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Short communication

The effect of kinetic heat shock on bovine oocyte maturation and subsequent gene expression of targeted genes

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Summary

The exposure of oocytes to heat stress during the maturation process results in harmful effects to their internal organelles, low fertilization capability and higher embryonic losses. In the present experiment the effect of heat shock (HS) during the maturation process was assessed. In Assay 1, oocytes from winter (December–March; $n = 100$) and summer (June–September; $n = 100$) months were collected and matured to analyse their HS tolerance. Total RNA was extracted from matured oocytes and cDNA synthesis was performed, followed by qPCR for selected genes (*Cx43*, *CDH1*, *DNMT1*, *HSPA14*), compared with two reference genes (*GAPDH* and *SDHA*). In Assay 2, oocytes collected during the winter were subjected to kinetic HS by stressing them at 39.5°C for 6, 12, 18 or 24 h and were afterwards matured at control temperature (38.5°C), and subsequently subjected to the previously described gene analysis procedure. Results of Assay 1 show that summer-collected oocytes exhibited lower maturation rate than winter-collected oocytes, which may be due to the down-regulation of the *HSPA 14* gene. Assay 2 showed that 6 h of HS had no effect on gene regulation. *CDH1* and *DNMT1* up-regulation was observed starting at 12 h, which may represent the effect of heat shock on oocyte development.

Keywords: Apoptosis, Gene quantification, Kinetic heat shock, Maternal heat stress

Introduction

Heat stress, which is caused by a rise in body temperature above its fixed set point, is an important factor that disrupts reproductive performance (Badinga *et al.*, 1985; Pavani *et al.*, 2015b), compromising the functioning of germ cells and the viability of early stage embryos (Hansen, 2009). HS has deleterious effects on the internal organelles, on oocyte maturation rate and fertilization capability, and eventually on embryonic development (Payton *et al.*, 2004; Pavani

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et al., 2015b). Our previous research (Pavani *et al.*, 2015b) demonstrated that both short-term and long-term exposure of oocytes to heat stress led to aberrant nuclear maturation, a process dependent upon extensive rearranging of the cytoskeleton (Gallicano, 2001; Shin & Kim, 2003), which is composed of microtubules and microfilament, that were altered in mature bovine oocytes and 2-cell embryos exposed to temperatures of 41–41.5°C (Tseng *et al.*, 2004; Rivera *et al.*, 2004). Stressed cells release heat shock proteins (HSPs), which facilitate correct folding of newly synthesized proteins and help proteins to cross cellular membranes (Kang *et al.*, 1990). The effects of heat stress are mostly detrimental to oocytes during the first 10–12 h of maturation (Putney *et al.*, 1989) at the time when cumulus cells are intimately associated with the oocyte. Gap junction intercellular communication (GJIC) or connexin 43 (Cx43) between cells can regulate cell death, proliferation and differentiation (Russell & Robker, 2007). Furthermore, oocyte and embryo development is associated with DNA methylation, an epigenetic mechanism for differentially marking the parental alleles of imprinted genes (Denomme & Mann, 2012). It occurs at cytosine residues, mainly within CpG dinucleotides, and is catalyzed by a family of DNA methyltransferases (DNMT). DNMT1 also plays a crucial role in mammalian preimplantation embryo development (Golding & Westhusin, 2003).

In previous research, gene expression analysis of embryos developed in the summer as opposed to in the winter developed aberrant expression patterns in *DNMT1*, *Cx43*, *GDF9*, *POU5F1* and *HSPA14* genes (Gendelman *et al.*, 2010; Pavani *et al.*, 2016). A similar pattern was also observed in the embryos that were developed from heat stressed oocytes, providing evidence that the development and growth of embryos depend on maternal mRNA and proteins stored in the oocytes. The current experiment consolidates our previous study (Pavani *et al.*, 2016), in which gene expression of the targeted gene was investigated to evaluate the kinetics of heat-shocked oocytes and of oocytes matured in the summer or in the winter.

