Heat Stress Reduces Milk Protein by Inducing Apoptosis in Dairy Cow Mammary Cells (Post-Print)

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Abstract

This study aimed to investigate the relationship between heat stress-induced reductions in milk protein content and yield and lactation-related hormones, milk protein synthesis-related signaling pathways, and mammary cell apoptosis, and to elucidate the underlying mechanisms for the decline in milk protein content and yield caused by heat stress. Four multiparous healthy Holstein dairy cows with similar days in milk, body weight, and milk yield were selected as experimental animals. A 2×2 crossover experimental design was adopted, comprising two periods of 18 days each (including a 9-day preliminary period and a 9-day experimental period), separated by a 30-day interval. The preliminary period was conducted under thermoneutral conditions with ad libitum feeding. During the experimental period, cows were randomly assigned to two groups (n=2), namely a heat stress group and a pair-fed group. The results demonstrated: 1) Heat stress extremely significantly decreased milk protein content and yield ($P<0.01$). 2) Heat stress exerted no significant effect on the tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway or -casein (CSN3) gene expression ($P>0.05$). 3) Heat stress significantly increased mTOR gene expression in the mammalian target of rapamycin (mTOR) signaling pathway ($P<0.05$), and tended to increase ribosomal protein S6 kinase (S6K1) gene expression ($0.05 P<0.10$). 4) Heat stress increased the expression of apoptosis-related cysteine protease 3 (CASP3) and cyclooxygenase-2 (COX2) genes in mammary cells ($P<0.05$), and tended to increase B-cell lymphoma-2-associated X protein (BAX) gene expression ($0.05 P<0.10$). Collectively, these findings indicate that heat stress may affect milk protein content and yield not by regulating the capacity of individual mammary cells to synthesize milk protein, but rather by inducing apoptosis of mammary cells, thereby reducing the number of mammary cells available for milk protein synthesis.

Full Text

Heat-Stress Decreases Milk Protein through Induction of Mammary Cell Apoptosis in Dairy Cows

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Abstract

This experiment was conducted to investigate the relationship among the decrease in milk protein content and yield during heat stress, lactation-related hormones, signaling pathways involved in milk protein synthesis, and mammary cell apoptosis, and to elucidate the underlying causes of milk protein decline induced by heat stress. Four healthy multiparous Holstein cows with similar days in milk [(101±10) d], body weight [(574±36) kg], and milk yield [(38.0±2.4) kg/d] were selected as experimental animals. A 2×2 crossover design was employed, consisting of two experimental periods, each lasting 18 days (with 9 days for pretrial and 9 days for trial), separated by a 30-day interval. During the pretrial period, cows were maintained in a thermoneutral environment with ad libitum feeding. In the trial period, cows were randomly divided into two groups (n=2): a heat stress group and a pair-restricted feeding group.

The results showed that: (1) Heat stress significantly decreased milk protein content and yield ($P<0.01$). (2) Heat stress had no significant effect on the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway or -casein (CSN3) gene expression ($P>0.05$). (3) In the mammalian target of rapamycin (mTOR) signaling pathway, heat stress significantly increased mTOR gene expression ($P<0.05$) and tended to increase ribosomal protein S6 kinase (S6K1) gene expression ($0.05 P<0.10$). (4) Heat stress increased the expression of apoptosis-related genes in mammary cells, including caspase-3 (CASP3) and cyclooxygenase-2 (COX2) ($P<0.05$), and tended to increase B-cell lymphoma-2-associated X protein (BAX) gene expression ($0.05 P<0.10$). In conclusion, heat stress may not affect milk protein content and yield by regulating the capacity of individual mammary cells to synthesize milk protein, but rather by inducing apoptosis of mammary cells, thereby reducing the number of mammary cells available for milk protein synthesis.

