Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.002

RESEARCH ARTICLE

Heat Stress Negatively Influence Mammary Blood Flow, Mammary Uptake of Amino Acids and Milk Amino Acids Profile of Lactating Holstein Dairy Cows

Shuangming Yue[1], Jing Qian[1], Jianguo Du[1], Xiaowan Liu[1], Hui Xu[1], Haoxiang Liu[1], Jingjing Zhang[1], and Xiaochun Chen[2]*

[1]Department of Bioengineering, Sichuan Water Conservancy Vocational College, Chengdu 611231, China
[2]Institute of Animal Science, Chengdu Agricultural College, Chengdu 611130, China
*Corresponding author: chenxiaochun163@163.com

ARTICLE HISTORY (22-277)

Received: August 12, 2022
Revised: November 26, 2022
Accepted: December 07, 2022
Published online: January 11, 2023

Key words:
Amino acids
Dairy cows
Heat stress
Milk protein synthesis
Udder

ABSTRACT

The current study was planned to evaluate the effects of heat stress (HS) on mammary blood flow and the profile of circulating amino acids (AA) in blood and milk of lactating Holstein dairy cows. For this purpose, twenty dairy cows with similar body conditions, parity, and milk production were divided into two treatments of 10 animals each. The first treatment group was exposed to HS and reared in summer season, and second treatment group were reared in thermoneutral environment (TN) during spring season. Results showed that HS resulted in high level of total AA (TAA), glycine, serine (Ser), alanine ($P<0.05$) predominantly while decrease concentrations of methionine (Met) and tryptophan (Trp) predominantly ($P<0.05$) in external pudic artery and mammary vein of experimental cows. Methionine, arginine (Arg), Trp and threonine (Thr) ($P<0.05$) supply to the mammary gland by pudic artery and lower amount of Met and Trp in mammary vain output ($P<0.05$) was observed in HS cows. Similarly, the uptake of most AA in the udder was decreased due to HS ($P<0.05$) and resulted in a lower ($P<0.05$) amount of TAA, glutamine, phenylalanine, Met, lysine, leucine, tyrosine, isoleucine, Ser, Thr, valine, and histidine in milk. A highly significant decrease ($P<0.01$) in amount of Arg and Trp in milk was observed in HS cows. Therefore, it is concluded that during HS blood AA use in dairy animals is shifted away from milk protein synthesis by lower mammary blood flow and AA uptake by mammary gland.

To Cite This Article: Yue S, Qian J, Du J, Liu X, Xu H, Liu H, Zhang J, Chen X. 2023. Heat stress negatively influence mammary blood flow, mammary uptake of amino acids and milk amino acids profile of lactating holstein dairy cows. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2023.002

INTRODUCTION

In summer, high temperature exerts heat stress in dairy cattle and lead to huge losses on commercial dairy farms especially in term of milk yield (Yue *et al.*, 2020). Heat stress not only decrease milk yield but also affect the components and the quality of milk (Berman *et al.*, 1985; Yue *et al.*, 2020).

In the bovine mammary glands, amino acids (AA) of blood are known to be the primary substrates for the synthesis of milk protein. The blood circulating AA can easily be changed significantly under catabolic conditions (Mantha *et al.*, 2018). It has been reported that during heat stress the level of blood AA, alanine (Ala), glutamine (Glu), aspartic acid (Asp), and glycine (Gly) known for gluconeogenesis, increase whereas the level of Lys, known for milk protein synthesis, decrease in lactating dairy cows (Guo *et al.*, 2018). Furthermore, milk synthesis is also dependent on substrate level in the blood and the rate of mammary blood flow (MBF) and lower rate of MBF is also known reduce rate of milk synthesis (Ardalan *et al.*, 2022). It has been reported that rate of MBF could be altered by many factors, such as heat stress, fasting, milking intervals, nutrient infusion (Cai *et al.*, 2018; Davis and Collier, 1985). A recent study has demonstrated that the distribution of blood flow in the cardiovascular system was affected by heat stress and the portal plasma flow has a 14% reduction in heat-stressed cows (Ríus, 2019). Literature had provided the evidence that MBF decreased in the heat stressed animals (Tao *et al.*, 2020). However, few experiments have been executed to study the effect of HS on MBF and the profile of circulating AA in lactating dairy cows. Therefore, the objective of the present study was to evaluate the direct effect of HS on mammary gland blood flow, blood amino acid profile, mammary uptake of AA and other physiological measurements in Holstein dairy cows.

