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Molecular Characterization and Gene Expression Profiling of Cultured Theca Cells from Iraqi Buffalo in Relation to Steroidogenic Pathway Genes

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Abstract

The present study investigates the molecular characteristics, survivability, and steroidogenic function of cultured theca cells (TCs) isolated from Iraqi buffalo ovaries under heat-stress conditions. Since global warming has been on the rise, affecting livestock fertility, there is a need to comprehend the regulations of thermal tolerance in buffalo reproductive cells to enhance the in vitro reproductive technologies. Primary TCs in both optimal conditions (37°C) and heat stress (40.5°C) conditions were subjected to 24, 48, and 72 hours of culture. Estradiol (E2), progesterone (P4), insulin-like growth factor-1 (IGF-1), and total antioxidant capacity (TAC) of conditioned media and TC pellets. mRNA expression of metabolic (CPT2, ATP5F1A, SREBP1) and stress-responsive genes (SOD2, NFE2L2, TNF-alpha) were analyzed. Buffalo TCs were found to have good thermal resilience with a viability of more than 87 percent in all treatments. Exposure to heat brought about time-dependent hormonal changes such as high secretion of E2 at 72 hours, temporary reduction of P4 at 48 hours, and progressive decrease in the levels of IGF-1. TAC is reduced at 48 hours but recovers in 72 hours, which suggests the activation of antioxidant compensation measures. Analysis of gene expressions revealed that CPT2 and NFE2L2 were downregulated, SREBP1 and TNF-alpha were upregulated in the middle of the exposure, and both SOD2 and ATP5F1A were expressed steadily, indicating selective metabolic and oxidative changes. These data indicate that there is a coordinated response in the genes to allow buffalo TCs to endure moderate heat stress and to regulate steroidogenesis, metabolism, and antioxidant signaling. The findings are helpful to improve buffalo reproduction under hot weather conditions and present possible goals to increase the heat resistance of in vitro culture systems.

Keywords:

Buffalo theca cells, gene expression analysis, heat stress, inflammatory response, oxidative stress genes.

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Introduction

Ovarian follicular development depends heavily on the coordinated actions of somatic cells, particularly cumulus cells and mural theca cells (TCs), which produce a range of regulatory factors essential for the growth, maturation, and developmental competence of oocytes (Uyar et al., 2013; Gonzalez & El-Sayed, 2024). Theca cells also play an important role in the synthesis of steroid hormones, the release of growth factors, and the provision of metabolic substrate that guarantees subsequent follicular development and later reproduction (Liu et al., 2015; Salman et al., 2023). It has been demonstrated that the reciprocal communication between the oocyte and the surrounding TCs regulates the meiotic maturation, cytoplasmic development, fertilization, and early embryogenesis in various mammalian species, highlighting the essential endocrine and paracrine roles that these cells play in the follicular physiology (Chappel, 2013; Yamamoto et al., 2014; Reddy & Qureshi, 2024). Together, these processes form a favorable microenvironment, which improves the quality of oocytes and increases embryo viability (Casillas et al., 2014; Quinby & Yannas, 2025).

Contemporary challenges, such as global warming, introduce additional complexity to reproductive biotechnology by exerting harmful effects on animal fertility and physiological efficiency (Kumar & Sunil, 2024). Heat stress disrupts several biochemical pathways, including follicular aromatase activity, which consequently alters the secretion patterns of estradiol and progesterone in cattle (Khodaei-Motlagh et al., 2011; Samir et al., 2017). Thermal stress on ovarian development in buffalo has been reported to cause impairment in the ability to grow a follicle and have a significant effect on the gene expression profile of both oocytes and the cumulus cells surrounding them (El-Sayed et al., 2018; Faheem et al., 2021a; Faheem et al., 2021b). These results indicate that high temperature has a direct negative impact on follicle steroidogenic potential and changes the relative distribution of mRNA transcripts that relate to genes that are essential in cellular survival, metabolic regulation, and hormone production (Khadrawy et al., 2019; Regan et al., 2018; Maghsoodi, 2014).

Nevertheless, the co-culture systems with TC monolayers or conditioned medium revealed positive effects on embryo development due to the ability of the system to detoxify the culture environment, control physicochemical conditions, and release bioactive factors that reinforce cellular organelle structures and promote maternal-to-embryonic genomic transition (Casillas et al., 2014; Uyar et al., 2013). Nevertheless, under conditions below optimum culture conditions, the behaviour of TCs remains unknown (Quinby & Yannas, 2025). The given discontinuity is a fact in the example of the tropical and subtropical species, e.g., the Iraqi buffalo, which has some adaptive mechanisms that could potentially influence the cellular processes to heat stress (Gamal et al., 2015; Kumari, 2023). Understanding how these cells behave in vitro under thermal stress is crucial for improving reproductive technologies and ensuring optimum follicular support in environments characterized by high ambient temperatures.

