



## RESEARCH ARTICLE

### Effects of Cold Stress on mRNA Level of Uncoupling Protein 2 in Liver of Chicks

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#### ABSTRACT

The uncoupling protein 2 (UCP2) gene belong to the superfamily of electron transport carriers of the mitochondrial inner membrane which is involved in thermogenesis and determining the functional evolution of UCP2 gene is important to understand the evolution of thermo-regulation in chickens. The aim of this study was to investigate the effects of cold stress on the mRNA level of UCP2 in liver of chickens. In this study, 120 15-d-old male chickens were randomly allocated into 12 groups (10 each group) for exposure to acute (up to 24 h) and chronic (up to 20 days) cold stress ( $12\pm 1^{\circ}\text{C}$ ). This study detected UCP2 mRNA levels in liver, glucagons (GLU) content in plasma, insulin (INS), blood glucose (BG) and free fatty acid (FFA) content in serum. The results showed that during acute cold stress, UCP2 mRNA levels gradually increased the INS and FFA content showed fluctuant change, GLU content gradually increased, and BG content first increased and then decreased. During chronic cold stress, UCP2 mRNA levels significantly increased compared with the control group, and the content of INS, GLU, BG and FFA were all gradually increased with the time lapsing. The results indicated that both acute and chronic cold stress could enhance the energy metabolism and the UCP2 mRNA levels in liver of chicks.

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#### INTRODUCTION

Exposure to various environmental factors, is responsible for affecting multiple biochemical regulatory systems and triggering various disorders (Epel, 2009), especially effect of energy metabolism. Poultry production faces serious problems when chickens are exposed to extreme thermal conditions such as stress. Stress is one of the basic factors in the etiology of disease and the cold stress, as one of the stressors, commonly exists in the cold region (Zhang *et al.*, 2011). Cold stress occurs when the surrounding temperature drops below  $18^{\circ}\text{C}$ , the body may not be able to warm itself, and occurs cold-related illnesses, permanent tissue damage and death (Dhanalakshmi *et al.*, 2007). Indeed, neonatal chicks cannot maintain their body temperature in cold environment until they acquire for thermogenesis and mature thermogenic organs (Mujahid and Furuse, 2009). Studies have shown that acute cold stress can cause the heat production, plasma glucose, muscle glycogen and lipid oxidation increased. Moreover, studies also proved that cold stress could significantly influence the energy metabolism in mice, human and chicken (Cichon *et al.*,

2002; Hangalapura *et al.*, 2006). In particular, cold exposure is accompanied by oxygen consumption stimulation in various tissues (Iossa *et al.*, 2001), coupled with an increase in levels of non-esterified fatty acids in serum (NEFA) (Peralta *et al.*, 2003).

The uncoupling protein (UCP) genes belong to the superfamily of electron transport carriers of the mitochondrial inner membrane. The uncoupling protein family is involved in thermogenesis and it's important to understand the functional of UCP genes in thermo-regulation in vertebrates. Also, the physiological role of UCP1 is restricted to thermogenesis (Ricquier and Bouillaud, 2000). UCP2 and 3 have been involved in a number of postulated functions in energy regulation (Zhang *et al.*, 2001). UCP2 facilitates uncoupling oxidative phosphorylation to reduce ATP synthesis (Busquets *et al.*, 2001; Rousset *et al.*, 2004). It has been suggested that UCP2 plays a major role in the regulation of reactive oxygen species (ROS) production (Duval *et al.*, 2002). Although its precise function has yet to be determined, it has been suggested that UCP2 could be involved in the regulation of lipid utilization as a fuel substrate, as a potentially important protein for the cold-

induced change of the energy metabolism (Abe *et al.*, 2006; Mujahid and Furuse, 2009).

The aim of this study was to investigate the effect of cold stress on UCP2 expression in liver of chickens, as well as INS, BG, FFA concentrations in chickens' serum and GLU concentrations in chickens' plasma.

