Cell Stress and Chaperones (2016) 21:467–475
DOI 10.1007/s12192-016-0673-9

ORIGINAL PAPER

The effect of heat stress on gene expression, synthesis of steroids, and apoptosis in bovine granulosa cells

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Received: 10 December 2015 / Revised: 21 January 2016 / Accepted: 22 January 2016 / Published online: 5 February 2016

Abstract Summer heat stress (HS) is a major contributing factor in low fertility in lactating dairy cows in hot environments. Heat stress inhibits ovarian follicular development leading to diminished reproductive efficiency of dairy cows during summer. Ovarian follicle development is a complex process. During follicle development, granulosa cells (GCs) replicate, secrete hormones, and support the growth of the oocyte. To obtain an overview of the effects of heat stress on GCs, digital gene expression profiling was employed to screen and identify differentially expressed genes (DEGs; false discovery rate (FDR)≤0.001, fold change ≥2) of cultured GCs during heat stress. A total of 1211 DEGs including 175 upregulated and 1036 downregulated ones were identified, of which DEGs can be classified into Gene Ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The results suggested that heat stress triggers a dramatic and complex program of altered gene expression in GCs. We hypothesized that heat stress could induce the apoptosis and dysfunction of GCs. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate the expression of steroidogenic genes (steroidogenic acute regulatory protein (Star), cytochrome P-450 (CYP11A1), CYP19A1, and steroidogenic factor 1 (SF-1)) and apoptosis-related genes (caspase-3, BCL-2, and BAX). Radio immunoassay (RIA) was used to analyze the level of 17β-estradiol (E_2) and progesterone (P_4). We also assessed the apoptosis of GCs by flow cytometry. Our data suggested that heat stress induced GC apoptosis through the BAX/BCL-2 pathway and reduced the steroidogenic gene messenger RNA (mRNA) expression and E_2 synthesis. These results suggest that the decreased function of GCs may cause ovarian dysfunction and offer an improved understanding of the molecular mechanism responsible for the low fertility in cattle in summer.

Keywords Bovine · Granulosa cells · Heat · Follicles

Introduction

Heat stress induces infertility in dairy cows and represents a major source of economic loss to the livestock sector (Wilson et al. 1998; de Torres-Júnior JR et al. 2008; Kobayashi et al. 2013; Shehab-E-Deen et al. 2011). The decrease in animal fertility is caused by elevated body temperature that influences ovarian functions, oestrous expression, oocyte health, and embryonic development (Wakayo et al. 2015). Ovarian follicle development is a complex process. During follicle development, granulosa cells (GCs) replicate, secrete hormones, and provide a critical microenvironment for follicular growth (Petro et al. 2012). Evidence indicates that multiple granulosa cell dysfunctions lead to disordered ovulatory and ovarian function (Tatone et al. 2008). Investigations of the effects of various agents on granulosa cells show that mycotoxin deoxynivalenol induced bovine ovarian granulosa cell apoptosis (Guerrero-Netro et al. 2015), and cigarette smoke impairs granulosa cell proliferation in young Swiss mice (Paixão et al. 2012). Furthermore, while much is now known about the

Electronic supplementary material The online version of this article (doi:10.1007/s12192-016-0673-9) contains supplementary material, which is available to authorized users.

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effects of various factors on normal granulosa cells (Hutt and Albertini 2007; Paul et al. 2011), the gene expression changes that contribute to granulosa cell dysfunction under thermal stress remain to be elucidated. Our first objective was to explore the global effect of thermal stress on gene expression. Our second and main objective was to identify the effects of heat treatment on the synthesis of progesterone (P_4) and 17β-estradiol (E_2) and apoptosis, infer how heat stress would affect GC-related functions and follicular development microenvironment.

Result

Analysis of differentially expressed genes

The global gene expression profiles in bovine granulosa cell samples representing heat treatment (HT) and normal control (NC) were identified with digital gene expression (DGE) technique. We used false discovery rate (FDR) ≤0.001 and fold change ≥2 as a threshold to identify differentially expressed genes during the heat treatment course. Total 1211 genes altered expression as compared to the control samples, of which, 175 genes were upregulated and 1036 genes were downregulated (Table S1).

