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Fu Zi Prevent Extreme Cold Stress in Broilers Through Anti-Oxidative Activity

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Abstract: Radix aconiti laterlis preparata (Fu Zi in Chinese), a Chinese traditional medicine has been widely used for Yang deficiency. Recent clinical studies have shown that Fu Zi could ameliorate stressed condition but the mechanisms underlying the anti-stress effect of Fu Zi remains unknown. In this study, researchers examined the effects of Fu Zi on the treatment of broilers exposed to Extreme Cold Stress (ECS). The levels of Nitric Oxide (NO) and creatine kinase in plasma were measured and Hsp70 mRNA expression in different tissues was analyzed. Broilers subjected to ECS exhibited increased NO content before 3 h and increased Hsp70 expression under a time and tissue-dependent manner. Biochemically, Fu Zi reversed ECS-induced increases in NO and Hsp70 mRNA and the results suggest that Fu Zi supplemented broilers kept an elevated plasma NO content through anti-oxidative activity during ECS.

Key words: Radix aconiti laterlis preparata, nitric oxide, extreme cold stress, broiler, plasma

INTRODUCTION

Low environmental temperature exerts a negative influence on the performance of poultry and causes the loss of revenue that ranges into millions of dollars each year. Combinations of low environmental temperature and humidity impair the thermoregulatory processes of chickens and effectively exacerbate heat preservation and dissipation during cold stress. Therefore, as ambient temperatures decrease, feed consumption and growth rate decrease and mortality rates increase (Ahmad and Mitsuhiro, 2012). Numerous studies have been conducted to examine the effects of dietary supplements on induction of cold resistance in chicks (Mahmoud *et al*., 2004; Sahin *et al*., 2006, 2003). One method that has been used to minimize poor performance and/or cold related mortality is dietary supplementation of Chinese medicine.

In traditional Chinese Medicine (CM), traditional Chinese prescriptions and formulae have been used by CM practitioners in China for thousands of years and are based on principles aimed to produce either synergism or antagonism; this medical approach has played an important role in the prevention and treatment of many discomforts in Chinese history (Kang *et al*., 2005; Wang *et al*., 2007). Traditional Chinese prescriptions have been commonly recognized as safe and effective in the treatment of various depressive disorders in China (Jordan and Tu, 2008). Fu Zi, a famous traditional Chinese medicine has been widely used to treat Yang-deficiency. Recent studies also showed that Fi Zi has definitely effects against cold stress (Kam and Liew, 2002). However, the mechanisms underlying anti-cold stress of Fu Zi remains unknown.

To address this issue, researchers examined the effects of the Fu Zi in broilers exposed to extreme cold stress. The anti-cold stress potential of Fu Zi was then evaluated by measuring biochemical index, Creatine Kinase (CK) and nitric oxide which was then compared with group under same condition except without Chinese medicine addition and normal group. Broilers with CM addition could maintain NO content and Hsp70 mRNA expression in a relatively smooth level. The results suggest that Fu Zi supplementation in broilers kept elevated plasma NO content in a balanced level through anti-oxidative activity during extreme cold stress.

MATERIALS AND METHODS

About 600, 4 weeks old mail Hauinan partridge chickens, an indigenous breed from Feixi the Old Hens Farming Co., Ltd. were randomly placed and housed in six coops (100 birds per coop, 0.044 m^2/bird) in a humidified atmosphere at 20±2°C. Chickens were fed on a commercial starter diet. At the 1st day of 5th week, dietary CM (200 mg kg^{-1}) was supplemented to half of the chickens in their feed throughout the experimental period and half received no supplement (N-CM). After 2 weeks supplementation, the room temperatures were suddenly decreased from 20±2-2±2°C in 2 h. About six chickens were randomly selected in each group at each of the

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Table 1: Primer sequences of Hsp70 and GAPDH