## MATERIALS AND METHODS

**Birds and Tissue Collection:** All procedures used in the present experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Chick model of cold stress was developed as described in our previous articles (Zhang *et al.*, 2011). 120 15-d-old male chickens (purchased from Weiwei Co. Ltd., Harbin, China) were randomly allocated into 12 groups (10 each group) for exposure to acute (1, 3, 6, 12 and 24 h) and chronic (5d, 10d and 20d) cold stress ( $12\pm 1^\circ\text{C}$ ), while the control groups were maintained at  $28^\circ\text{C}$ . The chickens were maintained in our animal facility, kept under a natural light-dark cycle (16L: 8D) and given free water and regular quantitative diet. The chicks were killed by cervical dislocation after stress termination. The liver were collected, immediately frozen on dry ice, and then stored at  $-70^\circ\text{C}$  for RNA isolation.

**Determination of serum FFA, INS, BG and blood plasma GLU:** Serum FFA concentrations were determined by enzymatic color test. Using the NEFA kit (A042, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's recommendations using 0.2 mL of serum. Serum INS, BG concentrations and blood plasma GLU concentration were determined by using the commercial kits (I055, F006, I057, Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's recommendations.

**Quantification of UCP2 mRNA:** Total RNA of liver was isolated by using a Trizol reagent (Invitrogen, Carlsbad, CA) as per protocol devised by the manufacturer. After quantification, these RNA samples were subjected to reverse transcription reaction. The expression levels of UCP2 gene was determined by real-time quantitative reverse transcription PCR by using SYBR Premix Ex Taq<sup>TM</sup> (Takara, China), and real time PCR system was ABI PRISM 7500 real-time PCR system (Applied Biosystems). To design primers, we used the chicken UCP2 mRNA GenBank sequence with accession number of AF287144. Chicken  $\beta$ -actin (GenBank accession number L08165) as a housekeeping gene was used as an internal reference. Primers (Table 1) were designed using the Oligo 6.0 Software, and were synthesized by Invitrogen Biotechnology Co. Ltd. in Shanghai, China. Real-time quantitative reverse transcription PCR was used to detect the expression of UCP2 gene in liver. The program was 1 cycle at  $95^\circ\text{C}$  for 30 s and 40 cycles at  $95^\circ\text{C}$  for 5 s and at  $60^\circ\text{C}$  for 34 s. Dissociation curves were analyzed by Dissociation Curve 1.0 Software (Applied Biosystems) for each PCR reaction to detect and eliminate the possible primer-dimer and non-specific amplification. The mRNA relative abundance was calculated according to the method of Pfaffl (Pfaffl, 2001).

**Table 1:** Gene-specific primers for  $\beta$ -actin, UCP2 used in the real-time quantitative reverse transcription PCR

Gene	Accession number	Primer (5'→3')	Product size, bp
UCP2	AF287144	Forward: CGCCCGCAACTCCATCATT	251
		Reverse: GCTGCCTTTGGTCCCTCAT	
$\beta$ -actin	L08165	Forward: ACCGCAATGCTTCTAAACC	93
		Reverse: CCAATCTCGTCTTGTATATGC	

**Statistical analysis:** Statistical analysis of all data was performed using SPSS for Windows (version 13; SPSS Inc., Chicago, IL, USA). When a significant value ( $P<0.05$ ) was obtained by one-way analysis of variance (acute cold stress groups), further analysis was carried out. All data showed a normal distribution and passed equal variance testing. Differences between means were assessed using Tukey's honestly significant difference test for post hoc multiple comparisons. Data are expressed as the mean $\pm$ SD. Differences between means (Chronic cold stress groups) were assessed using two-tailed paired Student's T-test.

## RESULTS

**Effects of cold stress on GLU, INS, BG and FFA levels:** Effects of acute cold stress on GLU, INS, BG and FFA levels are presented in Table 2. During acute cold stress, the content of INS and FFA showed fluctuant change compared to that in the control group. GLU content significantly increased ( $P<0.05$ ) compared to that in the control group and GLU content in 24h group increased by approximately 50% compared to that in the control group. BG content first increased and then decreased.

Effects of chronic cold stress on the GLU, INS, BG and FFA are presented in Fig. 1. The content of INS, GLU, BG and FFA were all significantly increased ( $P<0.05$ ) with the time lapsing compared to that in the corresponding control ones.

