

Intermittent mild cold stimulation improves the immunity and cold resistance of spleens in broilers

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ABSTRACT In order to investigate the effect of intermittent mild cold stimulation (IMCS) on immune function of spleens and adaptability to cold stress in broilers, 400 healthy 1-day-old Ross-308 chickens were divided into 5 groups: CC (control) reared in normal thermal environment from 1 to 49 d; CS3, CS4, CS5, and CS6 (treatments) raised at 3°C below the temperature of CC for 3, 4, 5, or 6 h at 1-d intervals from 15 to 35 d, respectively. Subsequently, CS3-6 was raised at 20°C from 36 to 49 d. At 50 d, all groups were exposed to acute cold stress (ACS) for 12 h. The spleen immunity index at 22, 29, 36, 43, and 49 d, expression levels of toll-like receptors (TLRs), cytokines and immunoglobulins at 22, 43, and 49 d and heat shock proteins (HSPs) before and after ACS at 50 d were examined. The spleen index of broilers aged 22 to 49 d did not differ between CS and CC ($P > 0.05$), and the spleen index of CS5 was higher

than that of CS3 at 49 d ($P < 0.05$). The mRNA levels of TLR5, TLR15, TLR21, and IL-2 in CS3, TLR3, TLR4, TLR15, TLR21, IL-2, IL-6, and IFN- γ in CS4, TLR1, TLR3, TLR4, TLR21, IL-2, IFN- α , IFN- γ , IgA, and IgG in CS6, but all TLRs, immunoglobulins and cytokines except IFN- γ in CS5 differential expressed stably compared with CC at 43 and 49 d ($P < 0.05$). Compared with Pre-ACS, the mRNA levels of HSP60, HSP70, and HSP90 were upregulated in CS after ACS ($P < 0.05$). Except for HSP90 mRNA and HSP70 protein in CS6, and HSP90 protein in CS3, the levels of HSPs after ACS in all treatment groups were higher than those in CC ($P < 0.05$), and the highest HSPs levels after ACS were found in CS5. We concluded that IMCS could enhance immunity of spleens and adaptability to ACS in broilers, besides CS5 was the optimal program.

Key words: broiler spleen, immune regulation, cold adaptation, heat shock protein

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INTRODUCTION

Cold is the main stressor for animals in northern areas. Cold stress generally occurs at ambient temperatures lower than 18°C, inducing decreased egg production rate, growth retardation, lower feed conversion rate, and higher morbidity and mortality of poultry (Tsiouris et al., 2015; Hu and Cheng, 2021). Therefore, the development of methods to establish adaptability to low temperature is a major task in animal production. Repeated cold exposure allows poultry generate cold acclimation through endocrine, metabolic and automatic regulation and behavioral mechanisms (Li et al.,

2017; Liu et al., 2020). Cold adaptation can enhance immune function, antioxidant capacity and resistance to cold stress of organisms, and mitigate the damage caused by adverse low environmental temperature to organisms (Li et al., 2017; Su et al., 2020). Of these, the change of immune function can be used as a measurement index of animal's adaptability to cold. After 2 wk of cold adaptation training at 2°C, cell-mediated immune function and disease resistance of mice were activated (Xu et al., 1992). Broilers built up cold adaptation after 14 d of cold stimulation at 10 to 12°C and their resistance to *Asporgillus flavus* was improved to a certain extent (Manning and Wyatt, 1990). Spleen, the largest peripheral immune organ in body, is settlement region of T and B lymphocytes, center of cellular and humoral immunity and main lymphatic organ that responds immunologically to antigens circulating in blood. The immune levels of birds were directly determined by development and function state of immune organs (Wang et al., 2010; Dharmaraj et al., 2017). It was pointed out by Selye (1936) that immune organs

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and tissues such as spleen, thymus, and lymph nodes shrank significantly when animals were subjected to intense stress. Cold and heat stress could result in blocked growth and development of poultry immune organs, further leading to lower development levels than normal and decreased immunity (Ren et al., 2011; Liu et al., 2014). While Maslov et al. (2016) found that intermittent chronic cold exposure (4°C, 8 h/d, 4 wk) increased the spleen weight of rats. It can be seen that severe stress environment can cause adverse effects on animal immune organs, while moderate cold stimulation generally does not.