Although prior investigations have documented the adverse influence of heat stress on oocyte- and cumulus-cell gene expression in buffalo, minimal information is available regarding the molecular characterization and steroidogenic gene expression profiles of cultured theca cells themselves. The literature lacks comprehensive data on how heat stress affects steroidogenesis-related genes such as CYP11A1, CYP17A1, STAR, and HSD3B in buffalo TCs, as well as systematic evaluations of their viability and endocrine function under elevated temperatures (Faheem et al., 2021a; Faheem et al., 2021b). Also, the molecular identity and behavior of cultured buffalo TCs, especially in the southern part of Iraq, have not been adequately studied despite other species demonstrating that granulosa and theca cells can quickly lose functional identity in in vitro conditions without proper control (Yenuganti & Vanselow, 2017; Baufeld & Vanselow, 2018; Elis et al., 2015).

It is on these gaps that the current research will undertake the detailed molecular characterization of cultured theca cells obtained from Iraqi buffalo ovaries, their survival and functionality to thermal stress, and expression analysis of important genes of the steroidogenic pathway under optimal and heat-stress conditions. The hypothesis is that thermal stress will cause great change in the profile of the molecular dynamics, survivability, and steroidogenic gene expression within the Indian buffalo TCs, thereby influencing the steroidogenic ability and overall physiological functioning of the buffaloes. The previous studies prove this hypothesis by showing that heat stress interferes with the functioning of follicular cells, mitochondrion formation, and hormone production courses in buffalo and other livestock species (Habersetzer et al., 2013; Roth, 2015; Seo et al., 2016; Fawzy et al., 2021a).

The key contributions of this study include providing the first detailed molecular characterization of cultured theca cells from Iraqi buffalo, establishing gene expression profiles of essential steroidogenic genes under normal and heat-stress conditions, demonstrating how thermal stress influences TC survivability and endocrine functionality, and generating novel insights that may enhance reproductive biotechnology programs including in vitro maturation and embryo-production systems under the increasing environmental challenges posed by rising global temperatures.

Although there is an increasing body of evidence that heat stress interferes with the ovarian functioning in buffalo, the direct molecular reactions of theca cells, such as their steroidogenic capacity, adaptations to metabolic changes, and stress-response mechanisms, are not well-illustrated. No extensive research has yet typified the response of the cultured theca cells of the Iraqi buffalo to high temperatures at hormonal, biochemical, and transcriptional levels of the cell. This gap in knowledge needs to be filled in order to enhance the in vitro reproductive systems to support the growing and increasingly demanding climatic conditions experienced by the buffalo in the tropical and subtropical areas. Thus, the current research provides a study of survivability, functional activity, and expression of major steroidogenic and stress-relevant genes in buffalo theca cells exposed to a regulated thermal stress. The given work seeks to produce the background knowledge by offering the first detailed molecular characterization of Iraqi buffalo theca cells under the heat-stress conditions that might guide the way toward raising awareness of the methods of improving follicular support, oocyte competence, and reproductive efficiency in hot surroundings.

To further investigate, the article describes the methodology, explains the isolation of theca cells from buffalo, and examines their response under the controlled heat-stress environment. Then the result contains the findings of the changes in cell viability, antioxidant capacity, steroid hormone secretion, and the differential expression of key stress- and metabolism-related genes. The results were discussed as the cellular mechanisms underlying heat-induced alteration are interpreted in the context of reproductive physiology. At last, the conclusion summarizes the results and highlights the changes in heat stress on buffalo ovarian function.

Materials and Methods

Design of Experiments

Primary theca cells (TCs) obtained using Iraqi buffalo follicles were cultured in one of two experimental conditions, one being an optimal control temperature of 37°C using 5 percent CO2, and the other was heat-stress at 40.5°C over 24, 48, and 72 hours. Heat-shock temperature and exposure times were chosen according to the prior cellular heat-stress models in buffalo granulosa cells that had exhibited high transcriptomic and steroidogenic changes at comparable heating conditions (Faheem et al., 2021a; Fawzy et al., 2021b).

During each incubation period, conditioned media were collected and used to determine estradiol (E2), progesterone (P4), insulin-like growth factor-1 (IGF-1), and total antioxidant capacity (TAC) at the conclusion of the period. Parallel TC cultures were harvested to assess the relative level of mRNA transcripts of cellular metabolism (CPT2, ATP5F1A, SREBP1) and cellular stress response (SOD2, TNF-alpha, NFE2L2) genes. These biochemical and molecular endpoints were chosen in order to describe the steroidogenic activity and stress-adaptive response of cultured theca cells in high temperature conditions (see Figure 1).