**Effects of cold stress on the mRNA levels of UCP2 in liver of chickens:** Effects of acute and chronic cold stress on the mRNA levels of adiponectin gene in liver are presented in Fig. 2A and 2B. The mRNA levels of UCP2 gene in liver of the 6h and 12h acute cold stressed chicks were significantly increased ( $P<0.05$ ) compared to that in the control group. But the mRNA levels of UCP2 gene in liver of 24h group decreased to the normal level compared to that in the control group.

The mRNA levels of UCP2 gene in liver of the 5d group had little change compared to that in the corresponding control ones. The mRNA levels of UCP2 gene in liver of the 10d and 20d chronic cold stressed chicks were significantly increased ( $P<0.05$ ) compared to that in the corresponding control ones.

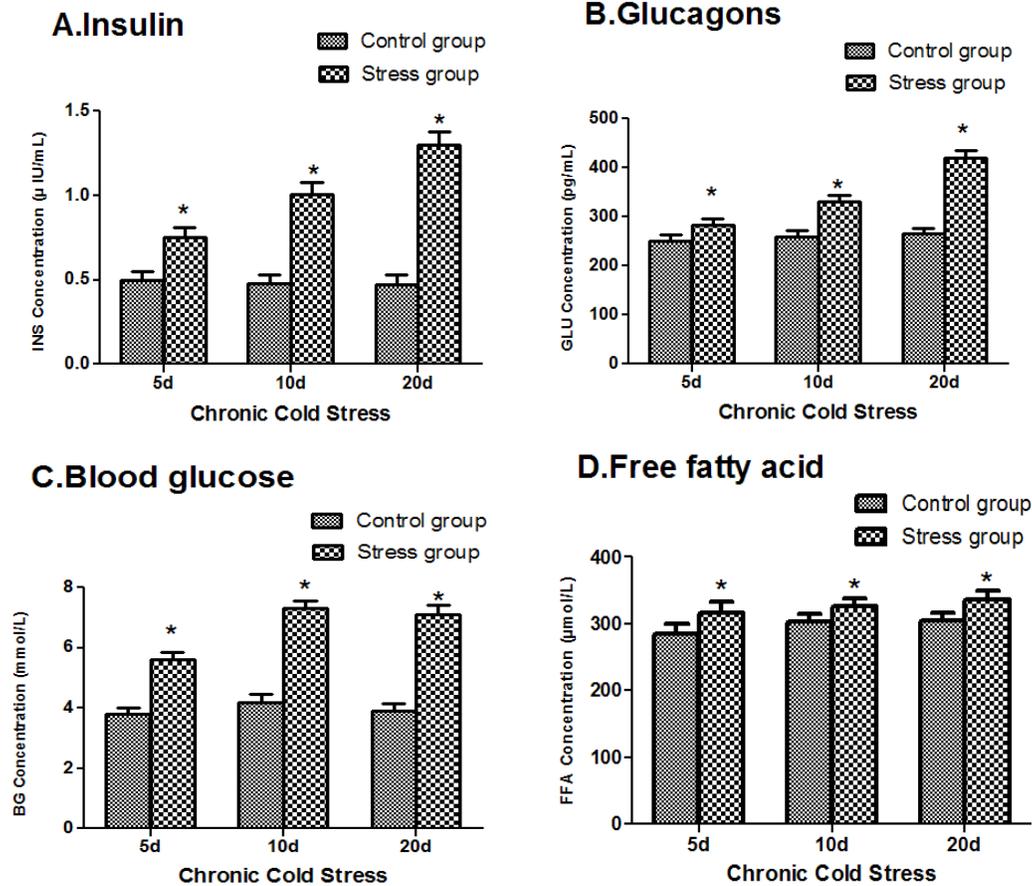
## DISCUSSION

Cold stress provokes physiological responses and induces changes in energy production and subsequent reactive oxygen species (ROS) production (Zhang *et al.*, 2011). It has been suggested that UCP could be involved in the regulation of lipid utilization as a fuel substrate, to be a potentially important protein for the cold-induced