Materials and Methods

Experimental design

For the first experiment, 25 cumulus–oocyte complexes (COCs) per replication, totalling 100 COCs/season, were collected in the summer (June, July, August, and September) and in the winter (December, January, February and March) and were matured and stored for later genetic analysis. Mean Temperature Humidity Index (THI) in Terceira Island, Azores, Portugal (38°43′N, 27°12′W) was 71.7 ± 0.7 in the summer and 62.8 ± 0.2 in the winter months, respectively. For the second experiment, 500 COCs collected during the winter months were divided into five groups of 100 COCs (25 COCs per replication): a control group which COCs matured *in vitro* (IVM) at 38.5°C for 24 h and four HS groups COCs in which IVM was performed at 39.5°C for 6, 12, 18 or 24 h, respectively, and placed back into control temperature (38.5°C) for completing the 24 h maturation period (except for the 24 h HS group). COCs matured at different heat exposure times were washed in DEPC water and stored at −80°C for further analysis.

Oocytes collection and *in vitro* maturation

All the oocyte collection and IVM procedures were performed as previously described by Pavani *et al.* (2015b). Briefly, COCs were collected by aspiration from antral follicles and the ones covered by at least four layers of compacted cumulus cells and evenly granulated ooplasm were washed twice in Tissue Culture Medium 199 (TCM-199), supplemented with 2% fetal bovine serum (FBS), 0.3 mg/ml glutamine and 50 μg/ml gentamycin. The COCs collected in the summer and the winter months of the first experiment were stored, and further subjected to genetic analysis. Similarly, COCs collected during the winter months and matured at HS (39.5°C) at the different exposure times were also stored for gene expression analysis.

Total RNA extraction

All matured oocytes were divided according to their heat shock treatment and seasonal differences, were washed three times with diethylpyrocarbonate (DEPC) treated water and stored as pools of 25 COCs at −80°C. Total RNA was extracted as previously described by Pavani *et al.* (2015a).

Single-strand cDNA synthesis, specific primer design and standard curves

Single-strand cDNA synthesis, primer design and standard curves were performed accordingly to Pavani *et al.* (2016). Specific primers for four chosen genes and two reference genes are shown in the Table 1.

Quantitative real-time polymerase chain reaction (qPCR)

Real-time PCRs were performed as previously described for embryos (Pavani *et al.*, 2016). Briefly, the template cDNA corresponded to oocytes samples from summer and winter months, control and the four HS treatment groups. A non-template control was also included. The amplification program included a pre-incubation step at the holding stage (50°C for 2 min and 95°C for 10 min), followed by 40 three-step

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Table 1 Oligonucleotide primers used for quantitative real-time polymerase chain reactions (qPCR)

Gene name	Direction	Sequences 5′→3′	Product length (base pair)	T_m (°C)	GenBank accession no.
CDH1	Forward	GACACTGGAGGTATCAGCGCAC	185	56	NM_001002763.1
	Reverse	CCAGCGACTTAGGAAATGGGC			
CX43	Forward	GCGATCCTTACCACACTACCAC	508	54	NM_174068
	Reverse	CCACCTCCAATGAAACAAAATGAAC			
DNMT1	Forward	AGCAATGGGCAGATGTTCCA	268	54	NM_182651.2
	Reverse	ATCTCGCGTAGTCTTGGTCG			
HSPA14	Forward	GGAGTTGATGAATCAGGAGC	190	51	NM_001046388
	Reverse	AATCTTGGAGCACAACCTGT			
GAPDHA	Forward	GCACAGTCAAGGCAGAGAAC	109	54	NM_001034034.1
	Reverse	TACTCAGCACCAGCATCACC			
SDHA	Forward	CTGCAGAACCTGATGCTTTGTG	188	55	NM_174178.2
	Reverse	ACGTAGGAGAGCGTGTGCTT			

amplification cycles consisting of a 15 s denaturation at 95°C, 1 min at 54°C and 30 s at 95°C. A melting curve for the verification of amplification product specificity was recorded at the final dissociation stage to confirm that there was no contamination from primer dimmers. The quantification was carried out using the comparative cycle threshold (Ct) method, with the results expressed in relation to endogenous reference genes and a control group. In this experiment, the reference genes were *GAPDH* and *SDHA*, and the targeted gene were *Cx43*, *HSPA14*, *CDH1*, and *DNMT1*.