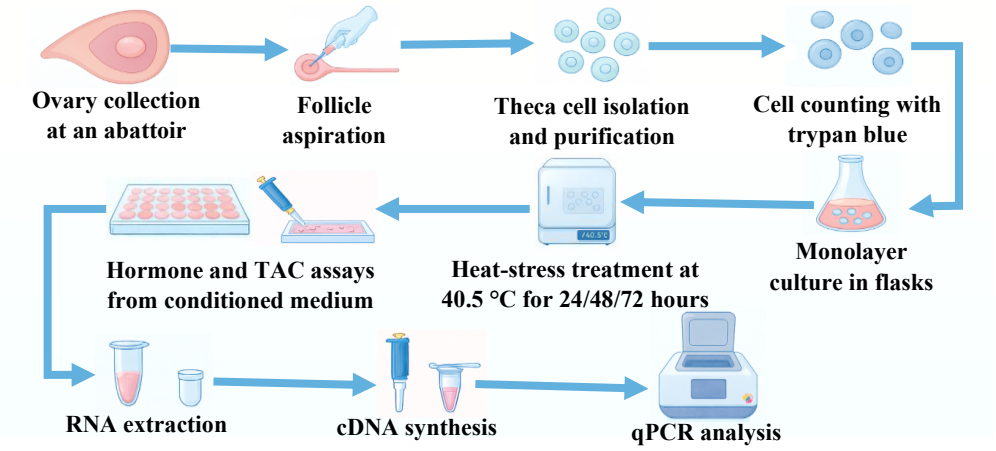

Figure 1. Experimental workflow for theca cell isolation, culture, heat treatment, and downstream analyses

Figure 1. Experimental protocol of the overall process of ovary collection, theca cell isolation, the primary culture formation, heat-stress treatment, conditioned-media experiments, and gene-expression testing.

All the media components and supplements were of tissue-culture grade unless otherwise noted and supplied by Sigma-Aldrich (USA). All the preparations during the experiment were done with sterile technique: preparation of fetal bovine serum (FBS), antibiotics, and culture reagents.

Preparation of Theca Cells and Collection of Ovaries

The ovaries used were collected in a local abattoir, southern Iraq, where the buffalo were in a healthy clinical condition. The collection of all ovaries was carried out in the middle of the estrus period, as confirmed by an overseeing veterinarian. They included only ovaries with visible corpora lutea and between 2- and 8-mm follicles, which did not satisfy the view of not visible follicles or those that were more than 1.8 cm according to already determined morphological parameters of buffalo follicle classification (Kumari, 2023).

Ovaries were then immediately transported to the laboratory within 2 hours in a thermally insulated container with Dulbecco phosphate-buffered saline (DPBS) that was kept at a temperature of 3437 °C. Ovaries were washed with 0.9% NaCl warmed in pre-warmed solution and then washed briefly with 70% ethanol for 30 seconds, as well as two more saline washes to ensure that the surface was decontaminated.

An 18-gauge needle was used to aspirate follicular fluid, and cumulus-oocyte complexes (COCs) were removed and discarded. The suspension was centrifuged at 177g/10 minutes to obtain a remaining suspension. The resulting TC pellet was washed, gently dissociated by pipetting, and centrifuged again at 277g for 5 minutes. After discarding the supernatant, the pellet was resuspended in TCM-199 (HEPES) supplemented with 1.5% penicillin–streptomycin. Cell number and viability were assessed using 0.4% trypan blue exclusion on a hemocytometer.

Thermal Treatment and Theca Cell Culture

Theca cells were seeded at a density of 1×10^6 cells/mL in TCM-199 HEPES medium supplemented with 10% FBS and 1.5% antibiotics. Cultures were maintained in humidified air with 5% CO_2, and medium was refreshed every 48 hours until cells reached ≈90% confluence (typically 5–7 days).

Control TCs were maintained at 37 °C, whereas heat-treated TCs were incubated at 40.5 °C for 24, 48, or 72 hours without changing the culture medium during the heat-stress period. After treatment, cell viability was re-evaluated, and adherent TCs were collected using 0.25% trypsin–EDTA for downstream RNA extraction. Conditioned media from each group were stored at –80 °C for hormonal and enzymatic analyses.

Analysis of Hormones and Antioxidant Enzymes

Concentrations of P4, E2, and IGF-1 were quantified using commercial ELISA kits (Sino Gene Clon Biotech Co., China) according to the manufacturer's instructions. Optical density (OD) readings were measured at 450 nm using a BioTek ELX808 microplate reader. The assay sensitivities were 8.57 pg/mL, 40 pg/mL, and one ng/mL for P4, E2, and IGF-1, respectively.

Total antioxidant capacity (TAC) was measured colorimetrically using a kit supplied by BioDiagnostic (Egypt). Absorbance was read at 505 nm using a Sunostk SBA-733 Plus spectrophotometer. All assays were performed using ≥3 biological replicates per treatment.

RNA Isolation and cDNA Synthesis

The extract of total RNA in TCs was obtained with the help of Gene JET RNA Purification Kit (Thermo Fisher Scientific) according to the instructions offered by the manufacturer. RNase-free DNase I was used to eliminate genomic DNA contamination, and the purity and concentration of RNA were determined spectrophotometrically with A260/A280 ratios of ~1.8 being acceptable.

The samples were normalized on RNA quantities before cDNA was synthesized. The synthesis of complementary DNA was carried out by the use of the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) that comprises MultiScribe™ Reverse Transcriptase and random primers.

Quantitative Real-Time PCR (qRT-PCR)

The design of gene-specific primers was done with Primer3 software, according to the reference sequences of GenBank, and all the properties of the primers are shown in Table 1. Amplification reactions were 10 µL Power SYBR Green Master Mix, 2 µL forward and reverse primers, 7.5 µL nuclease-free water, and 2 µL cDNA.

