

Research Article

Association of FOXO3a with HSP70-1 and HSP90AA1 Expression in Bovine Mammary Epithelial Cells

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Received 10 Sep 2026, Accepted 04 Oct 2026, Available online 05 Oct 2026, Vol.16 (2026)

Abstract

This study aimed to investigate whether the FOXO3a gene responds to heat stress and whether its expression is associated with the regulation of HSP70 and HSP90 in bovine mammary epithelial cells. Primary bovine mammary epithelial cells were isolated and cultured in vitro from bovine mammary tissue samples. Following the 11th passage, the cells were subjected to heat stress by incubation at 42 °C for 1 h in a water bath, followed by recovery at 37 °C. The kinetics of HSP70-1, HSP90AA1, and FOXO3a gene expression were evaluated at 2, 4, and 8 h after heat stress and compared with those of non-stressed cells. The results showed that HSP70-1 mRNA expression increased progressively and reaching peak levels at 4 and 8 h post-stress. Similarly, HSP90AA1 expression increased significantly from 2 to 8 h of recovery following heat exposure. FOXO3a expression increased following heat exposure and reached a highly significant peak at 4 h of recovery. These findings suggest that FOXO3a is responsive to thermal stress and may be involved in the molecular mechanisms regulating heat shock protein expression and cellular protection.

Keywords: HSP70, HSP90, FOXO3a, Gene Expression and Heat Stress

Introduction

Rising environmental temperatures, particularly when accompanied by intense solar radiation and high humidity, can impair an animal's ability to dissipate excess body heat effectively. When the accumulated heat load exceeds the animal's thermoregulatory capacity, a physiological condition known as heat stress (HS) develops, resulting in reduced productivity, poor health, and substantial economic losses. Dikmen and Hansen (2009) defined heat stress as the combined effect of environmental factors that elevate body temperature and trigger a series of physiological responses. At the cellular level, heat stress induces the heat shock response, a highly conserved adaptive mechanism that enables cells to maintain homeostasis under adverse environmental conditions (Arya *et al.*, 2007). This protective response is primarily mediated by heat shock proteins (HSPs), a family of highly conserved molecular chaperones that are widely expressed in both prokaryotic and eukaryotic organisms (Hassan *et al.*, 2019).

Heat shock proteins play essential roles in maintaining protein homeostasis by facilitating protein folding, preventing protein denaturation and aggregation, and promoting the repair or degradation of damaged proteins during cellular stress (Feder and Hofmann, 1999). The response of bovine mammary epithelial cells (BMECs) to heat stress is of particular importance because these cells are responsible for maintaining mammary gland function and milk synthesis (Li *et al.*, 2015). Heat stress exerts rapid effects on BMECs, with cellular responses occurring within minutes of exposure (Collier *et al.*, 2006, 2008). Exposure of BMECs to elevated temperatures (42°C) has been shown to induce extensive changes in gene expression, including the upregulation of genes involved in cellular stress responses, protein folding, and repair mechanisms, while suppressing genes associated with cellular metabolism, morphogenesis, and mammary-specific biosynthetic functions (Collier *et al.*, 2006). Furthermore, BMEC gene networks respond to environmental heat loads exceeding the thermoneutral zone through coordinated intracellular and extracellular signaling pathways that integrate cellular responses with whole-animal metabolic adaptations (Li *et al.*, 2015). Among the various cellular defense mechanisms activated during heat stress, HSP70 and

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DOI: <https://doi.org/10.14741/ijab/v.16.1.3>