Keywords: heat stress; milk protein; JAK-STAT; mTOR; apoptosis

Introduction

Heat stress can reduce milk protein content in dairy cows, thereby decreasing milk quality during summer months [1-2]. Elucidating the direct causes of milk protein decline induced by heat stress is crucial for improving milk quality during summer heat stress periods. Under heat stress conditions, dairy cows adapt by reducing dry matter intake (DMI) and altering endocrine hormone balance [3]. Milk protein synthesis is regulated and driven by hormones [4], primarily including growth hormone (GH), prolactin (PRL), insulin (INS), and insulin-like growth factor-1 (IGF-1) [5]. These hormones regulate protein synthesis mainly through signaling pathways such as Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and mammalian target of rapamycin (mTOR), acting on the initiation and elongation stages of transcription and translation [5].

Heat stress can reduce cell viability and induce apoptosis [6-8]. Studies using in vitro cultured mammary epithelial cells have shown that high temperature inhibits normal cell growth and promotes apoptosis [7]. Additionally, heat stress upregulated the expression of the pro-apoptotic gene B-cell lymphoma-2-associated X protein (BAX) in mammary cells, while the anti-apoptotic gene B-cell lymphoma-2 (Bcl-2) was initially upregulated and then downregulated [9]. Furthermore, by establishing a pair-restricted feeding group with feed intake consistent with the heat stress group, researchers have excluded the effect of decreased DMI on milk protein content during heat stress and found that the reduction in DMI during heat stress could only explain part of the decline in milk protein content, with the remaining portion being directly caused by heat stress [10-12].

While numerous studies have reported the effects of heat stress on production performance and milk composition, and in vitro studies have demonstrated that heat stress promotes apoptosis in cultured mammary cells [7], it remains unclear whether heat stress affects milk protein synthesis through the JAK-STAT and mTOR pathways in live dairy cows or whether it promotes apoptosis in mammary cells in vivo. The objective of this study was to investigate the relationship among decreased milk protein content during heat stress, lactation-related hormones, signaling pathways involved in milk protein synthesis, and apoptosis in mammary tissue, and to exclude the indirect effects of DMI reduction during heat stress through restricted feeding of non-heat-stressed cows, thereby revealing the direct causes of milk protein decline induced by heat stress and providing a theoretical basis for alleviating this decline.

Materials and Methods

1.1 Experimental Design and Animal Management Four healthy multiparous Holstein cows with similar days in milk [(101±10) d], body weight [(574±36) kg], and milk yield [(38.0±2.4) kg/d] were selected and randomly assigned to four large animal environmental control chambers (kooland, Beijing

Kooland Technology Co., Ltd.; 4 m×3 m×2.5 m; temperature 15-40°C; relative humidity 25%-85%; light intensity 0-800 lx, continuously adjustable). A 2×2 crossover design was employed, with two experimental periods, each lasting 18 days (9 days pretrial and 9 days trial), separated by a 30-day interval. During the pretrial period, all four cows were maintained in a thermoneutral environment (temperature 20°C; relative humidity 55%; temperature-humidity index [THI] 65.5; 12 h light) with ad libitum feeding. In the trial period, cows were randomly divided into two groups (n=2): a heat stress (HS) group [temperature: 06:00-18:00, 36°C; 18:00-06:00 (next day), 32°C; relative humidity 55%; THI 84.5; 12 h light] and a pair-restricted feeding (PRF) group (temperature 20°C; relative humidity 55%; THI 65.5; 12 h light). The HS group was fed ad libitum with sufficient diet provided. The daily feed intake of the PRF group was maintained at the same percentage of their pretrial DMI average as the HS group' s DMI from the previous day (ranging between 26.8%-61.0%). In other words, the PRF group started and ended the trial and sample collection one day later than the HS group, ensuring that daily DMI remained consistent between groups throughout the trial period. THI settings were based on heat stress thresholds [13].