Thermal cycling conditions were:

- 95 °C For 10 Min (Initial Denaturation)
- 40 Cycles Of
- 95 °C For 15 S
- 60 °C For 20 S
- 72 °C For 30 S
- Final Extension At 60 °C For 1 Min

qPCR was performed using an Applied Biosystems Step OnePlus™ system.

The ΔΔCt method was used to compute the relative transcript abundances of CPT2, ATP5F1A, SREBP1, SOD2, TNF-alpha, and NFE2L2, which were normalized to GAPDH in MIQE guidelines (Chappel, 2013).

Table 1. Primer sequences used for quantitative real-time PCR

Genes Names	Gene bank accession numbers	Primers sequences	size of Fragment (bp)
CPT2	NM_001011678	F: 5′-CTCTTGAGTCGTGGTGTGCG-3′R: 5′-CCTGATTTGGCTGATAAACGTG-3′	186
ATP5F1A	NM_001045889	F: 5′-CCGGATGAATAGCAGCTC-3′R: 5′-GCGTAGAATCTGTTTGAAGG-3′	150
SREBP1	NM_174684.2	F: 5′-TAAAACAGGAGTGGGTACCT-3′R: 5′-GAGACATTCGCTTTTAGA-3′	160
SOD2	NM_201527	F: 5′-GTGATACCATCGGGAAGATG-3′R: 5′-AAGGCCACTCAAGAACCTT-3′	165
NFE2L2	AF011927	F: 5′-CGCTTTCTCTGGGATGG-3′R: 5′-ATGTGGAGGAGGAGGTGA-3′	260
GAPDH	NM_001113302	F: 5′-GTGAGGAGGAGGAGGAGG-3′R: 5′-TCTAACAGGGGAGAGGG-3′	172
TNFα	NM_001034034	F: 5′-AGGTGGCAGAGGAGGAG-3′R: 5′-GGAGATGGAGGAGGCTT-3′	220

This table lists the gene-specific primers designed for evaluating metabolic and stress-responsive transcripts in cultured theca cells from Iraqi buffalo. Primer sequences were designed on the basis of GenBank reference accessions and optimized to be used in the qPCR amplification.

Data Analysis

The data on the hormone levels, enzymatic activity, and TC viability were compared by using one-way ANOVA, and the results were presented in the form of mean ± SEM. Statistical significance was set at $p < .05$. Normality and variance homogeneity were verified using the Shapiro–Wilk and Levene's tests, respectively. Relative gene expression data were analyzed using the GLM procedure and Duncan's multiple range test to determine significant differences among treatment groups. All tests were done with SPSS v22.0 and SAS (2004).

Results

Effects of Thermal Stress on Theca Cell Viability

The viability of Iraqi buffalo theca cells subjected to heat stress is summarized in Table 2 and illustrated in Figure 2. Across all experimental conditions, cell viability remained high, with values exceeding 87%. Control cultures maintained at 37 °C showed a viability of 88.5 ± 1.4%, which was statistically comparable to the 48-hour (91.0 ± 1.5%) and 72-hour (89.5 ± 2.0%) heat-stress groups. However, a significant difference was observed between the 24-hour treatment (87.0 ± 1.0%) and the 48-hour group, indicating a transient improvement in cell tolerance at mid-term heat exposure. Despite this variation, no significant declines were recorded at any time point, demonstrating that exposure to 40.5 °C did not induce cytotoxicity in theca cells (Table 1).

The following Table 2 illustrates the percentage viability of primary theca cells when cultured under the ideal conditions (37 °C) and high temperature (40.5 °C) after 24, 48, and 72 hours. A standard colorimetric viability assay was also used to determine cell viability, and the values are provided in the form of mean ±

SEM. The statistical significance of treatment (a, b), which was found at p = 0.05, indicates that the cells subjected to heat stress for 48 hours had a significantly greater viability than other groups.

Table 2. Viability of cultured theca cells from iraqi buffalo exposed to different heat-stress durations

Condition	Viability (%)
C 37°C	88.5±1.4 (ab)
24 h	87.0±1.0 (b)
48 h	91.0±1.5 (a)
72 h	88.0±1.5 (ab)

All in all, the data indicate that theca cells are rather viable in all treatments, with moderate and significant variations, depending on the time of exposure to heat. These findings show that the implemented heat-stress condition affected cellular activity without affecting fundamental survival. The preservation of high viability indicates that the later hormonal and transcriptional alteration can be explained by the functional heat-modulation as opposed to the damaged cell integrity.