HSP90 are the principal molecular chaperones involved in protecting BMECs from thermal injury. Their expression is rapidly induced following heat exposure and is essential for preserving cellular integrity and function (Collier *et al.*, 2006). Kapila *et al.* (2013) demonstrated that, under in vitro heat stress conditions, HSP mRNA expression in buffalo mammary epithelial cells increased significantly within 30 minutes after heat exposure and remained elevated for up to 12 hours, with maximal expression occurring between 2 and 4 hours' post-treatment. Similarly, Hu *et al.* (2016) reported significant increases ($P < 0.05$) in the mRNA expression of HSP27, HSP70, and HSP90 following heat shock in BMECs, with HSP70 exhibiting the highest level of induction after one hour of exposure to 42°C. Forkhead box O (FOXO) transcription factors belong to the large Forkhead (Fox) superfamily, which comprises 19 subclasses of Fox genes (Calnan and Brunet, 2008; Burgering, 2008). FOXO proteins are expressed in most mammalian tissues, although each member exhibits a degree of tissue-specific expression (Li, 2015). These transcription factors regulate numerous biological processes, including oxidative stress resistance, apoptosis, cell cycle progression, DNA repair, metabolism, and longevity (Van der Horst, 2007). Among them, FOXO3a has received considerable attention because of its central role in coordinating cellular adaptation to environmental stress. Previous studies have demonstrated that FOXO3a regulates the expression of numerous stress-responsive genes. In *Caenorhabditis elegans*, the FOXO homolog DAF-16 controls the transcription of a large number of genes involved in stress resistance (McElwee *et al.*, 2003). Furthermore, DAF-16/FOXO functions as a central regulator within a complex signaling network involving multiple upstream pathways and downstream target genes, including several heat shock proteins (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003). Few recent primary studies (review articles) have also suggested functional interactions between FOXO proteins and heat shock proteins in regulating cellular responses to environmental stress. Nevertheless, the role of FOXO transcription factors in the heat stress response of bovine mammary epithelial cells remains poorly understood. Therefore, the present study was designed to investigate the expression of FOXO3a in response to heat stress and to determine its relationship with the heat shock proteins HSP70-1 and HSP90AA1 in bovine mammary epithelial cells under in vitro conditions.

Material and methods

Data Collection

Bovine mammary tissue sample was collected from disease-free animal udder at slaughterhouse. To maintain the structural integrity of the alveoli and preserve the viability of the mammary epithelial cells (MECs), specimens were excised under aseptic conditions and transported to the laboratory facility in a thermal insulator maintained at a constant temperature of 4°C.

Establishment of bovine MECs primary culture

Mammary tissue samples cut to smaller pieces of 1mm³ cubes were transferred onto collagen coated cell culture dishes (Corning, USA) containing DMEM/ F-12 (Hyclone, Utah) supplemented with 10 % FBS and 100 IU/ml penicillin/ mg/ml streptomycin (Gibco Life Technologies, Carlsbad, CA, USA) and incubated at 37 °C, 5 % CO₂. For establishment of primary cell culture of MECs, cells were continuously cultured in DMEM/F-12 1:1 (1X) growth media was made till homogenous MECs were obtained. Cells were seeded in a six-well-plate and re-cultured for 48 hours until they reached to 80%-90% confluence.

Heat treatment to bovine MECs

The cells after 11th passages the experimental cell cultures were subjected to heat stress condition by incubation at 42 °C for 1 hour at water bath. Subsequently, upon completion of the heat stress treatment cells allowed to recover at 37 °C, 5% CO₂. To analyze the temporal dynamics of gene expression, cells were harvested by TRIzol reagent. The kinetics of HSP70-1, HSP90AA1 and FOXO3a genes expression were evaluated after 2h, 4h, 8h post-stress and compared against non-stressed control samples. Throughout the experimental period, the culture plate marked as control was kept at 37°C.

RNA Isolation

Total mRNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, California). The concentration, purity and integrity of the RNA's were confirmed on the NanoDrop 1000 UV-Vis Spectrophotometer. The total RNA was reverse transcribed using a reverse transcription level kit (TaKaRa, Dalian, China) according to the manufacturer description. Then the complementary DNA (cDNA) was stored at -20 °C for further use.

Primer Designing and Quantitative RT- PCR.

In accordance with established molecular protocols, primers for the target genes were designed using the National Center for Biotechnology Information (NCBI) Primer-BLAST tool. The specific primers were derived from the following GenBank accession numbers: HSP70-1 (GeneBank accession No. NM_ 174345.4), HSP90AA1 (GeneBank accession No. NM_001012670.2), FOXO3a (GeneBank accession No. NM_ 001206083.1) and ACTIN (GeneBank accession No. NM_173979.3), the latter serving as an internal reference control. The designated primer sets were optimized to yield distinct amplicon lengths of 88 bp, 167 bp, 103 bp, and 116 bp, respectively are documented in Table1.

