Animal Reproduction Science 205 (2019) 94–104

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

# Extracellular vesicles of follicular fluid from heat-stressed cows modify the gene expression of *in vitro*-matured oocytes

Felipe Morales Dalanezi[a], Henry David Mogollon Garcia[a], Rodrigo de Andrade Ferrazza[a], Fernanda Fagali Franchi[b], Patricia Kubo Fontes[b], Anthony Cesar de Souza Castilho[b], Marcelo Fábio Gouveia Nogueira[c], Elizabeth Moreira dos Santos Schmidt[a], Roberto Sartori[d], João Carlos Pinheiro Ferreira[a,*]

[a] *School of Veterinary Medicine and Animal Science, São Paulo State University, Rua Prof Dr Walter Mauricio Correa, sn, 18.618-681, Botucatu, Brazil*
[b] *Institute of Biosciences, São Paulo State University, Botucatu, São Paulo, Brazil*
[c] *Faculty of Sciences and Languages of Assis, São Paulo State University, Assis, Brazil*
[d] *Luiz de Queiroz College of Agriculture, – ESALQ, University of São Paulo, Piracicaba, Brazil*

ARTICLE INFO

*Keywords:*
Dairy
Reproduction
Biotechnology
Exosomes

ABSTRACT

The effect of heat stress (HS) on cattle reproduction is deleterious with respect to ovarian follicular development and oocyte quality. The objective of this study was to investigate the effect of follicular fluid extracellular vesicles (EVs) obtained from cows maintained in thermoneutral (TN) or HS conditions on *in vitro* oocyte maturation. Nonlactating cows were estrous synchronized. Immediately after ovulation day (D1), the cows were randomly assigned to TN or HS environments. Follicular fluid from all follicles from each treatment was pooled, and EVs were obtained. Pools of 20 cumulus oocyte-complexes (COCs), were allocated to the following treatments: Control ($n$ = 4 COC pools): matured in base medium; TN ($n$ = 4 COC pools): matured in base medium supplemented with TN EV suspension; and HS ($n$ = 4 COC pools): matured in base medium that was supplemented with the HS EV suspension. All treatments were conducted at 38.5 °C for 24 h in a humid atmosphere with 5% CO2. After maturation, the COCs were evaluated for meiotic progression, DNA integrity and oocyte quality-related gene expression. When the experimental groups were compared with the control group, a treatment effect was not observed for meiotic progression and DNA integrity. In the cumulus cells of TN group, there was relatively lesser expression of the IGFBP4 gene. In the oocytes of the TN as compared with the HS group, the IGFBP2, BMP15, GDF9, CDCA8, HAS2, RPL15, STAT3 and PFKP genes were expressed to a lesser extent. The findings indicated that oocytes matured in the presence of EVs from the follicular fluid of cows collected when there were TN conditions, however, there was a lesser expression of genes related to oocyte quality.

## 1. Introduction

A reduction of the reproductive performance of lactating cows during the summer months and associated economic losses has

---

[*] Corresponding author.
*E-mail address:* joao.cp.ferreira@unesp.br (J.C. Pinheiro Ferreira).

https://doi.org/10.1016/j.anireprosci.2019.04.008
Received 5 November 2018; Received in revised form 19 February 2019; Accepted 22 April 2019

been well documented. The biological mechanisms by which heat stress (HS) affects reproduction include a decrease of follicular growth and circulating estradiol, impairment of follicular dominance, delay of luteolysis (Wilson et al., 1998), and inhibition of fertilization, embryonic development and maternal recognition of pregnancy (Sartori et al., 2002; De Rensis and Scaramuzzi, 2003).

Extracellular vesicles (EVs) are present throughout an organism, have pleiotropic biological functions (Mathivanan et al., 2010) and are categorized by size, shape, membrane proteins, structural lipids and cell of origin (Taylor and Gercel-Taylor, 2013). Microvesicles originate from the plasma membrane, are irregularly shaped and have diameters ranging from 100 to 1000 nm; and exosomes (EXOs) originate from late-developing endosomes and have diameters ranging from 50 to 100 nm (Taylor and Gercel-Taylor, 2013). One of most important characteristics of EVs released into body fluids is the capacity of these compounds to be transferred to target cells, which allows for the detection of EVs in multiples fluids and tissues (Koga et al., 2005; Aliotta et al., 2010). The movement of EVs through tissue allows for the intercellular transport of important messengers, such as protein, miRNA and mRNA (Taylor and Gercel-Taylor, 2013; Di Pietro, 2016). These vesicles function to maintain physiological mechanisms and mediate pathogenic pathways (Quesenberry and Aliotta, 2010; Ratajczak et al., 2012). In addition, the contents of EVs can induce angiogenesis, apoptosis and cell proliferation in target cells because of surface interactions with cells of embryo recipient females (EL Andaloussi et al., 2013).

Several factors can restrict *in vitro* embryo production. Appropriately matured oocytes are required for development of *in vitro*-derived embryos with desirable morphological characteristics (Araújo et al., 2014). Thus, communication between oocytes and the surrounding cells (theca and granulosa) is important for development of female gametes (Eppig et al., 2002). Small-extracellular vesicles released by granulosa cells provide for important intra-follicular communication pathways that affect oocyte and embryonic development and metabolism (da Silveira et al., 2015, 2017; Machtinger et al., 2016).

When there are stressful environmental events, there is modification of cellular contents and increase the cellular secretion of EVs that can result in an enhancement of target cell defenses and prevention of cellular death (Eldh et al., 2010; Carver and Yang, 2016). Exosomes released by granulosa cells when oxidative stress is prevailing not only contain more antioxidants compared with normal exosomes but the contents have functions in improving the production of defensive substances in target cells (Saeed-Zidane et al., 2017). In the present study, there was investigation of the effect of adding EVs from the follicular fluid of Holstein cows housed in thermoneutral (TN) or HS conditions on meiotic progression, DNA integrity and gene expression of *in vitro*-matured oocytes.

## 2. Material and methods

### 2.1. Animals

Eighteen nonlactating adult clinically healthy Holstein cows were used in this experiment. A detailed description of the cows, feed and treatments has been previously reported Ferrazza et al. (2017). The experiment was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), Brazil (Permit number: 86/2013 - CEUA).

### 2.2. Experimental design

Of 18 cows, 12 were randomly selected and treated to be estrous synchronized using the Ovsynch protocol (Pursley et al., 1995) as well as a progesterone intravaginal device (Sincrogest, Ouro Fino, Brazil). On Day 2 (Day 0 = day of second injection of gonadotropin-releasing hormone (GnRH) when imposing the Ovsynch protocol), ovulation was confirmed by ultrasonography (MyLab Five, Esaote, Italy), and six cows that had ovulations were randomly assigned to two environments: 1) TN environment ($n = 3$): temperature of 24 °C with a temperature-humidity index (THI) of 75 (Armstrong, 1994) or 2) HS environment ($n = 3$): temperature of 38 °C with a THI of 88 (Armstrong, 1994).

The experiment was conducted in four replicates. Initially, three cows were subjected to the TN treatment and three were subjected to the HS treatment. After the cows were subjected to the HS treatment, cows were removed from the experiment.

On Day 9, the follicular fluid from the two largest ovarian follicles was individually aspirated using a sonography device (MyLab Five, Esaote, Italy) with a 7.5 MHz microconvex transducer coupled to a biopsy guide for transvaginal use. All remaining visible follicles were subsequently ablated. The development of a new wave of follicular development was subsequently monitored, and when the largest follicle was 8.5 mm, the fluid from the two largest follicles was aspirated.