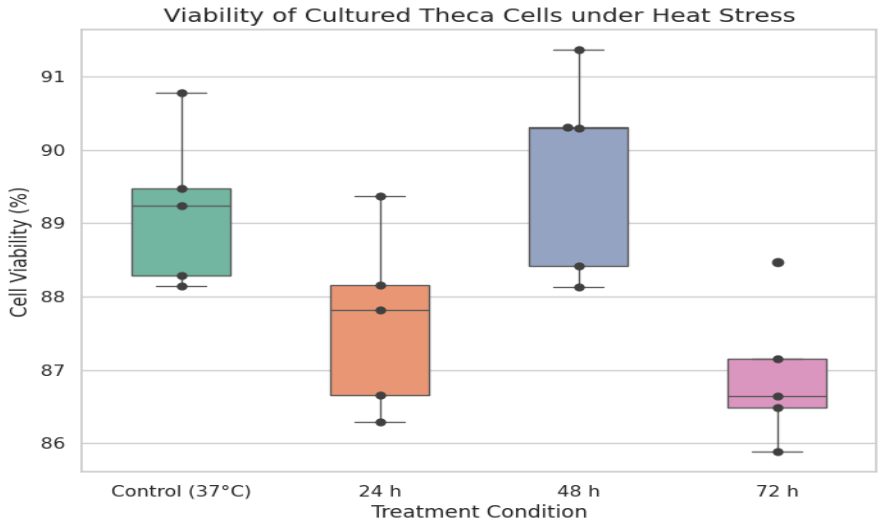

Figure 2. Viability of cultured theca cells from iraqi buffalo under heat stress

Effects of Heat Stress on Estradiol, Progesterone, IGF-1, and TAC Secretion

The effect of the thermal exposure was significant on the secretory pattern of the cultured theca cells, as the parameters of hormones and antioxidants showed specific time dynamics (Table 3; Figure 3).

The levels of estradiol (E2) were mostly the same in the control group (475 ± 50 pg/mL) and the treatment group (24 hours) (400 ± 40 pg/mL). Nevertheless, a sharp rise was observed at 72 hours (620 ± 80 pg/mL), which suggests an amplification of estrogenic activity at the time of extended exposure to heat.

Progesterone (P4) secretion was of a different pattern. Control (168 ± 10 pg/mL) and the 24-hour cultures were similar in values (170 ± 25 pg/mL). The reduction was significant at 48 hours (115 ± 15 pg/mL), which was a temporary blockage of luteogenic activity in the heat stress that occurred during the mid-term. At 72 hours, the P4 returned to normal (135 ± 5 pg/mL), but at a lower level when compared with controls.

The levels of IGF-1 gradually decreased as the duration of exposure to heat increased. The highest control cultures (30.5 ± 4.5 ng/mL) were realized, and the lowest concentration was realized after 72 hours

(21.5 ± 1.5 ng/mL). This blockage represents attenuated anabolic signaling and potential metabolic stress during extended thermal challenge.

Total antioxidant capacity (TAC) fluctuated across treatments. The lowest value was recorded at 48 hours (0.13 ± 0.03 mM/L), indicating increased oxidative stress at this time point. At 72 hours, TAC had risen to 0.19 ± 0.06 mM/L, which was higher than control and mid-term values, indicating that compensatory antioxidant processes were triggered towards the end of the exposure duration.

Taken together, these hormonal and antioxidant reactions indicate that heat stress significantly adjusts steroidogenic activity and oxidative homeostasis, with the most significant changes recorded at mid- to long-term periods of exposure.

Table 3. Effects of thermal stress on estradiol (e2), progesterone (p4), igf-1, and total antioxidant capacity (TAC) in cultured buffalo theca cells

Condition	Estrogen (E2) (pg/ml)	Progesterone (P4) (pg/ml)	IGF1 (ng/ml)	TAC (mM/L)
C 37°C	475±50 (ab)	168±10 (ab)	30.5±4.5 (a)	0.16±0.025 (ab)
24 h	400±40 (b)	170±25 (ab)	24.5±2.5 (ab)	0.18±0.015 (ab)
48 h	455±60 (ab)	115±15 (c)	23.0±3.0 (ab)	0.13±0.03 (b)
72 h	620±80 (a)	135±5 (bc)	21.5±1.5 (b)	0.19±0.06 (a)

Figure 3 shows the concentrations of Estradiol (E2, A), Progesterone (P4, B), Insulin-like Growth Factor-1 (IGF-1, C), and Total Antioxidant Capacity (TAC, D) in cultured buffalo theca cells exposed to 37 °C (control) or 40.5 °C for 24, 48, and 72 hours. Data are presented as mean ± SEM from three independent experiments. Taken together, these hormonal and antioxidant reactions indicate that heat stress significantly adjusts steroidogenic activity and oxidative homeostasis, with the most significant changes recorded at mid- to long-term periods of exposure.