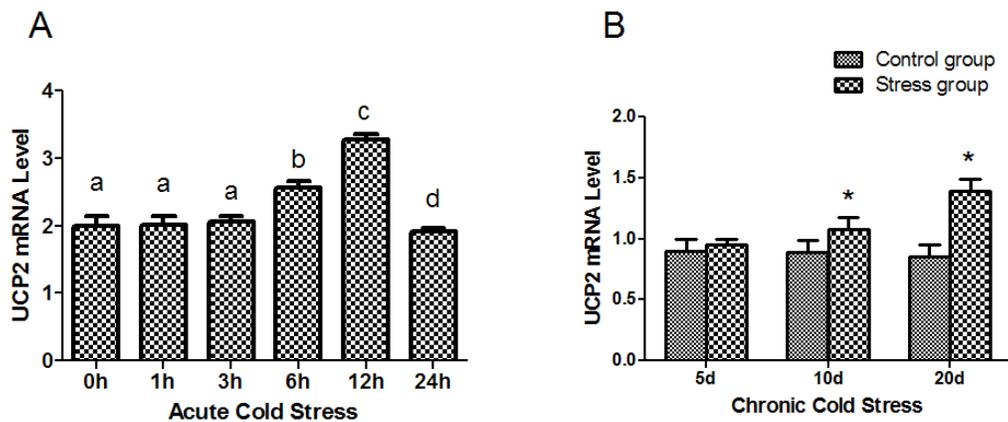
**Table 2:** Effects of acute cold stress on the insulin (INS) in plasma and glucagons (GLU), blood glucose (BG) and free fatty acid (FFA) in serum of chickens

Parameter	Units	Acute Cold Stress (Hours)					
		0	1	3	6	12	24
Insulin	$\mu$ IU/mL	0.43 $\pm$ 0.02 <sup>a</sup>	0.68 $\pm$ 0.03 <sup>b</sup>	0.74 $\pm$ 0.04 <sup>c</sup>	0.23 $\pm$ 0.01 <sup>d</sup>	0.58 $\pm$ 0.03 <sup>b</sup>	1.42 $\pm$ 0.03 <sup>e</sup>
Glucagons	Pg/mL	252 $\pm$ 11.2 <sup>a</sup>	311 $\pm$ 8.3 <sup>b</sup>	325 $\pm$ 12.4 <sup>c</sup>	337 $\pm$ 11.8 <sup>d</sup>	387 $\pm$ 13.1 <sup>e</sup>	391 $\pm$ 14.2 <sup>e</sup>
Blood glucose	mmol/L	4.1 $\pm$ 0.08 <sup>a</sup>	4.9 $\pm$ 0.09 <sup>b</sup>	22.3 $\pm$ 0.88 <sup>c</sup>	25.1 $\pm$ 0.83 <sup>d</sup>	9.1 $\pm$ 0.23 <sup>e</sup>	5.8 $\pm$ 0.15 <sup>f</sup>
Free fatty acid	$\mu$ mol/L	298 $\pm$ 8.9 <sup>a</sup>	207 $\pm$ 7.3 <sup>b</sup>	329 $\pm$ 6.9 <sup>c</sup>	486 $\pm$ 12.5 <sup>d</sup>	603 $\pm$ 13.2 <sup>e</sup>	176 $\pm$ 5.8 <sup>f</sup>

The different letters in a row indicated significant differences ( $P < 0.05$ ).



**Fig. 1:** Effects of chronic cold stress on the insulin (INS) in plasma and glucagons (GLU), blood glucose (BG) and free fatty acid (FFA) in serum of chickens. Panel A, B, C and D were the INS, GLU, BG and FFA content, respectively. \*In panel A-D indicated that there were significant differences ( $P < 0.05$ ) between the control group and the stress group at the same time point. Each value represented the mean $\pm$ SD of 5 individuals.



**Fig. 2:** Effects of acute and chronic cold stress on the mRNA levels of UCP2 gene in liver. Panel A was the result of effects of acute cold stress on the mRNA levels of UCP2 gene, the relative mRNA levels from the 0h control group were used as the reference values, the different letters indicated that there were significant differences ( $P < 0.05$ ) between any two chicks. Panel B was the result of the effects of chronic cold stress on the mRNA levels of UCP2 gene, the relative mRNA levels from the 5, 10, and 20 d control group were used as the reference values. \* in panel B indicated that there were significant differences ( $P < 0.05$ ) between the control and the stressed chicks at the same time point. Each value represented the mean $\pm$ SD of 5 individuals.

thermogenesis, and may play a role in the control of reactive oxygen species (ROS) production (Toyomizu *et al.*, 2002). The reactive mechanism of cold stress is very complicated, and the results from different studies are not consistent. These may be attributed to the duration time of cold exposure, the temperature of cold exposure, the genetic background of experimental animal, and so on. A number of studies have proved that cold stress could influence the energy metabolism (Cichon *et al.*, 2002).