### 2.3. Isolation of extracellular vesicles

The follicular fluid recovered from each experimental group ($n = 24$) was combined to form two pools: TN and HS. Each pool was individually centrifuged successively at 300*g* for 10 min, 2000*g* for 10 min and 16,500*g* 30 min at 4 °C. The supernatant was then placed in ultracentrifuge tubes (Sorvall MTX 150 - Micro-ultracentrifuge, Thermo Scientific, Waltham, Massachusetts, USA) and ultracentrifuged at 120,000*g* for 70 min at 4 °C. The pellet was washed in PBS (pH = 7.4) and ultracentrifuged again at 120,000*g* for 70 min at 4 °C. The pellet was diluted in PBS (100 μLof PBS per mL of centrifuged follicular fluid) and stored in a freezer at −80 °C.

### 2.4. Validation of the experimental model

Ten microliters of the solution from each experimental group was placed on a nickel grid and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde. After fixation, the grids were washed with PBS and stained with 2% uranyl acetate and 0.13% methylcellulose supplemented with 0.4% uranyl acetate. The grids were subsequently analyzed using transmission electron microscopy (Tecnai Spirit Electronic - FEI Company) to confirm the presence of EVs in the pellet extracted from the follicular fluid.

### 2.5. *Effect of adding EVs on oocyte maturation in vitro*

Ovaries, predominantly from *Bos indicus* females obtained from a commercial slaughterhouse, were transported to the laboratory (average time of 40 min) in a thermal container with 0.9% NaCl solution heated to 30 °C. In the laboratory, the ovaries were washed sequentially with 0.9% NaCl solution and 70% ethanol, and follicles with diameters between 3 and 8 mm were aspirated. The recovered follicular fluid was placed in a conical tube and incubated in a dry bath at 35 °C for 20 min. The pellet and 5 mL of follicular fluid were then aspirated and placed in a petri dish (90 mm). Only cumulus-oocyte complexes (COCs) graded as 1 and 2 (Stojkovic et al., 2001) were selected for use in the present study.

Pools of 20 COCs were *in vitro* matured in 90-μL drops of base medium composed of TCM-199 medium with Eagles' salts, glutamine and $NaHCO_3$ (Sigma) supplemented with pyruvate (22 μg/mL), amikacin (75 μg/mL), bovine serum albumin (4 mg/mL; Sigma) and follicle stimulating hormone (FSH, 1 μg/mL; folltropin, Vetoquinol). The COCs were matured at 38.5 °C for 24 h in a humid and gaseous atmosphere with 5.5% $CO_2$ according to the following experimental groups:

- Control group ($n$ = four pools): matured in 90 μL of base medium;
- TN group (n = four pools): matured in 81 μL of base medium plus 9 μL of EV suspension extracted from the follicular fluid of cows maintained in the TN environment.; and
- HS group (n = 4): matured in 81 μL of base medium plus 9 μL of EV suspension extracted from the follicular fluid of cows maintained in the HS environment.

After maturation, the meiotic progression and DNA fragmentation (apoptosis) of oocytes and gene expression were evaluated in the oocytes and cumulus cells.

### 2.6. *Evaluation of nuclear maturation and apoptosis in the oocytes*

The COCs were denuded by repeated pipetting in PBS containing 1 mg/mL polyvinylpyrrolidone (PBS-PVP) and were assessed by terminal transferase-mediated deoxynucleotidyl dUTP nick-end labeling (TUNEL) and Hoechst 33,342 staining to evaluate DNA fragmentation and meiotic progression, respectively, as described by Paula-Lopes et al. (2007) and Long et al. (1994).

### 2.7. *Evaluation of relative abundance of mRNA*

After 24 h IVM matured oocytes and cumulus cells were mechanically separated by vortex and stored at −80 °C freezer. The total RNA from 20 oocytes that were separated from the respective cumulus cells was extracted using the MicrokitRNeasy® (Qiagen, Mississauga, ON, CA) (four replicates/cell type for each group) according manufacturer's protocol. After purification, RNA samples were eluted in 14 μl of RNAse free water. The total RNA (200 ng/reaction for cumulus cells samples, entire RNA sample for oocytes and embryos) was incubated with DNAse I (1 IU/μg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using a random primer and conducting the protocol provided for the High Capacity kit (Applied Biosystems). After reverse transcription, 1 μL of each sample from control group was mixed to be used as calibrator sample to qPCR analyses (one pool for cumulus cells and another one for oocyte).

Relative abundances of mRNA transcripts for specific genes in the oocytes and cumulus cells were analyzed using the TaqMan® Gene Expression Assay (20×, Applied Biosystems) specific for *Bos taurus*. The genes analyzed are described in Table 1. Initially, the samples were subjected to the following preamplification protocol: 1.25 μL of assay mix (TaqMan assay concentrated to a final concentration of 0.2 × for each assay), 2.5 μL of PreAmp TaqMan Master Mix (Applied Biosystems) and 1.25 μL of cDNA. Reactions were activated at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s and annealing and amplification at 60 °C for 4 min for 14 cycles. The pre-amplified products were diluted five times after preamplification. For gene expression analysis, the sample solution prepared consisted of 2.25 μL cDNA (pre amplified products), 2.5 μL of TaqMan Universal PCR Master Mix (2X, Applied Biosystems) and 0.25 μL of 20X GE Sample Loading Reagent (Fluidigm); and the assay solution: 2.5 μL of 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 μL of 2X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used for data collection. After priming, the chip was loaded with 5 μL of each assay solution and 5 μL of each sample solution. The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm, South San Francisco, CA, USA) using the protocol TaqMan GE 96 × 96 Standard, that consisted of one stage of Thermal Mix (50 °C for 2 min, 70 °C for 20 min and 25 °C for 10 min) followed by a Hot Start stage (50 °C for 2 min and 95 °C for 10 min), followed by 40 cycles of denaturation (95 °C for 15 s), primer annealing and extension (60 °C for 60 s). The analysis was performed in duplicate, and a value for samples with a variation of less than 0.3 between raw Ct values was accepted for proceeding with the analysis. The Ct values were calculated using the system's software (Biomark Real-time PCR Analysis, Fluidigm). The value for relative abundance of mRNA transcripts for each gene was calculated using the ΔΔCt method (Pfaffl, 2001), with the cyclophilin A as reference gene (Machado et al., 2009), and the pool of cumulus cells and oocytes (control group) were used as a calibrator sample, respectively for cumulus and oocyte samples. Fold changes were calculated as $2^{-\Delta\Delta Ct}$.