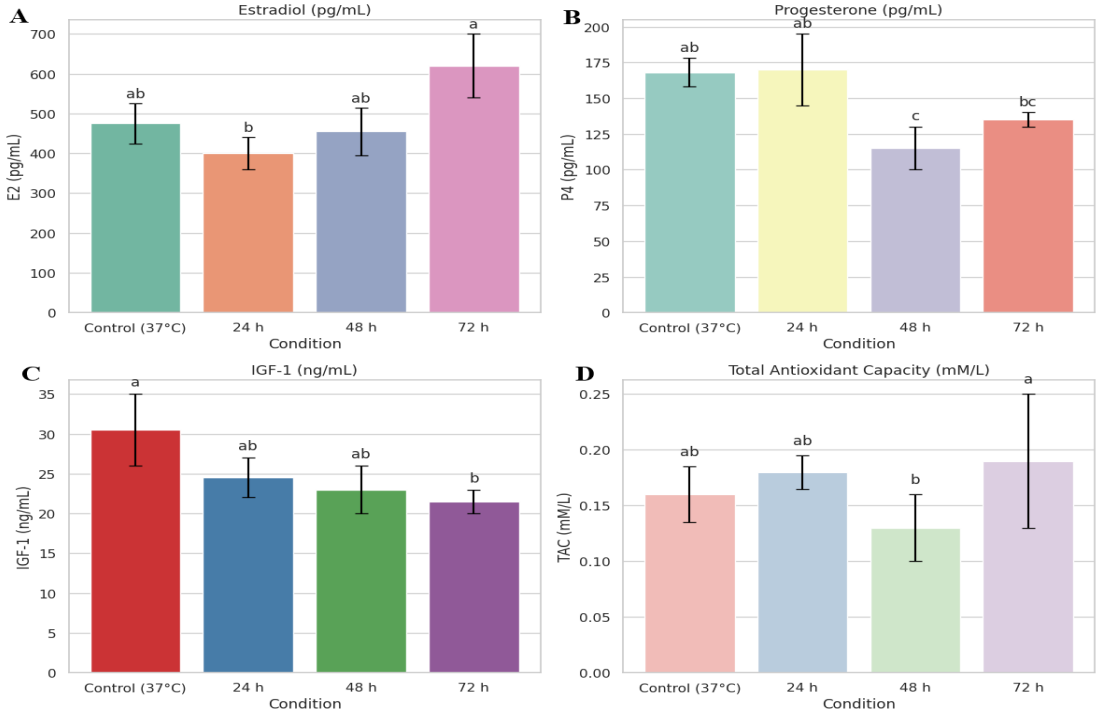

Figure 3. Time-dependent effects of heat stress on estradiol, progesterone, IGF-1, and total antioxidant capacity in cultured iraqi buffalo theca cells

Differential Abundance of Stress- and Metabolism-Related mRNA Transcripts

Heat exposure induced significant transcriptional adjustments in genes governing oxidative defense, inflammation, and cellular metabolism (Figure 3A–D). The SOD2 expression was also similar in all groups, indicating that the capacity to eliminate superoxide in mitochondria did not change during high temperatures. However, NFE2L2, a major master regulator of antioxidant defense, was greatly suppressed at 24 and 72 hours, indicating the inhibited induction of the Nrf2-mediated oxidative stress response during early and long-lasting exposure to heat.

An elevation of TNF-α was observed at 48 hours, which indicates the intermediate stimulation of pro-inflammatory pathways. This response is consistent with the expected cellular responses to moderate thermal stress responses in which TNF-α is frequently an early mediator of adaptive inflammatory responses. There was also evident modulation of metabolic gene expression that was dependent on heat. The mitochondrial 2 2-oxidation regulator (CPT2) was also found to be significantly suppressed at all heat-exposure intervals, which suggests inhibition of fatty-acid transport and oxidative metabolism. SREBP1, on the other hand, showed a significant increase in expression 48 hours post-heat-stress, and this may indicate an improved lipid-biosynthesis signaling, possibly in response to changes in cellular energetics. The stability of ATP5F1A, a subunit of mitochondrial ATP synthase, was constant across all groups and suggests that core ATP-generating capacity was maintained despite thermal challenge.

All of these transcriptional signatures point to the fact that the fundamental mitochondrial viability and ATP production have no impact, yet heat stress induces significant changes in inflammatory signaling, antioxidant regulation, and lipid/energy-metabolism pathways.

Table 4. Relative mRNA expression of stress- and metabolism-related genes in cultured buffalo theca cells under heat stress

Condition	SOD2	NFE2L2	TNFα	CPT2	SREBP1	ATP5F1A
C 37°C	1.15±0.1	8.0±1.5 (a)	0.9±0.4 (b)	3.0±0.35 (a)	0.45±0.1 (a)	1.65±0.65
24 h	1.85±0.5	2.1±1.0 (c)	2.0±0.6 (b)	0.35±0.05 (b)	5.0±3.0 (ab)	1.3±0.3
48 h	1.15±0.35	6.7±1.0 (ab)	4.8±0.3 (a)	0.5±0.15 (b)	8.0±2.8 (b)	0.95±0.1
72 h	2.4±0.8	3.4±1.8 (bc)	1.0±0.3 (b)	1.0±0.4 (b)	6.2±2.2 (ab)	1.7±0.9