Gene ontology and pathway analysis

To categorize biological processes that are overrepresented in control and heat-treated cells, we classified all known differentially expressed genes using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis available in the Database for Annotation, Visualization and Integrated Discovery (DAVID) [http://david.abcc.ncifcrf.gov/home.jsp]. One hundred seventy-five upregulated genes (fold change≥2) were analyzed by GO analysis then filtered to top 10 records by selecting only the most specific subcategories of GO terms under biological process which was considered significant by the Benjamini-Yuketeli test ($P<0.05$) were ranked (Fig. 1a). The major processes that exhibit higher levels of gene expression as a consequence of heat-treated GCs include GO terms related to translation, electron transport chain, oxidation reduction, and regulation of cell apoptosis and death. GO analysis of 1036 downregulated genes resulted in 160 records (Table S2), of which 10 highly enriched GO terms were selected under biological process. These processes that were significantly downregulated by heat-treated GCs as shown by functional analysis included protein catabolic process, protein localization, and protein transport (Fig. 1b). All differential genes were performed with KEGG pathway analysis (Table S2), and 14 pathways were filtered. These pathways included biosynthesis, degradation, and TGF-β signaling pathway (Fig. 1c).

Validation of DGEs by real-time qPCR

To confirm changes in the expression of genes identified by DGE analysis, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses of eight representative genes were performed on the same samples. Gene expression profiling of granulosa cells revealed that some genes associated with HSP family-related genes were active during cell response to heat (Tables 1 and 2). Similar to that observed in DGE analysis, the expressions of HSP family genes such as HSP90B1and apoptotic-related genes CASP3, BAX, and BCL-2 were significantly increased in heat-treated granulosa cells compared to control group (Fig. 2a). Some downregulated genes including steroidogenic genes SF1 (3.3-fold), STAR (4.8-fold), CYP11A1 (2.5-fold), and CYP19A1 (3.7-fold) were also identified (Fig. 2b).

Effects of heat treatment on E_2 and P_4 secretion of GCs

The concentration of E_2 (Fig. 3a) in the heat-treated group was lower than the control group in the culture media ($P<0.01$). There is no significant difference in P_4 level between control and heat group (Fig. 3b).

Effects of heat treatment on granulosa cell apoptosis

We estimated the apoptotic rate by flow cytometry (FCM), and the apoptotic rate of GCs was significantly higher in the HT group (Fig. 4a, d). Moreover, the active caspase-3 was also estimated by ELASA, and the caspase-3 activity of GCs was significantly higher in the HT group (Fig. 4c). Meanwhile, the ratio of BAX/BCL-2 was significantly higher in the HT group (Fig. 4b). Caspase-3 regulates cell apoptosis, and enhanced caspase-3 expression increased apoptosis. As shown in Fig. 2, the expression level of pro-caspase-3 in the bovine granulosa cells under heat treatment was significantly higher than the control group.

Discussion

Summer heat stress (HS) is a major factor in decreased reproductive performance in dairy cattle, possibly by affecting the metabolism capacity of ovarian follicles (Alves et al. 2014; Roth 2008). The current study offers the first thorough insight into the transcriptome analysis of primary bovine GCs during heat stress using DGE technology. The granulosa cells of the ovarian follicle support and nurture the oocyte and secrete estrogens which establish a functional bidirectional crosstalk with the oocyte through gap junctions and paracrine factors, and GCs are necessary for normal reproductive function (Langhout et al. 1991; Petro et al. 2012). Several studies have been published, demonstrating that the gene expression of

