control	4/73	5.6	Reference	–	–
	Heat stress	21/78	26.9	6.4	2.1–19.6	0.001
	Overmaturation	7/20	35.0	9.3	2.4–36.3	0.001

Likelihood ratio test 17.88; 2 d.f., $p < 0.0001$. Nagelkerke $r^2 = 0.154$.
[a]Confidence interval for the odds ratio.

No significant interactions were found. Based on the odds ratio, oocytes in HSO and OMO groups were, respectively, 14.5 and 5.4 times more likely to showed anomalous MII morphology after the IVM than those matured under control conditions.

Effects of heat stress on cortical granule distribution

Figure 2 shows laser confocal microscopic images of the equatorial section of FITC–LCA-labelled bovine oocytes showing representative CG distribution patterns and the number of oocytes for each pattern and group of study.

Logistic regression analysis indicated no significant effect of replicate or MII morphology on CG distribution pattern IV. Table 2 shows the adjusted odds ratios of the variable finally included in the logistic model. No significant interactions were found. Based on the odds ratio, the likelihood for an oocyte of showing CG distribution pattern IV was 6.3 and 9.3 times higher for HSO and OMO groups, respectively, than for the CO group.

Effects of heat stress on oocyte maturation considering both nuclear and cytoplasmic maturation

Logistic regression analysis indicated no significant effect of replicate on anomalous maturation considering both MII anomalous morphology and CG distribution pattern IV. Table 3 shows the adjusted odds ratios of the variable finally included in the logistic model. No significant interactions were found. Based on the odds ratios, the risk of undergo anomalous oocyte maturation was 17.1 and 18 times greater in oocytes cultured in HSO and OMO groups, respectively, than those in CO group.

Discussion

Heat stress proved to be valuable in aging oocytes. For this purpose nuclear and cytoplasmic maturation for each oocyte were assessed simultaneously. Similar figures of aged oocytes (Szollosi, 1971, 1974, 1975a) were registered for heat stressed and overmaturated oocytes. By using logistic regression procedures, the models were highly explanatory and significant. Three consecutive analyses showed how dramatically heat stress advanced age for nuclear and cytoplasmic processes. Including both nuclear and cytoplasmic maturation measurements, and based on the odds ratio, heat stressed and overmaturated oocytes were 17 and 18 times, respectively, more likely to age than controls, used as reference.

Heat stress during the last period of IVM resulted in a high percentage of oocytes showing a pattern IV of CG distribution. Pattern IV, which is characterized by the loss of the continuous layer of CG, was firstly described

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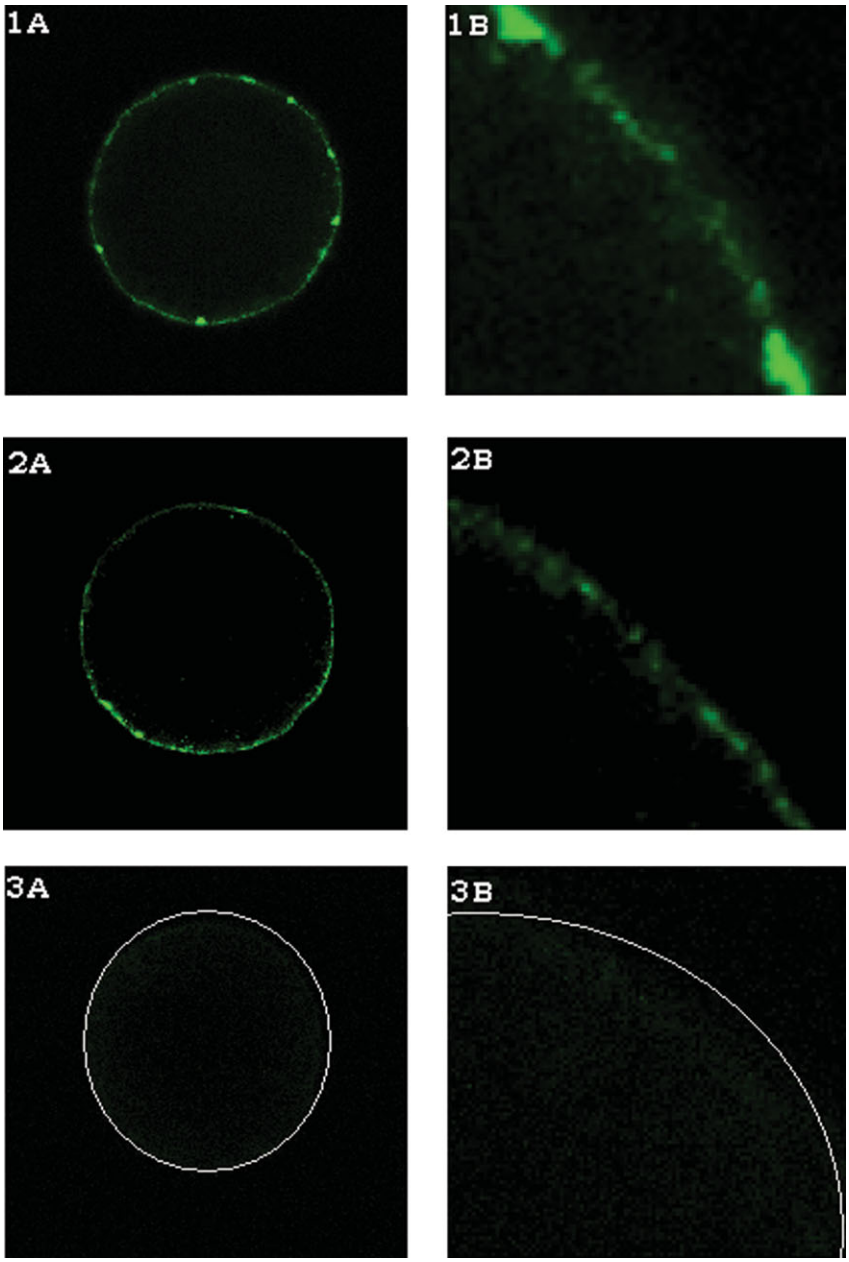