Genes	Forward primer	Reverse primer
Hsp70	TTTGACCTAACAGGCATCCCC	TTGTCCACAGCACTGACGTTC
GAPDH	AAAGTCCAAGTGGTGGCCATC	TTTCCCGTTCTCAGCCTTGAC

following time period: 0, 0.5, 1, 2, 3, 6, 12, 24, 72 and 144 h. To regulate the temperature, the thermometer was monitered at the centre and four side of each coop. During cold stress birds were provided access to a commercial broiler feed and water ad libitum. When the cold stress period was over in each time, birds were humanely euthanized by cervical dislocation for sampling. Blood samples (3 mL) were obtained, transferred into blood collection tubes containing heparin anti-coagulant (50 IU mL^{-1}) and immediately chilled on ice. The plasma samples for subsequent enzyme determination were obtained following centrifugation of whole blood at 3500 rpm for 10 min at 4°C after 20 min water bath at 37°C and stored at -20°C. The tissues (heart, liver, spleen and muscle) from broilers were quickly dissected (approximately 1.0 g) and placed into 1.5 mL tubes with 0.5 mL RNA later used for quantifying the Hsp70 mRNA expression levels. All the experimental procedures followed the guidelines of the regional Animal Ethics Committee. Samples were synchronously.

The activity of plasma Creatine Kinase (CK) (CK9231, Beijing Leadman Biochemistry Co., Ltd.) was measured by enzyme coupling spectrophotometry. Nitric oxide was assessed using a commercial kit modified for use with a multiwell plate spectrophotometer.

Samples were dissolved in TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA) for total RNA extraction and separated electrophoretically on agarose gels under denaturing conditions in order to confirm the integrity of ribosomal RNA bands.

Single-strand cDNA synthesis was carried out from 1 mg of total RNA by the Reverse Transcription (RT) reaction. After denaturation at 70°C for 10 min, RNA samples were incubated in 1 ×PCR buffer (10 mM Tris-HCl, pH 9.5, 50 mM Kcl, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 1 mM dNTPs mix, 500 ng oligo (dT) primer and 200U SuperScriptII enzyme (Gibco/BRL, Gaithersburg inc.) in a final volume of 20 mL. This reaction was maintained at 42°C for 50 min and subsequently incubated with 1U of RNase H for 20 min at 37°C for RNA template digestion. Amplification reactions were conducted using the sense and antisense primers shown in Table 1. PCRs were performed with 10% of total reverse transcription reaction volume, 0.5 mM specific primers (Table 1), 1 ×PCR buffer (10 mM Tris-HCl, 1.5-2.5 mM $MgCl_2$, 50 mM KCl) and 1U Taq DNA polymerase (Takara). These reactions were denatured at 94°C for 30 sec followed by annealing for 40 sec and extension corresponding to 72°C for 40 sec. Annealing temperatures of *GAPDH* and *Hsp70* genes was 61°C in a total of 25 cycles.

All statistical analysis was carried out using SAS9.1 edition unless otherwise indicated. Statistical analyses were carried out by standard analyses of variance or Student's t-test as appropriate. Differences between experimental groups were considered significant at $p<0.05$ or as otherwise indicated. All data were expressed as mean±SD.

RESULTS AND DISCUSION

Analysis of the data showed a significant effect of environmental temperature on plasma CK activity and NO content in N-CM group (Fig. 1). Plasma CK increased after 1 h cold stress and NO content significantly increased after 3 h cold stress in N-CM group. Broilers given the CM supplemented feed had a smoothly CK activity and NO content during the whole cold stress time.

Cold exposure significantly increased hsp70 mRNA expression levels over those of N-CM broilers in the ECS environment. The level of hsp70 expression showed time- and tissue-dependent in N-CM broilers. Hsp70 expressed in a significantly high level before 3 h and after 72 h cold stress in tissues except in spleen in the N-CM broilers. Broilers with 200 mg kg^{-1} CM supplementation showed no obvious change in hsp70 expression in all tissues measured (Table 2).

Nitric Oxide (NO) is a free radical gas with well-characterized signaling roles in mammalian systems acting as a second messenger during vasorelaxation, neurotransmission, immunity and cytotoxicity (Neill *et al*., 2002). Reports also suggested that NO can be synthesized during stress responses and it may be that cellular effects reflect responses to NO. It is possible that NO, itself a free radical $NO^{\cdot}$ can react with $O_2^{\cdot -}$ to form the highly reactive peroxynitrite anion, $ONOO^-$ (Clarke *et al*., 2000). Such oxidation will increase the number of Reactive Oxygen Metabolites (ROM) that can attack and irreparably damage membrane composition and permeability (Mahmoud *et al*., 2004). The elevation of plasma CK is indicative of skeletal muscle damage and is a consequence of the disruption in muscle cell membrane function and permeability.