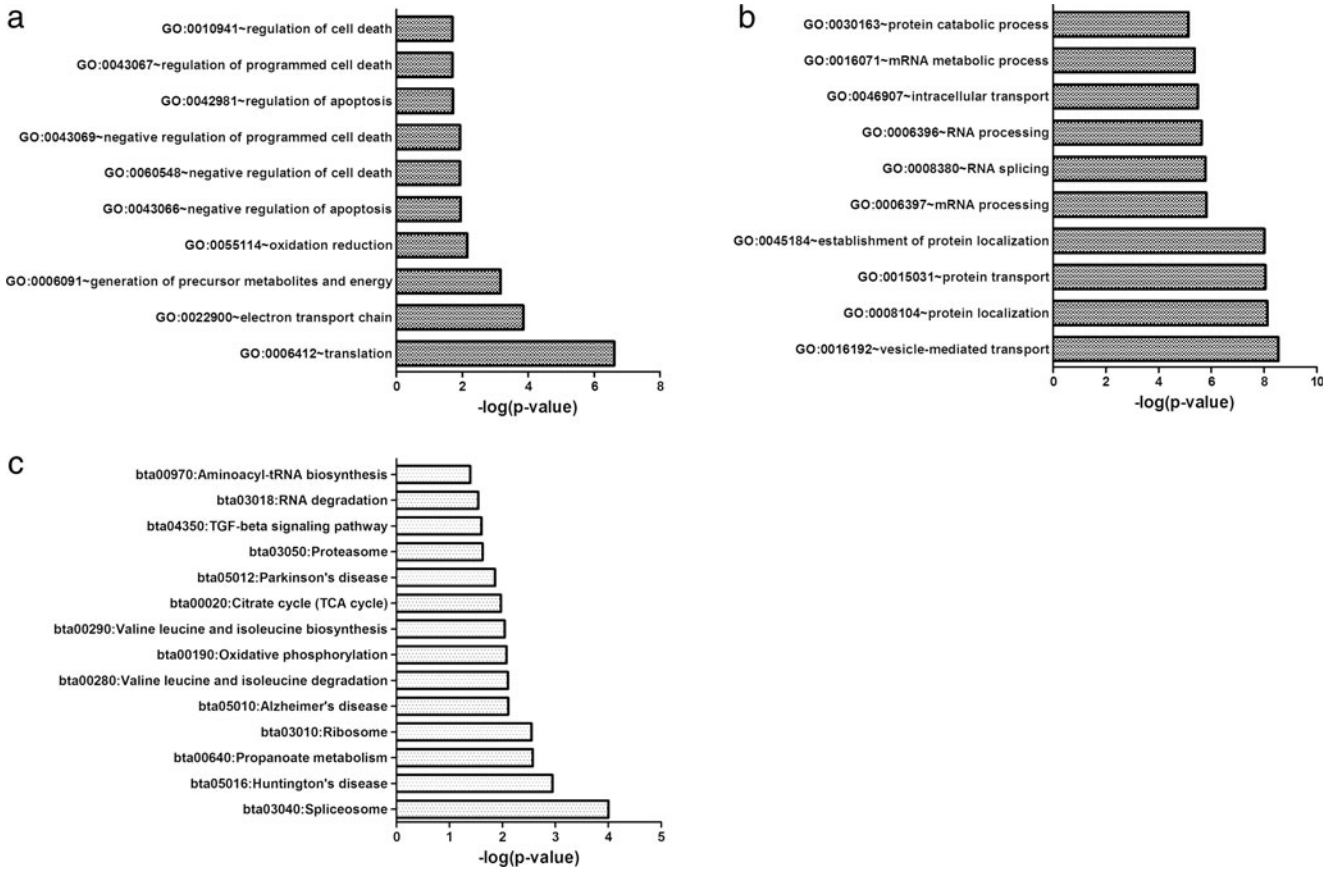

Fig. 1 Highly enriched GO terms analyzed of upregulated (**a**) and downregulated (**b**) genes that changed fold ≥2 by Gene Ontology analysis (*P* values <0.05). Only the most specific subcategories of GO terms under biological process which was considered significant by the Benjamini-Yuketeli test ($P < 0.05$) were ranked. Biological process analyzed (**c**) of regulated genes (up and down genes) that changed fold ≥2 folds by Gene KEGG analysis

GCs may differ in relation to the potential development of the embryo (McKenzie et al. 2004; Wathlet et al. 2011). Heat stress induced the alternation of progesterone production in cumulus cells which are surrounding the bovine oocyte during

Table 1 Primer sequences for RT-PCR

Gene	Primer sequence (5′–3′)	NCBI reference sequence
SF-1	F: CATCCTCTTCAGCCTGGATTTG R: CCTTCTCCTGAGCGTCTTTCAC	NM_174403.2
STAR	F: CCCATGGAGAGGCTTTATGA R: TGATGACCGTGTCTTTTCCA	NM_174189.2
CYP11A1	F: CTGGCATCTCCACAAAGACC R: GTTCTCGATGTGGCGAAAGT	NM_176644.2
CYP19A1	F: GGTTAGCTCCAGGAGTGCAG R: CTTGGCTTAGGGTCATGGAA	NM_174305.1
BAX	F: AGACACCTGAGCTGACCTTG R: GTCCCGAAG TAGGAGAGG AG	NM_173894.1
BCL-2	F: CTGGGG ATGACTTCTCTCG R: GGAGAA ATC AAA CAG AGG TC	NM_001166486.1
CASP-3	F: CTGGACTGTGGCATT GAGAC R: GCA AAG GGACTGGA GAACC	NM_001077840.1
HSP90B1	F: TTGCCTTCCAAGCTGAAGTT R: TGACATGCAGCAGGTTCTTC	NM_174700.2
18S rRNA	F: TCCAGCCTTCCTTCCTGGGCAT R: GGACAGCACCGTGTTGGCGTAGA	NM_174841.2

Table 2 Effects of heat stress on the expression of selected genes involved in the heat shock protein family (fold change ≥2-fold)