**Table 1**
List of genes analyzed in the cumulus cells and oocytes.

| Gene | Code |
|---|---|
| Insulin-like growth factor binding protein 2 (IGFBP2) | Bt03215063_m1 |
| Insulin-like growth factor binding protein 4 (IGFBP4) | Bt03259498_m1 |
| Insulin-like growth factor receptor (IGFR1) | Bt03649217_m1 |
| BCL2-associated X protein (BAX) | Bt03211775_g1 |
| Caspase 9 (CASP9) | Bt04282453_m1 |
| B-cell CLL/lymphoma 2 (BCL2) | Bt04298952_m1 |
| Mitochondrial translational initiation factor 3 (MTIF3) | Bt03231841_m1 |
| NLR family, pyrin domain containing 5 (MATER) (NLRP5) | Bt03218031_m1 |
| Signal transducer and activator of transcription 3 (STAT3) | Bt03259875_g1 |
| Ribosomal protein S25 (RPS25) | Bt03220437_g1 |
| Transcription factor A, mitochondrial (TFAM) | Bt03260080_m1 |
| Ribosomal protein L15 (RPL15) | Bt03288449_g1 |
| Phosphofructokinase, platelet (PFKP) | Bt04316544_m1 |
| Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA) | Bt04307507_m1 |
| Bone morphogenetic protein 15 (BMP15) | Bt03286494_u1 |
| Growth differentiation factor 9 (GDF9) | Bt03223996_m1 |
| Oocyte-secreted protein 1 (OOSP1) | Bt03233531_g1 |
| Catalase (CAT) | Bt03228716_m1 |
| Carnitine palmitoyltransferase 1B (CPT1B) | Bt03244638_m1 |
| Carnitine palmitoyltransferase 2 (CPT2) | Bt03233823_m1 |
| Hyaluronan synthase 2 (HAS2) | Bt03212695_g1 |
| Pentraxin 3, long (PTX3) | Bt03249012_m1 |
| Cyclin D2 (CCND2) | Bt03249250_m1 |
| Cell division cycle associated 8 (CDCA8) | Bt03257047_m1 |
| Cyclin-dependent kinase 6 (CDK6) | Bt04311261_m1 |
| Crowth arrest and DNA-damage-inducible, alpha (GADD45 A) | Bt03225650_m1 |
| Vascular endothelial growth factor A (VEGFA) | Bt03213282_m1 |
| Heat shock transcription factor 1 (HSF1) | Bt03249686_m1 |
| Heat shock protein 90 kDa alpha (cytosolic), class A member 1 (HSP90AA1) | Bt03218068_g1 |
| Chloride intracellular channel 3 (CLIC3) | Bt03263041_g1 |
| Glycine amidinotransferase (GATM) | Bt03237897_g1t |
| Lumican (LUM) | Bt03211921_m1 |
| Follicle stimulating hormone receptor (FSHR) | Bt03212670_m1 |
| Gremlin 1 (GREM1) | Bt03255355_m1 |
| Mitogen-activated protein kinase 1 (MAPK1) | Bt03216714_m1 |

### *2.8. Statistical analysis*

Statistical analyses were performed to evaluate the meiotic progression, apoptosis rate and gene expression of the COCs matured *in vitro*. These consisted of ANOVAs followed by use of a Tukey's test, with $P < 0.05$ as the significance level. If the data were not normally distributed, the data sets were log transformed before performing the statistical analyses. Data were expressed as the means ± SEM of replicates. All procedures were conducted using JMP® software (SAS Institute).

## 3. Results

The presence of EVs in the final pellet extracted from follicular fluid of HS and TN cows was confirmed by transmission electron microscopy (Fig.1). There were no treatment effects ($P > 0.05$) on the proportion of oocytes that developed at the metaphase II stage [Control = 80.0% ± 7.2 (48/60); TN = 77.4% ± 2.9 (96/124); HS = 66.4% ± 7.0 (85/128)] or on the integrity of oocyte DNA [Control = 51.5% ± 21.5 (33/64); TN = 66.1% ± 16.9 (82/124); HS = 57.1% ± 17.0 (72/126)].

With regard to the gene transcriptional profile of COCs, in the cumulus cells, there was no mRNA transcript detected for the gene encoding for *GDF9* and only relative abundance of IGFBP4 mRNA transcript was greater ($P < 0.05$) in the HS group compared to the TN group (Fig. 2). In the oocytes, the relative mRNA abundance of CDCA8, GDF9 and HAS2 was less in the TN group ($P < 0.05$), but there were no differences in transcript abundances between the HS and TN treatment groups (Fig. 3). The relative mRNA abundances for the BCL2, HSF1, PTX3 and *CPT1B* genes were greater in the HS when compared with the TN groups ($P < 0.05$), but there were no differences between these two treatment groups and the control group (Figs. 3 and 4). For BMP15, RPL15, STAT3 and IGFBP2, relative mRNA abundance was less in the TN group ($P < 0.05$), and for PFKP the abundance was less for the TN and HS groups ($P < 0.05$; Figs. 3 and 4). For BAX and IGFR1, the mRNA abundance was less in the TN than HS group ($P < 0.10$; Fig. 5). The mRNA for IGFBP4 and FSHR was less than that which could be detected using the procedures performed in the present study.

## 4. Discussion

The results of the present study indicated, for the first time, that EVs isolated from the follicular fluid of cows maintained in TN or

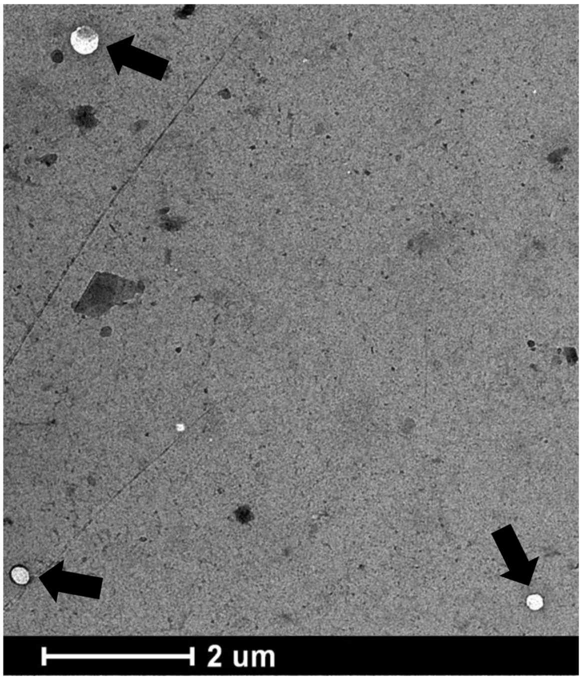


**Fig. 1.** Extracellular vesicles (black arrows) present in the final pellet extracted from follicular fluid of cows in the heat stress groups detected by transmission electron microscopy.