MATERIALS AND METHODS

Experimental animals, design and treatments: The experimental protocol and design has already been described in our published paper (Yue *et al.*, 2020). Briefly, twenty Holstein dairy cows with similar parity and milk production were divided into two treatments of 10 animals each. The first treatment group was exposed to heat stress (HS) and reared in summer season and second treatment group were reared in thermoneutral environment (TN) during spring season. The data of HS group was obtained from mid-July to late August in 2018 (THI was increased from 72.5 to 86.9 over 1 month and remained stable at 80 for 1 week). The data of TN group was obtained in mid-March to late April in 2018 (THI was from 52 to 65 over 1 month). Diets were prepared according to the recommended nutrients basis as described in the previous studies (Chen *et al.*, 2021; Qiu *et al.*, 2018). All managemental practice were ideal as described in the previous studies (Imran *et al.*, 2021; Riaz *et al.*, 2021).

Measurements, sampling, and analyses: All cows in each period were used for estimating mammary blood flow, using the method as reported in the previous study (Pacheco-Rios *et al.*, 2001). Simply, on the last four days of each period, cows were fitted with catheters in right external pudic artery and the mammary veins. Cows were tranquilized and prior catheterization a local subcutaneous anesthetic was injected. Animals were ensured to stand for at least 10 min prior to blood sampling and blood samples were collected from the right external pudic artery and the mammary vein for each of the animals over a 12-h period (from 0800 h to 2000 h). Blood samples were collected every 2 h as 1-h integrated samples (i.e. Continuous sampling for 1 h, no sampling for 1 h) at a rate of 1 mL/min, into plastic centrifuge tubes containing EDTA and maintained in ice.

The amino acid profile of whole blood was analyzed according to the operation manual by using a Hitachi amino-acid analyzer L-8800 (Tokyo, Japan).

Calculations: The MBF (liters per hour) was determined by the following formula Arteriovenous difference (AVD) = arterial-venous

$$\text{MBF (L/d)} = \frac{0.965 \times \text{AA output in milk (g/d)}}{\text{A}-\text{V of AA(g/L blood)}}$$

The supply of amino acid in pudic artery (g/d)=the level of amino acid in pudic artery × MBF

The output of amino acid in mammary vein

(g/d)= the level of amino acid in mammary vein × MBF

Mammary uptake (g/d)=AVD (g/L) × MBF (L/d)

Amino acid output in milk = amino acid (%) × milk yield (kg)

AAD: The differences between AA entrapped in udder and AA secreted in milk, calculated as: ADD = amino acid entrapped in udder - amino acid secreted in milk

Statistical analysis: The experimental data of MBF, blood to milk ratios, AA entrapped in udder, and surplus rate of AA in udder are reported as means with SEM. Other data is represented as means with standard deviation. Differences between experimental groups are analyzed by the independent two-sample t-test using the SPSS 17.0 and significance was considered at $P<0.05$.