HSP family	Gene symbol	Full gene name	Fold change
HSP32	HMOX1	heme oxygenase 1	3.7
HSP60	HSPD1	heat shock protein family D (Hsp60) member 1	2.6
HSP70	HSPA4	heat shock protein family A (Hsp70) member 4	3.5
	HSPA5	heat shock protein family A (Hsp70) member 5	3.1
	HSPA9	heat shock protein family A (Hsp70) member 9	3.7
	HSPA13	heat shock protein family A (Hsp70) member 13	4.9
	HSPA14	heat shock protein family A (Hsp70) member 14	3.0
HSP90	HSP90AA1	heat shock protein 90 kDa alpha family class A member 1	2.6
	HSP90B1	heat shock protein 90 kDa beta family member 1	3.2
HSP105	HSPH1	heat shock 105 kDa/110 kDa protein 1	3.7

maturation (Rispoli et al. 2013), and thermal stress could induced sheep granulosa cell apoptosis (Fu et al. 2014). Heat stress could induce metabolic changes, which compromise the number of ovarian follicles and the follicular environment, thus resulting in morphologically damaged oocytes (Alves et al. 2014). In the ovary, GCs are responsible for establishing the follicular microenvironment; any factors that impair GCs may disrupt oocytes (Guerrero-Netro et al. 2015; Rawan et al. 2015). So, we hypothesized that heat stress may induce the change of gene expression, finally leading to disordered ovarian function. In the present study, using DGE profiling of granulosa cells, we have demonstrated that thermal stress triggers a dramatic and complex program of altered gene expression in GCs similar to some patterns reported in other cell types exposed to thermal stress (Collier et al. 2008; Li et al. 2015). Some reported that thermal stress can regulate the RNA processing/translation, increase degradation of proteins, disrupt the cytoskeletal components, and alter metabolism process (Collier et al. 2006; Sonna et al. 2010). We found that GCs are susceptible to heat according to the GO analysis result. In the present study, the transcriptome profile indicated upregulation of genes involved in translation, electron transport chain, oxidation reduction, and regulation of cell apoptosis and death. Of the downregulated genes, the majority were involved in protein catabolic process, protein localization, and protein transport. The fact that substantially more genes were downregulated than upregulated in heat-treated GCs could indicate that reduction rather than activation in additional pathways occurs as GCs suffering heat stress. The majority of the differentially expressed genes were found to be associated with important biological processes, many being classified in biosynthesis, degradation, and TGF-β signaling pathway.

Nuclear steroidogenic factor 1 (SF-1) is considered to be a master regulator of steroidogenesis (Hattori et al. 2015), and it promotes estrogen biosynthesis through the regulation of P450 aromatase (CYP19A1) in the ovarian follicle (Mendelson et al. 2005). In our study, the messenger RNA (mRNA) expression of SF-1 decreased in CGs and the level of E_2 in the culture medium significantly decreased after heat treatment. Similarly, the mRNA expression of CYP19A1 was decreased in granulosa cells by real time-rtPCR. Based on these facts, it is reasonable to suggest that the downregulated expression of SF-1 may inhibit estrogen biosynthesis through the regulation of CYP19A1 expression in ovarian granulosa cells, and this in turn induces a low E_2 level. Progesterone is