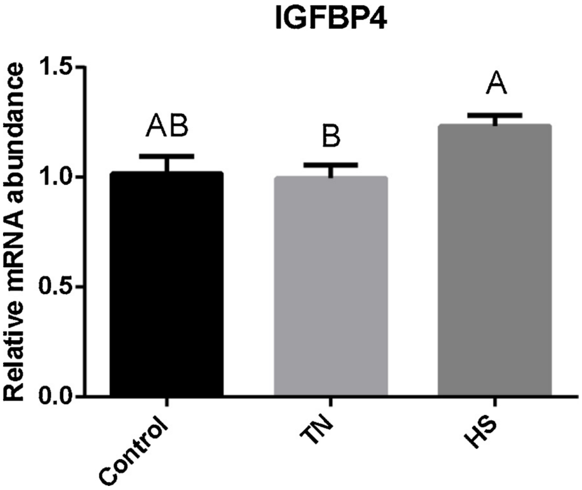
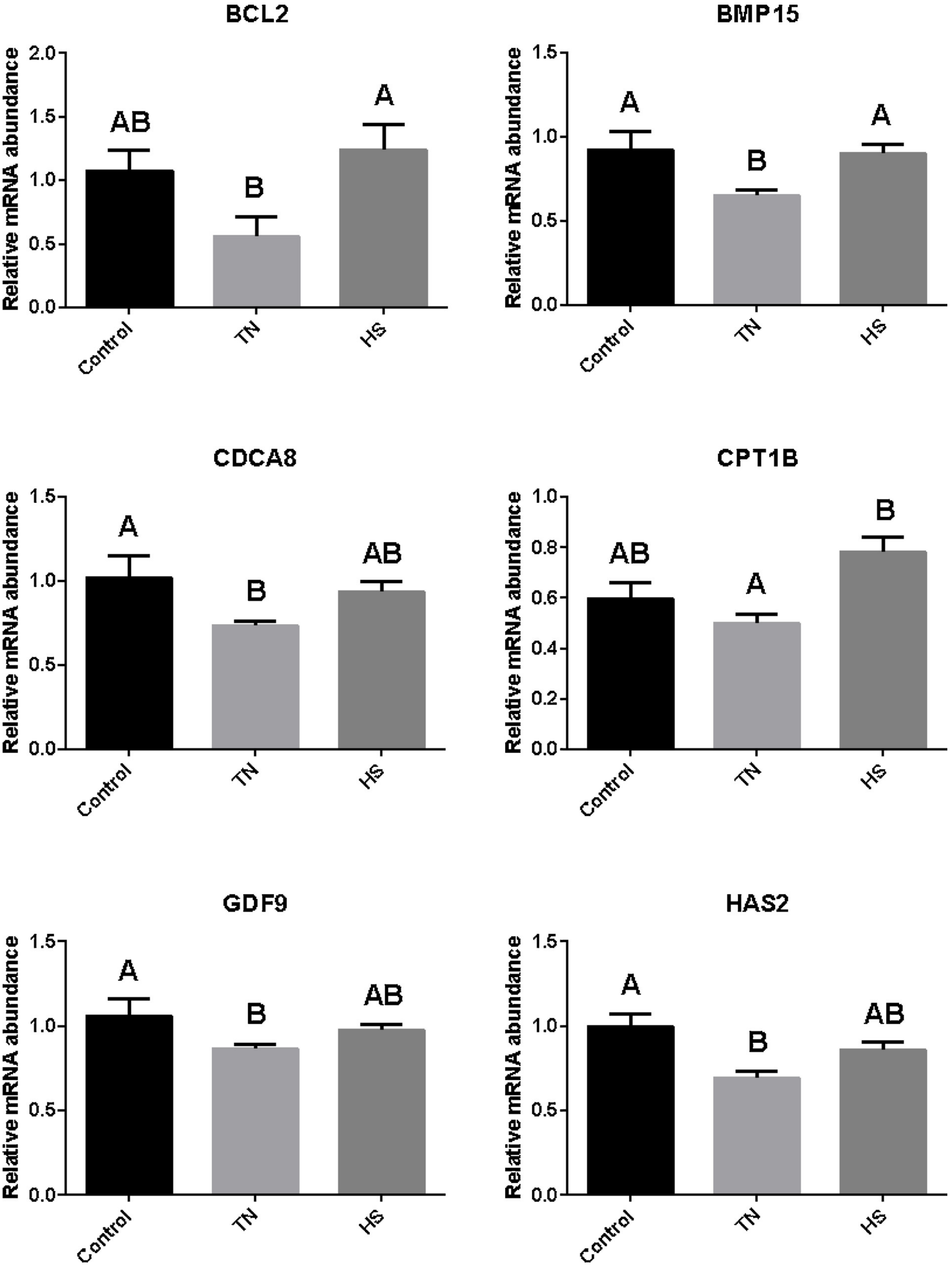


**Fig. 2.** Effects of EVs from the follicular fluid of cows in the thermoneutral (TN) or heat stress (HS) groups on the messenger RNA (mRNA) transcript abundance for genes regulating IGFBP4 production in cumulus cells of cattle; Data are presented relative to mRNA abundance (ΔΔCt), normalized using transcript abundance values for cyclophilin A as a reference gene; Data were derived from four independent replicates, each one containing a pool of cells from 20 COCs; Bars with different letters are different ($P < 0.05$).

HS conditions has constituents that modulate COC gene expression during *in vitro* maturation. Interestingly, in the present study, cumulus cells were affected to a moderate extent by the addition of EVs because only the IGFBP4 gene expression was modified by the EVs from the follicular fluid of Holstein cows subjected to HS when compared to TN treatment, which indicates that oocytes are the primary COC component affected by HS. Even though there were modifications in COC gene expression as a result of treatments in the present study, there were no differences between groups in the morphological analyses of oocytes. Only the oocyte maturation stage was, however, studied, and the observed changes in the transcription factors could affect subsequent embryonic characteristics and *in vitro* maturation (IVM) as well as *in vitro* fertilization (IVF) success rates.

The functions of insulin-like growth factors (IGFs) have been confirmed by the presence of IGF-1 and IGF-1R in the oocytes of cattle (Luciano et al., 2000; Satrapa et al., 2013a). There was a greater expression of *IGFBP4* and *IGFBP2* genes in the cumulus cells and oocytes, respectively, from the cows of the HS group as well as the tendency for a greater expression of the *IGFR1* gene in oocytes



(caption on next page)

**Fig. 3.** Effects of EVs from the follicular fluid of cows in the thermoneutral (TN) or heat stress (HS) groups on the messenger RNA (mRNA) transcript abundance for BCL2, BMP15, CDCA8, CPT1B, GDF9 and HAS2 genes in cattle oocytes; Data are presented as relative mRNA abundance (ΔΔCt), normalized using transcript abundance values for cyclophilin A as a reference gene, Data were derived from four independent replicates, each one containing a pool of cells from 20 COCs; Bars with different letters are different ($P < 0.05$).

($P = 0.07$). This finding indicates that oocytes could contain small amounts of bio-available free IGF, as proposed based on results where oocytes of Holstein cows had a greater abundance of *IGF1*, *IGF1R*, *IGFBP2* and *IGFBP4* than cumulus cells (Satrapa et al., 2013b; Lopes et al., 2017).

In oocytes matured *in vitro*, the addition of EVs had an intriguing effect with there being a decrease in expression of HSF1, *BMP15,* BCL2, IGFBP2, RPL15, STAT3*, PTX3* and *CPT1B* genes in the TN group relative to the HS group. In the TN group, there was a lesser relative abundance of *HSF1* gene transcripts, which encodes for heat shock protein (HSP) after cellular exposure to thermal or proteotoxic stressors (Pirkkala et al., 2001). Furthermore, the HSF1 protein functions as an anti-apoptotic factor in cellular responses and DNA repair (Page et al., 2006). In addition, with the TN group, there was a lesser relative abundance of *BCL2* and *STAT3* mRNA transcripts which also leads to protecting cells against oxidative stress (Sutton-McDowall et al., 2015). These results indicate that the addition of EVs from the follicular fluid of cows of the TN group resulted in attenuation of the protective gene responses generally observed when there is exposure of cells to toxic agents and/or stressors.