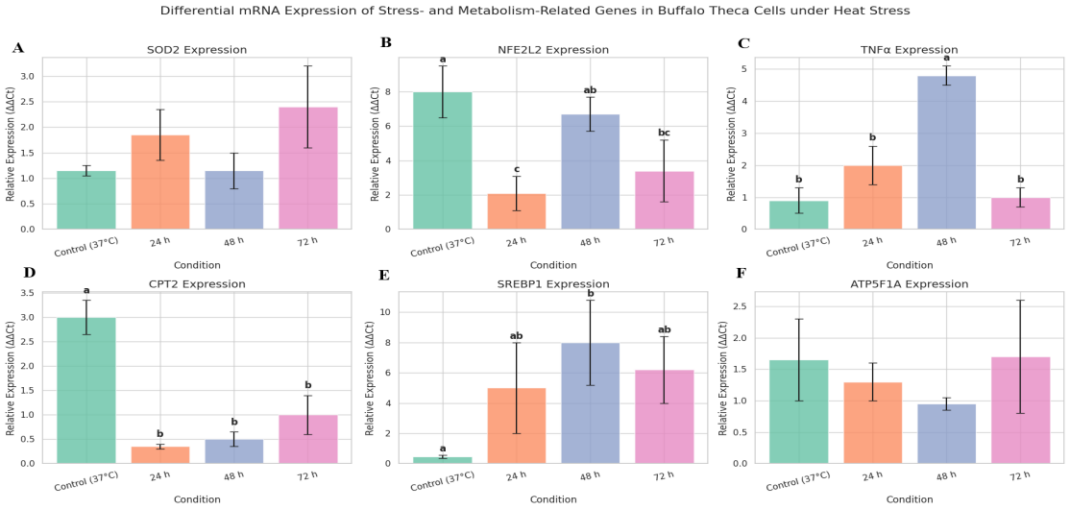

Figure 4. Heat-induced modulation of stress- and metabolism-related mRNA transcripts in cultured buffalo theca cells

Figure 4 and Table 4 present the expression of the mRNA of oxidative stress (SOD 2, A; NFE2L2, B), inflammation (TNF- 0), and metabolism (CPT2, D; SREBP1, E; ATP5F1A, F) in cultured Iraqi buffalo theca cells at 37°C (Control) and 40.5°C (24, 48, and 72 h). Data are presented as mean ± SEM ($n \geq 3$). Different letters above bars indicate statistically significant differences among treatment groups ($P < 0.05$, one-way ANOVA with Duncan's multiple range test). Heat stress results in time-dependent changes in antioxidant defense, inflammatory signaling, and lipid metabolism, and core mitochondrial ATP production does not change.

Discussion

In the current research paper, it is shown that Iraqi buffalo theca cells are strongly viable during moderate heat stress conditions and that there are time-dependent changes in steroidogenic activity, metabolic signaling, antioxidant capacity, and stress-responsive gene expression. In all the experimental conditions, the cell viability was above 87, and the transient variation, including a slight rise at 48 hours, is indicative of the body adapting to overcome the heat stress at mid-term. The preservation of high viability is a reflection that subsequent downstream modulation in the hormone secretion and gene expression is due to functional response to heat, and not to cytotoxicity, which is manifested as a real cellular adaptation. The estradiol, progesterone, and IGF-1 release to heat exposure showed different time trends. Estradiol increased to great heights during 72 hours, thus indicating that estrogenic activities become more active under long treatments with thermal conditions, as a result of aromatase or other steroidogenic enzyme stimulations. At 48 hours, the progesterone concentrations were reduced, and at 72 hours, they returned to some degree, which was an indication of temporary inhibition of luteogenic functionality during mid-term heat stress. The level of IGF-1 gradually decreased in the course of continuous exposure, which indicated a lack of attenuated anabolic signaling and metabolic stress. The total antioxidant capacity showed a biphasic reaction with a non-persistent decline in its level at 48 hours and a partial recovery at 72 hours, which was in line with the activation of the compensatory antioxidant system in the presence of oxidative stress. The stress- and metabolism-related gene transcriptional profile showed the selective modulation with time. The level of SOD2 was stable, which means that the mitochondrial superoxide detoxification was preserved, but NFE2L2, a regulator of antioxidant defense, was decreasing at 24 and 72 hours, which is one of the arguments for the partial inhibition of Nrf2-mediated pathways in the early and prolonged stress. TNF-2 expression rose at 48 hours, which was an indication of mid-term activation of pro-inflammatory signaling, whereas CPT2 was steadily suppressed, denoting inhibition of fatty-acid 2-oxidation in mitochondria. SREBP1, on the other hand, was increased after 48 hours, indicating increased lipid-biosynthesis signaling as an adaptive metabolic process. ATP5F1A maintained its stability, which may indicate that the core ATP-generating capacity does not diminish due to heat-induced adjustment of metabolic pathways. These transcriptional and functional alterations, when combined, reveal a concerted response that ensures viability, as well as regulates oxidative, inflammatory, and metabolic responses. The results are helpful to understand buffalo theca cells' adaptation to moderate levels of thermal stress, which is especially important due to the fact that reproductive physiology is very susceptible to the increasing levels of environmental temperature. The changes in steroidogenic activity, the IGF-1 signaling, and the antioxidant capacity may have an impact on follicular development, oocyte quality, and general fertility during heat stress conditions. The transcriptional changes observed point to possible molecular targets to be used to develop interventions geared towards enhancing heat resilience in buffalo reproductive tissues. The research could be applied in the future by creating nutritional, pharmacological, or genetic interventions that can increase the cellular ability to endure thermal stress, including dietary antioxidant or lipid metabolism modulators. Future research would examine cross-talk between the granulosa and theca cells in heat stress and the in vivo consequences of the follicular development and ovulation. Long-term studies can also determine epigenetic

alterations caused by repetitive exposures to high temperatures and the possible transgenerational consequences on procreation. All these results created a base on how to develop evidence-based approaches to reduce the negative effect of heat stress on buffalo reproductive performance, and what the future of livestock management in warmer climates should be.

