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Transcriptional, Mitochondrial Activity, and Viability of Egyptian Buffalo's Granulosa Cells *In Vitro* Cultured under Heat Elevation

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ABSTRACT

It is documented that heat stress caused impairment on the reproductive performance of dairy animals. However, there are few reports that have focused on the molecular and intracellular responses of *in vitro* cultured buffalo granulosa cells during heat elevation. The present study was conducted to investigate the effect of heat elevation during *in vitro* culture of buffalo granulosa cells on their viability, quality, mitochondrial activity, and transcriptional activity. Granulosa cells were harvested after aspiration of cumulus-oocytes complexes that were collected from abattoir ovaries. The granulosa cells were cultured *in vitro* either at a normal physiological temperature suitable for oocyte maturation and embryo development (38.5°C) or exposed to the elevated temperature of 40.5°C on day 3 of culture (the first two days were for confluence) for two hours of culture then continued at 38.5°C up to day 7 of culture. The viability of granulosa cells was measured using trypan blue and quality was estimated by measuring the level of intracellular reactive oxygen species (ROS) on day 7. Moreover, metabolic activity was performed by measuring the fluorescent intensity of mitochondria. Moreover, transcriptional activity was done by profiling four selected candidate genes using quantitative real-time PCR. The results indicated that the granulosa cells viability rate significantly decreased in the heat stress group (25.1 ± 3.7), compared to the control group (36.6 ± 5.3) on confluence day (day 3). In addition, the viability rate on the last day of culture (day 7) decreased in heat stress, compared to control (83.7 ± 4.5 and 97.4 ± 0.4, respectively). On the other hand, there was a nonsignificant difference in ROS profile between the control ($21.7*10^4 \pm 1.3$) and the heat-stressed group (15.7 ± 0.7) on day 7 of culture. However, the mitochondrial fluorescent intensity was higher in the control (21.9 ± 1.9) than in the heat-stressed group (15.4 ± 0.8) on day 7 of culture. The expression of cellular defense (HSF1) and apoptosis-inducing gene (P53) were significantly up-regulated in granulosa cells exposed to heat elevation, compared to the control group. On the other hand, the steroidogenesis-regulating gene (StAR) was down-regulated in granulosa cells cultured under heat shock, compared to the control group. In conclusion, heat stress reduced the viability of granulosa cells by inducing the expression of an apoptosis-related gene (P53) and compromised expression of genes regulating the steroid biosynthesis, which resulted in up-regulation of cell defense gene (HSF1) in an attempt to ameliorate the deleterious effect of heat stress on the biological activity of the granulosa cells.

Keywords: Apoptosis, Granulosa, Heat stress, Gene expression

INTRODUCTION

There are many different challenges that the livestock sectors face in developing countries, including nutrition deficiency, poor management, and heat stress. Among the environmental stressors, heat stress (HS) has a negative impact on animal reproductive performance causing great economic losses (Sammad et al., 2020). The HS impairs both ovarian functions and the developmental competence of oocytes (Sammad et al., 2020). The mammalian ovarian follicle consists of an oocyte that is surrounded by granulosa (GCs) and theca cells producing molecules, hormones, and nutrients to maintain the oocyte development potential, ovulation, and preimplantation embryo development (Albertini et al., 2001).

Granulosa cells are ovarian cells that enclose the follicle cavity and have a cross talk with oocytes through physical contact with zona pellucida and gap junctions, which facilitate the exchange of biological factors (Jancar et al., 2007). This crosstalk allows GCs to control oocyte maturation and its transcription activity (Carabatsos et al., 2000). Indeed, granulosa cells play a critical role in oocyte maturation and subsequent embryonic development (Gilchrist et al., 2004) by providing growth factors, amino acids, ions, and hormones (estrogen and progesterone). In addition, the ruptured follicle forms the corpus luteum after ovulation, and luteinized GCs become the main source for progesterone synthesis, which is the key to placenta development and pregnancy maintenance (Denkova et al., 2004; Matsuda et al., 2012). The steroidogenic activity of granulosa cells is controlled by many genes such as Steroidogenic Acute Regulatory Protein (StAR), Cytochrome P450 17A1 (CYP17A1), and 3-beta-Hydroxysteroid dehydrogenases (HSD3B2). In bovine, HS compromises follicular development, *in vitro* maturation, and fertilization of oocytes by impairing steroidogenic activity and viability of granulosa cells (Roth et al., 2001a; Roth et al., 2001b).