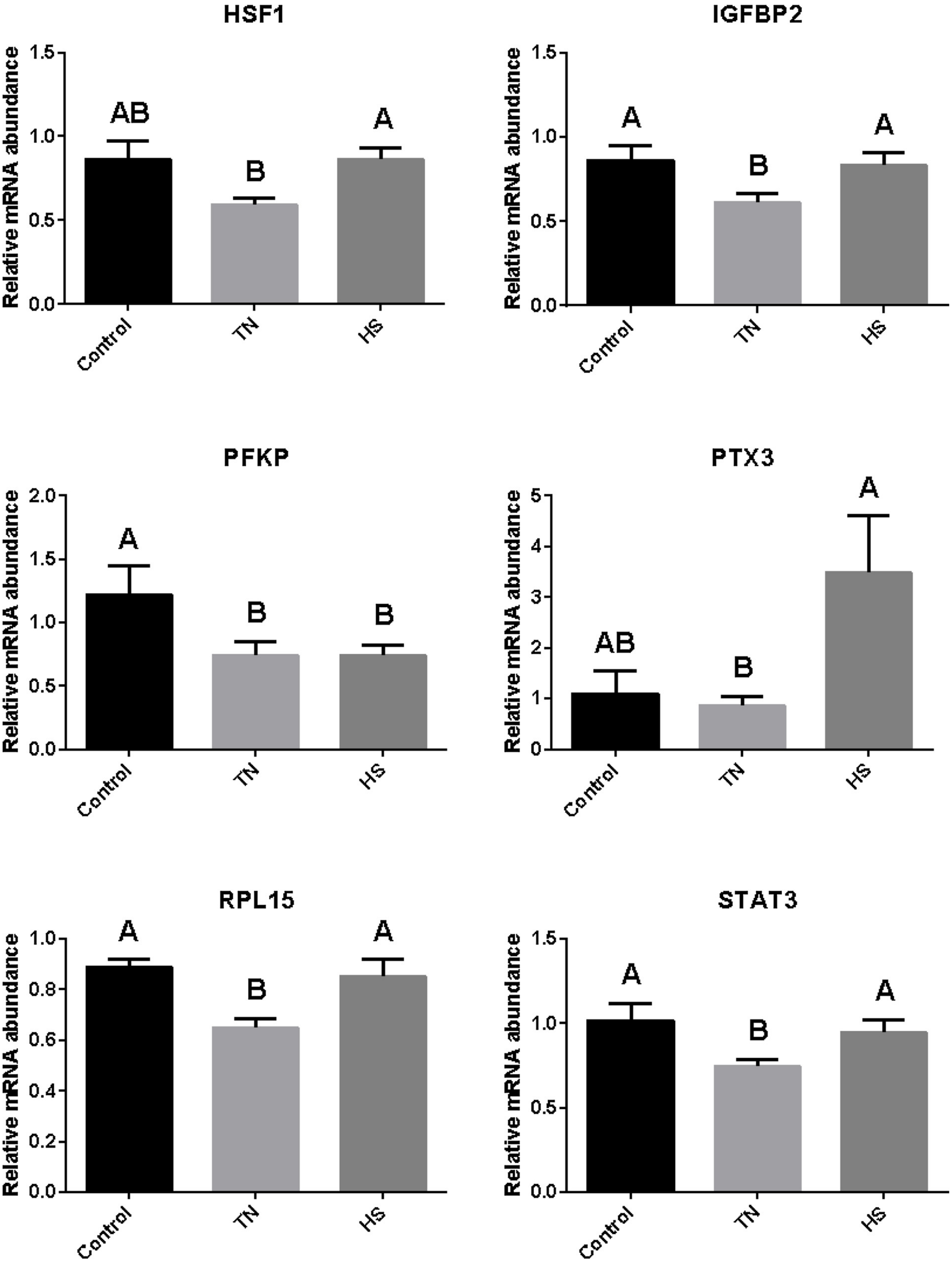
The *PTX3* protein is also involved in cumulus expansion and there was greater abundance of the mRNA transcript for *PTX3* for cows in the HS than TN group. This transcription factor encodes a protein that binds to tumor necrosis factor α-induced protein 6 (TNFAIP6), thus forming a multimolecular complex that has a high affinity for hyaluronic acid and is integral for matrix stabilization and full cumulus expansion (Carrette et al., 2001). Furthermore, PTX3 is also produced when there is a stimulus of primary inflammatory mediators (lipopolysaccharides and tumor necrosis factor α [TNFα]) (Breviario et al., 1992; Lee et al., 1993, 1994; Introna et al., 1996; Bottazzi et al., 1997) and HS increases serum concentrations of inflammatory mediators, such as TNFα (Min et al., 2016).The increased *PTX3* gene expression, therefore, could be reflective of an initial inflammatory condition in follicles of cows in the HS group. Energy metabolism of the COC is regulated by a set of genes, including *BMP15*, *PFKP* and *CPT1B* (Dunning et al., 2010). The *BMP15* and *PFKP* gene products are involved in glucose uptake and glycolysis, respectively, which are important for producing pyruvate that is metabolized as a result of the Krebs cycle (Caixeta et al., 2013), whereas CPT1B transfers fatty acids to the mitochondrial interior and consequently functions in producing energy as a result of β-oxidation (Dunning et al., 2010). The lesser *BMP15* gene expression in the TN group and the lesser *PFKP* gene expression in the TN and HS groups indicate that reduced glycolytic pathway activation occurs in the COCs of both groups. In the COCs of the HS group, there was compensaton for this reduction by increasing the *CTP1B* gene expression. The same response did not occur in the TN group, likely because of the adaptation of the follicular environment in cows of the HS group in ways that there was an increased energy for metabolic functions.

The *RPL15* gene encodes for a ribosome protein (Yao et al., 2004), and *CDCA8*, in turn induces the synthesis of a protein that functions in integration of the protein complex responsible for modulation of the chromosomal structures and cytoskeleton during cell division (Gassmann et al., 2004; Sampath et al., 2004). These two proteins are involved in sustaining the cell cycle and meiotic progression in oocytes (Sun et al., 2010). The lesser expression of these genes in the TN than HS group could also indicate there is attenuation of processes involved with meiotic progression in the oocytes, however this effect was not observed in the present study.

In the present study, the hypothesis was that EVs from the follicular fluid of cows in the TN and HS groups would have important effects on the expression of genes related to oocyte viability (Moussa et al., 2015). Perhaps the EVs of the TN group were involved in modulation of the expression of genes that were stimulated by stressor factors. Stress can lead to transcription of genes related to apoptosis and cellular death (Li et al., 2016) which may explain why the oocytes matured with EVs from cows of the TN group had a lesser mRNA transcript abundance for apoptotic genes. The observed effect was paradoxical because there were primarily gene modulations in the TN group, where the expression of these genes was predominantly attenuated. Apparently, the contents of the EVs of the TN group attenuated processes related to increased energy metabolism, cellular protection and meiotic progression. The follicular environment of cows in the TN group may have modulated EV production in granulosa cells so that the energetic milieu of these cells allowed for optimal metabolic functioning. This molecular information on the substances present in the EVs could be used in clinical practices to enhance oocyte development and ultimately quality.

Various types of stresses result in lesser oocyte quality (Ju et al., 1999; Agca et al., 2000; Rivera and Hansen, 2001; Mullen et al., 2004, 2007; Tseng et al., 2006). Results of several studies indicate, however, that sublethal stressor effects may not lead to induction of apoptosis (Pribenszky et al., 2010). Responses to stress could even result in improved oocyte viability (Du et al., 2008a, b; Pribenszky et al., 2008; Isom et al., 2009;) because of the activation of cellular protective mechanisms when stress conditions prevail (Bock et al., 2016). Nevertheless, the results of the present study for the HS treatment group were similar to those of the control group except the *PTX3* gene was expressed to a greater extent in cows of the HS group. The COCs for the present experiment were obtained from commercial slaughterhouses during the summer months, and procedures related to animal transport (Maziero et al., 2011) and pre-slaughter handling are stressors that result in an increase in blood cortisol and stress-related gene expression (Vijayan et al., 2003; Maheshwari et al., 2014). Thus, gene responses for adapting to a suboptimal environment could have already been activated in the oocytes of the control group, which could have led to similar gene expression in this group relative to the HS group. Furthermore, the maturation medium could also be a stressor because oocytes were removed from the optimal environment of the follicular fluid and transferred to an environment with free radicals or factors that facilitate the formation of these radicals, which may lead to damage of DNA and a decrease in oocyte quality (Combelles et al., 2009; Nikseresht et al., 2017).

