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RESEARCH ARTICLE

Effects of Traditional Chinese Medicines on the Milk Performance, Antioxidant Capacity and Immune Status of Dairy Cattle

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ABSTRACT

To study the effects of Traditional Chinese medicine (TCM) on milk performance, antioxidant capacity and immunity of dairy cattle, twenty healthy lactating Holstein cows were randomly divided into four groups (A, B, C and D) and supplemented with 0, 50, 100 and 150 g/d of TCM, respectively. The temperature-humidity index (THI) and milk yield were recorded. The mRNA expression of GSH-Px, CAT, Bcl-2 and p53 genes was detected using RT-PCR. Meanwhile, the apoptosis rate of lymphocytes was determined through flow-cytometry. The results showed that the THI exceeded 72 during all experimental periods. Compared to group A (10.66±0.75 Kg), the milk yields of the cattle in group B, C and D were increased by 20.26 ($P<0.05$), 24.20 ($P<0.05$) and 16.98% ($P>0.05$), respectively. The somatic cell count in three treatment groups was lower than those in group A by 21.68 ($P<0.05$), 29.88 ($P<0.05$) and 24.22% ($P<0.05$), in group B, C and D respectively. The mean expression of GSH-Px and CAT genes were increased in groups B, C and D. The apoptosis rates of lymphocytes in the three treatment groups on the 40th day were lower than that of the control group by 27.45 ($P<0.01$), 27.36 ($P<0.01$) and 24.55% ($P<0.01$) in group B, C and D, respectively. The mean expression of Bcl-2 gene in groups B, C and D was increased, and the mean expression of p53 gene was decreased in three treatment groups. Thus, it is concluded that TCM can improve milk performance, antioxidant capacity and immune status of dairy cattle under heat stress, which can help dairy cattle to resist heat stress in hot seasons.

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INTRODUCTION

The dairy industry is an important part of the economy; however, it continues to suffer severe economic losses due to environmental factors such as the high ambient temperature and humidity (Carabano *et al*., 2016). In fact, heat stress is detrimental to dairy production and causes an increase in body temperature, a decrease in food intake and milk production (West, 2003, Strong *et al*., 2015). In a previous study, the temperature-humidity index (THI) was used to estimate the degree of heat stress in dairy cattle (Dash *et al*., 2016). When the THI exceeds 72, there are many physiological changes in the cattle. One of the prominent changes under heat stress is oxidant stress (Cheng *et al*., 2016). The body performs its antioxidant functions through enzymes glutathione peroxidase (GSH-Px) and catalase (CAT). They can protect cells when faced with oxidative damage caused by free radicals (Wu *et al*., 2016) Therefore, the mRNA expression level of the GSH-Px and CAT genes could be influenced by heat stress (Liu *et al*., 2016). Another change that occurs during heat stress is the reduction of immune function. A previous study has demonstrated that heat stress can reduce immune function because heat stress stimulates the release of corticosterone and catecholamines and initiates lipid peroxidation in

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lymphocyte membranes (Ma *et al*., 2005). Meanwhile, heat stress leads to cell damage through reactive oxygen species, which results in cell death and apoptosis of lymphocytes (Liu *et al*., 2016). Apoptosis is a complex process and p53 can activate cell apoptosis by the intrinsic pathway which triggered by proapoptotic members of Bcl-2. Thus, the expression of Bcl-2 increases when the temperature rises (Alvarez *et al*., 2006; Song *et al*., 2016).

Traditional Chinese medicine (TCM) prescriptions are formed by combinations of Chinese herbs according to the prescription-composing principles (Song *et al*., 2017). TCM comes from different parts of perennial herbs, which have heat stress- relieving activities and immune-enhancing effects for human and animals (Song *et al*., 2014, Liu *et al*., 2015). In addition, TCM will not cause excessive residues or toxicity because of their natural origin (Guo *et al*., 2011). Therefore, TCM has become popular as a feed additive in recent years (Song *et al*., 2014). However, the effects of all previously studied TCM on the heat stress response, milk performance, antioxidant and anti-apoptosis capacity of dairy cattle have not been clarified. Thus, the objective of this study was to evaluate the potential positive effects of TCM as feed additives on milk performance, antioxidant and anti-apoptosis capacities of dairy cattle under heat stress conditions.

MATERIALS AND METHODS

Preparation of traditional Chinese medicine prescriptions: The heat stress-releasing formula was made of Chinese herbs (Table 1), which were purchased from Hengyang, Hunan Province. Dried herbs were mixed, crushed, and passed through a 3~5mm screen sieve, and then given to treatment groups in dosages of 0, 50, 100, 150 g/day/cow.