Data analysis

Data analysis was performed as described by Faheem *et al.* (2014) and Pavani *et al.* (2016) for embryos. For comparisons between genes, data from GV stage COC's collected during winter were used as a calibrator group for relative gene expression analysis. The mRNAs levels of the *Cx43*, *CDH1*, *DNMT1* and *HSPA14* genes were compared between the summer and winter matured oocytes and exposure time (control and 6 h, 12 h, 18 h, 24 h HS samples) using *GAPDH* and *SDHA* as reference genes for normalization.

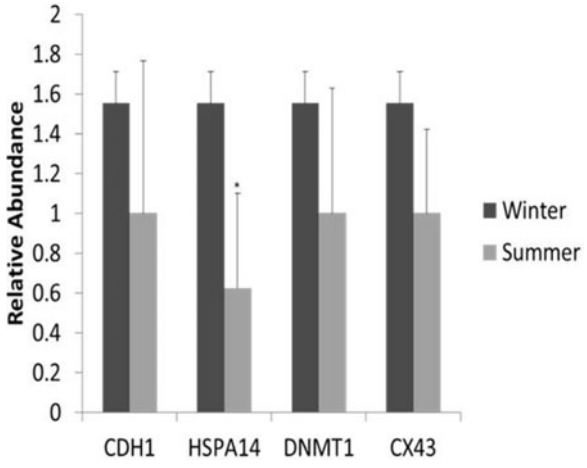

Figure 1 Gene expression transcripts of *in vitro* bovine oocytes in warm months compared with cold months. Columns with (*) represent significant differences ($P < 0.05$) within the gene. Bars indicate standard error of the mean. Mean THI (Temperature Humidity Index) in warmer months (June, July, August and September) was 71.7 ± 0.7 and in cold months (December, January, February and March) was 62.8 ± 0.2.

Results

Assay 1: *In vitro* maturation effect on gene expression during summer and winter months

The analysis of gene quantification of matured COCs during summer as compared with winter showed a significant ($P < 0.05$) down-regulation in HSPA14 with respect to reference genes *GAPDH* and *SDHA* (Fig. 1). As for the *CDH1*, *DNMT1*, and *Cx43* genes, no difference was observed when compared with data collected for the winter months ($P > 0.05$), which indicates no seasonal effect on gene expression.

Assay 2: Effect of heat shock on gene expression of *in vitro* matured oocytes

After examining gene expression in samples exposed to different heat shock times (Fig. 2), it was found that prolonged heat stress altered gene regulation. No difference in gene expression was seen ($P > 0.05$) in heat shock oocytes at 6 h with respect to the control group (Fig. 2*a*). Samples exposed to 12 h to 24 h exhibited a constant up-regulation of CDH1 genes ($P < 0.05$) (Fig. 2b–d). An up-regulation ($P < 0.05$) of the