RESULTS

Blood amino acid profile of mammary vein and pudic artery: Results of blood amino acid profile of mammary vein and pudic artery represents that HS influenced some AA in mammary vein (Fig. 1). Heat stress resulted in high level of total AA, urea, Gly, Ala, leucine (Leu), isoleucine (Ile), serine (Ser) and threonine (Thr) ($P<0.05$) in external pudic artery of dairy cows. However, the concentrations of methionine (Met), lysine (Lys) and tryptophan (Trp) ($P<0.05$) were decreased due to HS in external pudic artery of dairy cows. Moreover, no difference was observed in Glu, Glu-NH_2, phenylalanine (Phe), arginine (Arg), tyrosine (Tyr), valine (Val) and histidine (His) concentrations in external pudic artery of dairy cow due to HS. Results of blood amino acid profile of mammary vein represents that HS influenced some AA in mammary vein (Fig. 1). The concentrations of TAA, Ala, Gly and Ser were higher in mammary vein of heat-stressed cows than cows in TN dairy animals ($P<0.05$). A significantly decreased concentration of Met, and Trp has been observed in mammary vein of heat-stressed cows than cows in the TN animals ($P<0.01$). Furthermore, no difference was observed in urea Glu, Phe, Asp, Arg, Lys, Tyr, Val, Leu, Ile, and His concentrations in mammary vein of dairy cows between TN and HS group.

Mammary blood flow: Mammary blood flow (liters per minute) was affected by HS (Table 1). The MBF obtained from A-V differences of labeled AA (Phe and Tyr) were low ($P<0.05$) in HS animals than in TN animals.

Supply of amino acid in external pudic artery and output of amino acid in mammary vein: The pudic artery amino acid supply g/d and mammary vein amino acid output g/d in TN and HS group is presented in Fig. 2. Results showed that the amount (g/d) of some AA supply to the mammary gland was affected by HS. The supply of Met, Arg, Trp and Thr ($P<0.05$) to the mammary gland by pudic artery was lower in HS cows while the amount of urea and Ser supply was higher in the mammary gland by pudic artery was higher in HS cows ($P<0.05$). No difference was observed in the amount of Asp, Phe, Gly, Ala, Glu, Leu, Ile, Lys, Val and His supply to the mammary gland of HS and TN group cows by pudic artery. Compared with the cows of TN group, HS cows had a lower amount of Met and Trp in mammary vain output ($P<0.05$). There was a higher amount of Ser, His, Leu and Lys in mammary vein output of the cows in HS group ($P<0.05$).

Uptake and uptake rate of amino acid by udder: Amino acids uptake by mammary gland of experimental dairy cows was significantly affected by HS (Fig. 3). The uptake of most AA (TAA, Ala, Arg, Met, Gly, Lys, Leu, Ile, Trp, Thr and Val) in the udder was significantly decreased due to HS ($P<0.05$), while the uptake of Glu, and Ser in the udder was increased due to HS. His, Phe, Tyr and Asp were not affected by HS. However, there was no effect on the amino acid uptake rate between the two treatments.

Milk amino acid profile: Amino acids profile secreted in milk is presented in Fig. 4. Results of AA secreted in milk showed that HS group had a lower ($P<0.05$) amount of TAA,

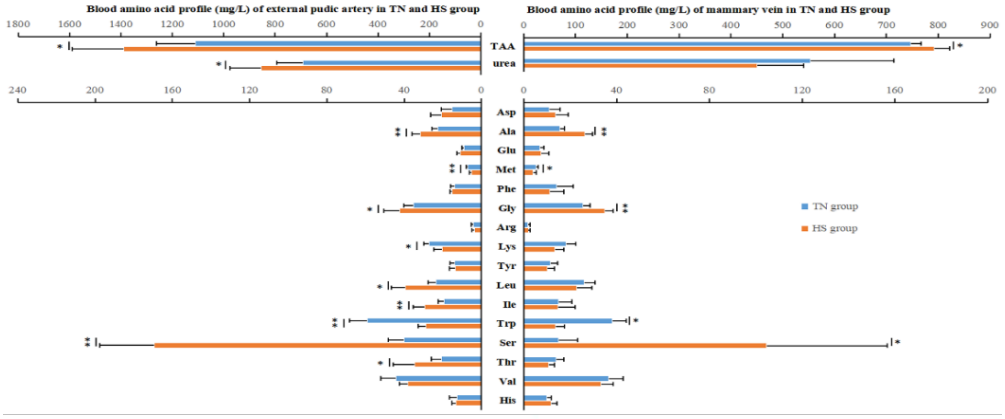

Fig. 1: The effect of HS on blood amino acid profile (mg/l) of external pudic artery and mammary vein. Data are mean ± sd, n = 10. *p<0.05, **p<0.01. taa=total amino acid.