Table 1: Composition and content of traditional Chinese medicine prescription (air dry basis)

Botanical name	Used part	Content (g)
Vaccaria hispanica	Seed	50
Dioscorea opposita	Rhizome	50
Fallopia multifiora	Rhizome	50
Leonurus artemisia	Leaves and stem	50
Crataegus pinnatifida	Fruit	35
Alisma plantago-aquatica	Rhizome	35
Cuscutae chinese	Seed	35
Clinopodium megalanthum	Seed	35
Stemmacantha uniflora	Root	30
Cynanchum otophyllum	Root	30
Lindera aggregate	Root	30
Astragalus membranceus	Root	30

Animals, diets, and experiment design: All animals were cared for according to the principles of the Hunan Agricultural University Animal Care and Use Committee. Twenty Holstein dairy cows (38-39 months old, 680±20kg BW) were randomly assigned to four groups. The four groups were A (control group, fed on basal diet, Table 2), B (basal diet+50 g/d/cow TCM), C (basal diet+100 g/d/cow TCM), and D (basal diet+150 g/d/cow TCM). They had free access to water. The experimental period consisted of seven days for adaption and 40 days for sampling. The temperature and humidity of the dairy farm were recorded daily and the average THI was calculated. Meanwhile, the average daily milk yield was also recorded. Milk samples were collected in clean plastic bottles and analyzed with a fully-automatic milk analyzer (FOSS, Denmark) for fat and protein contents, and somatic cell count. The anticoagulant-treated blood was collected from the jugular vein on the 0, 20 and 40th days for RNA extraction and lymphocyte separation.

Measurement of lymphocyte apoptosis: The cells were prepared as a single cell suspension in washing buffer. The cells were transferred into a 1.5 ml polypropylene tube on ice and 1 ml of cold (0°C) absolute ethanol was added. The cells were incubated (10min, 37°C) and then were subsequently washed twice with PBS for fixation (1ml, ice-bath). Later, 1 ml of propidium iodide (PI, Beyotime Biotechnology Institute) staining solution (40 μg/ml PI in PBS) was added to the single cell suspension and mixed well. Finally, the apoptosis rate of the lymphocyte was analyzed by a flow-cytometer (Beckman Coulter, EPICS XL/MCL).

Determination of GSH-px, CAT, Bcl-2 and p53 mRNA levels: Total RNA was isolated from liver samples using Trizol reagent (ISOGEN-LS, Japan), and then was reverse transcribed into cDNA. Primers were designed using Primer Premier Software (PREMIER Biosoft International, CA, USA). The primer sequences and Genbank accession numbers were obtained from Genbank (Table 3). The expression of GSH-Px, CAT, Bcl-2 and p53 was quantified by RT-PCR.

The program used for the amplification of the genes consisted of a denaturation step at 95°C for 15 ses, followed by 35 cycles of 60°C for 15 sec, 72°C for 45 sec, and extension at 72°C for 7min. All reactions were carried out using the ABI 7500 real-time PCR machine (Applied Biosystems, Beijing, China), then the resultant amplification data were analyzed using the 7500 Sequence Detection System (SDS) software (Applied Biosystems, Beijing, China). Relative gene expression level was calculated based on qPCR efficiency (E) and the threshold cycle difference (△Ct) between treatment and control groups for both target and reference genes using Relative

Table 2: Feed formula and nutrient component

Feedstuff	Composition (%)	Nutrient Levels (% of DM)		Premix	
Corn	52.00	DM	86.98	Vitamin A	1500000 IU
Wheat bran	12.00	CP	18.97	Vitamin D3	300000 IU
Soybean Meal	10.00	NE, MJ/kg	7.09	Vitamin E	1800000 IU
Cottonseed meal	14.00	NDF	18.01	Nicotinic acid	2500 mg
Rapeseed Meal	5.00	ADF	8.45	Copper	1500 mg
Shell meal	1.60	Calcium	0.95	Iron	5600 mg
Salt	1.00	Phosphorous	0.62	Manganese	4500 mg
$CaHPO_4$	1.40			Zinc	9600 mg
$NaHCO_3$	2.00			Iodine	150 mg
Premix	1.00			Selenium	40 mg
Total	100.00			Cobalt	60 mg

Nutrient levels were calculated values.