To cope with different environment stimulation, cells may support efficient stress responses through regulation of gene transcription, protein expression, and enzyme activity or proceed into the cell-death pathway due to activation of stress signaling cascades (Phuong et al., 2015). Toll-like receptors (TLRs), acting as sentinels of pathogens, are characterized in mammals by recognition of pathogen-associated molecular patterns (PAMPs) and activation of antiviral, antibacterial, antifungal, and antiparasitic innate immunity (Ramasamy et al., 2011). In addition, TLRs mediate inflammatory responses or acquired immune responses via the production of cytokines due to activation of NF- κ B downstream signaling signals (Gan et al., 2017). Paul et al. (2015) found that upregulation of almost all TLR1-10 mRNA levels in immune cells under heat stress could provide the means to fight against viral infection, while all TLR genes except TLR3 and TLR8, and activities of dendritic cells and macrophages were down-regulated in goat submitted to long-term cold stress in winter. Cytokines, a class of soluble polypeptides with small molecular weights, are believed to participate in many biological processes like immunomodulation, cell growth, and damaged tissue repair as intercellular mediators by stabilizing specific mRNAs and facilitating their translation. Previous studies have shown that the secretion of cytokines contributes to regulating stress/stimulation related immune disorders (Wei et al., 2018; Liu et al., 2020). Su et al. (2018) reported that rapidly decreasing temperature by 10°C enhanced expression levels of IL-4 and IL-6, but inhibited release of IFN- γ , resulting in immunodissonance of Th1/Th2 in ileum of cold-stressed broilers. Immunoglobulin, an important class of immune effector molecules, can be converted into antibodies by antigens induction. As the first barrier of humoral immunity, immunoglobulins play an important role in maintaining immune function in animals. According to Choi et al. (2017), decreased IgM level in serum of black rockfish (*Sebastes schlegelii*) was detected as a result of low water temperature. Analogously, in chickens, a low-temperature environment at 12°C for 21 d was found to reduce serum IgM and IgG contents (Olfati et al., 2018). At present, the effects of heat and cold stress on immune molecules in broilers have been studied deeply, but little is known about the regulation mechanism of mild cold stimulation on broiler's immune.

Heat shock proteins (HSPs) are a series of highly conserved molecular chaperones being capable of modulating folding/unfolding of proteins and the assembly/disintegration of protein complexes to protect stressed cells (Qian et al., 2014). HSPs play a protective role in immune system by triggering T cell regulation and repressing the expression of cytokines such as IL-6, and TNF- α (Srivastava and Pramod, 2002). Acute cold stress makes body more susceptible to bacterial and viral infection generally, and HSPs are crucial molecules adjusting stress-induced dysfunction (Fu et al., 2014; Wei et al., 2018). In chickens, overexpression of HSPs (HSP27, HSP40, HSP60, HSP70, and HSP90) can protect the immune organs from oxidative stress due to cold stress (Fu et al., 2014). Thus, the change of HSPs is a good marker to judge the antistress ability of an organism.

Previous studies have found that continuous cold stimulation of 3°C below usual temperature improve immune function and ability to withstand cold stress during the later growth stages (Su et al., 2018; Wei et al., 2018; Su et al., 2019). As for IMCS, a more energy-efficient strategy, our previous studies demonstrated that 21-d IMCS at 3°C below feeding temperature for applied 3 h at 1-d intervals or 6 h at 2-d intervals, would not impact the production performance but enhance antioxidant capacity (Wang et al., 2016; Li et al., 2017). Therefore, more nuanced IMCS schemes (3°C below the temperature of CC for 3, 4, 5, or 6 h at 1-d intervals from 15 to 35 d of age) were set in this study based on the previous results to further evaluate the impact of potential acclimation strategies for coping cold stress in broiler production and determine the optimal program. The spleen organ index, the mRNA levels of TLRs, cytokines, immunoglobulins and HSPs, and the protein levels of HSPs were analyzed to determine an IMCS scheme that maximizes spleen immune function and cold resistance of broilers. Results of this study provide a scientific basis for potential acclimation strategies for coping cold stress in poultry production and supply a theoretical basis for revealing the immune-modulatory mechanism of animals under cold stimulation.