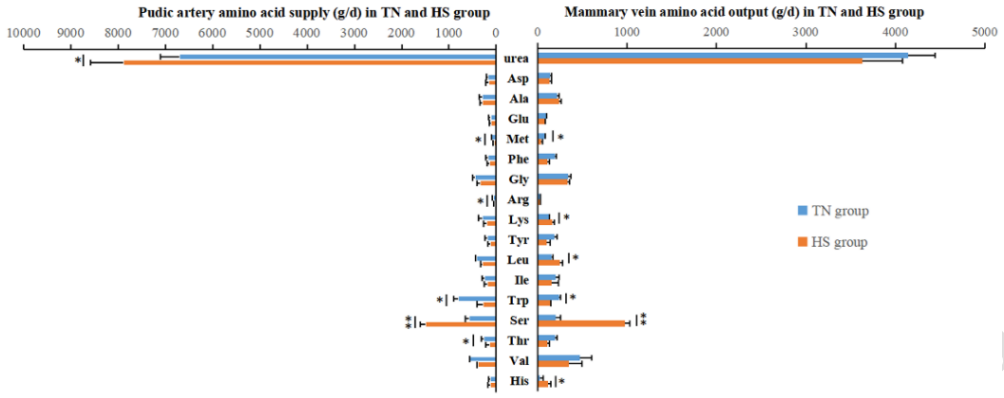

Fig. 2: The effect of HS on pudic artery amino acid supply (g/d) and mammary vein amino acid output (g/d). Data are mean ± SD, n = 10. *P<0.05, **P<0.01. TAA=Total amino acid.

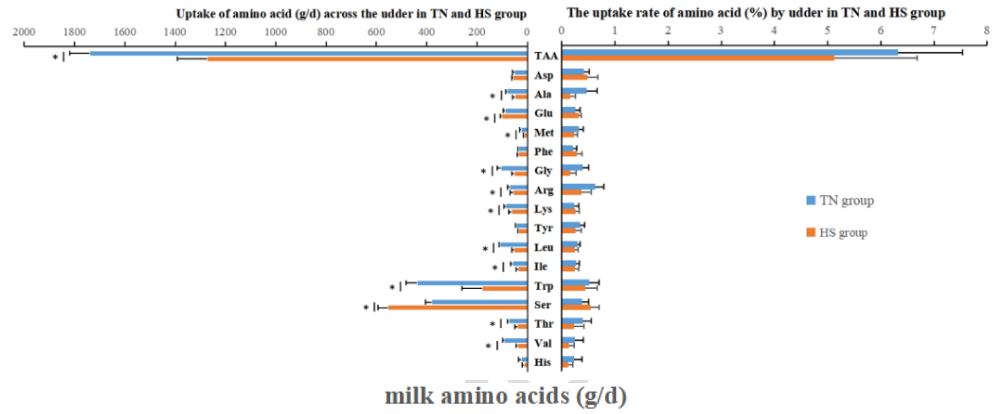

Fig. 3: The effect of HS on uptake and uptake rate of amino acid (g/d) across the udder. Data are mean ± SD, n = 10. *P<0.05, **P<0.01. TAA=Total amino acid

