Expression of heat shock and apoptosis genes in riverine buffalo (*Bubalus bubalis*) cumulus–oocyte complexes during *in-vitro* maturation under thermal stress conditions

Beshoy S.F. Khalil[a,b], Salah El-Din El-Assal[a], Nasser Ghanem[c]

Departments of [a]Genetics, Faculty of Agriculture, [c]Animal Production, Faculty of Agriculture, [b]Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University, Cairo, Egypt

Correspondence to Nasser Ghanem, PhD, Department of Animal Production, Faculty of Agriculture, Cairo University. El-Gamaa Street, Giza, Cairo 12613, Egypt. Tel: +20 100 298 6157; fax: +20235717355; e-mail: nassergo@agr.cu.edu.eg

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Background and objectives
Thermal stress arising from climate change is a crucial issue that threatens the livestock worldwide. It has various and wide range of effects on livestock's reproductive performance. Buffalo is a main livestock in the Egyptian agricultural sector, and its' susceptibility to the ambient temperature negatively affects its reproductive performance. Thus, it is important to study how the thermal stress affects the bubaline oocytes at both cytological and molecular levels. The current study aimed to investigate the effects of thermal stress for two different periods on the maturation of bubaline oocytes under *in vitro* conditions and screen the expression of various genes responsible for some mechanisms related to thermal stress alleviation, cumulus expansion, and apoptosis.
Materials and methods
Cumulus–oocyte complexes (COCs) were retrieved from buffalo ovaries and divided into three groups (C, T_1, and T_2) and underwent in-vitro maturation after being examined for quality. During the first 2/6 h of in-vitro maturation, good-quality COCs were exposed to 40.5°C and thereafter continued their maturation at 38.5°C. The COCs were denuded from the surrounding cumulus cells 22–24 h after maturation and were either preserved for RNA isolation in –80°C freezer or fixed for molecular maturation evaluation using Hoechst staining. The total RNA was isolated from three biological replicates of the three COC groups (C, T_1, and T_2) using Pico-pure RNA isolation kit, followed by cDNA synthesis for the genes of interest using real-time PCR (qPCR). Statistical analysis was performed for the obtained results for discussion and conclusion.
Results
The nuclear maturation declined more in the oocytes exposed to longer period of thermal stress than those exposed to short period of thermal stress. The longer the oocytes exposed to thermal stress, the higher was the expression of heat shock genes. The expression of heat shock genes was more expressed in cumulus cells in different groups than their corresponding oocytes. Moreover, expression of apoptosis-inducing gene (*BAX*) increased more in COCs exposed to long period of thermal stress than those in short period and control groups. This effect was also visible more in cumulus cells than in their corresponding oocytes. Although the cumulus expansion showed no significant change in pattern, the cumulus marker genes showed reverse relation with the period of the thermal stress, suggesting alteration in extracellular matrix proteins.
Conclusion
Heat stress affected negatively the nuclear maturation of buffalo oocytes by downregulation of cumulus expansion (*PTX3*, *TNFAIP6*, and *HAS2*) genes and upregulation of proapoptotic (*BAX*) gene under *in vitro* conditions. In response to this harmful situation, the cumulus cells surrounding oocytes undergo complex molecular mechanisms to adapt to the thermal shock by upregulation of heat shock transcripts (*HSF1*, *HSF2*, *HSP90*, and *HSP70*) and antiapoptotsis gene (*BCL2*) to provide protection against thermal stress and sustain oocyte viability.

Keywords:
chaperones, cumulus–oocyte complex, gene expression, heat stress, buffalo, in-vitro maturation, qPCR

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Introduction

Great attention is paid nowadays toward climate change issue and its subsequent influences on environmental events, as it is an integrated issue of many composite effects and various stresses. Ascension

of ambient temperature leads to excrescent heat gain, which results in heat stress that has a negative effect on animal production sector by disturbing the reproduction state through declining fertility and conception rates [1–3].

Riverine buffalo (*Bubalus bubalis*) is the principal multipurpose ruminant animal in the agricultural activities in Egypt, with contribution of 2 230 060 tons of raw milk production and 333 471 tons of meat production [4]. Buffalo is a polyestrous animal, which is well adapted to harsh environmental conditions of tropical and subtropical countries such as Egypt. Despite that, seasonality has a powerful negative effect on buffalo than cows [5]. Principal reasons of this sensitivity are that the buffalo's body has low density of sweat glands and dark skin color, hindering heat loss [6,7].

Under Egyptian hot summer conditions and its subsequent thermal stress events, female buffaloes' reproductive performance suffers from various physiological disorders such as silent estrus [8,9] and anestrous [10] due to poor ovarian activity [11] and irregular ovarian cycles [12]. It is documented that heat stress correlates with the increase of vaginal temperature by 2°C [13], which explains the decline in the average number of follicles per ovary in buffalo during summer (3.19±0.35) in comparison with winter (7.22±1.07) [14]. Moreover, the reduction of follicle cohort per ovary during hot season is explained by alterations of the hypothalamus–pituitary–ovarian axis, represented in perturbations of gonadotropins' surge [15,16].