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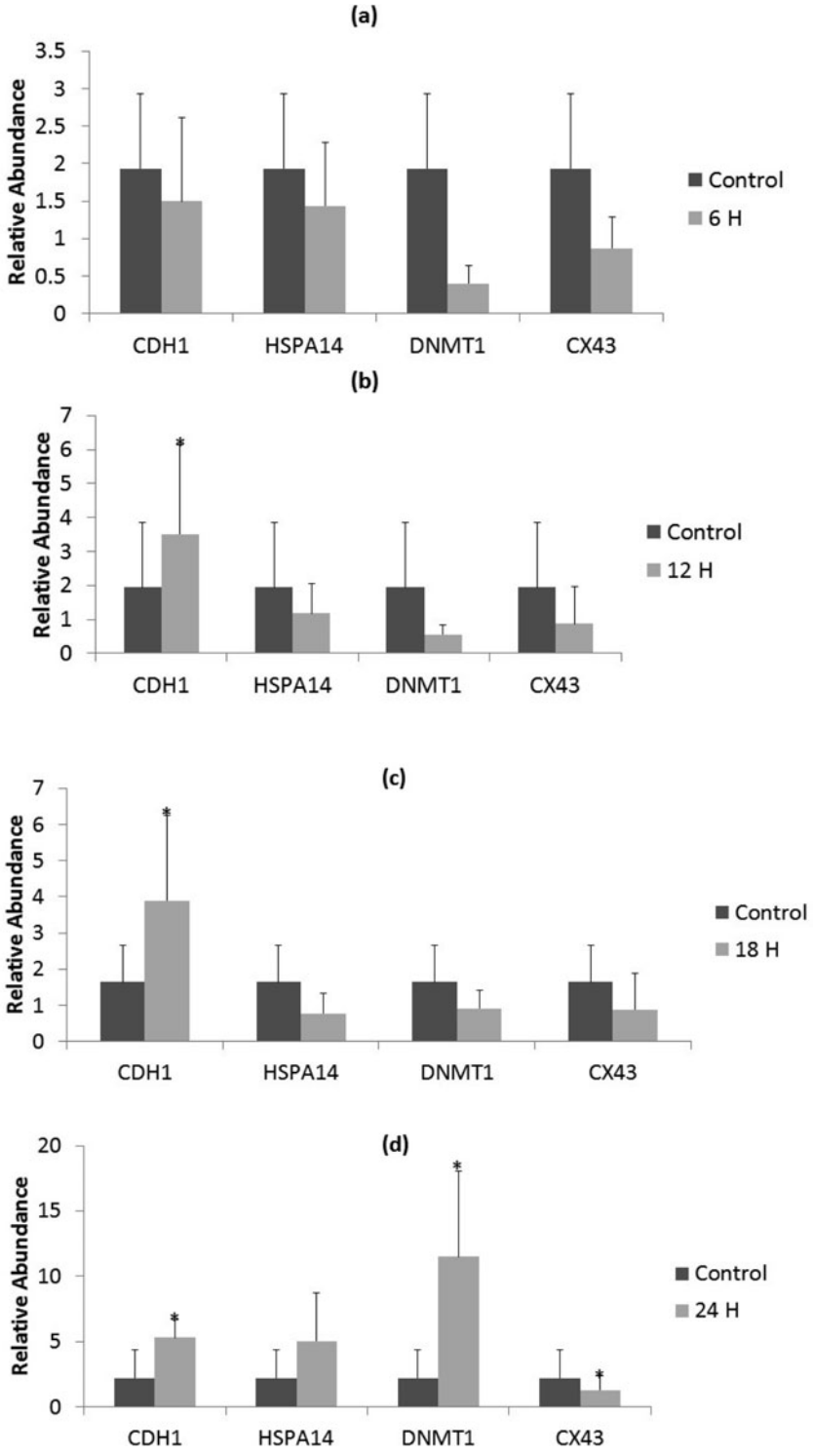

Figure 2 Gene expression transcripts of *in vitro* bovine oocytes matured under different kinetic heat stress (39.5°C) exposure times with reference to the oocytes matured at control (38.5°C): (*a*) Control versus 6 h. (*b*) Control versus 12 h. (*c*) Control versus 18 h. (*d*) Control versus 24 h. Columns with (*) represent significant differences ($P < 0.05$) within the gene. Bars indicate standard error of the mean.

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DNMT1 gene was identified in samples exposed to 24 h of heat stress (Fig. 2*d*). No altered expression was observed in *HSPA14* and *Cx43* genes for any of the HS treatments.

Discussion

Environmental stress induces an alteration in the ovarian pool of follicles and their oocytes, which seems to be a cause of reduced fertility in dairy cows during the warmest months of the year (Roth *et al.*, 2008). A short-term, mild heat shock enhances the thermotolerance of many organisms (Craig & Gross, 1991; Georgopoulos & Welch, 1993) including mammalian embryos (Ryan *et al.*, 1992; Arechiga *et al.*, 1995). However, prolonged heat exposure may be detrimental to oocyte and embryo viability. In previous experiments (Pavani *et al.*, 2015b, 2016) we found that prolonged heat stress did affect oocyte maturation and blastocyst development. The present experiment confirms that embryos developed from oocytes under maternal heat stress (obtained in summer months) had alterations in some of the targeted developmental genes, in agreement with previous observations that prolonged heat shock of bovine oocytes at 39.5°C and 40.5°C led to slow embryo development and gene alteration (Pavani *et al.*, 2016).