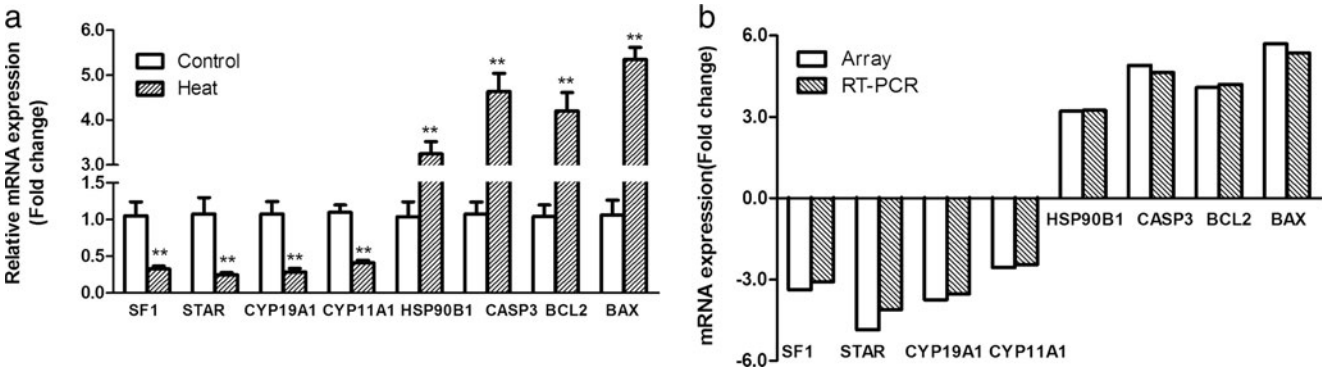

Fig. 2 **a** Validation of differentially expressed genes by real-time qPCR. Relative quantification of seven representative genes was performed. qRT-PCR values were determined from the $^{\Delta\Delta}$Ct for the target genes relative to 18S. Significantly different results for treatments used one-way ANOVA with Fisher's LSD post hoc test; $P < 0.05$. A *single asterisk* indicates a statistical difference of $P < 0.05$, and a *double asterisk* indicates a statistical difference of $P < 0.01$. **b**. Comparison of RT-PCR findings to DGE profiling results

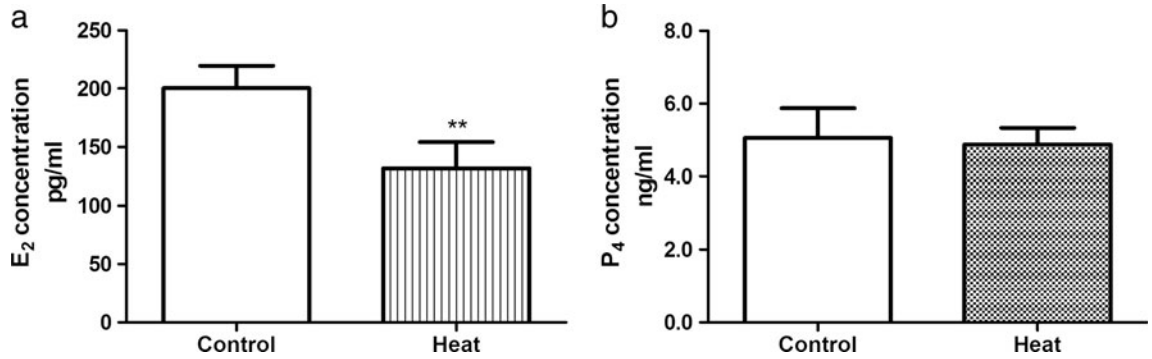

Fig. 3 Effect of heat stress on E_2 (**a**) and P_4 (**b**) in culture media. A *single asterisk* indicates a statistical difference of $P < 0.05$, and a *double asterisk* indicates a statistical difference of $P < 0.01$

one of the fundamental steroid hormones for the regulation of the bovine estrous cycle, and its biosynthesis is attributed to the increased expression of steroidogenic acute regulatory protein (Star) and cytochrome P-450 (CYP11A1) (Mosa et al. 2015; Zhang et al. 2015). The mechanism of progesterone biosynthesis involves CYP11A1 catalyzing the conversion of cholesterol to pregnenolone and Star transporting cholesterol from the outer to the inner mitochondrial membrane (Pescador et al. 1996; Rekawiecki et al. 2005). In this study, we found that the mRNA expression of CYP11A1 and Star decreased, but the P_4 level has no significant difference between control and heat treatment group. The higher temperature induced an oversecretion of ovarian hormones in porcine ovarian granulosa cells (Sirotkin 2010). But some studies show that heat stress diminishes gonadotropin receptor expression and attenuates estrogenic activity in rat granulosa cells (Shimizu et al. 2005). Our study is the first that demonstrated the effect of heat stress on gene expression and steroid hormone synthesis in bovine ovarian granulosa cells.

The lack of estradiol leads to granulosa cell apoptosis (Shimizu et al. 2005). Thus, we hypothesized that apoptosis-related genes might be expressed in heat treatment GCs. According to the gene expression profile, we confirmed the expression levels of BCL-2 and BAX mRNAs in granulosa cells by real time RT-PCR. Our findings indicate that the mRNA expression levels of antiapoptotic gene (BCL-2) and