MATERIALS AND METHODS

Animal Care and Experimental Design

All experiments and procedures performed in this study were approved and conducted according to the Institutional Animal Care and Use Committee of the Northeast Agricultural University (IACUC-NEAU20150616). A total of 400 healthy 1-d-old Ross-308 male broilers were randomized into 5 equal groups: a control group (CC) and 4 cold stimulation groups (CS3, CS4, CS5, and CS6), and each had 5 replicates (16 chicks/replicate). All birds were reared in battery cages (180 cm \times 80 cm \times 60 cm) in climate control chambers and the experimental thermal conditions were shown in Figure 1. The control group was managed

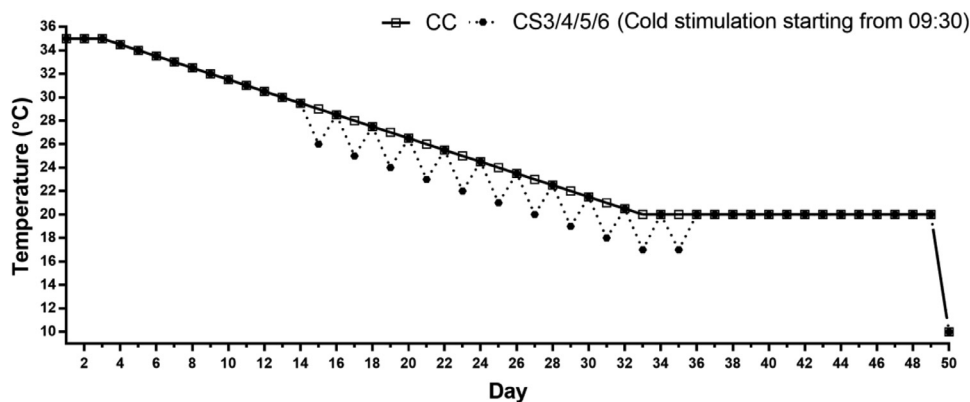


Figure 1. Experimental thermal conditions used in this study.

under normal room temperature scheme, namely 35°C for the first 3 d then reduced gradually by 1°C every 2 d until it dropped to 20°C at 32 d of age and maintained at that temperature till 49 d. The birds in CS3, CS4, CS5, and CS6 were exposed to 3°C below the temperature used in CC starting at 09:30 am every other day for 3, 4, 5, and 6 h, respectively, from 15 to 35 d of age. All broilers were subjected to acute cold stress (ACS) of 10°C for 12 h starting at 07:00 am at 50 d. During the entire duration of the experiment, the birds had free access to water, a complete starter diet (21% crude protein [CP] and 12.10 MJ/kg metabolizable energy [ME] for the first 3 wk of age), a grower diet (19.00% CP and 12.60 MJ/kg ME for 4–6 wk of age) and a finishing diet (17.50% CP and 13.20 MJ/kg ME for 7 wk of age). The composition of the commercial diets (Baishicheng Animal Husbandry, Harbin, China) included corn, soybean meal, vitamin A, vitamin D3, copper sulfate, calcium chloride, etc. All broilers were exposed to artificial light (0 h dark for the first 3 d and 23 h light:1 h dark from 4 d onward) and stable humidity (60–70% for first 2 wk and 40–50% for 4–7 wk). Meanwhile, the broilers were immunized with *Newcastle disease* and *Avian influenza virus* vaccines according to conventional immunization procedures.

One broiler from each replicate per group was euthanized and weighed at 08:00 am at 22, 29, 36, 43, 49 d and after 12 h acute cold stress at 50 d, then their spleens were quickly removed, washed with deionized water, weighed and stored at -80°C .

Spleen Index Determination

The spleen index of each broiler was calculated by the equation as follows:

$$\text{Spleen index}(\%) = \text{Spleen weight}/\text{Body weight} \times 100\%$$

RNA Extraction and Reverse Transcription

Total RNA was extracted from the collected frozen tissues using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's protocol. Dried RNA samples were dissolved in 40 μL of 1% DEPC water.