During the trial, cows were fed twice daily at 05:00 and 17:00, with milking conducted simultaneously. Milk yield was recorded for each cow. Residual feed was collected and weighed before morning feeding on day 2. All animals had free access to water. Diets were formulated according to NRC (2001) recommendations for dairy cattle [14] with a concentrate-to-forage ratio of 50:50. Diet composition and nutrient levels are shown in Table 1 , and diets were provided as total mixed rations (TMR). Metabolic chamber sanitation management followed standard procedures of the State Key Laboratory of Animal Nutrition.

1.2 Sample Collection During both pretrial and trial periods (with the PRF group starting one day later than the HS group), rectal temperature and respiration rate of each cow were recorded three times daily (07:00, 14:00, 22:00), along with chamber temperature and humidity. Milk samples (50 mL) were collected daily (25 mL each in the morning and evening), preserved with potassium dichromate (0.6 mg/mL), mixed thoroughly, and stored at 4°C. On days 2, 4, 6, and 8 of both periods, 10 mL of blood was collected from the tail artery/vein 2-3 h post-feeding. After standing for 30 min, blood was centrifuged at 1,500×g for 20 min at 4°C, and serum was collected in 1.5 mL EP tubes and stored at -20°C. Diet samples were collected on days 2, 4, 6, and 8, and residual feed samples were collected before morning feeding on day 2. On day 9 of the trial period, mammary tissue samples were collected in vivo using methods described in "Research Methods for Ruminant Nutrition" [15].

1.3 Measurements Diet and residual feed samples were dried at 60°C for 48 h to determine moisture content (GB/T 6435-1986, forced convection oven UFE400, Germany MEMMERT). Dried samples were ground through a 40-mesh sieve and stored at -20°C for subsequent analysis. Conventional feed analysis

was performed according to national standards: crude ash (GB/T 6438-1992, box-type resistance furnace SRJX-8-13, Tianjin Taisite Instrument Co., Ltd.), crude fat (GB/T 6433-2006, Soxtec™ AVANTI 2043, Denmark FOSS), and crude protein (GB/T 6432-1994, automatic Kjeldahl nitrogen analyzer FOSS KJELTEC 2300, Denmark). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to Van Soest et al. [16]. Approximately 0.5 g of sample was encapsulated in special filter bags for fiber analysis (College of Animal Science and Technology, China Agricultural University) and washed using an automatic fiber analyzer (A2000i, ANKOM, USA) with 2 drops of amylase added during washing.

Milk protein content was analyzed using infrared spectrophotometry (automatic milk composition analyzer Minor-78110, FOSS, Denmark). Serum GH, INS, and PRL concentrations were determined using kits from Nanjing Jiancheng Bioengineering Institute. Serum IGF-1 concentration was measured using a kit from Shanghai Enzyme-linked Biotechnology Co., Ltd.

1.4 Real-Time Quantitative PCR Analysis of Gene Expression

1.4.1 Total RNA Extraction from Cells Mammary tissue was ground in liquid nitrogen to extract total RNA. RNA purity and the ratio of absorbance at 260 and 280 nm (OD260 nm/OD280 nm) were detected using NanoDrop 1000. Values between 1.8-2.0 were considered suitable for reverse transcription. Concentration was adjusted to 500 ng/ L for subsequent reverse transcription.

1.4.2 Reverse Transcription Reverse transcription was performed using the Prime Script™ RT reagent Kit with gDNA Erase (TaKaRa, Japan). The reverse transcription system (10 L) contained: 5×Prime Script RT Master Mix 2 L, total RNA 1 L, and RNase-free water 7 L. Reaction conditions were: 37°C for 15 min, 85°C for 5 s, and storage at 4°C. The resulting cDNA was stored at -20°C.