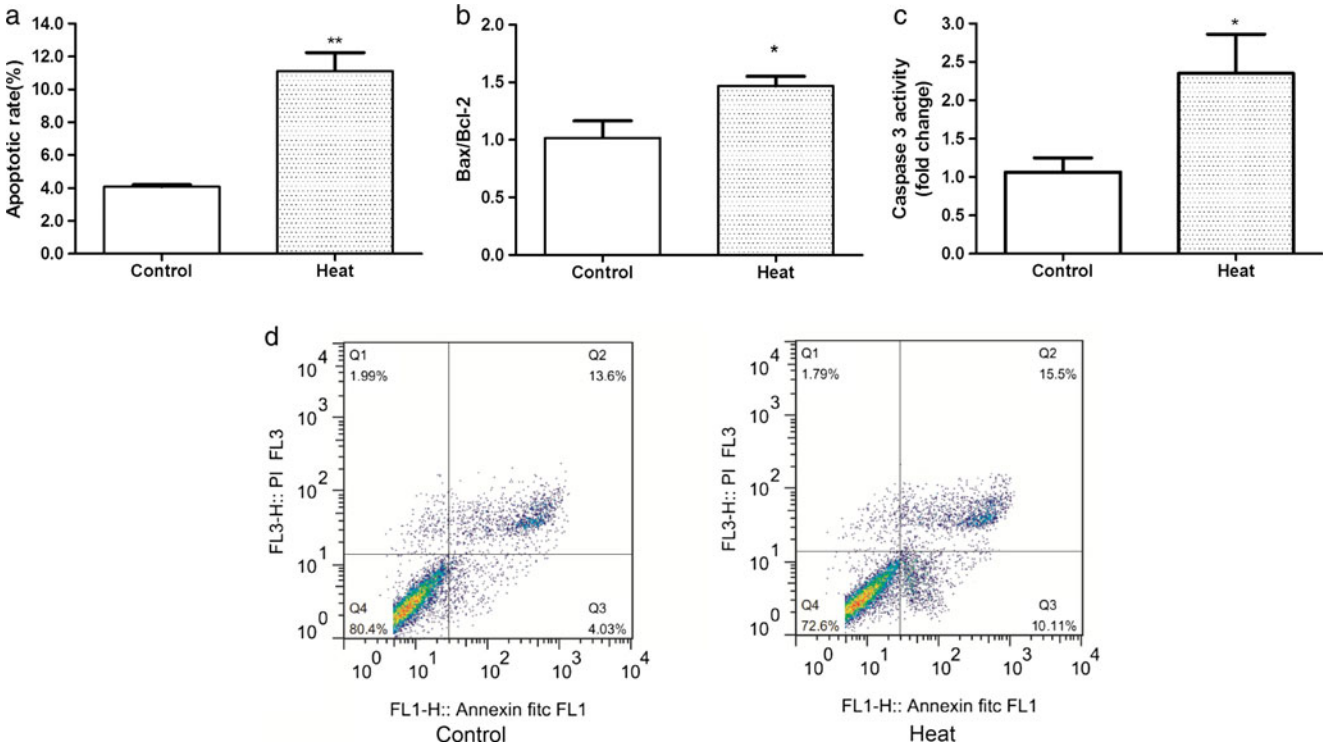

Fig. 4 The effect of heat treatment on cell apoptosis. After cells were treated with heat stress, the relative number of cells undergoing apoptosis was determined by flow cytometry after annexin V/propidium iodide staining and the apoptosis rates were analyzed (**a**, **d**). The ratio of BAX/BCL-2 (**b**) and the activity of caspase-3 in GCs (**c**). A *single asterisk* indicates a statistical difference of $P < 0.05$, and a *double asterisk* indicates a statistical difference of $P < 0.01$

proapoptotic gene (BAX) were increased, but the ratio of BAX to BCL-2 mRNA was higher in heat treatment group. There is a delicate balance between anti- and proapoptotic BCL-2 family members that exists in each cell, and the relative concentrations of these two groups of proteins determine whether the cell survives or undergoes apoptosis (Setroikromo et al. 2007). In addition, we observed the executioner caspase-3 of apoptotic pathway and found that caspase-3 activity was affected by heat stress. Meanwhile, the increased apoptotic rate of the GCs also confirmed this result.

The HSP family-related genes are known to be active during cell response to various stressors (Setroikromo et al. 2007). In this study, some HSP family-related genes involved in HSP32, HSP60, HSP70, HSP90, and HSP105 family were upregulated by heat treatment. In all HSPs, the most pronounced stress-related changes and involvement in granulosa cell functions have been demonstrated for HSP70, and small HSP-HSP32 (HMOX1, HO-1). The high temperature can stimulate the transcription of HSP70.2, HSP72, and HSP105/110 genes in ovarian follicular granulosa cells (Sirotkin 2010; Sirotkin et al. 2011). Inducible HO-1 is likely to be important autocrine/paracrine factors that regulate apoptosis in porcine GCs (Harada et al. 2004). HO-1 may play a stimulatory role of endogenous carbon monoxide in the production of ovarian steroids in the rat ovary (Alexandreanu and Lawson 2003). Under heat stress, the synthesis of HSP proteins is a cellular adaptive response to help maintain cellular homeostasis.