Expression Software Tool (REST 2008, Corbett Research, Sydney, Australia). Therefore, all target gene transcriptions were expressed as n-fold differences relative to the calibrator. For each gene, standard curves were used to calculate the individual real-time PCR efficiencies (E=10[−1/slope]). β-actin was used as an internal control to normalize the results.

$\Delta\Delta Ct = (Ct_{target} - Ct_{\beta\text{-}actin})_t - (Ct_{target} - Ct_{\beta\text{-}actin})_c$

Statistical analysis: Results are presented as mean ± SE and analyzed by SPSS Statistical Software 17.0 (Chicago, IL). Comparisons between groups were analyzed by one-way ANOVA followed by Duncan's multiple range tests. Differences were considered significant if $P<0.05$ and highly significant if $P<0.01$.

RESULTS

Effects of TCM on milk performance of dairy cattle: On average, the THI exceeds 72 (73.5±1.5) during the experimental period. Compared to the control group, the milk yield (Table 4) increased by 20.26 ($P<0.05$), 24.20 ($P<0.05$) and 16.98% ($P>0.05$) in groups B, C and D, respectively. In addition, the somatic cell count was decreased significantly in TCM treatment groups. They reduced by 21.68, 29.88 and 24.22% ($P<0.05$) in group B, C and D. However, there were no significant changes in the milk fat and milk protein contents ($P>0.05$).

Effects of TCM on mRNA expression of GSH-Px and CAT genes: The results (Fig. 1) showed that there were no significant differences among the four groups before TCM treatment. On the 20th and 40th days, the mRNA expression of GSH-Px was increased ($P>0.05$) in group B, C and D. The results for CAT mRNA expression (Fig. 1) showed that there were no significant differences in these groups before TCM treatment. On the 20th and 40th days, the mRNA expression of CAT was increased in groups B, C and D.

Table 3: Primer sequences for GSH-Px, CAT, Bcl-2, P53 and β-actin

Gene	Primer sequence (5'-3')	Product (bp)
GSH-Px	F: AGGAAAACGCCAAGAACGAGGAG R: GGGGACCAGGTGATGAACTTAG	213
CAT	F: GGCCTCCGCGATCTTTTCAATG R: GGGCCGTCACGCTGGTAGTTG	392
Bcl-2	F: GATGACCGAGTACCTGAACC R: GAGACAGCCAGGAGAAATCA	122
p53	F: CTTTGAGGTGCGTGTTT R: CAGTGCTCGCTTAGTGC	118
β-actin:	F: TCCAGCCTTCCTTCCTGGGCAT R: GGACAGCACCGTGTTGGCGTAGA	116

Table 4: Effect of traditional Chinese medicine on milk performance of dairy cattle

Groups	Milk yield (kg)	Milk fat percentage (%)	Milk protein content (%)	Somatic number (×10^5)
A	10.66±0.75^b	3.61±0.24	3.27±0.20	5.12±0.33^a
B	12.82±0.60^a	3.49±0.14	3.26±0.17	4.01±0.25^b
C	13.24±0.69^a	3.32±0.18	3.50±0.18	3.59±0.36^b
D	12.47±0.59^{ab}	3.79±0.16	3.13±0.09	3.88±0.41^b

$^{a-c}$Means within a column (on the same day) with different superscripts significantly differ ($P<0.05$). Group A (control group, fed on basal diet), B (basal diet+50g/d/cow TCM), C (basal diet+100g/d/cow TCM), and D (basal diet+150g/d/cow TCM).

Effects of TCM on mRNA expression of Bcl-2 and P53 genes: Before TCM treatment, the results (Fig. 2) showed that there were no significant differences in the mRNA expression of Bcl-2 gene in peripheral blood lymphocytes (day 0). However, the expression of the Bcl-2 gene increased in group B and C on the 20th day ($P<0.01$). On the 40th day it was increased in group B, C and D ($P<0.01$). However, the expression of p53 gene (Fig. 2) was decreased on the 20 and 40th days in groups B, C and D ($P<0.01$).

Effects of TCM on apoptosis rate of lymphocytes in peripheral blood: The apoptosis rate of lymphocyte in peripheral blood was detected by flow cytometry. The results (Table 5) showed that there were no significant differences among these groups before TCM treatment. However, compared to group A, the apoptosis rate of lymphocytes was significantly lower ($P<0.05$) on the 20th day in groups B and C. On the 40th day, the apoptosis rate had decreased by 27.45 ($P<0.01$), 27.36 ($P<0.01$) and 24.55% ($P<0.01$) in groups B, C and D, respectively.