Findings in the present study substantiate that EVs from exogenous follicular fluid modulate oocyte gene expression during IVM of



*(caption on next page)*

**Fig. 4.** Effects of EVs from the follicular fluid of cows in the thermoneutral (TN) or heat stress (HS) groups on the messenger RNA (mRNA) transcript abundance for the HSF1, IGFBP2, PFKP, PTX3, RPL15 and STAT3 genes in cattle oocytes; Data are presented as relative mRNA abundance (ΔΔCt), normalized using the transcript abundance values for cyclophilin A as a reference gene; Data were derived from four independent replicates, each one containing a pool of cells from 20 COCs; Bars with different letters are different ($P < 0.05$).

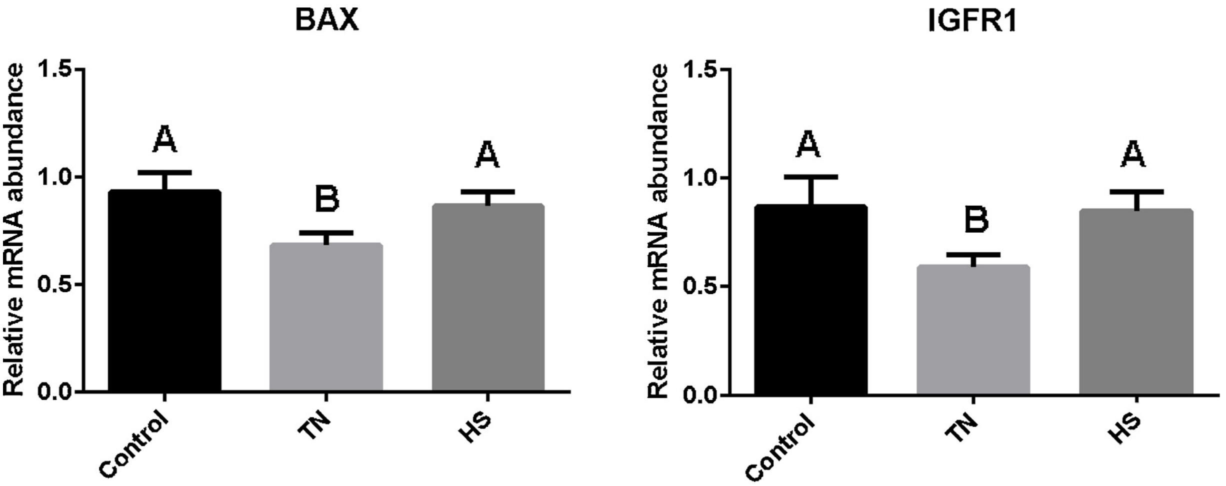


**Fig. 5.** Effects of EVs from the follicular fluid of cows in the thermoneutral (TN) or heat stress (HS) groups on the messenger RNA (mRNA) transcript abundance for BAX and IGFR1 genes in cattle oocytes; Data are presented as relative mRNA abundance (ΔΔCt), normalized using the transcript abundance values for cyclophilin A as a reference gene; Data were derived from four independent replicates, each one containing a pool of cells from 20 COCs; Bars with different letters are different ($P < 0.07$).

oocytes. Furthermore, oocytes that were matured in the presence of EVs from the follicular fluid of cows of the TN group had a lesser expression of genes related to oocyte quality.

## Funding

The São Paulo Research Foundation (FAPESP) funded the study (Grant # 2012/18297-7).

## Conflicts of interest

No potential conflicts of interest are reported by the authors.

## References

Agca, Y., Liu, J., Rutledge, J.J., Critser, E.S., Critser, J.K., 2000. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol. Reprod. Dev. 55, 212–219. https://doi.org/10.1002/(SICI)1098-2795(200002)55:2<212::AID-MRD11>3.0.CO;2-M.

Aliotta, J.M., Pereira, M., Johnson, K.W., de Paz, N., Dooner, M.S., Puente, N., Ayala, C., Brilliant, K., Berz, D., Lee, D., Ramratnam, B., McMillan, P.N., Hixson, D.C., Josic, D., Quesenberry, P.J., 2010. Microvesicle entry into marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription. Exp. Hematol. 38, 233–245. https://doi.org/10.1016/j.exphem.2010.01.002.

Araújo, V.R., Gastal, M.O., Figueiredo, J.R., Gastal, E.L., 2014. In vitro culture of bovine preantral follicles: a review. Reprod. Biol. Endocrinol. 12 (78). https://doi.org/10.1186/1477-7827-12-78.

Armstrong, D.V., 1994. Heat stress interaction with shade and cooling. J. Dairy Sci. 77, 2044–2050. https://doi.org/10.3168/jds.S0022-0302(94)77149-6.

Bock, I., Raveh-Amit, H., Losonczi, E., Carstea, A.C., Feher, A., Mashayekhi, K., Matyas, S., Dinnyes, A., Pribenszky, C., 2016. Controlled hydrostatic pressure stress downregulates the expression of ribosomal genes in preimplantation embryos: a possible protection mechanism? Reprod. Fertil. Dev. 28, 776–784. https://doi.org/10.1071/RD14346.

Bottazzi, B., Vouret-Craviari, V., Bastone, A., De Gioia, L., Matteucci, C., Peri, G., Spreafico, F., Pausa, M., D'Ettorre, C., Gianazza, E., et al., 1997. Multimer formation and ligand recognition by the long pentraxin PTX3. J. Biol. Chem. 272, 32817–32823. https://doi.org/10.1074/jbc.272.52.32817.

Breviario, F., d'Aniello, E.M., Golay, J., Peri, G., Bottazzi, B., Bairoch, A., Saccone, S., Marzella, R., Predazzi, V., Rocchi, M., 1992. Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. J. Biol. Chem. 267, 22190–22197.

Caixeta, E.S., Sutton-McDowall, M.L., Gilchrist, R.B., Thompson, J.G., Price, C.A., Machado, M.F., Lima, P.F., Buratini, J., 2013. Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. Reproduction 146, 27–35. https://doi.org/10.1530/REP-13-0079.

Carrette, O., Nemade, R.V., Day, A.J., Brickner, A., Larsen, W.J., 2001. TSG-6 is concentrated in the extracellular matrix of mouse cumulus oocyte complexes through hyaluronan and inter-alpha-inhibitor binding. Biol. Reprod. 65, 301–308. https://doi.org/10.1095/biolreprod65.1.301.

Carver, K.A., Yang, D., 2016. N -Acetylcysteine amide protects against oxidative stress–Induced microparticle release from human retinal pigment epithelial cells. Investig. Opthalmology Vis. Sci. 57 (360). https://doi.org/10.1167/iovs.15-17117.

Combelles, C.M., Gupta, S., Agarwal, A., 2009. Could oxidative stress influence the in-vitro maturation of oocytes? Reprod. Biomed. Online 18, 864–880. https://doi.org/10.1002/ana.22528.Toll-like.

da Silveira, J.C., Winger, Q.A., Bouma, G.J., Carnevale, E.M., 2015. Effects of age on follicular fluid exosomal microRNAs and granulosa cell transforming growth factor-β signalling during follicle development in the mare. Reprod. Fertil. Dev. 27, 897–905. https://doi.org/10.1071/RD14452.

da Silveira, J.C., Andrade, G.M., Collado, M., del, Sampaio, R.V., Sangalli, J.R., Silva, L.A., Pinaffi, F.V.L., Jardim, I.B., Cesar, M.C., Nogueira, M.F.G., Cesar, A.S.M., Coutinho, L.L., Pereira, R.W., Perecin, F., Meirelles, F.V., 2017. Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One 12, 1–25. https://doi.org/10.1371/journal.pone.0179451.