No difference on gene transcription was seen between summer and winter for CX43, CDH1, DNMT1, but HSPA14 had a significantly lower expression in summer. This could explain the lower oocyte maturation observed in the summer (44.3%) compared with winter (78.4%) (Pavani *et al.*, 2015b), considering that high expression of HSPA14 results in augmented proteins synthesis that may aid oocyte survival (Zhang *et al.*, 2011).

We had previously demonstrated that oocyte maturation rate decreased significantly due to high temperature and exposure time (Pavani *et al.*, 2016). The present study evaluated gene regulation of kinetic heat stressed oocytes. As no gene expression differences were observed between control and the 6 h HS samples, it can be assumed that oocytes are resistant to a 1°C rise from the control temperature for a 6 h period. Edwards & Hansen (1997) showed that oocytes matured at 41°C for the first 12 h had a reduction in blastocyst rate of 30–65%. Our results support these findings, as after heat exposure at 39.5°C, oocytes exhibited up-regulation of *CDH1* across the 12 h, 18 h, and 24 h exposure groups accompanied by low maturation rates (Pavani *et al.*, 2016). *CDH1* is an important tumour suppressor gene that plays a role in suppressing migration and invasion of key transmembrane proteins essential for the maintenance of intercellular junctions in epithelial tissues (Pfeiffer *et al.*, 2011). It also functions as a regulator of cell proliferation by participating in CDK inhibitor-dependent G1 arrest and DNA damage-induced G2 arrest (Sudo *et al.*, 2001). This function can explain the constant up-regulation of *CDH1* as an attempt to protect oocytes from apoptosis, because *CDH1* is up-regulated when there are signs of DNA damage due to heat stress (Eguren *et al.*, 2013) and is associated with the protection of cells under different stress conditions. HSPA14 remained unexpressed possibly due to its minor effect on heat stressed bovine oocytes. Similarly, no gene regulation of Cx43 was observed in response to heat stress in all heat exposure time groups.

Up-regulation in *DNMT1* was only observed after 24 h of heat shock exposure, which can be explained by the fact that its up-regulation is associated with the chromatin of metaphase II stage (Cirio *et al.*, 2008; Kurihara *et al.*, 2008) which appears only towards the end of the maturation period (24 h). Furthermore, *DNMT1* alone is sufficient to maintain the methylation of the imprinted genes (Hirasawa *et al.*, 2008), and prolonged heat stress could cause DNA damage, which is typically associated with *CDH1* and *DNMT1*. The higher expression of *CDH1* along with *DNMT1* is also an indicator of apoptosis (Palii *et al.*, 2008).

Conclusion

The combination of both experiments provided a better understanding of the effects of heat stress on oocyte maturation and gene expression. The maturation rates obtained from our previous work (Pavani *et al.*, 2015b) were 78.4 ± 8.0% versus 44.3 ± 8.1% ($P < 0.001$) for colder and warmer months, respectively. Comparing those maturation rates with the gene expression analysis carried out, a parallelism is evident between the lower maturation rates obtained during summer months and the altered expression of *HSPA14*. The gene quantification of *in vitro* matured oocytes subjected to HS at different temperatures provided additional evidence that the low maturation rate starting at 12 h of heat shock previously detected (Pavani *et al.*, 2016), may be due to the constant up-regulation of *CDH1* starting at 12 h and an up-regulation of *DNMT1* in samples exposed to 24 h of HS. Overall, the gene regulation analysis of *CDH1* and *DNMT1* supports the view that after prolonged heat shock, *in vitro* matured oocytes could experience apoptosis. It is therefore logical to assume that such heat stress conditions could have deleterious effects on oocyte growth, protein synthesis, or the formation of transcripts, all of which are required for subsequent embryo development.

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Conflict of interest

None of the authors has any conflict of interest to declare.

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