The exposure of buffalo to thermal stress during hot season, when temperature range is between 40 and 44°C, reduces the quality of the oocytes and their developmental competence, reflecting the consequences on the resultant early-cleaved embryos represented in compromising their ability to reach the blastocyst stage [17]. The cumulus–oocyte complexes (COCs) recovered from heat-stressed buffaloes during hot season, when temperature could reach 40°C, and underwent in-vitro maturation (IVM) under 38.5°C showed darker ooplasm and degenerated cumulus-granulosa cells in comparison with cold season [18]. Buffalo embryos produced *in vitro* from heat-stressed groups of oocytes that were matured, fertilized, and subjected to embryo culture till day 8 at 39.5°C or 40.5°C for 2 h once every day showed poor ability to reach blastocyst stage [19]. In line with previous trend, the retrieved COCs during cold season that were subjected to thermal shock at various temperatures during various intervals of times of IVM showed reduction in nuclear maturation rate [20] and were significantly higher in reactive oxygen species due to the induced oxidative stress [21]. The previous results in bubaline showed consistency with the studies that were applied *in vitro* on different models employed in cows, as in the exposure of oocytes to thermal stress [22] and embryos at different developmental stages [23–27]; all showed decline in survival and reaching blastocyst stage.

On cytological level, it was demonstrated that during cold season the oocyte's membrane contains 2.2 times more polyunsaturated fatty acids in comparison with hot season [28], which affects the fertility. Thermal stress disturbed cytoplasmic and nuclear maturation events of oocytes; in addition, absence of a continuous layer of cortical granules during maturation under thermal stress conditions was reported [29]. Moreover, it caused disorganization of spindle microfilaments of the oocytes, which affected the cytoskeleton, leading to deformably chromatin segregation [30]. Oocytes that underwent thermal stress were arrested at metaphase I through metaphase II stages and faced fertilization failure [31].

Cumulus cells are important somatic cells as they adjoin the oocytes, and their expansion is regulated by oocyte-secreted factors [32]. Their exposure to elevated temperatures disturbed their functions, as in synthesizing proteins involved in extracellular matrix during IVM [33,34] in bovine. Hyperthermia caused reduction in the number of trans-zonal projections of COCs obtained from stressed porcine ovaries [35], and impairment of gap junction communication in bovine COCs [36], which means the obstruction of extracellular matrix formation, compromising oocytes' quality and prevention of resuming meiotic division.

Heat shock transcription factors (HSFs) lead the triggering of transcription of heat shock proteins (HSPs) to mediate thermotolerance response and returning to homeostasis [37]. Activity of heat shock factor 1 (*HSF1*) initiates the response to the heat shock by its binding to the 5' promoter regions of the HSP genes such as *HSP70* and *HSP90* [38]. During thermal stress, *HSF1* and *HSP90* showed upregulation in their expression in bubaline oocytes, whereas *HSF2* and *HSP70* showed low transcript numbers; however, the transcripts of the same four genes were increased in their number in the cumulus cells of the COCs exposed to the same trial [20].

This study was divided into two main experiments. Experiment 1 goal was to examine the effects of thermal shock on developmental competence of bubaline oocytes, whereas Experiment 2 goal was to examine quantitative expression of some genes related to various groups using quantitative real-time PCR (qRT-PCR) according to their functions, such as thermotolerance genes (*HSF1*, *HSF2*, *HSP90*, and *HSP70*), apoptotic genes (*BAX* and *BCL2*), and cumulus expansion marker genes (*TNFAIP6*, *PTX3*, and *HAS2*).

Materials and methods

Chemicals and reagents

All chemical, reagents, and medium used in the current study were purchased from Sigma-Aldrich (St Louis, Missouri, USA), except those mentioned in the text.

Ethical approval

This work was done with the ethical approval of Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) under the approval number: CU-II-F-8-20.

Experimental design

This study was carried out in two experiments. The first experiment was designed for studying bubaline oocytes' competency under one level of temperature through two different periods at the beginning of IVM. In this experiment, the retrieved COCs (150 oocytes/group) during winter were divided into three groups. The first group that was exposed to 38.5°C was considered as control (C), the second group that was exposed to 40.5°C for the first 2 h was considered as treatment 1 (T_1), and the third group that was exposed to 40.5°C for the first 6 h was considered as treatment 2 (T_2). After each temperature treatment, the treated groups (T_1 and T_2) continued the IVM at 38.5°C for 22–24 h. The second experiment was based on using qRT-PCR to investigate the expression of the genes related to thermotolerance (*HSF1*, *HSF2*, *HSP90*, and *HSP70*), cumulus expansion marker genes (*TNFAIP6*, *PTX3*, and *HAS2*), and pro-apoptosis gene (*BAX*) and anti-apoptosis gene (*BCL2*) in the oocytes and their adjoined cumulus cells.

Collection of ovaries and oocytes recovery

Buffalo ovaries were obtained from nearby slaughter houses shortly after the slaughtering process. The ovaries were transferred to the laboratory in a thermos filled with warmed (37°C) physiological saline solution (0.9% NaCl) supplemented with antibiotic (100 μg/ml streptomycin sulfate). The ovaries were then washed three times with physiological saline, twice with 70% ethanol, and finally with physiological saline twice. COCs were aspired from all visible ovarian follicles with size of 3–8 mm using 18-G needle attached to a 10-ml syringe containing aspiration medium (TCM-199-Hepes medium; supplemented with 2% inactivated fetal bovine serum, 0.3 mg/ml glutamine, and 50 μg/ml gentamycin). Evaluation of COCs was performed using a stereomicroscope (Leica, Bensheim, Germany), in which COCs were morphologically examined. Oocytes with homogenous ooplasm and multiple layers of cumulus cells were chosen according to their morphological criteria [39] for further processing after being examined.