In this study, broilers without CM addition showed a significant increase in NO content and CK activity after 3 and 1 h, respectively when submitted to ECS. Broilers

Table 2: HSP70 levels in the organs of stressed and control broilers in different times (fold change)

	Non CM dietary				200 mg kg^{-1} CM dietary			
Cold stress time	Liver	Heart	Spleen	Muscle	Liver	Heart	Spleen	Muscle
Control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.5	1.23	1.46	0.95	2.02	1.18	0.74	1.15	0.82
1	1.55	1.57	1.24	2.62	2.10	0.86	1.05	0.80
2	2.32	1.41	1.32	2.45	4.82	0.65	1.12	0.47
3	1.73	1.83	1.28	1.82	1.36	1.27	1.39	0.71
6	1.16	1.48	1.16	1.78	1.49	1.16	1.26	0.76
12	1.03	1.24	1.34	1.43	0.83	0.6	0.88	0.81
24	2.23	0.92	1.40	1.10	4.56	0.73	1.18	0.50
72	3.38	3.07	1.32	2.21	2.05	1.32	1.14	0.84
144	2.49	3.26	1.29	2.88	3.55	0.71	1.60	0.60

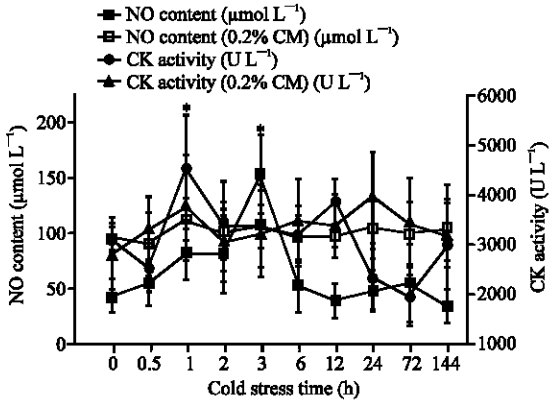

Fig. 1: The level of plasma CK and NO after different times of cold stress. Values indicated are the mean±SE. *Significant at 0.05 level when comparing the cold stressed groups with control

with CM supplementation showed no obvious change in NO and CK during the whole ECS. In CM addition group, broilers showed higher plasma NO content before ECS. The elevated plasma NO level would thus effectively maintain the plasma NO stability during ECS. Also, broilers in CM addition group showed a relatively CK stability during ECS.

From the results of HSP70 mRNA expression in liver, heart, spleen and muscle in N-CM group, HSP70 mRNA showed an obvious fold change before 3 h and after 72 h in liver, heart and muscle but no obvious change in spleen during the whole cold stress time. This suggested that Hsp70 mRNA expression is tissue and time-dependent during cold stress in broilers. Hsp70 mRNA expression in CM broilers had no obvious change during the whole ECS.

Increasing in NO content made the accumulation of ROM which together with the subsequent cellular damage caused by ROM activity has been suggested as a key factor that activates *Hsp* genes (Ananthan *et al*., 1986). Furthermore, broilers subjected to long time cold stress demand a high-energy supply provided by substrate oxidation, producing oxidative free radicals which might induce the latter Hsp70 high expression (Lindquist, 1986). These might responsible for the two peaks of Hsp70 expression in N-CM broilers during ECS.

CONCLUSION

Many studies have demonstrated that Chinese herbal medicine Fu Zi has strong reducing capacity, antioxidative activity on lipid-peroxidation and scavenging effects on hydroxyl radical (Guo *et al*., 2009; Kam and Liew, 2002; Yang *et al*., 2009). From the results of this study, we may thus suggest that Fu Zi could reduce cold stress in broilers through anti-oxidative activity to maintain elevated plasma NO content.

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