Figure 2 Laser confocal microscopic images of the equatorial section (*A*) and its detail (*B*) of fluorescein isothiocyanate *Lens culinaris* agglutinin (FITC–LCA)-labelled bovine oocytes showing representative patterns of CG distribution for the different groups: pattern II (1A, 1B: CO = 14/73, HSO = 12/78, OMO = 1/20), pattern III (2A, 2B: CO = 54/73, HSO = 45/78, OMO = 12/20), pattern IV (3A, 3B: CO = 14/73, HSO = 21/78, OMO = 7/20). CO, control oocytes; HSO, heat stress oocytes; OMO, overmaturation oocytes.

in bovine oocytes by Hosoe Shioya (1997) and is the most common pattern of CG distribution for oocytes that had underwent exocytosis after insemination or activation. Although heat stress advanced oocyte maturation in previous studies (Payton *et al.*, 2004; Edwards *et al.*, 2005), pattern IV of CG distribution related to the final maturation of oocyte was not described. We describe herein how heat treatment or overmaturation can result in a pattern IV of CG distribution in a large number of oocytes. These results suggest that heat stress applied at the end of the period of oocyte maturation can induce CG loss.

As observed in the present study, an anomalous progression to second meiotic metaphase was a feature of heat stressed oocytes. A modified behaviour of the cytoskeleton may be the underlying cause, as it has been described in previous studies (Tseng *et al.*, 2004; Ju *et al.*, 2005; Roth & Hansen, 2005). Nonetheless,

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Table 3 Odds ratios of variables included in the final logistic regression model for anomalous oocyte maturation, considering both anomalous patterns of metaphase II (MII) morphology and cortical granules (CG) distribution pattern.

		MII anomalous morphology and CG distribution pattern IV				
Factor	Class	*n*	%	Odds ratio	95% CI[a]	*p*-value
Group	Control	1/73	1.4	Reference	–	–
	Heat stress	15/78	19.2	17.1	2.0–133.5	0.007
	Overmaturation	4/20	20.0	18.0	1.9–172.0	0.012

Likelihood ratio test 16.45; 2 d.f., $p < 0.0001$. Nagelkerke $r^2 = 0.178$.
[a]Confidence interval for the odds ratio.

other aspects of nuclear behaviour may be modified by temperature stress, such as the rate and normality of chromosomal condensation (Roti, 2008). The behaviour of contractile proteins of the cytoskeleton will be perturbed by inappropriate temperature. Aspects that remain to be clarified are: (1) how much variation in temperature an oocyte can withstand; and (2) whether tolerance to shifts in temperature changes with the stage of meiotic maturation.

Overall, there is the question as to whether tolerance to temperature modifications *in vitro* differs from that in the living animal. There is already a body of evidence indicating that the temperature of preovulatory Graafian follicles is lower than temperature elsewhere in the ovary of rabbits (Grinsted *et al.*, 1980), women (Grinsted *et al.*, 1985), pigs (Hunter *et al.*, 1997, 2000, 2006) and perhaps cattle (Greve *et al.*, 1996). This finding suggests the existence of sensitive systems of temperature regulation *in vivo* that may be difficult to mimic *in vitro*.

A further experiment that would be relevant would be to culture individual Graafian follicles (Baker & Neal, 1972, 1974; Picton *et al.*, 2008) under different protocols of heat stress. Such a study might reveal greater perturbations in oocyte maturation when within a heat stressed follicle.

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