De Rensis, F., Scaramuzzi, R.J., 2003. Heat stress and seasonal effects on reproduction in the dairy cow - a review. Theriogenology. https://doi.org/10.1016/S0093-691X(03)00126-2.

Di Pietro, C., 2016. Exosome-mediated communication in the ovarian follicle. J. Assist. Reprod. Genet. https://doi.org/10.1007/s10815-016-0657-9.

Du, Y., Lin, L., Schmidt, M., Bøgh, I.B., Kragh, P.M., Sørensen, C.B., Li, J., Purup, S., Pribenszky, C., Molnár, M., Kuwayama, M., Zhang, X., Yang, H., Bolund, L., Vajta, G., 2008a. High hydrostatic pressure treatment of porcine oocytes before handmade cloning improves developmental competence and cryosurvival. Cloning Stem Cells 10, 325–330. https://doi.org/10.1089/clo.2007.0089.

Du, Y., Pribenszky, C.S., Molnár, M., Zhang, X., Yang, H., Kuwayama, M., Pedersen, A.M., Villemoes, K., Bolund, L., Vajta, G., 2008b. High hydrostatic pressure: a new way to improve in vitro developmental competence of porcine matured oocytes after vitrification. Reproduction 135, 13–17. https://doi.org/10.1530/REP-07-0362.

Dunning, K.R., Cashman, K., Russell, D.L., Thompson, J.G., Norman, R.J., Robker, R.L., 2010. Beta-oxidation I is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83, 909–918. https://doi.org/10.1095/biolreprod.110.084145.

EL Andaloussi, S., Lakhal, S., Mäger, I., Wood, M.J.A., 2013. Exosomes for targeted siRNA delivery across biological barriers. Adv. Drug Deliv. Rev. 65, 391–397. https://doi.org/10.1016/j.addr.2012.08.008.

Eldh, M., Ekström, K., Valadi, H., Sjöstrand, M., Olsson, B., Jernås, M., Lötvall, J., 2010. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS One 5, 1–8. https://doi.org/10.1371/journal.pone.0015353.

Eppig, J.J., Wigglesworth, K., Pendola, F.L., 2002. The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc. Natl. Acad. Sci. 99, 2890–2894. https://doi.org/10.1073/pnas.052658699.

Ferrazza, R., de, A., Mogollón, H.D.G., Vallejo, V.H.A., Nogueira, C., de, S., Veríssimo, C.J., Sartori, J.R., Sartori, R., Ferreira, J.C.P., 2017. Thermoregulatory responses of Holstein cows exposed to experimentally induced heat stress. J. Therm. Biol. 66, 68–80. https://doi.org/10.1016/j.jtherbio.2017.03.014.

Gassmann, R., Carvalho, A., Henzing, A.J., Ruchaud, S., Hudson, D.F., Honda, R., Nigg, E.A., Gerloff, D.L., Earnshaw, W.C., 2004. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J. Cell Biol. 166, 179–191. https://doi.org/10.1083/jcb.200404001.

Introna, M., Alles, V.V., Castellano, M., Picardi, G., De Gioia, L., Bottazzai, B., Peri, G., Breviario, F., Salmona, M., De Gregorio, L., Dragani, T.A., Srinivasan, N., Blundell, T.L., Hamilton, T.A., Mantovani, A., 1996. Cloning of mouse ptx3, a new member of the pentraxin gene family expressed at extrahepatic sites. Blood 87, 1862–1872. https://doi.org/10.1016/1043-4666(94)90299-2.

Isom, S.C., Lai, L., Prather, R.S., Rucker, E.B., 2009. Heat shock of porcine zygotes immediately after oocyte activation increases viability. Mol. Reprod. Dev. 76, 548–554. https://doi.org/10.1002/mrd.20975.

Ju, J.C., Parks, J.E., Yang, X., 1999. Thermotolerance of IVM-derived bovine oocytes and embryos after short-term heat shock. Mol. Reprod. Dev. 53, 336–340. https://doi.org/10.1002/(SICI)1098-2795(199907)53:3 < 336::AID-MRD9 > 3.0.CO;2-M.

Koga, K., Matsumoto, K., Akiyoshi, T., Kubo, M., Yamanaka, N., Tasaki, A., Nakashima, H., Nakamura, M., Kuroki, S., Tanaka, M., Katano, M., 2005. Purification, characterization and biological significance of tumor-derived exosomes. Anticancer Res. 25, 3703–3707.

Lee, G.W., Lee, T.H., Vileek, J., 1993. TSG-14, a tumor necrosis factor- and IL-1-Inducible protein, is a novel member of the pentaxin family of acute phase proteins. J. Immunol. 150, 1804–1812.

Lee, G.W., Goodman, A.R., Lee, T.H., Vilcek, J., 1994. Relationship of TSG-14 protein to the pentraxin family of major acute phase proteins. J. Immunol. 153, 3700–3707.

Li, L., Wu, J., Luo, M., Sun, Y., Wang, G., 2016. The effect of heat stress on gene expression, synthesis of steroids, and apoptosis in bovine granulosa cells. Cell Stress Chaperones 21, 467–475. https://doi.org/10.1007/s12192-016-0673-9.

Long, C.R., Damiani, P., Pinto-Correia, C., MacLean, R.A., Duby, R.T., Robl, J.M., 1994. Morphology and subsequent development in culture of bovine oocytes matured in vitro under various conditions of fertilization. J. Reprod. Fertil. 102, 361–369.

Lopes, A.C., Palhão, M.P., Fernandes, C.A.C., Sudano, M.J., Castilho, A.C.S., Caixeta, E.S., 2017. Differential expression of insulin-like growth factor family members in immature cumulus–oocyte complexes from dairy cows with different genotypes. Reprod. Domest. Anim. 52, 1067–1073. https://doi.org/10.1111/rda.13028.

Luciano, A.M., Modina, S., Gandolfi, F., Lauria, A., Armstrong, D.T., 2000. Effect of cell-to-cell contact on in vitro deoxyribonucleic acid synthesis and apoptosis responses of bovine granulosa cells to insulin-like growth factor-I and epidermal growth factor. Biol. Reprod. 63, 1580–1585.

Machado, M.F., Portela, V.M., Price, C.A., Costa, I.B., Ripamonte, P., Amorim, R.L., Buratini Jr., J., 2009. Regulation and action of fibroblast growth factor 17 in bovine follicles. J. Endocrinol. 202, 347–353. https://doi.org/10.1677/JOE-09-0145.

Machtinger, R., Laurent, L.C., Baccarelli, A.A., 2016. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum. Reprod. Update 22, 182–193. https://doi.org/10.1093/humupd/dmv055.

Maheshwari, M., Bhutani, S., Das, A., Mukherjee, R., Sharma, A., Kino, Y., Nukina, N., Jana, N.R., 2014. Dexamethasone induces heat shock response and slows down disease progression in mouse and fly models of Huntington's disease. Hum. Mol. Genet. 23, 2737–2751. https://doi.org/10.1093/hmg/ddt667.

Mathivanan, S., Ji, H., Simpson, R.J., 2010. Exosomes: extracellular organelles important in intercellular communication. J. Proteomics 73, 1907–1920. https://doi.org/10.1016/j.jprot.2010.06.006.

Maziero, R.R.D., Martins, A.C., Mollo, M.R., Martin, I., Bastos, M.R., Ferreira, J.C.P., Rumpf, R., Sartori, R., 2011. Ovarian function in cows submitted to acute stress during proestrus. Livest. Sci. 138, 105–108. https://doi.org/10.1016/j.livsci.2010.12.003.