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Therefore, the current study focused on the evaluation of granulosa cells viability and transcriptional activity when the cells were cultured *in vitro* under HS, compared to normal conditions.

MATERIAL AND METHODS

Experimental design

A constant concentration of granulosa cells was cultured in six-well cell culture plates. Cells were divided into two groups. The first group was the control that cultured under *in vitro* normal temperature 38.5^{o}C while the second treated group was exposed to heat stress at 40.5°C for 2 hours on day 3 of culture (granulosa cells were confluent), followed by normal temperature until day 7 of culture. Cells viability was measured using trypan blue and quality was estimated using intracellular reactive oxygen species successively on day 3 after heat treatment and day 7. Moreover, metabolic activity was performed by measuring the mitochondrial activity and transcriptional activity was done by profiling four selected candidate genes (HSF1, StAR, and P53, BCL2) using quantitative real-time PCR (Ghanem et al., 2020b).

Chemicals and reagents

Collection of ovaries and granulosa cells preparation

The collection of GCs was done according to Ghanem et al. (2020a). Ovaries were collected from local slaughtered houses in physiological saline supplemented with gentamycin and kept at approximately 37°C. Granulosa cells were aspirated from buffalo's follicles (2-8 mm). After oocyte selection, granulosa cells were centrifuged at 1500 rpm for 5 minutes. The pellet of granulosa cells then was re-suspended in the washing medium [TCM-199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, USA) and 1% antibiotic (streptomycin and penicillin)]. An 18-gauge needle mechanically broke the clumps of the cell. Finally, a total of 500000 cells were cultured per well under 38.5°C 5% CO2 and humidified air until the treatment.

Granulosa cells trypsinization

The trypsinization of GCs was performed according to Ghanem et al. (2020a). The medium was aspirated from each well slowly and the cell layer was washed twice with sterilized phosphate buffer saline (PBS). A total volume of 100 µl of trypsin EDTA (Sigma-Aldrich, St. Louis, MO, USA) solution (10%) was added to every well slowly then the plate was incubated at 38.5°C for 30 seconds. In the next step, 1 ml of washing medium was added and the suspension was centrifuged at 1500 rpm for 5 minutes. The pellets of GCs were mixed with 1 ml of washing medium.

Granulosa cells viability

Cells viability was determined using trypan blue (0.4%) according to Ghanem et al. (2020a). A total volume of 10 µl of cell suspension was mixed with 10 µl of trypan blue and incubated at room temperature for 1-2 minutes. Total cell count and viable cell count were calculated by hemocytometer using a magnification of 10 X (Inverted Microscope, Leica DMI 3000B, Wentzler, Germany).

Intracellular reactive oxygen species detection

Intracellular reactive oxygen species (ROS) were detected by 6-carboxy-2′, 7′-dichlorodihydro fluorescein diacetate (H2DCFDA; life technologies, California, USA) according to the protocol described by the manufacturer with some modifications according to Ghanem et al. (2020a). Granulosa cells from each group were incubated with 985 µl of 15 µl MH2DCFDA mixed with 970 µl PBS at 38.5°C for 45 minutes. The cells were washed with PBS and images were captured with a Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Tokyo, Japan) using a blue-fluorescence filter, and images were acquired by LAS Core software.

Mitochondrial activity

Mitochondrial activity of buffalo GCs was determined using MitoTracker® Green FM (M7514, life technologies) according to the manufacturer's instructions with some modifications according to Ghanem et al. (2020a). The GCs from each group were incubated with 200 µl MitoTracker green dye to 800 µl PBS for 45 minutes, followed by washing with PBS. The images were captured with a Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Tokyo, Japan) using a blue-fluorescence filter, and images were acquired by LAS Core software.

Image analysis after fluorescent staining

Captured images (13 images) per stain were processed using Image J software. The data of fluorescence intensity were presented as mean ± SD.