In vitro maturation and nuclear staining

COCs were IVM in maturation media composed of TCM 199-Hepes fortified with 10% fetal bovine serum inactivated, 5 μg/ml of follicle-stimulating hormone, 1 μg/ml of estradiol-17 (E2), 0.15 mg/ml of glutamine, 22 μg/ml of Na-pyruvate, and 50 μg/ml of gentamycin. The immature oocytes (60/drop or well) were cultured into 100 μl maturation drops covered with mineral oil in a 3-cm petri dish according to the experimental design. The culture dishes were incubated in an incubator (Binder, York, UK) at 100% humidity, 5% CO_2, 5% O_2, and 90% N_2 for 22–24 h. COCs matured at 38.5°C for 24 h were designated as the control group (C). Heat shock treatments were applied on COCs at 40.5°C for first 2 h (T_1) or 6 h (T_2) of IVM, and thereafter matured at 38.5°C for the remaining 22 h for T_1 and 16 h for T_2.

Denudation and fixation of different cells

Cumulus cells were removed mechanically under the stereomicroscope (Leica, Bensheim, Germany) by repeat pipetting of COCs into maturation medium drops fortified with 0.25% of hyaluronidase. Following fixation, oocyte nuclear progression was evaluated by staining with Hoechst 33258 at concentration of 0.01 mg/ml (Sigma-Aldrich, USA). The hoechst stain was diluted in 0.3% PBS-PVP and oocytes were stained for 10 min on the dark. The stained oocytes were washed three times with 0.3% PBS-PVP and finally loaded on clean glass slide. The number of stained oocytes in each nuclear stage (germinal vesicle, germinal vesicle breakdown, metaphase I, and metaphase II) was recorded in each experimental group.

RNA isolation from oocytes and cumulus cells

Total RNA was isolated from oocytes and their adjoined cumulus cells using the Arcturus picopure RNA isolation kit (Cat# 12204-01 Thermo Fisher

Scientific, USA) according to manufacturer's protocol from different biological replicates. Extraction buffer (100 µl) was added to the samples followed by pipetting. Samples were incubated for 30 min at 42°C to release RNA and then centrifuged at 3000 g for 2 min. Afterward, the supernatant was transferred in a new 1.5-ml RNA-free tube. A volume of 250 µl of conditioning buffer was added to the RNA-free tube, which was then incubated for 5 min at room temperature, and the tube was then centrifuged at 16 000 g for 1 min. Eventually, a volume of 100 µl of 70% ethanol was added to the RNA extraction followed by pipetting. The mixture was added to the column and centrifuged at 100*g* for 2 min. Samples underwent centrifugation again at 1600*g* for 30 s, followed by addition of 100 µl of wash buffer I and centrifugation at 8000 g for 1 min. A mixture of total volume of 40 µl composed of 5 µl of DNase I and 35 µl of RDD was added to the samples and then incubated at room temperature for 15 min, followed by the addition of 100 µl of wash buffer II. The samples underwent centrifugation at 16 000 g for 2 min, followed by another centrifugation at the same spinning force for 1 min. The columns were transferred to new 1.5-ml tubes, and a volume of 20 µl of elution buffer was added to each tube. At last, the samples underwent centrifugation two times, at 1000 g for 1 min and then at 16 000 g for another minute.

NanoDrop 2000C instrument (Thermo Scientific, Wilmington, Delaware, USA) was used to evaluate the concentration and quality of the total RNA extracted from all samples, taking into consideration that the readings are at the absorbance of A_{260}/A_{280} nm. The RNA was stored at −80°C in an ultra-cool freezer (Thermo Fisher Scientific) until further use.

cDNA synthesis

To eliminate any DNA contaminants from the total RNA isolated from the different types of cells, DNA digestion using DNA digestion kit (Thermo Scientific, Carlsbad, California, USA) was done by adding to RNA (1 µg), the mixture of 10× reaction buffer with $MgCl_2$ (1 µl), DNase I (1 µl), and nuclease-free water to reach 10 µl of total volume. The tubes were then incubated at 37°C for 30 min. Then, a volume of 1 µl 50 mM EDTA was added followed by incubation at 65°C for 10 min The prepared RNA was later used as a template for reverse transcription. The reverse transcription of RNA samples to cDNA was done using the Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific) according to Ghanem *et al*. [40]. The following chemicals were added: 5 µg of total RNA samples, 1 µl of oligo dt18 primer, 4 µl of PCR buffer, 2 µl of dNTPs, 1 µl of RNase inhibitor, 1 µl RNase inhibitor enzyme, and 1 µl of reverse transcriptase enzyme; they were gently mixed by pipetting. The PCR mixture was incubated in a PCR thermocycler (Thermo Fisher Scientific) at 42°C for 60 min, and then at 70°C for 5 min and at 4°C overnight.