In conclusion, the changes in the transcriptome of the heat-treated bovine granulosa cells indicated that the granulosa cells of follicle are more sensitive to effects of heat stress than other somatic cells (Collier et al. 2008). As a result, activation of transcription of genes and activation transport and localization of proteins which involved in thermal protection are enhanced and there are some indications of disruption in gene networks involved in follicle development. Our present results also show that heat stress can induce apoptosis of GCs as evidenced by the activation of caspase-3 activity and BAX/BCL-2 ratio and inhibit the estrogen synthesis. We have reported a gene expression analysis which could offer an improved understanding of the molecular mechanism responsible for the low fertility in cattle in summer (Fig. 5), but subsequent investigations should include the functional assessment of individual DEGs that may be involved in follicular growth process under heat stress to improve our understanding of the relationship between these molecules and the follicular growth.

Material and methods

Bovine ovaries and primary culture of granulosa cells

Eighteen ovaries were collected from nine Chinese Holstein cows (3 to 5 years of age) at a local abattoir in Nan Jing (from

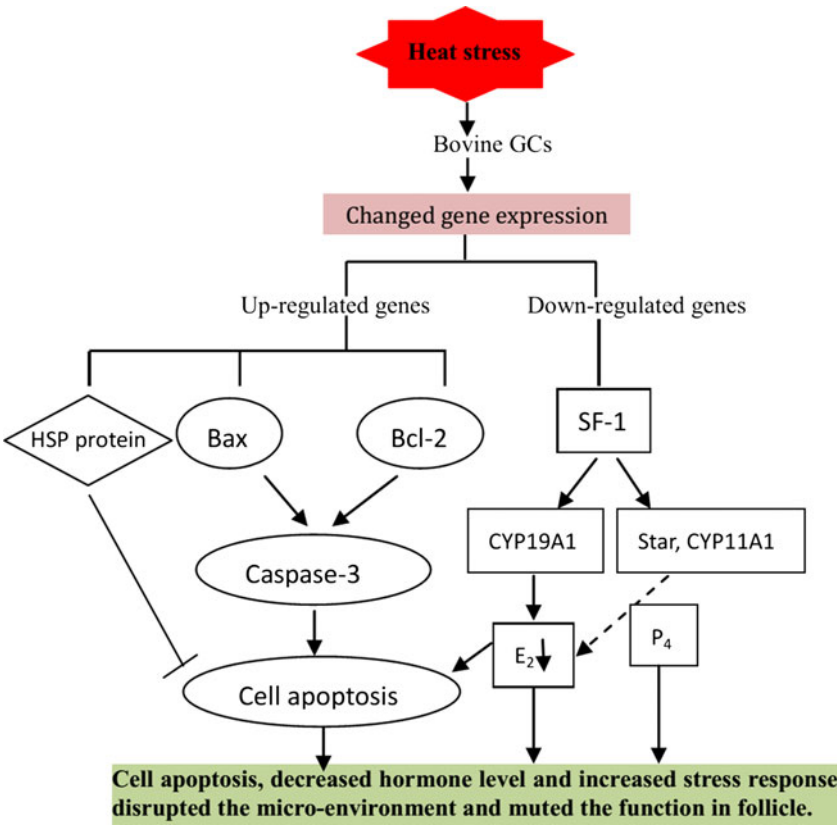

Fig. 5 Proposed mechanisms of regulating heat stress response related to follicular function within bovine ovary. Upregulated genes—BCL-2, BAX, and HSP—were involved in the regulating mechanism of GCs via induced or inhibited cell apoptosis. Downregulated genes—SF-1, CYP19A1, STAR, and CYP11A1—were involved in the secretion of E_2 and P_4. Moreover, the decline of E_2, in turn, might enhance the possibility of GC apoptosis and follicle function. *Black line arrows* indicate a directly stimulating effect (→); *black dashed arrows* indicate an indirectly stimulating effect (→); *black line* from HSP protein indicates an inhibiting effect (⊣)

non-pregnant cows, within 20 min of slaughter, and transported to the laboratory in Hank's balanced salt solution (HBSS) with $Mg^{2+}$and Ca^{2+} at 4 °C). The granulosa cells were removed from the remainder of the follicles (4–6 mm) by gentle rubbing with a glass Pasteur pipette, previously modified by heat sealing the tip into a rounded smooth surface. The $HBSS^{-/-}$ containing the granulosa cells was centrifuged at 500×*g* for 7 min at 4 °C, the medium was removed by aspiration, and the cells were washed twice in phosphate-buffered saline (PBS), pH 7.4. Finally, the cells were resuspended in DMEM supplemented with 10 % FBS, seeded at 5×10^5 cells/mL into dish, and incubated at 38 °C with 5 % CO_2 (Hatzirodos et al. 2015).