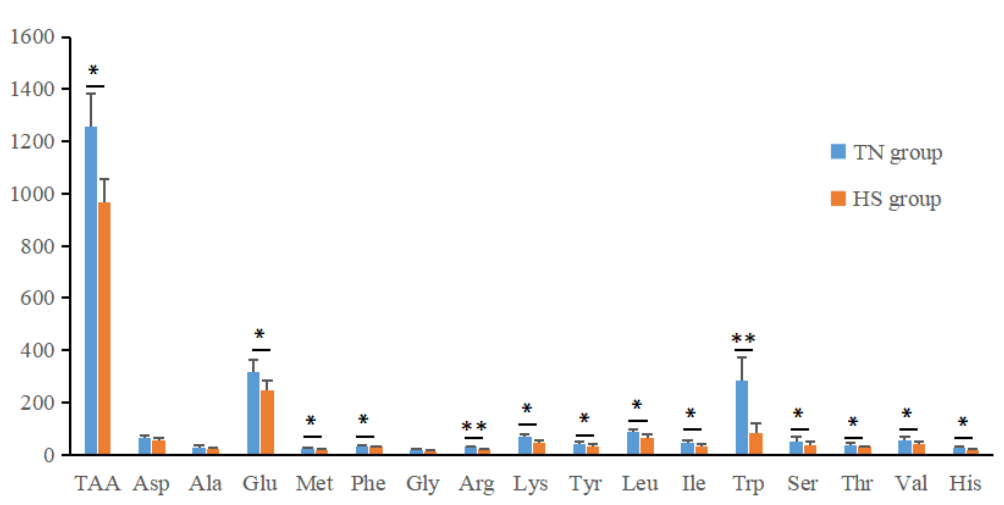

Fig. 4: The effect of HS on milk amino acid (g/d). Data are mean ± SD, n = 10. *P<0.05, **P<0.01. TAA=Total amino acid.

Glu, Met, Phe, Lys, Tyr, Leu, Ile, Ser, Thr, Val, and His in milk. A highly significant decrease (P<0.01) in amount of Arg and Trp was observed in heat-stressed cows. Moreover, no differences were observed in the amount of Asp, Ala, and Gly between the cows in the HS and TN groups.

The difference in amino acids entrapped in udder and secreted in milk: Heat stress significantly affected the AAD (Table 2). The AAD of TAA, Ala, Met, Gly, Leu, Ile, Trp, Thr, Val and His was lower in HS dairy cows (P<0.05). An increased AAD of Asp, Lys, and Ser was seen

in heat-stressed cows (P<0.05). However, HS did not affect the AAD of Arg, Phe and Tyr (P>0.05).

Surplus rate of amino acids in udder between amino acid input and amino acid output: Table 3 showed that the surplus rate of AA in udder between AA uptake and AA output of the cows in the TN and HS group. Heat stress reduced the surplus rate of Leu, Met, Thr, Val and His (P<0.05). The surplus rate of Asp, Glu, Lys, and Trp was elevated by HS (P<0.05). However, no significant differences were observed in the surplus of TAA, Ala, Phe, Gly, Arg, Tyr, Ile and Ser between the animals in the TN and HS group.

Table 1: The effects of HS on Mammary blood flow and blood to milk ratios.

Items	Treatment		SEM	P-value
	TN	HS		
Blood flow (L/d)	12350.5	7805.1	1815.51	0.026
[1]Blood to milk ratio	314.5	289.4	10	0.150

[1]Blood to milk ratio: The ratio of blood flow through the udder (L/d) to milk production (kg/d) per day.

Table 2: The effects of HS on amino acids entrapped in udder.

Amino acid	AAD[1]		SEM	P-value
	TN	HS		
TAA[2]	481.3	304.5	12.51	0.035
Asp	-11.9	4.4	4.31	<0.01
Ala	52.3	26.3	3.81	0.023
Glu	-274.3	-143.4	12.42	<0.01
Met	0.3	-5.8	5.32	0.035
Phe	1.4	1.9	0.52	0.067
Gly	88.3	37.9	1.61	0.023
Arg	40.8	35.5	2.11	0.067
Lys	14.5	20.9	2.53	0.035
Tyr	2.1	2.8	0.52	0.078
Leu	21.3	-10.5	1.21	0.021
Ile	15.3	4.6	0.32	<0.01
Trp	155.6	83.2	5.61	<0.01
Ser	330.3	516.3	8.22	<0.01
Thr	35.4	10.8	1.62	0.024
Val	50.2	-3.5	0.3	<0.01
His	0.2	-4	0.2	<0.01

[1]AAD: The differences between amino acids entrapped in udder and amino acids secreted in milk, calculated as: AAD = amino acid entrapped in udder - amino acid secreted in milk: [2]TAA=Total amino acid.