RNA isolation

Total RNA was extracted using GeneJet RNA Purification Kit (ThermoFisher Scientific, USA) from three different biological replicates of granulosa cells of each experimental group according to Faheem et al. (2021). First, a volume of

600 µL of Lysis buffer was supplemented with 12 µL of β-mercaptoethanol added to each sample tube, and mixed with vortex until homogenization was reached. The sample tubes were centrifuged at 16000 × g for 5 minutes. The mix was transferred into a new RNase-free microcentrifuge tube. After that 600 µL of ethanol (96-100%) were added and the solution was mixed by pipetting. Up to 700 µL of lysate were transferred to the GeneJET RNA Purification Column inserted in a collection tube. The columns were centrifuged at 12000 × g for 1 minute. The flow-through was discarded and the purification column was placed back into the collection tube. This step was repeated until all of the lysates were transferred into the column and centrifuged. The collection tube containing the flow-through solution was discarded and the GeneJET RNA Purification Column was placed into a new 2 mL collection tube. Afterwards, 700 µL of wash buffer 1 (supplemented with ethanol) was added to the GeneJET RNA purification column and centrifuged at 12000 × g for a minute. The flow-through was discarded and the purification column was placed back into the collection tube. Moreover, 600 µL of Wash Buffer 2 (supplemented with ethanol) was added to the GeneJET RNA purification column and centrifuged at 12000 × g for a minute. The flow-through was discarded and the purification column was placed back into the collection tube. The previous step was repeated using 250 µL of wash buffer 2 that was added to the GeneJET RNA purification column and centrifuged at 12000 × g for 2 minutes. The flow-through solution was removed and the purification column was moved to a new tube. Finally, RNA was eluted by adding 20 µL of nuclease-free to the center of the GeneJET RNA purification column membrane and centrifuged at 12000 × g for 1 minute. The DNA residue was removed by adding 1 µl of DNases and 1 µl of MgCl2 buffer (ThermoFisher Scientific, USA) to each RNA sample and incubated at 37°C for 30 minutes in a PCR instrument (ThermoFisher Scientific, USA) then 1µl of EDTA was added and incubated at 65°C for 10 minutes. The purification column was discarded and eluted total RNA was measured using a nanodrop spectrophotometer (ThermoFisher Scientific, USA) and purity was estimated using measurement at 260/280 ratio. The extracted total RNA was stored at -70°C in an ultra-cool freezer (ThermoFisher Scientific, USA) until further use.

The synthesis of cDNA

The reverse transcription of RNA samples to cDNA was done using RevertAid first-strand cDNA synthesis kit (ThermoFisher Scientific, USA) according to Ghanem et al. (2020b). The following chemicals were added to each of 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, 1 µl of reverse transcriptase enzyme were gently mixed by pipetting. The PCR mix was incubated in PCR thermocycler (Thermo Fisher Scientific, USA) at 42°C for 60 minutes, then at 70°C for 5 minutes and at 4°C overnight.

Quantitative real-time PCR analysis

Three replicates from each treatment were used for profiling selected candidate genes using quantitative real-time PCR analysis. Each pair of primers (Table 1) of selected candidate genes (HSF1 and StAR) and housekeeping gene (GAPDH) were designed using primers3 software (https://primer3.ut.ee/). The real-time PCR reaction mix was prepared by adding 12 ul of Maxima Sybr green PCR master mix (ThermoFisher Scientific, USA), 5.4 ul nuclease-free water, and 0.3 ul of forward and reverse primers which were incubated in StepOnePlus™ instrument (ThermoFisher Scientific, USA). The PCR mix was incubated at 50°C for 2 minutes, initial denaturation was done at 95°C for 10 minutes followed by 40 cycles of 95°C (Denaturation) for 15 minutes than at 60°C for 1 minute (annealing), and finally, melt curve at 95°C for 15 seconds and then 60°C for 1 minute. The expression data were analyzed using the delta-delta Ct method after normalization of the target genes with the housekeeping gene.