Total RNA integrity was ensured by horizontal electrophoresis on a 1.5% agarose gel. Total RNA concentration and purity were determined using a NanoDrop-2000 Spectrophotometer (Thermo Scientific, Wilmington, CA). RNA was reverse transcribed to synthesize complementary DNA (cDNA) by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) following the manufacturer's instructions, then diluted 5 times with DEPC water and stored at -80°C .

Quantitative Real-Time PCR Analysis

The specific primers listed in Table 1 were designed and synthesized by bioengineers (Sangon Biotech, Shanghai, China). THUNDERBIRD SYBR qPCR Mix (Toyobo) was used in quantitative real-time-PCR reactions on a LightCycler 480 II instrument (Roche, Basel, Switzerland) and each 10 μL final reaction mix was prepared from 5 μL SYBR Green I, 1 μL diluted cDNA template, 3.4 μL nuclease-free water, 0.3 μL of forward primer, and 0.3 μL of reverse primer. The PCR program was set as follows: 95°C for 1 min, repeated 40 cycles of 95°C for 15 s and 60°C for 1 min with fluorescence signal detected at the extension step.

The melting curve verified the single target amplification specificity. The relative abundance of mRNAs was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method. The housekeeping gene β -actin was used as an internal control for normalization of gene expression.

Western Blot Analysis

Total proteins were isolated from frozen spleen tissues (150 mg) with lysis buffer containing 1 mM PMSF (Biosharp, Beijing, China). The protein concentration was quantified using a BCA Protein Assay Kit (Biosharp) and adjusted to 5 mg/ μL . We detected the protein levels of HSPs according to the method of Su et al. (2018). Briefly, equal amounts of total protein (50 μg /condition) were fractionated by SDS-PAGE with 12% polyacrylamid gels and transferred to a nitrocellulose membrane (Biosharp, Hefei, China) in a semi-dry transfer equipment (Liuyi, Beijing, China). After sealing in a 5% milk TBST buffer at 37°C for 2 h, the blots with

Table 1. Gene-specific primers used in the real time RT-PCR analysis.

Gene	Reference sequence	Primer sequences (5'-3')
TLR1	NM_001081709	Forward: AGTCCATCTTTGTGTTCGCC Reverse: ATTGGCTCCAGCAAGATCAGG
TLR2	XM_001232192	Forward: GATTGTGGACAACATCATTGACTC Reverse: AGAGCTGCTTTCAAGTTTTCCC
TLR3	NM_001011691	Forward: TCAGTACATTTGTAACACCCCGCC Reverse: GGCGTCATAATCAAACACTCC
TLR4	NM_001030693.1	Forward: AGTCTGAAATGCTGAGCTCAAAT Reverse: GCGACGTTAAGCCATGGAAG
TLR5	NM_001024586	Forward: CCTGTGCTTTGAGGAACGAGA Reverse: CACCCATCTTTGAGAACTGCC
TLR15	NM_001037835	Forward: GTTCTCTCTCCAGTTTTGTAATAGC Reverse: GTGGTTTCATTGGTTGTTTTAGGAC
TLR21	NM_001030558	Forward: TGCCCTCCCACTGCTGTCCACT Reverse: AAAGGTGCCTTGACATCCT
IL-2	NM_204153.1	Forward: CTGTATTTCGGTAGCAATG Reverse: ACTCCTGGGTCTCAGTTG
IL-6	NM_204628.1	Forward: AAATCCCTCCTCGCAATCT Reverse: CCCTCACGGTCTTCTCCATAAA
IFN- α	XM_004937097.1	Forward: GGACATGGCTCCACACTAC Reverse: GGCTGCTGAGGATTTTGAAGA
IFN- γ	NM_205149.1	Forward: GAACTGGACAGGGAGAAATGAGA Reverse: ACGCCATCAGGAAGGTTGTT
IgA	NM_205287.1	Forward: TGCTAGTGGTTGTGGTGTGTG Reverse: CGGAGGCGGAGGAGACGATG
IgG	XM_025146241.1	Forward: CGATTCCAGCCTCAGCGTCAC Reverse: TAGGTGCCGTTGAAGTGTCTCTGG
HSP60	NM_001012916.1	Forward: AGCCAAAGGGCAGAAATG Reverse: TACAGCAACAACCTGAAGACC
HSP70	NM_001006685.1	Forward: CGGGCAAGTTTGACCTAA Reverse: TTGGCTCCACCTATCTCT
HSP90	NM_001109785.1	Forward: TCCTGTCTCGGCTTTAGTTT Reverse: AGGTGGCATCTCCTCGGT
β -actin	NM_205518.1	Forward: CACCACAGCCGAGAGAGAAAT Reverse: TGACCATCAGGGAGTTCATAGC