Table 3: The effects of HS on surplus rate of amino acids in udder between amino acid input and amino acid output.

Items	Treatment		SEM	P-value
	TN	HS		
TAA[1]	0.28	0.24	0.02	0.854
Asp	-0.23	0.08	0.06	<0.01
Ala	0.64	0.53	0.03	0.064
Glu	-3.13	-1.39	0.73	<0.01
Met	0.01	-0.47	0.04	<0.01
Phe	0.04	0.06	0.01	0.069
Gly	0.83	0.73	0.07	0.068
Arg	0.56	0.62	0.09	0.078
Lys	0.17	0.32	0.03	0.032
Tyr	0.05	0.07	0.04	0.067
Leu	0.21	-0.18	0.06	<0.01
Ile	0.26	0.12	0.07	0.056
Trp	0.36	0.54	0.06	0.045
Ser	0.87	0.93	0.05	0.082
Thr	0.49	0.27	0.06	0.031
Val	0.55	-0.09	0.04	<0.01
His	0.01	-0.29	0.05	<0.01

The surplus rate of amino acids (SRAA) in udder was calculated as SRAA = AAD/uptake, where AAD is the difference between amino acids entrapped in the udder and amino acids secreted in milk, and uptake is the amino acids entrapped in the udder: [1]TAA=Total amino acid.

DISCUSSION

Increase in temperature along with diet along with diet alteration have impact on cattle physiology, welfare, health, and reproduction (Li *et al.*, 2014; Qiu *et al.*, 2020; Qiu *et al.*, 2021). The rate of MBF was lower in HS animals in the current study which is similar with the findings of the previous studies of severely heat-stressed animals (Davis and Collier, 1985; Guo *et al.*, 2018; Tao *et al.*, 2020). It has been reported that lower feed intake, milk frequency and perfusion of nutrients (Ardalan *et al.*, 2022; Cai *et al.*, 2018; Yue *et al.*, 2020) reduce the MBF and published results of current study explored that dry matter intake in animals was decreased (Yue *et al.*, 2020) in HS dairy cows that could be the reason of low MBF in the current study. It has been reported in previous studies that the reduction of milk protein is due to HS that is related to limited supply of precursor of milk synthesis as a result of reduce MBF (Berman *et al.*, 1985; Guo *et al.*, 2018; Ríus, 2019; Tao *et al.*, 2020).

Methionine, Phe (+Tyr), Trp, which are uptaken by mammary gland are almost totally secreted into milk in the form of milk protein while Leu, Lys, Arg, Ile, Val and Thr, rate of uptake to output (U:O) is greater (Cai *et al.*, 2020; Davis and Collier, 1985; Guo *et al.*, 2018). The uptake to output of the AA in TN animals were consistent with previous studies (Doepel and Lapierre, 2011; Mabjeesh *et al.*, 2002). However, HS affected the U:O of the AA such as Met, Lys, Arg, Leu, Ile in HS cows. The U:O of Ala, Gly, Glu and Val was enhanced by HS. In the current study, higher level of Thr, Ser, Gly, and Ala were detected in the whole blood of the external pudic artery in animals reared in HS group. These findings are similar with other studies, and they reported that higher level of AA (Glu, Gly, Asp, Ser, and Val) for gluconeogenesis in animals suffered from heat-stresses and starvation respectively (Cai *et al.*, 2018; Guo *et al.*, 2018; Ríus, 2019; Verbeke and Peeters, 1965). It has been reported that in nutrient deficiency, the liver reinforces the catabolism of amino acid, and the concentration of amino acid increased in blood (Cai *et al.*, 2020; Cai *et al.*, 2018; Stahel *et al.*, 2014). In the current study, HS may reduce the nutrient intake and resulted in higher concentration of Thr, Ser, Gly, and Ala in blood of the external pudic artery. It is well documented that lower plasma glucose level in HS animals enhance the use of AA for gluconeogenesis (Cowley *et al.*, 2015; Nie *et al.*, 2018).