Heat treatment

First, the GCs were precultured in medium (serum-free conditions) at 38 °C (optimal and physiologically relevant temperature for cultured mammalian ovarian cells) under 5 % CO_2 in humidified air. After 48 h of preculture, when cells attached to the bottom of the wells, the medium was replaced with medium of the same composition. Then cells were cultured at temperatures 38 °C (control group) or 40.5 °C (heat treatment group) for 2 h, and then the cells were cultured at 38 °C for 12 h. Immediately after culture, the cells and culture media were collected for subsequent analyses (Li et al. 2015).

RNA isolation

Total RNA was extracted from the granulosa cells using Trizol reagent (Invitrogen, Carlsbad, USA), according to the manufacturer description. The absorbance values at 260 and 280 nm were checked to assess the RNA concentration and purity for protein impurities in the samples. The RNA integrity was checked by the electrophoresis on 2 % agarose gels (*m*/*v*).

Differential expression detection of genes

Construction of digital gene expression profiling and annotation of gene expression as previously described after obtaining the normalized gene expression level, the differential expression of each gene across samples was compared. The genes with false discovery rate (FDR) ≤0.001 and fold change ≥2 were defined as the differentially expressed genes (DEGs).

GO and KEGG pathway analysis

The Molecule Annotation System (http://david.abcc.ncifcrf.gov/) was used to analyze the differentially expressed genes (Huang et al. 2009). The Gene Ontology (GO) terms of biological process in DAVID were employed to categorize enriched biological themes in up- and downregulated gene lists.

Real-time reverse transcription-polymerase chain reaction

RT-PCR was performed to confirm the microarray results. Total RNA was extracted from control and heat-treated granulosa cells as described above, and total RNA was reverse transcribed using a reverse transcription level kit (TaKaRa, Dalian, China) according to the manufacturer protocols. The expression levels were checked for nine genes. The 18S ribosomal RNA (rRNA) gene was used as the invariant control. Primers were designed using Primer Premier 5.0 and are shown in Table 1S. RT-PCR was performed with SYBR®Premix Ex TaqTM (TaKaRa BiotechnologyCo., Ltd., Japan, DRR081A). The reaction solution was prepared on ice and comprised 10 μL of 2× SYBR®Premix Ex TaqTM, 0.8 μL of PCR forward primer (10 μM), 0.8 μL of PCR reverse primer (10 μM), 0.4 mL of 50× ROX reference dye, 2 μL of cDNA (100 ng μL^{-1}), and dH_2O to a final volume of 20 μL. The reaction mixtures were incubated in a 96-well plate at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicate. The gene expression levels in the normal and heat-treated GCs were analyzed with the $2^{-\Delta\Delta CT}$ method.

Determination of estradiol and progesterone by radio immunoassay

Radio immunoassay (RIA) was used to assay the levels of E_2 and P_4 in culture media. We collected all of the culture media in normal and heat treatment group, and then determinate the levels of E_2 and P_4. The concentrations of E_2 and P_4 were ranging from 172 to 235 pg/mL and from 4.1 to 6.2 ng/mL, respectively. The intra-assay CVs averaged less than 10 %. The results are expressed as the amount of steroids (pg/mL and ng/mL).

Analysis of GC apoptosis by flow cytometry

GCs were prepared by enzymatic digestion and washed with preheated PBS. Then the GCs were measured through APC-annexin V/PI double staining by utilizing the Annexin V Apoptosis Detection Kit APC (eBioscience) according to the manufacturer's instructions before being analyzed by flow cytometry.

Activated caspase-3 activity assay of GCs

Caspase-3 enzymatic activity of GCs was determined using a Caspase Apoptosis Assay Kit (Geno Technology Inc., St. Louis, MO, USA).

Statistical analysis

All data were obtained from one independent experiment carried out in triplicate. Main and interactive effects were analyzed by the independent sample *t* test using SPSS16.0 software. $P < 0.05$ was considered statistically significant.

Acknowledgments This research was supported by the National Natural Science Foundation of China (Grant No. 31501955) and the Fundamental Research Funds for the Central Universities (Grant No. KJQN201607).

Author contributions Lian Li conceived and designed the experiments. Lian Li and Yu Sun performed the experiments. Lian Li analyzed the data. Jie Wu, Yu Sun, and Man Luo contributed reagents/materials/analysis tools. Lian Li and Genlin Wang wrote the paper. Yu Sun and Man Luo prepared the materials. Jie Wu helped analyze the data and in RT-PCR.

Compliance with ethical standards

Ethics statement In the present experiment, animal care and procedures were approved and conducted under the established standards of Nanjing Agricultural University, Nanjing, China.

Conflict of interest The authors declare that they have no competing interests.

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