were treated at 4°C overnight with rabbit anti-mouse antibodies specific for HSP60 and HSP70 (1:5,000, presented by Professor Shiwen Xu, Northeast Agricultural University, Harbin, China), and HSP90 (1:1,200, WanleiBio, Shenyang, China) and β -actin (1:5,000, Abcam, United Kingdom). Subsequently, a HRP goat anti-rabbit IgG (1:4,000, ABclonal, Wuhan, China) was conjugated onto the primary antibodies. The protein bands were visualized using an ECL chemiluminescent kit (Biosharp) and scanned using a gray band scanner (GeneGnomeXRQ, Cambridge, UK). The optical density value (OD) of the bands was quantified using Image J software (NIH, Bethesda, MD) and the relative abundance of the HSPs proteins were expressed as the ratios of optical density of each of those proteins to that of β -actin.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 21 (IBM, Armonk, NY). The experimental data were tested for normal distribution using the Kolmogorov–Smirnov test before statistical analysis and all showed homogeneity of variance. The least-square mean and standard deviation (mean \pm SD) for immune organ index and gene expression level of spleen were calculated

Table 2. Spleen growth index of Ross-308 broilers.

Bird age	CC	CS3	CS4	CS5	CS6
D 22	0.98 ^y \pm 0.15	0.78 ^y \pm 0.14	0.85 ^y \pm 0.05	0.82 ^m \pm 0.10	0.94 ^y \pm 0.13
D 29	1.01 ^y \pm 0.10	1.13 ^s \pm 0.07	1.11 ^s \pm 0.09	1.12 ^z \pm 0.05	1.09 ^{xy} \pm 0.08
D 36	1.14 ^{xy} \pm 0.09	1.10 ^s \pm 0.17	1.11 ^s \pm 0.17	1.22 ^y \pm 0.05	1.24 ^s \pm 0.11
D 43	1.23 ^s \pm 0.04	1.21 ^s \pm 0.07	1.24 ^s \pm 0.10	1.30 ^{xy} \pm 0.03	1.23 ^s \pm 0.25
D 49	1.24 ^{abx} \pm 0.07	1.19 ^{bx} \pm 0.13	1.27 ^{abx} \pm 0.03	1.41 ^{ax} \pm 0.06	1.29 ^{abx} \pm 0.07

Note: Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c, d) and days of age (x,y,z).

by applying one-way analysis of variance (ANOVA). Intergroup and intragroup differences at the same and different time points were assessed by Duncan's multiple comparison at a significance threshold of 5%.

RESULTS

Organ Index

Table 2 showed the effect of intermittent cold stimulation on the spleen growth index of Ross-308 broilers. The spleen index of broilers aged 22 to 49 d did not differ between CS and CC ($P > 0.05$). When IMCS ended for 14 d (at 49 d), the spleen index of CS5 was the highest and significantly higher than that of CS3 ($P < 0.05$).

Relative Expression Levels of TLRs

The effect of intermittent cold stimulation on mRNA levels of toll-like receptors (TLR1, TLR2, TLR3, TLR4, TLR5, TLR15, and TLR21) in spleens of broilers was presented in Figure 2. In general, the mRNA levels of TLR1, TLR2, TLR3, and TLR4 were significantly lower while TLR5 and TLR21 were significantly higher in CS groups than CC group ($P < 0.05$) during IMCS (at 22 d). When IMCS ended for 7 and 14 d (at 43 and 49 d), lower levels of TLR1, TLR2, and TLR21 and higher levels of TLR3, TLR4, TLR5, and TLR15 were found in CS compared with CC ($P < 0.05$). The continuous differential expression of TLRs compared with CC in CS5 was the most stable, showing that all TLRs measured in CS5 but merely TLR5, TLR15, and TLR21 in CS3, TLR3, TLR4, and TLR21 in CS6, TLR1, TLR3, TLR4, TLR15, and TLR21 in CS4 had consistent differential expression compared with CC at 43 and 49 d ($P < 0.05$).