1.4.3 Real-Time Quantitative PCR Using cDNA as template, real-time quantitative PCR was performed according to the SYBR Green instructions (TaKaRa). The ubiquitously expressed transcript (UXT) gene was used as an internal reference. All gene primers were synthesized by BGI, with primer sequences shown in Table 2 . Primer sequences for prolactin receptor (PRLR), insulin receptor (INSR), JAK2, STAT5B, ETS domain transcription factor 5 (ELF5), mTOR, eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1), eukaryotic translation initiation factor 4E-binding protein 2 (eIF4EBP2), ribosomal protein S6 kinase (S6K1), and UXT were obtained from Bionaz et al. [5]. Primer sequences for caspase-3 (CASP3) and cyclooxygenase-2 (COX2) were obtained from De Moraes et al. [17]. Primer sequences for fatty acid synthase (FAS), Bcl-2, BAX, and caspase-8 (CASP8) were obtained from Kamemori et al. [18]. Primer sequences for tumor necrosis factor receptor 1 (TNFR1) were obtained from Okuda et al. [19].

The reaction system (20 L) contained: 10 L SYBR® Premix Ex Taq™ II (2×), 0.8 L forward primer, 0.8 L reverse primer, 2 L cDNA, and 6.4 L RNase-free water. The reaction program consisted of: stage 1 at 95°C for 30 s; stage 2 for annealing and extension at 95°C for 5 s and 60°C for 34 s, for 40 cycles. The melting curve program was 55°C for 30 s for 41 cycles. Synthesized cDNA was stored at -20°C. Cycle threshold (Ct) values were automatically read using IQ5 real-time quantitative sequence detection software (Bio-Rad versa Doc, Bio-Rad, USA), and the 2-ΔΔCT method was used to analyze relative gene expression.

1.5 Statistical Analysis Average values of respiration rate, rectal temperature, milk protein content, milk protein yield, DMI, and other major nutrient intakes during the pretrial period of each period were used as covariates for the trial period. Variance analysis was performed using the Mixed model in SAS 9.3, and least squares means were used for mean comparisons. $P<0.01$ was considered highly significant, $P<0.05$ was considered significant, and $0.05 P<0.10$ was considered a tendency.

Results

2.1 Environmental THI and Rectal Temperature, Respiration Rate, and Feed Intake of Cows As shown in Table 3 , THI values were 82.4 and 64.6 for the HS and PRF groups, respectively, with the HS group being significantly higher ($P<0.01$). Compared with the PRF group, the HS group significantly increased respiration rate and rectal temperature ($P<0.01$). As shown in Figure 1 [Figure 1: see original paper], daily mean THI values during the trial period were above 72 for the HS group, while those during both pretrial and trial periods were below 72 for the PRF group. Table 3 and Figure 2 [Figure 2: see original paper] show that there were no significant differences in DMI and other major nutrient intakes between the HS and PRF groups ($P>0.05$).

2.2 Effects of Heat Stress on Milk Protein Content and Yield As shown in Table 4 , compared with the PRF group, the HS group significantly decreased milk protein content and yield ($P<0.01$).

2.3 Effects of Heat Stress on Blood Hormone Concentrations Related to Lactation As shown in Table 5 , there were no significant differences in blood GH, INS, PRL, and IGF-1 concentrations between the HS and PRF groups ($P>0.05$).

2.4 Effects of Heat Stress on Gene Expression in JAK-STAT and mTOR Signaling Pathways in Mammary Cells As shown in Table 6 , compared with the PRF group, the HS group significantly increased PRLR gene expression ($P<0.05$) but had no significant effect on INSR gene expression ($P>0.05$). Heat stress had no significant effects on JAK-STAT pathway genes (JAK2, STAT5B, ELF5) ($P>0.05$) but significantly affected mTOR pathway

genes (mTOR, eIF4EBP1, eIF4EBP2, S6K1). Specifically, the HS group significantly increased mTOR gene expression ($P<0.05$) and tended to increase S6K1 gene expression ($0.05 P<0.10$), while having no significant effects on eIF4EBP1 or eIF4EBP2 gene expression ($P>0.05$).

2.5 Effects of Heat Stress on Expression of Apoptosis-Related Genes in Mammary Cells As shown in Table 7 , compared with the PRF group, the HS group increased the expression of CASP3 ($P<0.05$), COX2 ($P<0.05$), and tended to increase BAX ($0.05 P<0.10$) in mammary cells, while having no significant effects on FAS, Bcl-2, TNFR1, or CASP8 gene expression ($P>0.05$).