Alanine is synthesized in muscle by transamination of pyruvate and then released into the bloodstream. However, in case of lower intake, Ala hampers the activity of pyruvate kinase enzymes and enforce the regulation of gluconeogenesis and glycolysis (Hedges and Ryan, 2019). Therefore, higher plasma Ala level in the current study is also a indicator of insufficient cellular energy substrates for HS animals.

Lysine has often been identified as a first or second-limiting AA in many dairy rations (Cheng *et al.*, 2021) and Lys deletion decreased both milk and milk protein yields (Cheng *et al.*, 2021). It has also been reported that both the α- or ε-N of Lys is used for the mammary synthesis of Ala, Asp, Glu, and Ser (Lapierre *et al.*, 2009; Yoder *et al.*, 2020).

In the current study, HS reduced the uptake of Lys of mammary gland leading to reduce milk yield as reported in

previous study (Cheng *et al.*, 2021). However, we observed that the uptake of Ala, Gly, Glu, and Ser was increased in heat-stressed cows, and it has been reported that Lys is used for the mammary synthesis of Ala, Asp, Glu, and Ser (Lapierre *et al.*, 2009; Yoder *et al.*, 2020) and therefore, it implied that part of Lys could be used to synthesis non-essential AA to satisfy milk synthesis under HS.

It has been reported that in the enterochromaffin cells of the gut, 5-Hydroxytryptophan(5-HT) is synthesized from Trp and released into the blood (Gostner *et al.*, 2020) and during HS 5-HT is known to increase in rectal temperature in cattle (Sutoh *et al.*, 2018). It could be speculated that under HS, the absorbed tryptophan is primarily metabolized as 5-HT, which is involved in temperature regulation rather than milk synthesis. Leucine not stop protein degradation (Duan *et al.*, 2016) muscle protein but also stimulate the synthesis (Li *et al.*, 2007). We found that the uptake of Leu and the U:O of Leu was decreased by HS which could be one of the reasons for decreased milk protein synthesis in HS animals.

One of the main uses of Arg by the mammary gland is for conversion to ornithine and nitric oxide and the metabolic end-products of the conversion of Arg to ornithine are putrescine, spermidine, and spermine (Corl *et al.*, 2008; Doepel and Lapierre, 2011). It has been reported that Putrescine has positive effects on cell proliferation and inhibition of the Arg-ornithine pathway (via inhibition of arginase) reduced mammary cell proliferation (Wang *et al.*, 2017). In this study, we observed that HS resulted in the decline of absorption of Arg by mammary gland, which may depress the pathway of Arg-ornithine pathway and then reduce mammary cell proliferation.

It is known that milk AA are the most essential source of AA in humans. Consistent with our previous study (unpublished) and other reports (Tian *et al.*, 2016), we found that the U:O and level of the most of AA were reduced in heat-stressed cows. These AA may produce glucose through gluconeogenesis or energy by deamination and oxidation, which may reduce their distribution into milk via the mammary gland. Furthermore, AA in cells participate in a variety of metabolic reactions, producing carbon dioxide, urea, polyamines and non-essential AA. The increase of blood urea concentration in heat-stressed cows indicated that more AA were metabolized into urea rather than milk protein in mammary gland cells. Therefore, HS may affect not only the content of milk protein, but also the quality of milk protein.

Conclusion: In heat stress dairy cows, the mechanism of self-regulation of blood flow for thermoregulation and the mammary microvascular system are associated with the decreased mammary blood flow. Based on the results of the current study, it is concluded that during heat stress blood amino acids use in dairy animals is shifted away from milk protein synthesis by lower mammary blood flow and amino acid uptake by mammary gland. The present study may provide scientific experimental basis to reduce the negative effects of heat stress on blood flow and nutrient supply to mammary glands in dairy animals.

Acknowledgements: Authors acknowledge the help of undergraduate students for help during this experiment.

Authors Contribution: SY and JQ conceived and designed the experiment. JD, XL and HX carried out the manamgent the cows. SY and HL performed invivo experiments and amino acid analysis. JZ, XC and SY performed statistcal analysis. All authors reviewed the manuscript.

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