Relative Expression Levels of Cytokines

The mRNA levels of cytokines (IL-2, IL-6, IFN- α , and IFN- γ) in spleens of Ross-308 broilers during and after cold stimulation training were illustrated in the histogram in Figure 3. In response to IMCS, IFN- α , and IFN- γ mRNA levels in CS significantly increased compared to CC after 7 d of cold exposure ($P < 0.05$). Besides, IL-2 and IL-6 mRNA levels in CS3 were significantly lower ($P < 0.05$), while IL-2 levels in CS4 and CS5 were higher than those in CC on 22 d ($P < 0.05$). When the broilers

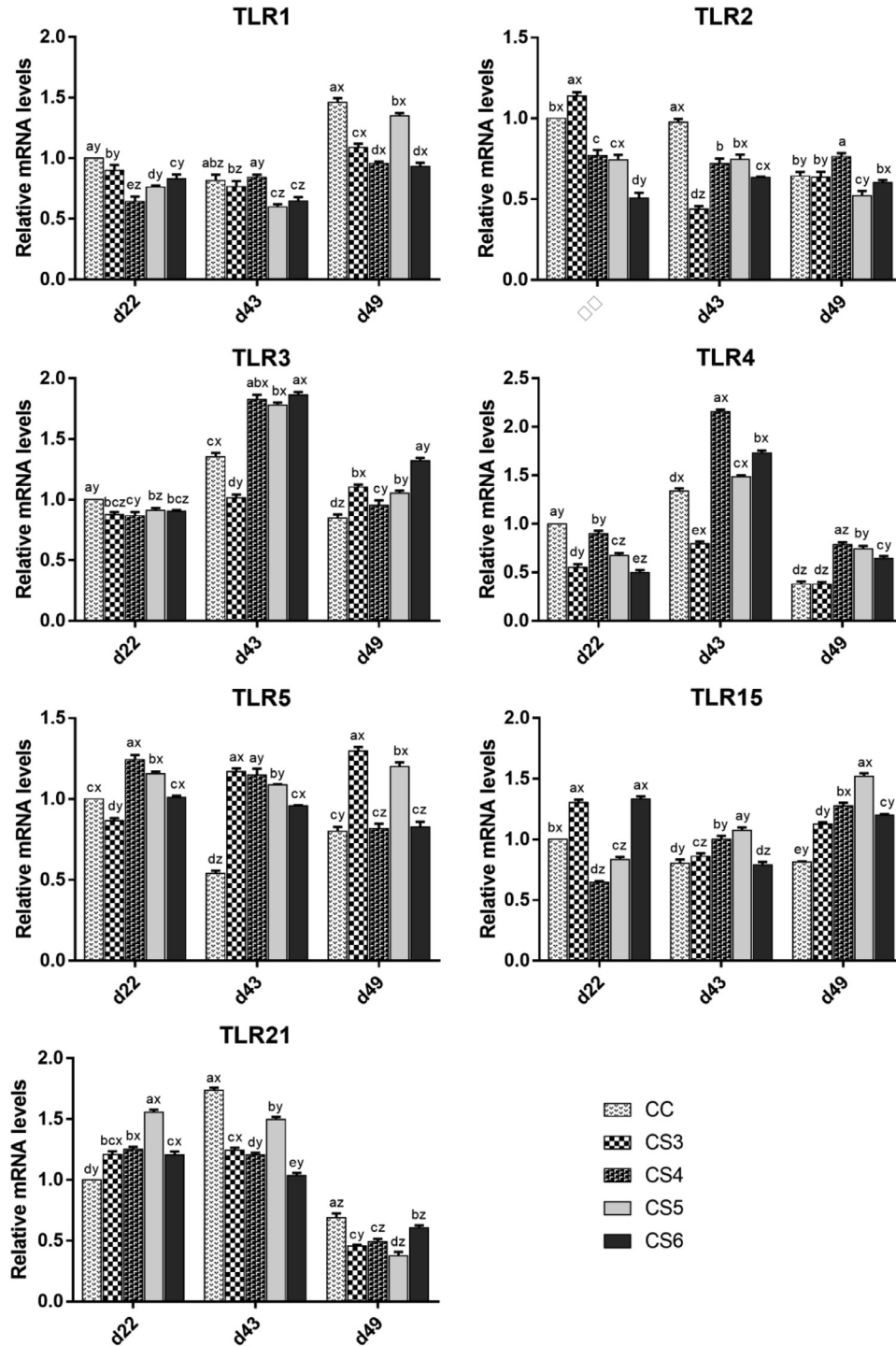


Figure 2. The mRNA levels of Toll-Like Receptors (TLR1, TLR2, TLR3, TLR4, TLR5, TLR15, and TLR21) in spleens of Ross-308 broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c, d) and d of age (x, y, z).

got rid of cold conditions for 7 and 14 d (at 43 and 49 d), cytokines maintained a steady trend generally, presenting as lower expression of IFN- α and higher expression of