Discussion

When THI exceeds 72, dairy cow performance begins to decline significantly, making 72 the established THI threshold for heat stress [13]. However, Zimbleman et al. [20] found that high-producing dairy cows begin to show heat stress responses when THI exceeds 68, with milk yield decreasing by 2.2 kg per 24 h at THI=68. In this study, the average THI during the trial period was 82.4 for the HS group, with daily mean THI values consistently above 72, while the PRF group remained below 68 during both pretrial and trial periods, satisfying the requirements for heat stress and non-heat-stress conditions. Compared with the PRF group, heat stress significantly increased respiration rate and rectal temperature. Collectively, these data confirm the successful establishment of heat stress and non-heat-stress models in this experiment.

Numerous studies have shown that THI above 72 causes decreased DMI [21-22] and reduced milk protein content [23], thereby decreasing milk quality. Further research has demonstrated that DMI reduction during heat stress can only explain part of the milk protein decline [10-12], with the remainder being directly caused by heat stress. In this study, both HS and PRF groups showed decreasing milk protein content and yield over time during heat stress, though the PRF group showed an increasing trend in milk protein content during the last two days of the trial period, possibly due to protein concentration following decreased milk yield. These data suggest that heat stress reduces milk protein through both DMI reduction and other direct pathways.

Milk protein synthesis is regulated and driven by hormones [4], primarily GH, PRL, INS, and IGF-1 [5]. GH plays an important role in promoting growth and lactation, mainly by regulating energy balance and increasing protein synthesis [24]. PRL primarily promotes mammary gland development and initiates and maintains lactation. INS, a protein hormone secreted by pancreatic -cells, is the only hormone that lowers blood glucose while promoting glycogen, fat, and protein synthesis. Studies have shown that increasing blood INS concentration significantly increases mammary blood flow and milk yield [25-26]. IGF-1 not only acts as an intermediary for GH function but also promotes mammary epithelial cell proliferation and alveolar formation through its receptor-mediated signaling pathway [27].

Current reports on the effects of heat stress on lactation-related hormones are inconsistent. Song et al. [28] found that serum INS concentration tended to decrease during heat stress in early and mid-lactation cows, consistent with Herbein et al. [29], while Itoh et al. [30] reported that heat stress significantly increased blood INS concentration in lactating cows. Song et al. [28] also found that serum PRL concentration was lower during heat stress than during non-heat-stress periods in early, mid, and late lactation, whereas Zhu et al. [31] reported opposite results. Studies on GH effects are generally consistent, showing that heat stress decreases or tends to decrease blood GH concentration [11,32-33]. Reports on IGF-1 are limited, with Rhoads et al. [11] suggesting heat stress decreases IGF-1 concentration, while McGuire et al. [33] found no significant effect. In this study, heat stress had no significant effects on blood GH, INS, PRL, or IGF-1 concentrations compared with the PRF group, possibly due to the small number of experimental animals.

Milk protein gene expression involves DNA transcription and mRNA translation. Studies have shown that PRL, INS, GH, glucocorticoids, and IGF-1 affect lactation directly or indirectly, primarily through JAK-STAT and mTOR signaling pathways that regulate protein synthesis by acting on transcription and translation initiation and elongation stages [5]. When PRL or GH binds to its receptor, receptor dimerization occurs, bringing receptor-coupled JAKs into proximity and activating them through reciprocal tyrosine phosphorylation. Activated JAKs then catalyze tyrosine phosphorylation of the receptor, creating "docking sites" that recruit STAT5 proteins. Finally, JAKs catalyze phosphorylation of receptor-bound STAT5, and activated STAT5 dimers enter the nucleus to bind -interferon activation sequence (GAS) sites, inducing transcription of ELF5, SOCS1, SOCS2, and milk protein genes, with ELF5 playing an important role in regulating STAT5 activity in mammary tissue [34-36]. In the mTOR pathway, the primary complex is mTOR complex 1 (mTORc1), which phosphorylates specific sites on eIF4EBP, causing dissociation from eukaryotic translation initiation factor (eIF) 4E and allowing eIF4E to bind with eIF4G and eIF4A to form the eIF4E • eIF4G • eIF4A complex. This complex is important for translating mRNAs with 5' -untranslated region (5' -UTR) secondary structures [37], as eIF4A can unwind these structures, facilitating scanning and enabling ribosomes to rapidly locate the mRNA start codon, thereby promoting translation. mTORc1 also mediates S6K1 phosphorylation, which can phosphorylate ribosomal protein S6 (rpS6), eIF4B, SKAR (S6K1 Aly/REF-like target), and eukaryotic elongation factor 2 (eEF2) kinase, affecting mRNA translation initiation and elongation [38].