Table1 Primer sequences and expected the size of HSP70, HSP90, FOXO3a and ACTIN genes

Primer	Upstream primer (5'-3')	Downstream primer (5'-3')	Expected segment size/bp
HSP70-1	GCCAATGGCATCCTTAACGT	TCAGACGACCTTTGTCATTGGT	88
HSP90AA1	AGAGCCCTTCTTTTGTCCC	TCCTCAGAATCCACCACACC	167
FOXO3a	CACTACGGCAACCAGACACT	TGCTGGCCTGAGACATCAAG	103
ACTIN	CGGCACCACCATGTACCCT	ACACGGAGTACTTGCGCTCAG	116

The quantification of mRNA expression was quantitative real-time PCR (qRT-PCR) utilizing Fast-Start Universal SYBR Green Master (Rox) in strict accordance with the manufacturer's specifications. The reaction solution was prepared in a total volume of 20 μ l was comprised of 10 μ l SYBR, 0.5 μ l each of 10 μ M forward and reverse primers, 7 μ l DNase/RNase-free water, and 2 μ l diluted cDNA template. The thermal cycling profile consisted of an initial denaturation and enzyme activation step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 sec. and a combined annealing/extension phase at 60 °C for 60 sec. The targeted genes data were normalized to the endogenous reference gene ACTIN by using an optimized comparative Ct ($2^{-\Delta\Delta C_t}$) value method [13], where the relative fold change was calculated based on the formula: $\Delta\Delta C_t = \Delta C_{t \text{ target}} - \Delta C_{t \text{ reference}}$.

Statistical analysis

The statistical significance of mRNA expression levels across different recovery time points was analyzed using SPSS statistical program (SPSS 19.0 for Windows; SPSS, Inc., USA). Comparative analysis within and between target gene groups was performed using a one-way analysis of variance (ANOVA) using the SPSS program. P-value of <0.05 was established as the threshold for statistical significance.

Results

The present research utilizes the bovine mammary epithelial cells (BMECs) as a representative *in vitro* model to examine the longitudinal temporal changes in mRNA expression profiles of HSP70-1, HSP90AA1 and FOXO3a genes under conditions of thermal stress.

HSP70-1 Gene Expression in BMECs

Fig 1 delineates the mRNA expression profile of the HSP70-1 gene in bovine mammary epithelial cells (BMECs) subjected to thermal stress. Results showed that the kinetic expression of HSP70-1 mRNA reached peak levels during the 4 and 8 h recovery intervals, where values were significantly elevated ($P < 0.05$) relative to both the 2 h recovery period and the basal control levels. Statistical analysis indicated no significant difference ($P > 0.05$) in mRNA abundance when comparing the 4 h and 8 h recovery phases and the initial 2 h recovery period did not deviate significantly ($P > 0.05$) from the basal control.

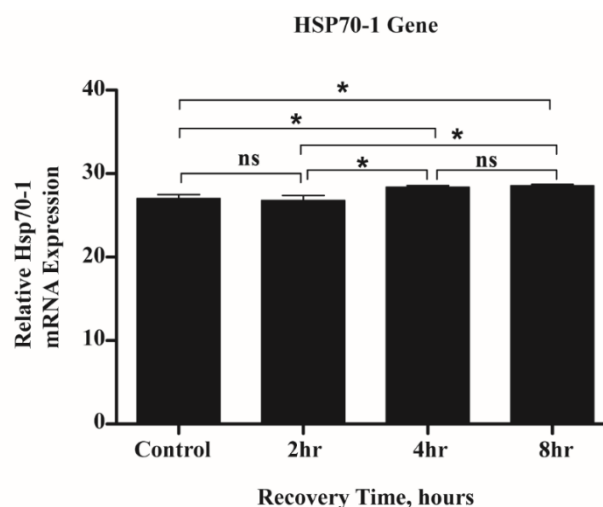


Fig 1. mRNA expression profile of HSP70-1 was determined following heat-shock treatment. Samples were collected at 2, 4, and 8 h post-treatment. Relative gene expression was quantified using the comparative $2^{-\Delta\Delta C_t}$ method and normalized to the endogenous reference gene ACTIN. Differences among treatment groups were analyzed by one-way analysis of variance (ANOVA) using SPSS software, with $P < 0.05$ considered statistically significant