Quantitative real-time PCR

A pair of primers specific to each target genes (Table 1) were designed using primers3 program (https://primer3.ut.ee/) from available gene sequence in NCBI Gene bank database. Additionally, GAPDH housekeeping gene was used for normalization and data analysis. For real-time PCR reaction, the following components were added in the qPCR tube: 12 µl of

Table 1 List of primers used for quantitative real-time PCR analysis

Genes	GenBank accession number	Product length (bp)	Primer sequences
GAPDH	NM_001034034.2	219	F: 5'- AGGTCGGAGTGAACGGATTC –3' R: 5'- GGAAGATGGTGATGGCCTTT –3'
HSF1	KC568561	170	F: 5'- CGACCACCCTCATTGACTCC –3' R: 5'- CATCTTTGGAGTGCGAGCCA –3'
HSF2	NM_001290919.1	226	F: 5'- GCCCTCTCATGTCTAGTGCC –3' R: 5'- TTCTCAGAATCAACAAGGAGGT –3'
HSP70	JN604432.1	182	F: 5'- GCAGGTGTGTAACCCCATCA –3' R: 5'- ATAGGGCAAGACCCAAGTCC –3'
HSP90	XM_006069300.1	177	F: 5'- ACAGCCGTTCTCTTGAGTCAC –3' R: 5'- TGGGCAATTTCTGCCTGGAA –3'
BAX	XM_006050928	232	F: 5'- GACTCTCCCCGAGAGGTCTT-3' R: 5'- AGATGGTCACTGTCCAACCAC –3'
BCL2	XM_010979993.	241	F: 5′ –ACATCCACTATAAGCTGTCG –3′ R: 5′ –TAGCGCCGAGAGAAGTCAT –3'
TNFAIP6	NM_001007813	186	F: 5'-TGTCCTGCTATGGGAAGAGG –3' R: 5'-TGCTTGTAGGTGGCAAGATG –3'
PTX3	NM_001076259	206	F: 5'-TTTATTCCCCATGCGTTCCA –3' R: 5' –CTCCACCCACCACAAGCATT –3'
HAS2	NM_174079	180	F: 5'-GATTCCTGGATCTCCTTCCTCA –3' R: 5' –TTGGCTGCCCATAAATTCTTG –3'

Maxima Sybr Green qPCR Master Mix (Thermo Fisher Scientific), 0.5 µl of forward primer (10 µM), and 0.5 µl of reverse primer (10 µM), 2 µl of template cDNA, and 5 µl nuclease-free water to get 20 µl as the total reaction volume. The PCR mix was incubated at 50°C for 2 min; initial denaturation was done at 95°C for 10 min followed by 40 cycles of 95°C for 15 min for denaturation. Afterward, annealing cycles took place at 60°C for 1 min, and finally, the melt curve at 95°C for 15 s and then 60°C for 1 min. The expression data were analyzed using the delta-delta Ct method after normalization of the target genes with the housekeeping gene.

Statistical analysis

Relative expression was calculated using the relative quantification method (2-ΔΔCT method) [40]. The expression profiles of selected target genes were analyzed using the SAS (SAS, 2004) using the general linear model procedure. Fluorescent intensity data of cells viability, metabolic activity, lipid content, mitochondrial activity and ROS level were analyzed by applying a one-way analysis of variance. The analyzed data were expressed as mean±SE of means. Comparisons were significantly different if *P* value less than 0.05. Statistical analysis of data was performed using the IBM SPSS Statistics 22 program (SPSS Inc., Chicago, Illinois, USA).

Results

Effect of thermal shock for two different periods on expansion and maturation rates of the oocytes

Our results revealed that culture at 40.5°C for the first 2 h (T_1) and 6 h (T_2) of IVM (T_1) did not lead to reduction in the number of expanded COCs in comparison with the control group (C) at 38.5°C. In terms of cumulus cell expansion, the rate of expansion was 98.95, 97.61, and 100% in C, T_1, and T_2, respectively, showing no significant difference ($P \leq 0.05$), as shown in Fig. 1.

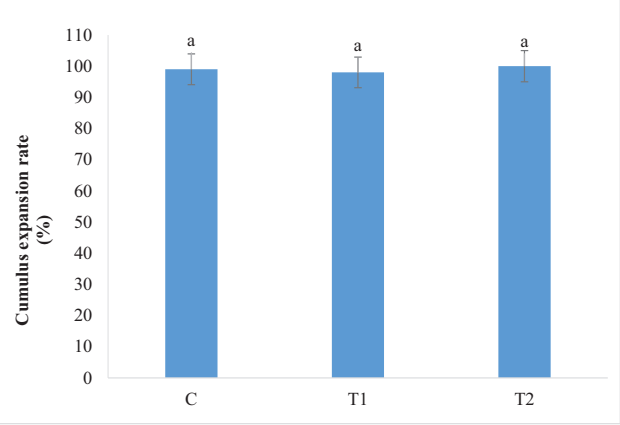
It was observed that thermal shock compromised ($P \leq 0.05$) the nuclear maturation in COCs cultured at 40.5°C for the first 2 (T_1) and 6 h of IVM (T_2), compared with the control group. Nuclear maturation percentages were 77.5% for C, 53.6% for T_1, and 41.2% for T_2, showing a significant difference ($P \leq 0.05$) among experimental groups. In addition, the observed decline in the nuclear maturation percentages of oocytes was at the higher temperature (40.5°C) for the longer period (first 6 h of IVM) compared with the same temperature for the shorter period (first 2 h of IVM), as shown in Fig. 2.