Min, L., Zheng, N., Zhao, S., Cheng, J., Yang, Y., Zhang, Y., Yang, H., Wang, J., 2016. Long-term heat stress induces the inflammatory response in dairy cows revealed by plasma proteome analysis. Biochem. Biophys. Res. Commun. 471, 296–302. https://doi.org/10.1016/j.bbrc.2016.01.185.

Moussa, M., Shu, J., Zhang, X.H., Zeng, F., 2015. Maternal control of oocyte quality in cattle "a review". Anim. Reprod. Sci. 155, 11–27. https://doi.org/10.1016/j.anireprosci.2015.01.011.

Mullen, S.F., Agca, Y., Broermann, D.C., Jenkins, C.L., Johnson, C.A., Critser, J.K., 2004. The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. Hum. Reprod. 19, 1148–1154. https://doi.org/10.1093/humrep/deh201.

Mullen, S., Rosebaum, M., Critser, J.K., 2007. The effect of osmotic stress on the cell volume, metaphase II spindle and developmental potential of in vitro matured porcine oocytes. Cryobiology 54, 281–289. https://doi.org/10.1038/nature13314.A.

Nikseresht, M., Toori, M.A., Rahimi, H.R., Fallahzadeh, A., Kahshani, I.R., Hashemi, S.F., Bahrami, S., Mahmoudi, R., 2017. Effect of antioxidants (β-mercaptoethanol and cysteamine) on assisted reproductive technology in vitro. J. Clin. Diagn. Res. 11, BC10–BC14. https://doi.org/10.7860/JCDR/2017/21778.9298.

Page, T.J., Sikder, D., Yang, L., Pluta, L., Wolfinger, R.D., Kodadek, T., Thomas, R.S., 2006. Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. Mol. Biosyst. 2, 627–639. https://doi.org/10.1039/b606129j.

Paula-Lopes, F.F., Boelhauve, M., Habermann, F.A., Sinowatz, F., Wolf, E., 2007. Leptin promotes meiotic progression and developmental capacity of bovine oocytes via cumulus cell-independent and -Dependent mechanisms. Biol. Reprod. 76, 532–541. https://doi.org/10.1095/biolreprod.106.054551.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT – PCR. Nucleic Acids Res. 29, 16–21.

Pirkkala, L., Nykänen, P., Sistonen, L., 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15, 1118–1131. https://doi.org/10.1096/fj00-0294rev.

Pribenszky, C., Du, Y., Molnar, M., Harnos, A., Vajta, G., 2008. Increased stress tolerance of matured pig oocytes after high hydrostatic pressure treatment. Anim. Reprod. Sci. 106, 200–207. https://doi.org/10.1016/j.anireprosci.2008.01.016.

Pribenszky, C., Vajta, G., Molnar, M., Du, Y., Lin, L., Bolund, L., Yovich, J., 2010. Stress for stress tolerance? A fundamentally new approach in mammalian embryology. Biol. Reprod. 83, 690–697. https://doi.org/10.1095/biolreprod.110.083386.

Pursley, J.R., Mee, M.O., Wiltbank, M.C., 1995. Synchronization of ovulation in dairy cows using PGF2-α and GnRH. Theriogenology 44, 915–923. https://doi.org/10.1016/0093-691X(95)00279-H.

Quesenberry, P.J., Aliotta, J.M., 2010. Cellular phenotype switching and microvesicles. Adv. Drug Deliv. Rev. 62, 1141–1148. https://doi.org/10.1016/j.addr.2010.06.001.

Ratajczak, M.Z., Kucia, M., Jadczyk, T., Greco, N.J., Wojakowski, W., Tendera, M., Ratajczak, J., 2012. Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies. Leukemia 26, 1166–1173. https://doi.org/10.1038/leu.2011.389.

Rivera, R.M., Hansen, P.J., 2001. Development of cultured bovine embryos after exposure to high temperatures in the physiological range. Reproduction 121, 107–115.

Saeed-Zidane, M., Linden, L., Salilew-Wondim, D., Held, E., Neuhoff, C., Tholen, E., Hoelker, M., Schellander, K., Tesfaye, D., 2017. Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. PLoS One 12, 1–24. https://doi.org/10.1371/journal.pone.0187569.

Sampath, S.C., Ohi, R., Leismann, O., Salic, A., Pozniakovski, A., Funabiki, H., 2004. The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. Cell 118, 187–202. https://doi.org/10.1016/j.cell.2004.06.026.

Sartori, R., Sartor-Bergfelt, R., Mertens, S.A., Guenther, J.N., Parrish, J.J., Wiltbank, M.C., 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J. Dairy Sci. 85, 2803–2812. https://doi.org/10.3168/jds.S0022-0302(02)74367-1.

Satrapa, R.A., Razza, E.M., Castilho, A.C.S., Simões, R.A.L., Silva, C.F., Nabhan, T., Pegorer, M.F., Barros, C.M., 2013a. Differential expression of igf family members in heat-stressed embryos produced in vitro from opu-derived oocytes of nelore (bos indicus) and holstein (bos taurus) cows. Reprod. Domest. Anim. 48, 1043–1048. https://doi.org/10.1111/rda.12211.

Satrapa, R.A., Castilho, A.S., Razza, E.M., Pegorer, M.F., Puelker, R., Barros, C.M., 2013b. Differential expression of members of the IGF system in OPU-derived oocytes from Nelore (Bos indicus) and Holstein (Bos taurus) cows. Anim. Reprod. Sci. 138, 155–158. https://doi.org/10.1016/j.anireprosci.2013.02.023.

Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Goncalves, P.B., Wolf, E., 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol. Reprod. 64, 904–909. https://doi.org/10.1095/biolreprod64.3.904.

Sun, S.-C., Lin, S.-L., Zhang, D.-X., Lu, S.-S., Sun, Q.-Y., Kim, N.-H., 2010. Borealin regulates bipolar spindle formation but may not act as chromosomal passenger during mouse oocyte meiosis. Front. Biosci. 2, 991–1000 (Elite Ed).

Sutton-McDowall, M.L., Purdey, M., Brown, H.M., Abell, A.D., Mottershead, D.G., Cetica, P.D., Dalvit, G.C., Goldys, E.M., Gilchrist, R.B., Gardner, D.K., Thompson, J.G., 2015. Redox and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone. Mol. Reprod. Dev. 82, 281–294. https://doi.org/10.1002/mrd.22470.

Taylor, D.D., Gercel-Taylor, C., 2013. The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids. Front. Genet. 4, 1–12. https://doi.org/10.3389/fgene.2013.00142.

Tseng, J.K., Tang, P.C., Ju, J.C., 2006. In vitro thermal stress induces apoptosis and reduces development of porcine parthenotes. Theriogenology 66, 1073–1082. https://doi.org/10.1016/j.theriogenology.2006.03.003.

Vijayan, M.M., Raptis, S., Sathiyaa, R., 2003. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen. Comp. Endocrinol. 132, 256–263. https://doi.org/10.1016/S0016-6480(03)00092-3.

Wilson, S.J., Marion, R.S., Spain, J.N., Spiers, D.E., Keisler, D.H., Lucy, M.C., 1998. Effects of controlled heat stress on ovarian function of dairy cattle. 1. lactating cows. J. Dairy Sci. 81, 2124–2131. https://doi.org/10.3168/jds.S0022-0302(98)75788-1.

Yao, J., Ren, X., Ireland, J.J., Coussens, P.M., Smith, T.P.L., Smith, G.W., 2004. Generation of a bovine oocyte cDNA library and microarray: resources for identification of genes important for follicular development and early embryogenesis. Physiol. Genomics 19, 84–92. https://doi.org/10.1152/physiolgenomics.00123.2004.