HSP90AA1 Gene Expression in BMECs

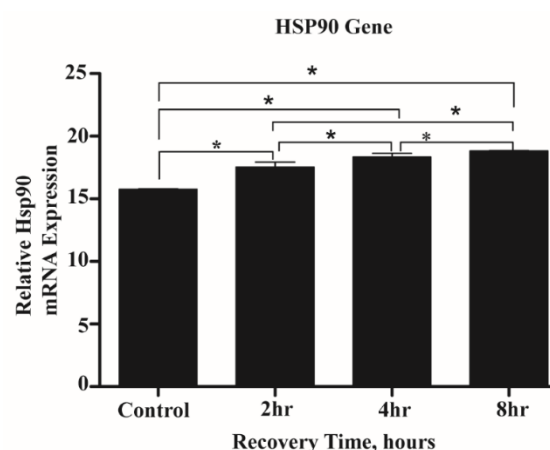


Fig 2. mRNA expression profile of HSP90AA1 was evaluated following heat-shock treatment. Samples were collected at 2, 4, and 8 h after exposure to heat stress. Relative HSP90AA1 expression was quantified using the comparative $2^{-\Delta\Delta C_t}$ method and normalized to the endogenous reference gene ACTIN. Statistical differences among treatment groups were assessed using one-way analysis of variance (ANOVA) in SPSS software, with $P < 0.05$ considered statistically significant

The experimental data provided, Fig 2 illustrates the temporal mRNA expression profile of *HSP90AA1* following heat stress and during subsequent recovery periods of 2, 4, and 8 h. The quantitative real-time PCR (qRT-PCR) analysis reveals a dynamic and significant upregulation of *HSP90AA1* mRNA levels throughout the recovery phase. During the initial 2 to 8 h following heat stress, mRNA expression levels gradual and statistically significant increase ($P < 0.05$) when compared to the basal (control) levels. The expression was significantly in the recovery phase 2 h relative to the control group. As the recovery progressed to the 4 h mark, a further significant increase was observed compared to the 2 h interval. This upward trend continued into the 8 h mark, where mRNA levels reached their peak significance relative to the 4 h period.

FOXO3a Gene Expression in BMECs

Fig 3 showed the kinetic mRNA expression profile of FOXO3a in bovine mammary epithelial cells (BMECs) following heat stress exposure reveals a distinct time-dependent regulatory pattern. During the initial 2 h period of heat stress treatment, the transcriptional levels of FOXO3a remained statistically stable, showing no significant deviation ($P > 0.05$) from the basal control levels. Data revealed that, at the 4h recovery period, FOXO3a expression reached a peak that was highly significant ($P < 0.05$) when compared to both the basal levels and other recovery time points (2 and 8 h). Following this peak, data indicated a significant reduction in mRNA levels by the 8 h recovery interval relative to the 4 h peak. Results revealed that there were no statistically significant differences ($P > 0.05$) between the basal control, 2 h and the 8 h recovery periods.

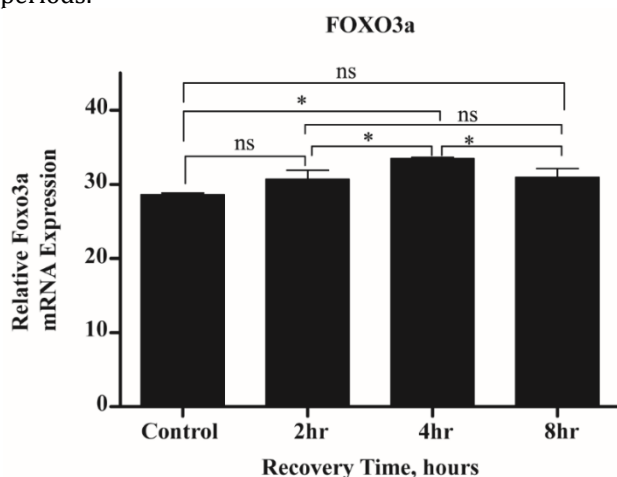


Fig 3. FOXO3a mRNA expression was evaluated following heat-shock treatment, with samples collected at 2, 4, and 8 h post-treatment. Relative expression was normalized to *ACTIN* gene and calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Differences among groups were analyzed by **one-way ANOVA** using **SPSS**, with $P < 0.05$ considered statistically significant.