Analysis of mRNA expression

Heat shock-related genes

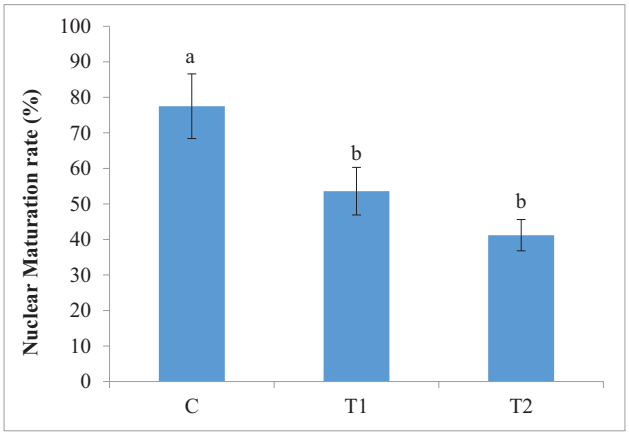
The effect of thermal stress for the two different periods during IVM on mRNA expression of heat shock-related genes (*HSF1*, *HSF2*, *HSP90*, and *HSP70*) of mature oocytes and their adjoined cumulus cells is illustrated in Figs 3–6, respectively. Our results revealed an increase in transcript abundance of the *HSF1*, *HSF2*, and *HSP90* genes in the oocytes and their adjoined cumulus cells of treated groups T_1 and T_2 in comparison with the control group (C). The expression of *HSP70* showed no significant increase ($P \geq 0.05$) between the short period stressed group and the control group, whereas there was a significant difference ($P \leq 0.05$) between control

Figure 1



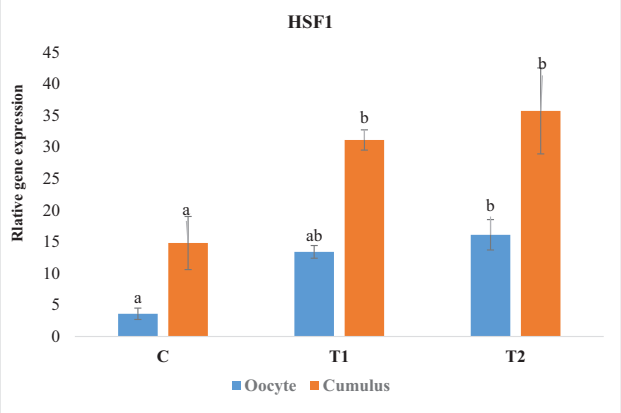
Cumulus expansion of buffalo mature cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

Figure 2



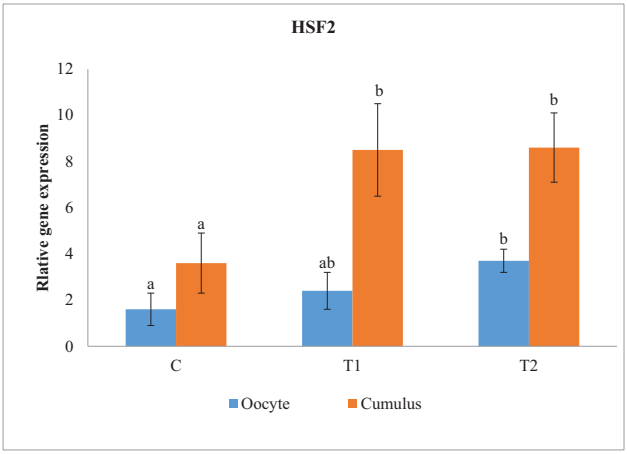
Nuclear maturation rate of buffalo cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

Figure 3



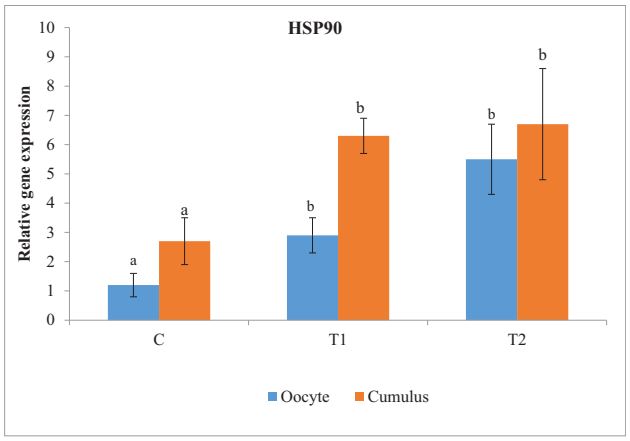
Gene expression profile of heat shock gene *HSF1* in buffalo mature cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