DISCUSSION

Heat stress has adverse effects on feed intake and milk performance in dairy cows, which results in economic losses for the dairy industry (Rhoads *et al*., 2009). Cows were exposed to heat stress (THI exceeding 72) during the experimental period, which has a detrimental effect on milk performance (Zimbelman *et al*., 2013). In this trial, milk yield was reduced at the beginning of the experimental period because heat stress can affect metabolism. It reduced nutrient intake and nutrient uptake by the mammary gland, which leads to a decline in milk yield (Caroprese *et al*., 2013). However, milk yield increased in three TCM treatment groups, indicating that milk performance may be improved by TCM under conditions of heat stress. Besides, it has been proven that heat stress lead to changes in milk composition (Cheng *et al*., 2014). Our results showed that the changes in the milk fat and protein were slight, which suggested that TCM could alleviate milk composition changes caused by heat stress. In addition, the somatic cell count of milk is an indicator of clinical mastitis (Havlin and Robinson, 2015). In this experiment, somatic cell count was decreased in the TCM treatment groups, which showed that TCM has positive effects on decreasing the somatic cell count.

Table 5: Effects of traditional Chinese medicine on apoptosis rate of lymphocytes in peripheral blood of dairy cattle

Groups	Time (d)			
	1	20	40	mean
A	5.09±0.34	10.28±0.64^a	11.00±0.65^I	8.79±1.86
B	5.48±0.43	8.12±0.72^b	7.98±0.71^{II}	7.19±0.86
C	5.82±0.68	7.85±0.54^b	7.99±0.46^{II}	7.22±0.70
D	5.36±0.53	9.42±0.47^{ab}	8.30±0.66^{II}	7.69±1.21

$^{a-c}$Means within a column (on the same day) with different superscripts significantly differ ($P<0.05$). $^{I-II}$Means within a column (on the same day) with different superscripts significantly differ ($P<0.01$). Group A (control group, fed on basal diet), B (basal diet+50 g/d/cow TCM), C (basal diet+100 g/d/cow TCM), and D (basal diet+150 g/d/cow TCM).

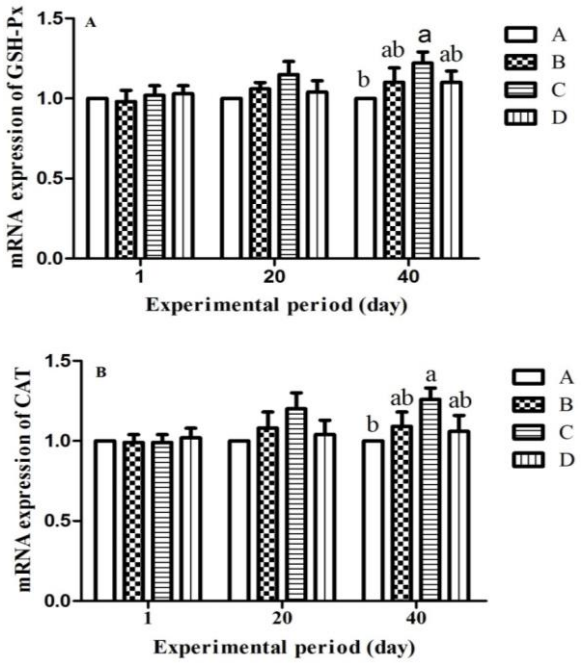

Fig. 1 The mRNA expression of GSH-Px gene (A) and CAT gene (B) in group A (control group, fed on basal diet), B (basal diet+50g/d/cow TCM), C (basal diet+100g/d/cow TCM), and D (basal diet+150g/d/cow TCM). [a–c]Means on the same day with different superscripts significantly differ ($P<0.05$).