Comparative Analysis of Gene Expression: Hsp70-1, Hsp90AA1, and Foxo3a

The comparative analysis of mRNA expression profiles for HSP70-1, HSP90AA1, and FOXO3a in BMECs during recovery from heat stress reveals distinct temporal kinetics and magnitude of induction. Result in Fig 4, showed the expression levels of these target genes were evaluated at basal levels (non-stress) and during recovery intervals of 2, 4, and 8 h. Statistical analysis indicates that during the initial recovery phase (2–8 h), the mRNA expression of FOXO3a was significantly higher ($P < 0.05$) than the expression levels of both HSP70-1 and HSP90AA1. The kinetic profile of the heat shock proteins showed that HSP70-1 displayed significantly higher ($P < 0.05$) mRNA levels compared to HSP90AA1 throughout the 2–8 h recovery periods. Data analysis revealed that, HSP70-1 levels remained significantly lower ($P < 0.05$) than those of FOXO3a throughout the 2–8 h observation periods. Conversely, results of HSP90AA1 demonstrated the lowest relative expression among the three genes, with its mRNA levels being significantly lower ($P < 0.05$) than both HSP70-1 and FOXO3a.

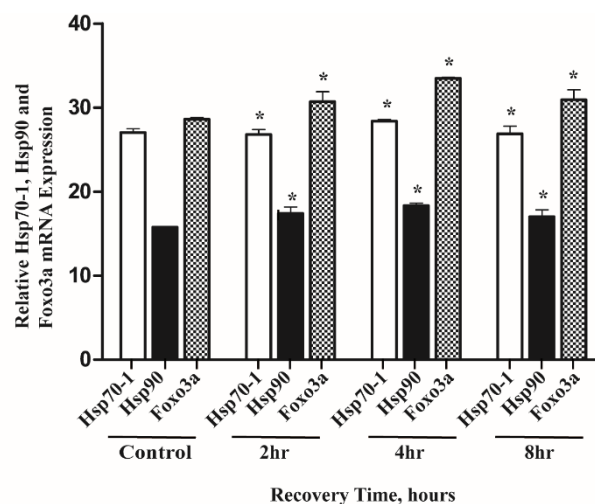


Fig 4. Comparison of HSP70-1, HSP90AA1, and FOXO3a mRNA expression following heat-shock treatment using one-way analysis of variance (ANOVA) in SPSS, with $P < 0.05$ considered statistically significant

Discussion

Heat stress is a major environmental challenge that adversely affects the productivity and economic sustainability of the dairy industry, particularly in high-producing lactating cows. Exposure to environmental temperatures exceeding the thermoneutral zone results in an elevation of core body temperature, triggering a series of detrimental physiological responses. These responses include reduced feed intake, decreased milk yield, and alterations in milk composition, all of which contribute to substantial economic losses (Armstrong, 1994; West, 2003; Collier and Collier, 2012). To counteract