Conclusion

In the current research paper, it is shown that Iraqi buffalo theca cells are strongly viable during moderate heat stress conditions and that there are time-dependent changes in steroidogenic activity, metabolic signaling, antioxidant capacity, and stress-responsive gene expression. In all the experimental conditions, the cell viability was above 87, and the transient variation, including a slight rise at 48 hours, is indicative of the body adapting to overcome the heat stress at mid-term. The preservation of high viability is a reflection that subsequent downstream modulation in the hormone secretion and gene expression is due to functional response to heat, and not to cytotoxicity, which is manifested as a real cellular adaptation. Different time trends were observed in the release of estradiol, progesterone, and IGF-1 to heat exposure. Estradiol levels rose to highs at 72 hours, which means that estrogenic activities are more active when subjected to long periods of thermal conditions, possibly through the stimulation of aromatase or other steroidogenic enzymes. At 48 hours, the progesterone concentrations were reduced, and at 72 hours, they returned to some degree, which was an indication of temporary inhibition of luteogenic functionality during mid-term heat stress. The level of IGF-1 gradually decreased in the course of continuous exposure, which indicated a lack of attenuated anabolic signaling and metabolic stress. The total antioxidant capacity showed a biphasic reaction with a non-persistent decline in its level at 48 hours and a partial recovery at 72 hours, which was in line with the activation of the compensatory antioxidant system in the presence of oxidative stress. The stress- and metabolism-related gene transcriptional profile showed the selective modulation with time. The level of SOD2 was stable, which means that the mitochondrial superoxide detoxification was preserved, but NFE2L2, a regulator of antioxidant defense, was decreasing at 24 and 72 hours, which is one of the arguments for the partial inhibition of Nrf2-mediated pathways in the early and prolonged stress. TNF-2 expression rose at 48 hours, which was an indication of mid-term activation of pro-inflammatory signaling, whereas CPT2 was steadily suppressed, denoting inhibition of fatty-acid 2-oxidation in mitochondria. SREBP1, on the other hand, was increased after 48 hours, indicating increased lipid-biosynthesis signaling as an adaptive metabolic process. ATP5F1A maintained its stability, which may indicate that the core ATP-generating capacity does not diminish due to heat-induced adjustment of metabolic pathways. These transcriptional and functional alterations, when combined, reveal a concerted response that ensures viability, as well as regulates oxidative, inflammatory, and metabolic responses. The results are helpful to understand buffalo theca cells' adaptation to moderate levels of thermal stress, which is especially important due to the fact that reproductive physiology is very susceptible to the increasing levels of environmental temperature. The changes in steroidogenic activity, the IGF-1 signaling, and the antioxidant capacity may have an impact on follicular development, oocyte quality, and general fertility during heat stress conditions. The transcriptional changes observed point to possible molecular targets to be used to develop interventions geared towards enhancing heat resilience in buffalo reproductive tissues. The research could be applied in the future by creating nutritional, pharmacological, or genetic interventions that can increase the cellular ability to endure thermal stress, including dietary antioxidant or lipid metabolism modulators. Future research would examine cross-talk between the granulosa and theca cells in heat stress and the in vivo consequences of the follicular development and ovulation. Long-term studies can also determine epigenetic alterations caused by repetitive exposures to high temperatures and the possible transgenerational consequences on procreation. All these results created a base on how to develop evidence-based approaches to

reduce the negative effect of heat stress on buffalo reproductive performance, and what the future of livestock management in warmer climates should be.

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Author Contributions

Sabreen Noori Dagman conceived and designed the study, performed the sample collection, cell culture, experimental analyses (hormone, antioxidant assays, and qPCR), analyzed and interpreted the data, drafted and edited the manuscript, and approved the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article. All research was conducted objectively and without any financial or personal relationships that could influence the results.

Ethical Consideration

All the experimental activities that touched on the collection of buffalo ovaries were conducted in line with the ethical requirements of using animal tissues in experimentation. Clinically healthy ovaries of the buffalo slaughtered in a local abattoir were used as part of regular meat production, so no animal was killed to conduct this study. Gathering and processing of ovarian tissues was conducted under the guidance of a licensed veterinarian to guarantee the welfare of animals and reduce stress before slaughter. All the laboratory processes involving primary cell isolation, culture, and molecular analyses were conducted in accordance with institutional requirements of biosafety and ethical research. Because there were no experimental interventions on animal subjects, a formal approval of an animal ethics committee was unnecessary, but all the protocols complied with the internationally accepted principles of the ethical use of animal materials in biomedical studies (e.g., the 3Rs - Replacement, Reduction, Refinement).

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