Figure 4



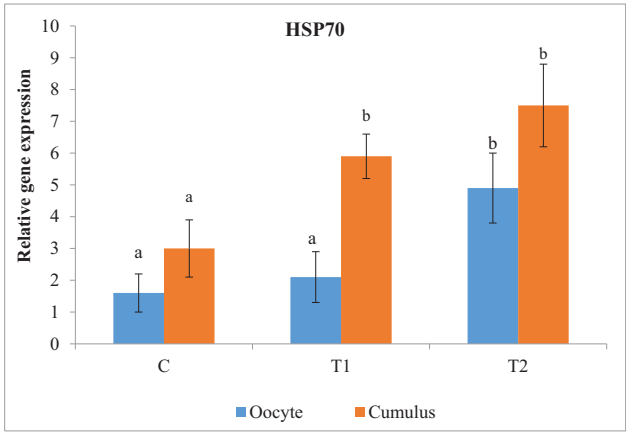
Gene expression profile of heat shock gene *HSF2* in buffalo mature cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

Figure 5



Gene expression profile of heat shock gene *HSP90* in buffalo mature cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

Figure 6



Gene expression profile of heat shock gene *HSP70* in buffalo mature cumulus–oocyte complexes cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05.

group and long period stressed group. The expression of the four previous genes in the cumulus cells showed a significant increase ($P \leq 0.05$) in the treatment groups in comparison with the control group.

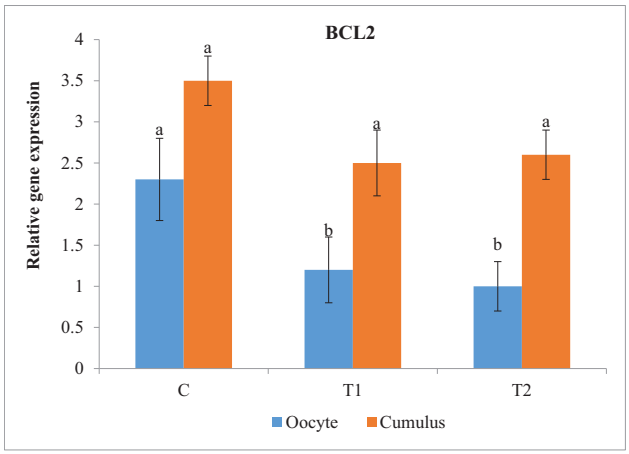
Apoptosis-related genes

The results of antiapoptotic gene *BCL2* and proapoptotic gene *BAX* are illustrated in Figs 7 and 8. The abundance in mRNA expression of *BCL2* gene showed a significant decrease ($P \leq 0.05$) in oocytes of the treated groups T_1 and T_2 in comparison with C group, whereas there was no significant difference ($P \geq 0.05$) in the expression in the adjoined cumulus cells in the three groups. The abundance of RNA transcript numbers of *BAX* gene showed a significant increase ($P \leq 0.05$) in the two cell types in the treated groups T_1 and T_2 in comparison with the C group.

Cumulus expansion marker genes

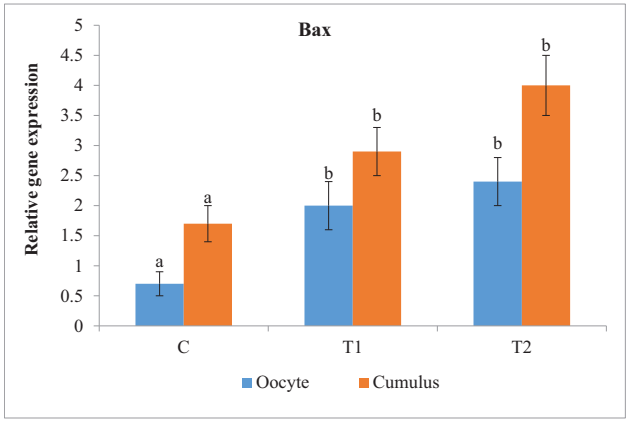
The current study revealed a biological difference in the expression of cumulus expansion marker genes *TNFAIP6*, *PTX3*, and *HAS2* genes. Despite that, there was no significant difference ($P \geq 0.05$) in the

Figure 7



Gene expression profile of apoptosis-related gene *BCL2* in buffalo oocytes recovered from COCs cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05. COC, cumulus–oocyte complex.

Figure 8



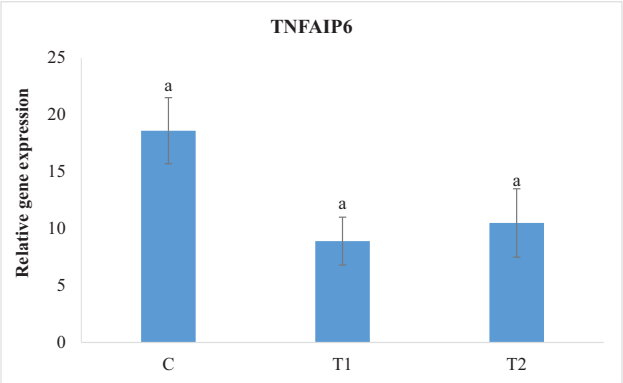
Gene expression profile of apoptosis-related gene *BAX* in buffalo oocytes recovered from COCs cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05. COC, cumulus–oocyte complex.

expression of the three genes in the cumulus cells of the three groups, as shown in Figs 9–11, respectively.