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

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)
StAR	XM_006183353.3 DQ062682.1	F:5′-CCATGGAGAGGCTTTATGAA-3′ R:5′-TCTTTCCCAATCTTCTGCAG-3′	103
HSF1	KC568561	F:5’-CGACCACCCTCATTGACTCC-3’ R:5’-CATCTTTGGAGTGCGAGCCA-3’	170
P53	XM_006175816.3	F:5′ - CCACCTGAAGTCTAAGAAGG-3′ R:5′ - AGTGCAGGTCAACTTCTTTA-3′	250
BCL2	XM_010979993.	F:5′ACATCCACTATAAGCTGTCG3′ R:5′ -TAGCGCCGAGAGAAGTCAT3′	241
GAPDH	NM_001034034.2	F:5′-TGCCCAGAATATCATCCCT-3’ R:5′- CTCATCATACTTGGCAGGTT-3’	166

Statistical analysis of data

The viability of granulosa cells, metabolic activity, and ROS level data were analyzed by applying a One-way Analysis of Variance (ANOVA). The analyzed data were expressed as mean ± standard error (SE) of means (SEM). Comparisons were significantly different if $p < 0.05$. Statistical analysis of data was performed using the IBM SPSS

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Statistics 22 program (SPSS Inc., Chicago, Illinois, USA). The expression profiles of selected target genes were analyzed using the SAS (SAS, 2004) using the general linear model (GLM) procedure. In addition, Duncan's test was used to detect differences among means of the two studied groups. Values of means were considered significant at $p < 0.05$.

RESULTS

Granulosa cells viability rate

At the beginning of the experiment, the viability rate was 88.3. The viability rate of granulosa cells showed a significant increase in control (36.6 ± 5.3), compared to the heat-treated group (25.1 ± 3.7) on day 3 of *in vitro* culture (Table 2). Moreover, there was a significant ($p < 0.05$) increase in granulosa cells viability rate in control (97.4 ± 0.4), compared to the HS group (83.7 ± 4.5) at the end of the culture period (day 7) as shown in Table 2.

Table 2. Viability rate of buffalo granulosa cells after heat shock at 40.5^{o}C for 2 hours on days 3 and 7 of *in vitro* culture

Items	Initiate	At confluence (day 3)	End of culture (day 7)
Control	88.3 ± 0.0	36.6 ± 5.3	97.4 ± 0.4^a
Heat-treated GCs	88.3 ± 0.0	25.1 ± 3.7	83.7 ± 4.5^b

GCs: Granulosa cells

Mitochondrial activity

Mitochondrial activity was detected at the end of the culture period (day 7). Moreover, the mitochondrial fluorescent intensity was higher ($p < 0.05$) in the control (21.9 ± 1.9) than in the heat-stressed group (15.4 ± 0.8) as shown in figures 1 and 2 (a and b).

Reactive oxygen species level

There was an insignificant difference between the control (21.7 ± 1.3) and the heat-stressed group (15.7 ± 0.7) on day 7 of culture figures 3 and 4 (a and b).

Gene transcriptional profile

Heat shock factor 1 expression

The transcriptional profile of HSF1 gene was significantly up-regulated in granulosa cells exposed to HS compared to that cultured under normal temperature (Figure 5).

Steroidogenic acute regulatory gene (star) expression

The expression of StAR gene was up-regulated significantly ($p < 0.05$) in the granulosa cells of the control, compared to the HS group (Figure 6).

Antiapoptosis-related gene

The expression of BCL2 was similar in the granulosa cells of the control group and HS group (Figure 7).

Apoptosis-related gene

The transcript abundance of P53 gene was increased significantly in the granulosa cells of control compared with that of the HS group (Figure 8).

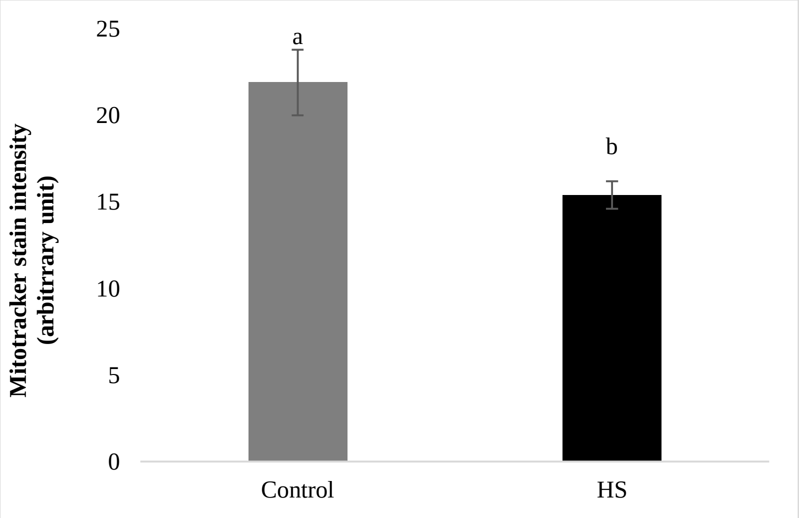

Figure 1. Mitochondrial fluorescent intensity of buffalo granulosa cells after heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture

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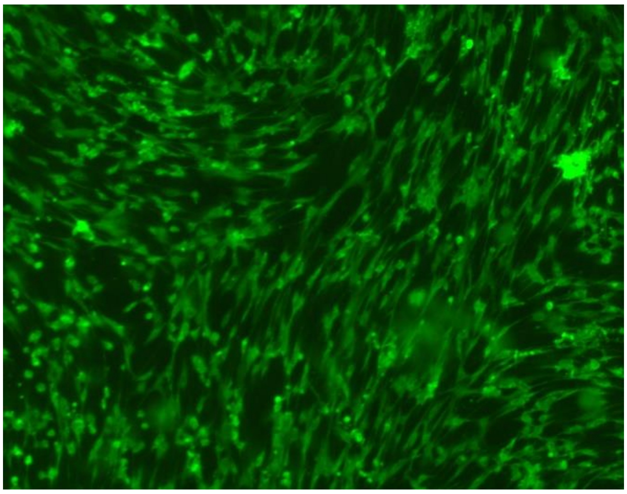

Figure 2a. Mitochondria of granulosa cells cultured *in vitro* under normal temperature and stained with Mitotracker

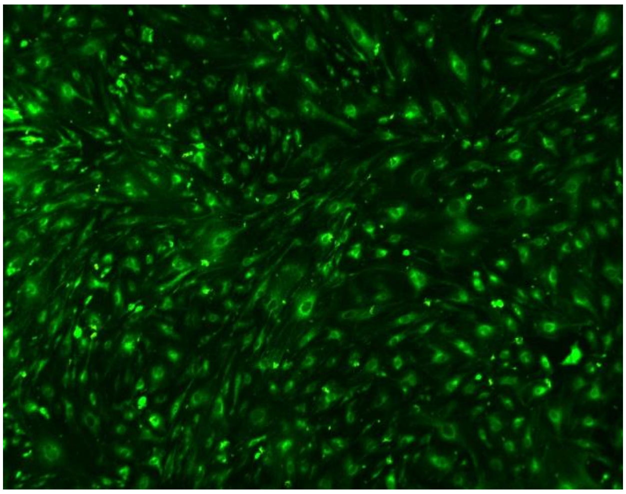

Figure 2b. Mitochondria of granulosa cells cultured *in vitro* under heat stress conditions and stained with Mitotracker

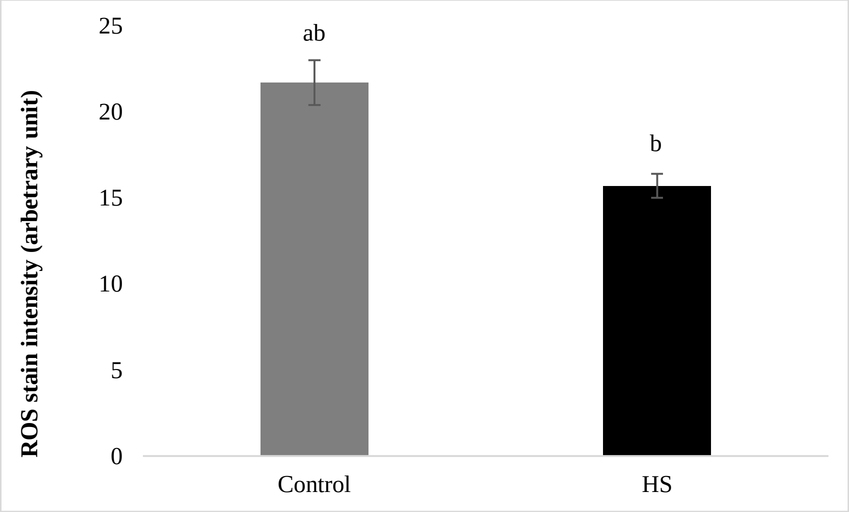

Figure 3. The level of reactive oxygen species of buffalo granulosa cells after heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture

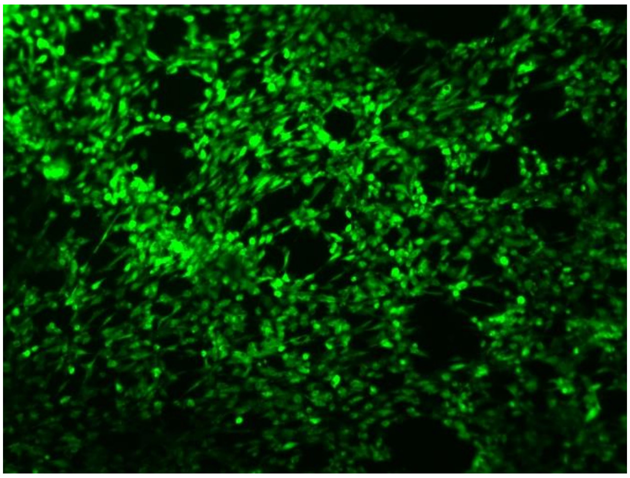

Figure 4a. Reactive oxygen species of granulosa cells cultured under normal condition and stained with MH2DCFDA

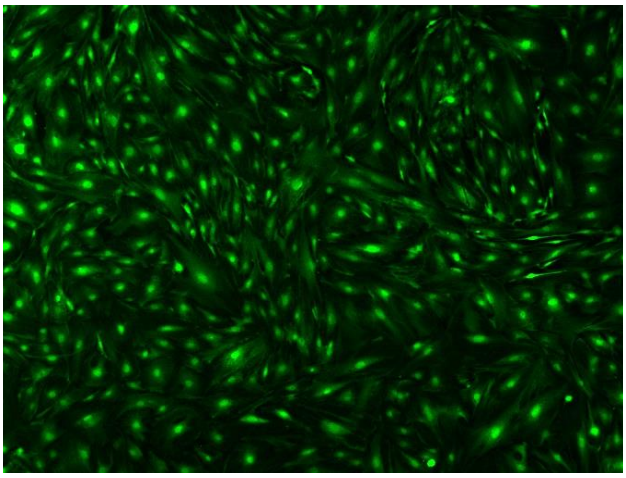

Figure 4b. Reactive oxygen species of granulosa cells cultured under heat stress and stained with MH2DCFDA

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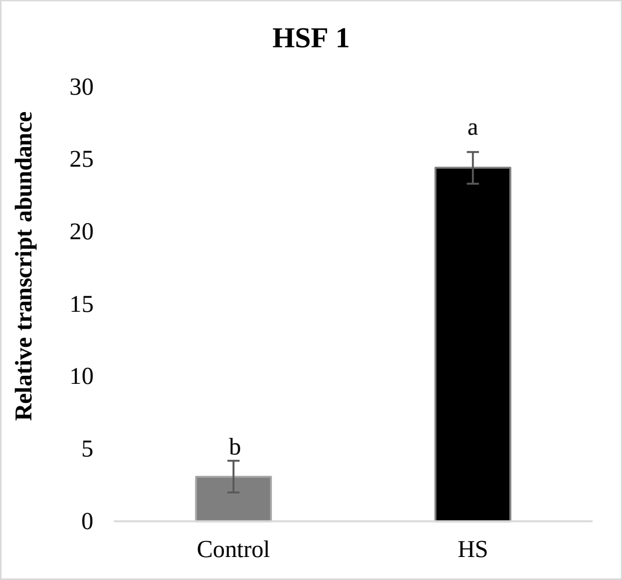

Figure 5. The expression profile of heat shock factor 1 of buffalo granulosa cells after heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture

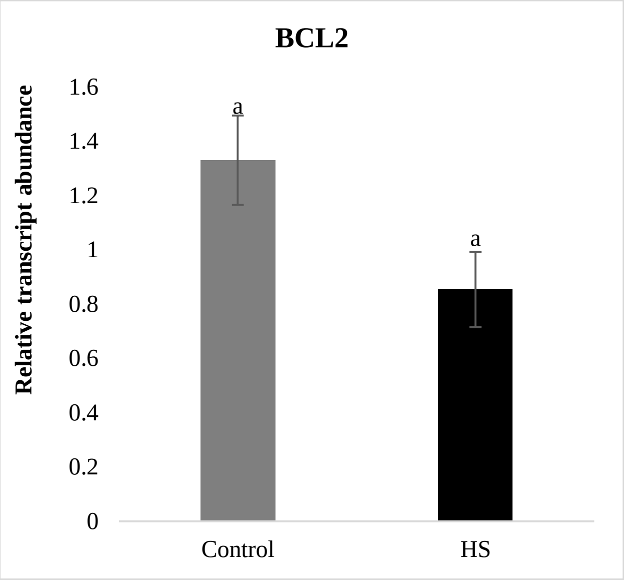

Figure 7. The expression profile of anti-apoptotic related transcript (BCL2) of buffalo granulosa cells following heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture

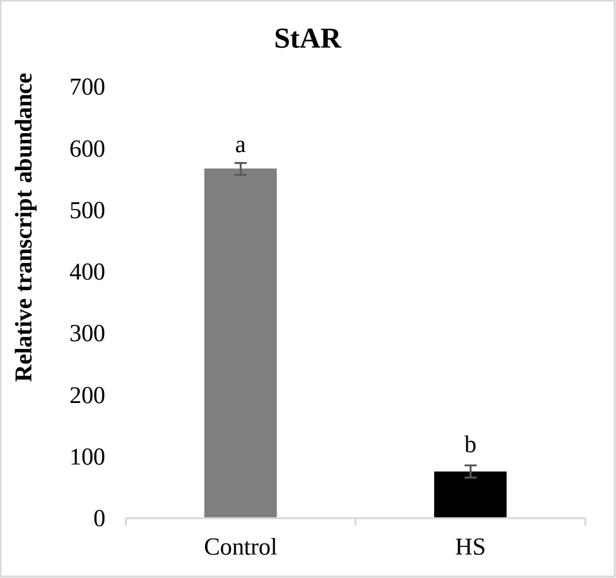

Figure 6. The expression profile of steroidogenic acute regulatory protein of buffalo granulosa cells after heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture. StAR: Steroidogenic Acute Regulatory

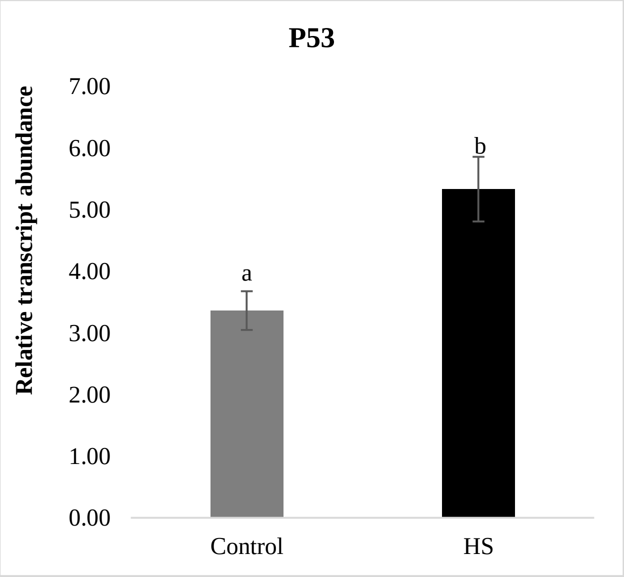

Figure 8. The expression profile of apoptosis-related transcript (P53) of buffalo granulosa cells after heat shock at 40.5°C for 2 hours on day 7 of *in vitro* culture

DISCUSSION

Elevation of ambient temperature caused HS on dairy animals and resulted in a reduction in fertility that manifested in impairment of follicle development, estradiol biosynthesis, ovulation, oocyte quality, and early embryonic development (Badinga et al., 1993; Wakayo et al., 2015; Li et al., 2016a). In the present study, the increased temperature on day 3 during *in vitro* culture of granulosa cells reduced the viability of granulosa cells, compared to the control group. However, Faheem et al. (2021) have observed that cultured GCs exposed to 40.5°C for different time durations (24, 48, and 72 hours) showed no significant differences in the GCs viability of the post-heat-treatment group, compared to the control group that was exposed to 37°C. In support to the current findings, it was demonstrated that exposure of bovine granulosa cells to heat shock at 39°C, 40°C, and 41°C significantly for 2 hours reduced the cell viability, increased incidence of apoptosis, and finally impaired steroidogenesis by reducing estradiol and progesterone levels (Khan et al., 2020). The results of the current study (Table 2) revealed that a reduction in cellular viability was linked with decreased metabolic activity after exposure to heat elevation at the end of the culture period (day 7). This could be a sign of an

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intracellular demise due to thermal stress through the incidence of apoptosis that compromises all biological activity of granulosa cells.