Sugar and fat is an important material of energy storage and function, both for the body to provide coordinated more than 70% energy source. In cold conditions, when metabolic heat sufficient to maintain body temperature, animal increase heat production by shivering. The main role of INS is to promote the synthesis in liver, muscle and fat tissue controlled substances in the energy metabolism and storage. In the process of cold stress, carbohydrate and FFA oxidation enhancement is well-known phenomenon, FFA may induce "decoupling" of oxidative phosphorylation. Indeed, Bravo's (Bravo *et al.*, 2001) research suggested that the marked diminution in respiration induced by albumin in cold-stressed mitochondria indicates the presence of FFA in the inner membrane of these mitochondria. Cold water stress could reduce levels of INS in human serum (Tikusis *et al.*, 1999). However, high FFA levels in cold-exposed chicks indicate that a compensatory response to attain homeothermy might be due to enhancement in lipolysis, reduction in oxidative capacity and/or insufficient enhancement of oxidative capacity to oxidize the available free FAs (McWilliams *et al.*, 2004).

In the chicken, lipogenesis occurs essentially in the liver, the adipose tissue being only a storage tissue (Diot and Douaire, 1999). In addition, unlike the majority mammalian species, FA synthesis occurs primarily in the liver of the chicken (as in the human) rather than adipose tissue (Ramachandran *et al.*, 2007). In our study, cold exposure could significantly increase GLU, INS, BG and FFA concentration in chickens. It indicated that cold stress could change the body energy metabolism and GLU might play an important role after exposed to cold in the body energy metabolism.

Since UCP2 is thought to be involved in the regulation of energy expenditure, body weight, and fatty acid metabolism (Ricquier *et al.*, 1999). Mori (Mori *et al.*, 2004) examined alterations in hepatic UCP2 gene expression by energy metabolism. An increased UCP2 mRNA expression has been reported in lipopolysaccharide-treated and transformed rat hepatocytes (Cortez-Pinto *et al.*, 1998). These data suggested that UCP2 expression could be induced in response to different stimuli in liver. We have used real-time PCR method to identify the changes in the mRNA expression of UCP2. After 6 h of cold exposure, we detect a marked increase in the expression of UCP2 mRNA in liver. Previously, we found cold stress could induce oxidative stress in chicken (Zhang *et al.*, 2011), which might also relate to the slowdown of cellular repair mechanisms in the cold. Oxidative phosphorylation is never fully coupled to ATP synthesis in vivo, conditional mainly on the existence of UCPs. UCPs catalyze proton conductance and dissipate the mitochondrial gradient

required for ATP production, which results in a reduction of mitochondrial membrane potential and mitigates ROS formation. Cold-induced oxidative stress and changed the body energy metabolism has been suggested to play key role in liver damage. UCP2 are induced by energy metabolism disorder and oxidative stress products and seem to be crucial for reducing the mitochondrial ROS production (Andrews *et al.*, 2005). The present paper is aimed at further investigating the effects of UCP2 in chicken liver, especially with respect to how UCPs are controlled under cold-induced oxidative stress in chicken. Tseng (Tseng *et al.*, 2011) observed stimulation of the UCP might help to prevent oxidative damage and to maintain metabolic balance and cellular homeostasis in zebrafish upon cold exposure. Thus, it is conceivable that cold stress promotes an increase in the expression of the UCP2 gene in hepatocytes. In our study, during the cold stress, the mRNA levels of UCP2 gene were increased about 1.5 times over control. These results indicated that the expression of UCP2 gene in liver could be influenced by cold stress, but because of little research, the effect of cold stress on the UCP2 gene needs to be further studied, especially in chickens.

In conclusion, our study demonstrated that both acute and chronic low temperature exposure could influence the energy metabolism and increase mRNA levels of UCP2 in liver of chickens. Additional work will be required to elucidate the exact mechanisms responsible for modulating the release of UCP2 and to characterize its metabolic roles in cold-induced response.

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