Discussion

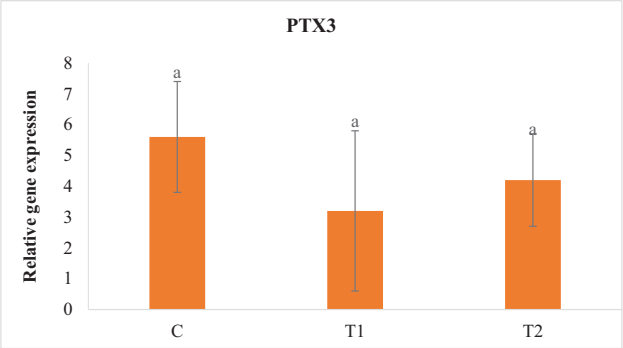
Genetic and environment are key factors that play a crucial role in controlling the fertility of farm animals. Climate change has become a concerning environmental challenge worldwide, causing negative effects on animal productive and reproductive performances. Elevating ambient temperature causes hyperthermia, which results in thermal stress and causes drastic changes in animal productivity [1,41].

Figure 9



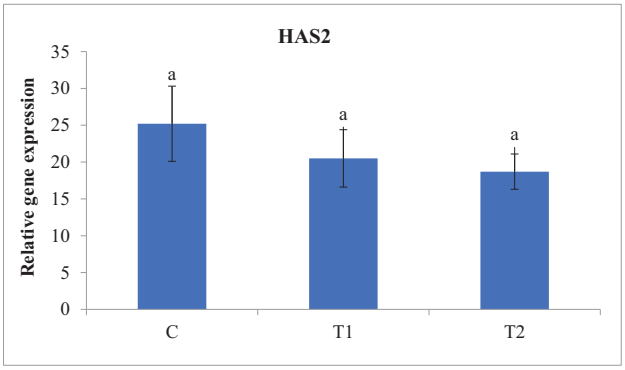
Gene expression profile of cumulus expansion marker gene *TNFAIP6* in buffalo cumulus cells recovered from COCs cultured under short (T_1) and long (T_2) heat shock at 40.5°C during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05. COC, cumulus–oocyte complex.

Figure 10



Gene expression profile of cumulus expansion marker gene *PTX3* in buffalo cumulus cells recovered from COCs cultured under short (T_1) and long (T_2) heat shock during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05. COC, cumulus–oocyte complex.

Figure 11



Gene expression profile of cumulus expansion marker gene *HAS2* in buffalo cumulus cells recovered from COCs cultured under short (T_1) and long (T_2) heat shock during in-vitro maturation. Bars with different superscripts (a, b, c) are significantly different at *P* value less than or equal to 0.05. COC, cumulus–oocyte complex.

The results of the present investigation indicated lower nuclear maturation rate of buffalo oocytes exposed to thermal stress for short and long periods during IVM, although there was no change in cumulus expansion rate. In accordance with our results, nuclear maturation of the bubaline oocytes was shown to be 50 and 56.9% during summer and autumn, respectively, in comparison with 74.6 and 71% during winter and spring, respectively [42]. COCs collected from porcine ovaries that were exposed to thermal shock at 41.5°C for 1 h before IVM showed delayed meiotic resumption and low polar body extrusion [43]. Bovine oocytes that were IVM at 39.5°C for 18 h were delayed in reaching metaphase II (MII) stage by 12.3±7.8%, whereas those exposed to 40.5°C for the same period reached by 9.1±1.4%, in comparison with the unstressed group (78.4±8.0%) [44]. Examination of heat-stressed cow oocytes at 40 and 41°C for the first 12 h of IVM revealed the blocking of nuclear maturation at metaphase I (MI), anaphase I (AI), or telophase I (TI) stages. Noteworthy, *in vivo* and IVM of COCs under thermal stress conditions disrupts the biological functions of the oocytes and causes alterations that are reflected on nuclear and cytoplasmic maturation [45].

Our results showed a reverse correlation between the period of thermal stress and the nuclear maturation of COCs in consistent with former publications in bovine [31,33,46–50], camels [51], porcine [35,43,52], and buffalo [17–20]. It was reported that the bovine oocytes' developmental competence was declined during summer and continued to remain low throughout autumn [53,54]. Investigations of the ovarian follicles and the recovered COCs during cold season were reported to be significantly higher in terms of number and quality than those recovered in the hot season [18]. Obviously, the oocytes that underwent thermal stress showed reduction in developmental competence and cleavage rate [20]. Exposure of bubaline COCs to thermal stress, at 40.5°C for the first 2 or 6 h of IVM, led to a significant decline in oocytes' nuclear maturation rate and extrusion of the first polar body in our study. Thermal stress was reported to disrupt the oocytes' phospholipid composition [28], rearrangement of cytoskeleton [31], morphology of microtubules [30], and formation of spindle fibers [55]. A counteractive effect was reported that thermal shock at 41°C did not diminish the oocytes' capability to undergo nuclear and cytoplasmic maturation; on the contrary, it hastened the maturation processes [56].

Consequently, COCs developed a defense mechanism by chaperones' activity to overcome the thermal stress conditions, as our investigation revealed an increase in heat shock-related genes in the treated COCs of this study. *HSF1* is known to be a highly conserved gene among eukaryotic organisms, which plays an important role not only in thermal tolerance as the principal heat stress transcription factor [57] but also in metabolism and development [58]. Its relative abundance was reported to be higher during hot season in buffalo compared with bovine [59]. Its transcription was reported to be upregulated in bubaline granulosa cells that were exposed to thermal stress for 2 h starting from day 3 till day 7 of cell culture [40].