The effects of heat stress on JAK-STAT and mTOR pathways in dairy cow mammary cells have not been previously reported. Yoshihara et al. [39] found that heat stress had no significant effects on mTOR and eIF4EBP1 gene expression in mice. Studies on human muscle cells showed that heat stress increased mTOR and S6K1 phosphorylation while decreasing eIF4EBP1 phosphorylation, though eIF4EBP1 phosphorylation returned to pre-heat-stress levels after 1 h [40]. In this study, heat stress had no significant effects on JAK-STAT pathway gene

expression but significantly increased mTOR gene expression and tended to increase S6K1 gene expression. Heat stress had no significant effect on CSN3 gene expression in this study, whereas Hu et al. [9] found that heat stress decreased S1-casein and -casein gene expression in in vitro cultured bovine mammary epithelial cells. These differences may be due to variations between in vivo and in vitro systems and differences in heat stress severity (42°C in Hu et al. [9] vs. average rectal temperature of 40.0°C in this study). Collectively, these data suggest that heat stress may not affect milk protein synthesis through JAK-STAT and mTOR pathways, and CSN3 gene expression may not be significantly affected by heat stress.

Numerous studies have shown that heat stress reduces cell viability and induces apoptosis [6-8]. Using in vitro cultured mammary cells, Zhou et al. [7] found that high temperature inhibited normal growth and promoted apoptosis, while Hu et al. [9] found that heat stress upregulated pro-apoptotic gene BAX expression, while anti-apoptotic gene Bcl-2 was initially upregulated then downregulated. Due to limitations in directly measuring apoptosis in mammary tissue from live cows, this study selected several apoptosis-related genes and used real-time quantitative PCR to analyze their expression to understand heat stress effects on mammary cell apoptosis. The results showed that heat stress significantly increased expression of apoptosis-related genes CASP3 and COX2 in mammary cells, with a tendency to increase BAX expression, while anti-apoptotic gene Bcl-2 expression was unaffected, suggesting that heat stress may reduce mammary cell viability and promote apoptosis in live mammary tissue.

In conclusion, compared with the PRF group, heat stress decreased milk protein content and yield without decreasing expression of genes in milk protein synthesis-related signaling pathways (JAK-STAT and mTOR), suggesting that heat stress may not affect the capacity of mammary cells to synthesize milk protein. Consistent with in vitro studies [9], heat stress increased expression of apoptosis-related genes (CASP3, COX2, BAX) in live mammary cells, indicating that heat stress may induce apoptosis in live mammary cells. Taken together, heat stress may not affect milk protein content and yield by regulating the capacity of individual mammary cells to synthesize milk protein, but rather by inducing apoptosis of mammary cells, thereby reducing the number of mammary cells available for milk protein synthesis.

However, due to current technical limitations in detecting apoptosis in mammary tissue from live cows, we cannot fully confirm that heat stress induces apoptosis in live mammary cells. Further improvements in research technology are needed to investigate the causes of milk protein decline during heat stress and provide a theoretical basis for developing measures to alleviate this decline.

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Note: Figure translations are in progress. See original paper for figures.