the adverse effects of heat stress, cattle activate a range of cellular defense mechanisms that promote survival and maintain cellular homeostasis. One of the most important protective responses is the induction of heat shock proteins (HSPs) in mammary epithelial cells (Kregel, 2002). Heat shock proteins function as molecular chaperones that facilitate proper protein folding, prevent the aggregation of misfolded proteins, and promote the repair or degradation of damaged proteins. Through these actions, HSPs preserve protein integrity and cellular homeostasis during thermal stress, thereby minimizing cellular injury and reducing production losses associated with heat-induced cellular damage (Mayer and Bukau, 2005). In the present study, we investigated the expression patterns of two major heat shock protein genes, HSP70-1 and HSP90AA1, in BMECs exposed to thermal stress. In addition, we evaluated the expression of the FOXO3a gene to determine its potential regulatory role in modulating the expression of HSP70 and HSP90 under heat stress conditions. Primary cultures of BMECs have been widely employed as an *in vitro* model to investigate the molecular mechanisms that enable mammary epithelial cells to maintain cellular homeostasis during environmental stress. Previous studies using this model have focused on signaling pathways involved in heat stress adaptation, protein synthesis, oxidative stress, and apoptosis induced by elevated temperatures (Du *et al.*, 2007; Du *et al.*, 2008; Hu *et al.*, 2009; Zhao *et al.*, 2010; Kapila *et al.*, 2013). Accordingly, the present study examined the expression profiles of HSP70, HSP90, and FOXO3a in BMECs isolated from Chinese Holstein Friesian cattle under *in vitro* heat stress conditions. Quantitative analysis demonstrated that HSP70-1 mRNA expression was significantly increased in heat-treated cells compared with the thermoneutral control (Fig1). This observation is consistent with the findings of Bhanuprakash *et al.* (2016), who reported that HSP70 expression is strongly associated with elevated environmental temperatures and may serve as a reliable molecular biomarker for evaluating the physiological effects of thermal stress in cattle. Although no significant difference in HSP70-1 expression was detected between the control and heat-stressed groups after a 2 h recovery period ($P > 0.05$), significant upregulation was observed following 4 and 8 h of recovery ($P < 0.05$). However, no statistically significant difference was detected between the 4 and 8 h recovery periods ($P > 0.05$), suggesting that HSP70-1 expression reached a plateau after its initial induction. Rapid induction of HSP70 following thermal stress has been consistently reported in several bovine studies. Bhanuprakash *et al.* (2013) demonstrated that HSP70 mRNA expression increased markedly during the first 6 h of heat and cold stress in peripheral blood mononuclear cells (PBMCs) from both Sahiwal and Frieswal (Holstein crossbred) cattle. Similarly, Hu *et al.* (2016) reported a rapid increase in both HSP70 mRNA and protein expression in bovine mammary epithelial

cells following heat stress. Comparable findings were reported by Kapila *et al.* (2013), who observed that HSP70 was the most responsive member of the heat shock protein family in buffalo mammary epithelial cells, with expression increasing within 30 min of heat exposure and remaining significantly elevated for up to 8 h. Moreover, several heat shock proteins continued to exhibit significant expression for as long as 12 h following thermal stress. Collectively, the findings of the present study agree with previous reports demonstrating that HSP70 is among the earliest and most responsive molecular chaperones activated during heat stress.

Similar to HSP70-1, HSP90AA1 encodes a highly abundant and evolutionarily conserved molecular chaperone that plays a pivotal role in numerous cellular processes. Quantitative analysis demonstrated a progressive increase in transcript abundance of HSP90AA1 mRNA throughout the recovery period (Fig 2), with significantly higher expression observed at 2, 4, and 8 h following heat exposure ($P < 0.05$). Moreover, HSP90AA1 expression differed significantly between each recovery interval, indicating a sustained and time-dependent transcriptional response to thermal stress. The coordinated upregulation of HSP70-1 and HSP90AA1 observed in the present study demonstrates the rapid activation of the cellular heat shock response in bovine mammary epithelial cells. Exposure to elevated temperatures stimulates the expression of these molecular chaperones as an immediate cytoprotective mechanism to preserve protein homeostasis, prevent protein misfolding and aggregation, and facilitate the refolding or degradation of damaged proteins. The marked increase in the expression of both genes observed in this study further emphasizes their importance in preserving mammary epithelial cell integrity, maintaining normal cellular function, and enhancing cell survival under heat stress conditions (Li *et al.*, 2015; Collier and Collier, 2012). To further investigate the molecular mechanisms underlying the heat stress response, we examined the expression profile of FOXO3a, a transcription factor known to regulate multiple cellular stress-response pathways. BMECs were subjected to the same heat treatment protocol used for the HSP genes (42°C for 1 h), and FOXO3a mRNA expression was subsequently quantified after recovery periods of 2, 4, and 8 h (Fig 3). Quantitative real-time PCR analysis revealed that FOXO3a expression increased following heat stress exposure. However, a statistically significant increase was detected only after 4 h of recovery ($P < 0.05$), whereas no significant differences were observed at the 2- or 8-h recovery periods relative to the control group ($P > 0.05$). These findings suggest that the transcriptional activation of FOXO3a occurs in a transient and tightly regulated manner during the cellular response to thermal stress. Fig 4 summarizes the relative mRNA expression profiles of all three genes during the post-heat stress recovery period. Overall, FOXO3a exhibited higher relative transcript