Our results showed that *HSF1* abundance was significantly high during IVM of the bubaline COCs under thermal stress for both short-term and long-term periods, which coincides with previous results [20,40,60]. In addition to role of *HSF2*, this gene seems to be important for biosynthesis of *HSP70* protein. At the early embryonic development, *HSF1* seems to be in control of thermotolerance; but further as the embryo develops, *HSF2* takes control. It was observed that reduced abundance level of *HSP70* was coupled with *HSF2* deficiency in blastocysts [61], which agrees with the obtained results of our study, reporting a low level of *HSF2* during the short thermal stress period in comparison with *HSF1* expression level.

HSP70 is a constitutively proapoptotic expressed protein that relates to harsh conditions such as thermal stress [59,62,63]. Its transcript abundance has been a sign for thermal stress in embryos of cow [64] and buffalo [17–19]. Our obtained results are in accordance with the previously mentioned studies in the increase of its increased transcription during thermal stress periods in embryos and cells, which may relate to its role in refolding the misfolded proteins for cell survival and functionality in blocking apoptosis [65–68].

Another substantial protein, *HSP90*, was reported to be highly expressed in our investigation during thermal stress periods, in accordance with some previous reports [69–71]. In contrary to *HSP70*, *HSP90* showed antiapoptotic functions necessary for survival of the mammalian cells [20,72]. *HSP90* role, as a main mediator of homeostasis [73], extends to oocytes during thermal stress to overcome the stress period and its deleterious effects on the developmental competence [74]. It was observed that upregulation of *HSP90* is protective for buffalo during thermal stress [75,76]. It appears that expression of *HSF1* and *HSP90* follows the same trend [20,77], as in the case of both *HSF2* and *HSP70* [61].

Failure of COCs to overcome thermal stress and its deleterious consequences induced apoptosis in our investigation. This was visible by upregulation of proapoptotic gene (*BAX)* in both cumulus and their enclosed oocytes, which is similar to the data reported during IVM of bubaline oocytes under thermal shock conditions [17,20]. In addition, *BAX* expression was reported to be upregulated during 8–16-cell stage and blastocyst of buffalo embryos [19]. However, *BAX* was suggested to interact with *HSP70* under stress conditions, which suppress apoptosis induction and its negative effect on maturation process [20,78]. However, the buffalo oocyte maturation rate was reduced as a consequence of thermal stress [19,79]. Our results indicated stable expression of antiapoptotic gene (*BCL2)* in cumulus cells under thermal stress and downregulation in their enclosed oocytes, which is opposite to the findings of Yadav *et al*. [19]. Indeed, *BCL2* is a well-known inner mitochondrial transcript that inhibits apoptosis in mammalian cells [80,81]. *BCL2* showed an increased expression in rat follicle at day 7 of recovery from thermal stress exposure, but the expression started to be downregulated at day 14 of recovery, indicating the activation of programmed cell death of oocyte [82]. Additionally, prominent downregulation of *BCL2* in bubaline of granulosa cells was reported [41]. Interestingly, *BCL2* was upregulated in camel oocytes exposed to acute thermal stress, whereas its transcription was downregulated in the cumulus cells of the same COCs [51], which may indicate the role of cumulus cells as a defense line of oocytes in facing stressful conditions and alleviating thermal stress.

Cumulus cells have a vital role in completion of nuclear and cytoplasmic maturation of oocyte, as their expansion is a sign for the formation of the extracellular matrix maturation completion of oocytes [83]. It was observed that *PTX3* and *HAS2* genes' expression level was not change during cold or hot seasons and the changes were observed for the *HSF1*, *HSF2*, *HSP90* and *HSP70* in pig's cumulus cells [71]. Our results are in agreement with the previous studies concerning the expression of *HAS2* gene, as no significant change in its abundance was observed in cumulus cells of the control and treated bubaline COCs [84,85]. Although expression level of *PTX3* and *TNFAIP6* decreased, this decline was not significant. It seems that *HAS2* had a constant level of expression in porcine endometrial cells, whereas *PTX3* and *TNFAIP6* expression levels were changed throughout different phases of *in vitro* culture, which led to the presumption that interaction between *PTX3* and *TNFAIP6* may be the reason to maintain the expression level of *HAS2* [84]. Moreover, a significant change in the expression of *PTX3* and *TNFAIP6* aroused in bovine cumulus cells between the *in vivo* and *in vitro* conditions, whereas the *HAS2* showed no change in its abundance in both cases [85]. Thus, further studies are required to understand the role of these cumulus expansion genes under *in vivo* and *in vitro* thermal stress conditions and its effect on oocyte developmental capacity.

Conclusion

The results indicated that reduction in nuclear maturation of buffalo COCs exposed to heat shock is due to defect in maturation process as shown by downregulation of cumulus expansion genes (*PTX3*, *TNFAIP6*, and *HAS2*), in addition to decrease in the viability of COCs, as shown by upregulation of apoptosis related gene (*BAX*). However, the cumulus cells enclosing oocytes undergo complex molecular mechanisms to adapt to the thermal shock conditions as shown by upregulation of heat shock transcripts (*HSF1*, *HSF2*, *HSP90*, and *HSP70*) and antiapoptotic gene (*BCL2*), which provide a line of defense to oocytes.

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Conflicts of interest

There are no conflicts of interest.

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