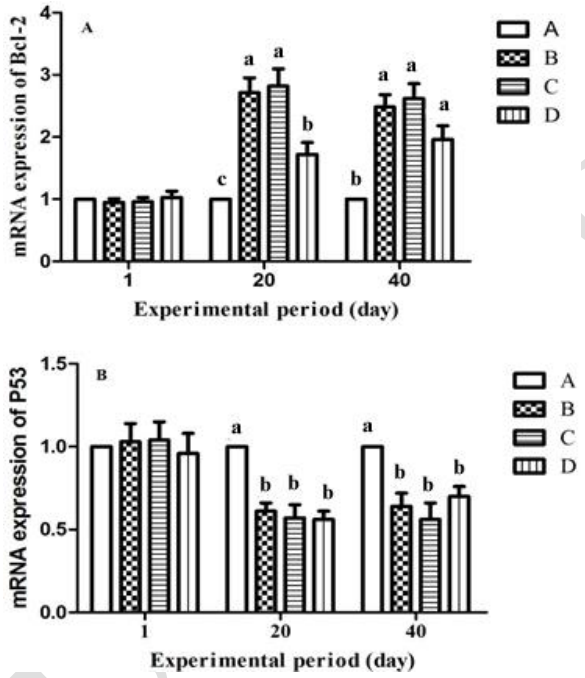

Fig. 2: The mRNA expression of Bcl-2 gene (A) and p53 gene (B) in group A (control group, fed on basal diet), B (basal diet+50g/d/cow TCM), C (basal diet+100g/d/cow TCM), and D (basal diet+150g/d/cow TCM). [a–c]Means on the same day with different superscripts significantly differ ($P<0.01$).

When dairy cows suffer high ambient temperature and humidity, they will form oxygen free radicals, which cause an imbalance between oxidants and antioxidants (Li *et al*., 2016). The production of oxygen free radicals and the accompanying damage to the organism are controlled by antioxidant defense systems (Aggarwal *et al*., 2013). Catalase (CAT) and glutathione peroxidase (GSH-px) are two important antioxidants in the body (Liu *et al*., 2016). A previous study has demonstrated that the activity of CAT and GSH-px is increased to remove a large amount of oxygen free radicals from an animal's body (Wu *et al*., 2016). In the present study, the mRNA expression of GSH-px and CAT was increased in TCM treatment groups, which indicated that TCM could regulate the activity of GSH-px and CAT to help resist heat stress by increasing the mRNA expression of antioxidants. Similar results were obtained in a previous study, which found that the mRNA expression of GSH-px and CAT was increased in suitable TCM treatment groups (Liu *et al*., 2016). The reasons for the increase of GSH-px and CAT expression caused by the addition of TCM may be that TCMs used in our experiment have anti-oxidant functions, such as *Astragalus membranceus* and hawthorn (Li *et al*., 2010, Chen *et al*., 2014).

It has been well established that heat stress also leads to lymphocyte apoptosis. The apoptosis rate of lymphocytes in peripheral blood was detected (Zhang *et al*., 2016). Our experiment showed that the apoptosis rate of lymphocytes was significantly decreased in TCM treatment groups, which proved that supplementation with TCM could reduce the apoptosis of lymphocytes in dairy cows under heat stress. This agrees with previous studies, which found that TCM plays an important role in the development of the immune response and promotes lymphocytes proliferation in animals through its effective constituents (Kong *et al*., 2004; Ma *et al*., 2005). When heat stressed, the lymphocytes (especially T and B lymphocytes) are damaged or undergo apoptosis, depressing the function of the system. Therefore, immune function could be measured by key related genes like p53 and Bcl-2 (Cheng *et al*., 2015). One of the functions of p53 is the activation of cellular apoptosis. Moreover, Bcl-2 has been reported to be an anti-apoptotic gene (Alvarez *et al*., 2006; Song *et al*., 2016). In our experiment, the expression of p53 decreased in TCM treatment groups. This is agreement with the changes in the apoptosis rate of lymphocytes in three TCM treatment groups, which indicated that TCM can improve immune function in dairy cows under heat stress. The apoptosis of lymphocytes was decreased because the activation of apoptosis function weakened when the expression of p53 decreased. Thus, TCM may improve the immune function through regulation of the apoptosis rate of lymphocytes and the expression of p53 (Alvarez *et al*., 2006). Thereby, TCM could protect lymphocytes from apoptosis through a down-regulation of p53 mRNA and an up-regulation of Bcl-2 mRNA, such that immune function is enhanced. Furthermore, it has been reported that the TCMs used in our experiment have immune enhancing function (Xue *et al*., 2008, Kim *et al*., 2014).

Conclusions: In summary, we concluded that dietary supplementation with TCM for dairy cows significantly improved milk performance, and alleviated heat stress by enhancing anti-oxidation and modulating immune activity through the regulation of the expression of associated genes. The dosage of 100 g/d was better than 50 or 150 g/d. These findings could help to find out more effective additives to resist heat stress under hot climatic conditions for dairy cattle.

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Authors contribution: PL conceived and designed the study. YH and ZZ executed the experiments. SY and JL analyzed the data. All authors interpreted the data, critically revised the manuscript for technical content and approved the final version.

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