abundance than either HSP70-1 or HSP90AA1 throughout the experimental period. In contrast, among the two heat shock protein genes, HSP70-1 consistently displayed higher mRNA expression levels than HSP90AA1, indicating that HSP70-1 may constitute the primary molecular chaperone involved in the early adaptive response of bovine mammary epithelial cells to thermal stress. Although the functions of FOXO transcription factors in regulating oxidative stress, apoptosis, metabolism, and longevity are well established, their role in regulating heat shock protein expression during thermal stress remains poorly understood. However, growing evidence suggests that FOXO proteins functionally interact with HSP70 and HSP90 to coordinate cellular stress responses by reducing oxidative stress, maintaining protein homeostasis, and inhibiting apoptosis (Concannon *et al.*, 2003; Lehtinen *et al.*, 2006; Garrido *et al.*, 2006; Wadhwa *et al.*, 2010; Donovan *et al.*, 2016).

The increased expression of FOXO3a observed in the present study suggests that this transcription factor may participate in the regulation of the heat shock response in bovine mammary epithelial cells. One possible mechanism is that FOXO3a may interact with heat shock factor-mediated signaling pathways, or co-regulate HSP70-1 and HSP90AA1 transcription during recovery from thermal stress. This finding is consistent with previous reports demonstrating functional crosstalk between FOXO signaling and heat shock protein regulation across multiple species. In *Caenorhabditis elegans*, the FOXO ortholog DAF-16 is rapidly activated following heat shock in parallel with the induction of hsp-12.6, thereby enhancing the expression of stress-resistance genes and improving cellular survival (Furuhashi and Sakamoto, 2014). Similarly, studies in *Drosophila melanogaster* identified several heat shock protein genes as direct transcriptional targets of dFOXO, which binds to their promoter regions and undergoes rapid nuclear translocation in response to environmental stress, including heat stress (Donovan *et al.*, 2016; Uliczka *et al.*, 2024). In human cells, Grossi *et al.* (2018) proposed the existence of an HSF1-FOXO3a regulatory axis that coordinates cellular adaptation to stress by integrating heat shock signaling with FOXO-mediated transcriptional responses. Furthermore, Yoshihara *et al.* (2016) demonstrated that heat stress induces FOXO3a expression in mice through an HSP72-dependent signaling pathway, providing additional evidence of the close relationship between FOXO transcription factors and heat shock proteins. Taken together, these findings support the hypothesis that FOXO3a contributes to the cellular adaptation of bovine mammary epithelial cells to thermal stress. The observed expression patterns, together with previous findings from other model systems, suggest that FOXO3a may function as an important upstream regulator within the molecular network governing the heat stress response.

Conclusion

The present findings indicate that FOXO3a is responsive to heat stress and may play an important role in the adaptive response of bovine mammary epithelial cells to thermal challenge. The transient but significant upregulation of FOXO3a following heat stress, together with the concurrent induction of HSP70-1 and HSP90AA1, suggests that FOXO3a may participate in the molecular network regulating heat shock protein expression and cellular protection against thermal damage. Further experiments are needed to conclude whether FOXO3a is required for Hsps expression, approaches such as gene knockdown or overexpression and promoter-binding analyses will be valuable and precise to clarify the direct regulatory relationship between FOXO3a and heat shock protein genes in response to thermal stress.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank the National Science Foundation of China (Grant No. 31372290) for funding this project.

Author contributions

Tarig Mohammed BADRI, designing, conducting laboratory experiences, analysis, and writing. **Murtada ALSIDDIG**: designing, experience monitoring, revision, and writing. **Ahmed AbdelGadir Adam**: results assessment and manuscript revision. **Egbal Suliman Abu Shulukh**: Results assessment and manuscript revision. **Li Lian and Cai Yafei** experience monitoring, Revision and fund facilities

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