Indeed, granulosa cells proliferate and synthesize hormones required for follicular growth and development (Petro et al., 2012). This ability depends on the antioxidant capacity of granulosa cells to sustain the optimum microenvironment inside the follicle. The results of the present study (Figures 3 and 4a, b) indicated a high capacity of granulosa cells to scavenge the ROS, which either produced by reducing endogenous metabolic activity or induced by heat stress as there was no significant difference between the control and heat-stressed group on day 7 of culture. These results were in accordance with those obtained by Faheem et al. (2021) who detected stability on the steroidogenic activity of GCs under heat elevation by stable expression of SOD2 and sustaining intracellular antioxidant capacity under heat elevation. However, the steroidogenic activity that was indicated in the present study by StAR expression (Figure 6) might be compromised due to the differences in the culture condition and low GCs concentration. In addition, heat elevation increased intracellular ROS level (Paul et al., 2009), subsequently induced apoptosis (Liu et al., 2015), and finally impaired the development competence of oocyte (Blondin et al., 1997). Similarly, the results of the current study indicated higher expression of apoptosis, inducing genes, namely P53 (Figure 8) in heat-stressed buffalo granulosa cells although there was no difference in the expression of anti-apoptotic related transcript (BCL2). Similarly, earlier investigations revealed apoptosis incidence in bovine granulosa cells coupled with increased expression of HO-1 (play role in the protective response to stress), however, the precise molecular mechanism is still unknown (Li et al., 2016b; Luo et al., 2016). Recently, heat stress caused apoptosis induction and incidence of oxidative stress and by increasing expression of NRF2 and HO-1 genes in *in vitro* cultured granulosa cells (Wang et al., 2019). Indeed, the maintenance of the cellular antioxidant system of granulosa cells under heat stress is regulated by the suppression of apoptosis and increased proliferative activity (Regan et al., 2018) however, when the cells cannot tolerate intense heat stress subsequently the viability of cells is compromised.

The HS impairs the development of the ovarian follicle and the cells increase the biosynthesis of heat shock proteins to repair cellular damaged proteins (Li et al., 2016b). In a study done in bovine granulosa cells, heat shock genes, such as HSP32, HSP60, HSP70, HSP90, and HSP105, were upregulated in response to heat shock (Li et al., 2016a). In agreement with this observation, the results of the current study showed increased expression of HSF1 (Figure 5) in buffalo granulosa cells exposed to heat elevation for 2 hours on day 3. Moreover, the GCs reduced the expression profile of gene-regulating steroidogenic activity (StAR). It was demonstrated a lower expression of the StAR gene in heat-stressed bovine granulosa cells (Khan et al., 2020). Additionally, the transcript abundance of HSF1 was upregulated in *in vitro* matured buffalo cumulus-oocyte complexes that developed under heat stress (El-Sayed et al., 2018). The elevation of heat during *in vitro* culture of granulosa cells, reduced viability that might attenuate steroidogenic activity by reducing expression of StAR. Moreover, the stability of ROS level and upregulation of HSF1 is the key cellular response of defense mechanism that might protect GCs functionality under suboptimal heat elevation conditions.

CONCLUSION

Based on the findings of the present study, heat stress reduced the viability of granulosa cells by inducing the expression of an apoptosis-related gene (P53) and compromised the expression of genes regulating the steroid biosynthesis. In response to this suboptimal intracellular condition, GCs up-regulated cell defense gene (HSF1) in an attempt to ameliorate the deleterious effect of heat stress on their biological activity.

DECLARATIONS

Competing interests

All authors declare that there is no conflict of interest.

Authors' contributions

All authors are contributed equally to the current manuscript by designing the experiment, writing, and revising it. All authors confirmed the final draft of this manuscript and data analysis.

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Ethical considerations

All ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked and approved by all authors.

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