IL-2, IL-6, and IFN- γ in CS compared with CC ($P < 0.05$). Concretely, with the exception of CS3 in which merely IL-2, there were 3 indicators respectively in the other CS groups (IL-2, IL-6, and IFN- γ in CS4, IL-2, IL-6, and IFN- α in CS5, IL-2, IFN- α , and IFN- γ in CS6) presented consistent differential trends compared with CC at 43 and 49 d ($P < 0.05$).

Relative Expression Levels of Immunoglobulins

The fluctuations in mRNA levels of Immunoglobulins (IgA and IgG) in spleens of Ross-308 broilers with or without cold stimulation training were shown in Figure 4. Compared with CC, IgA levels in all CS groups and IgG levels in CS4 and CS5 exhibited a marked increase, whereas IgG level in CS3 decreased after 7-d IMCS (at 22 d) ($P < 0.05$). When IMCS

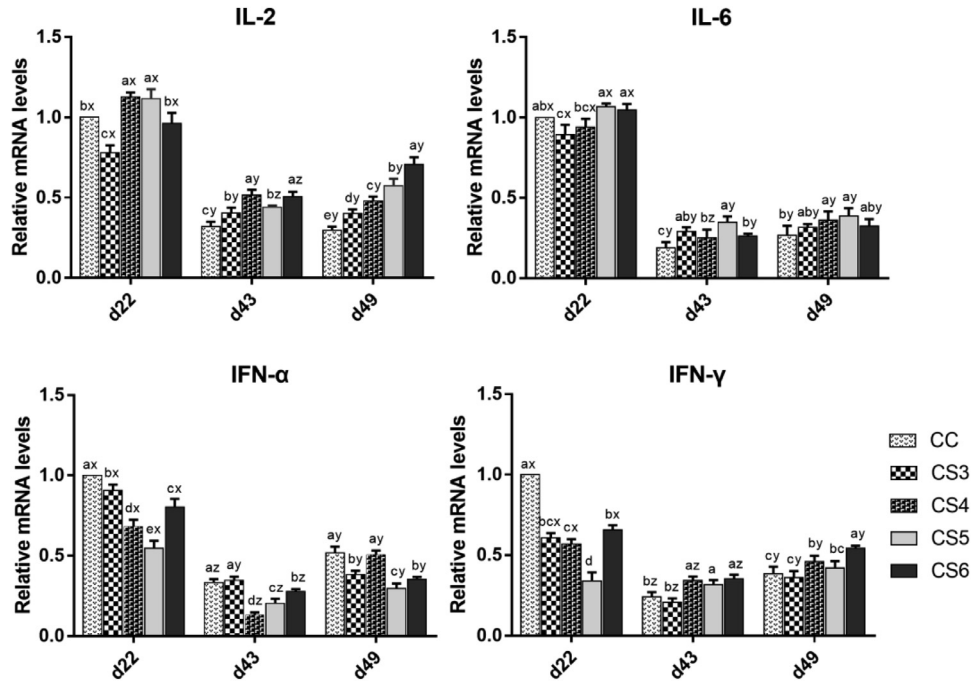


Figure 3. The mRNA levels of Cytokines (IL-2, IL-6, IFN- α , and IFN- γ) in spleens of Ross-308 broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c, d) and d of age (x,y,z).

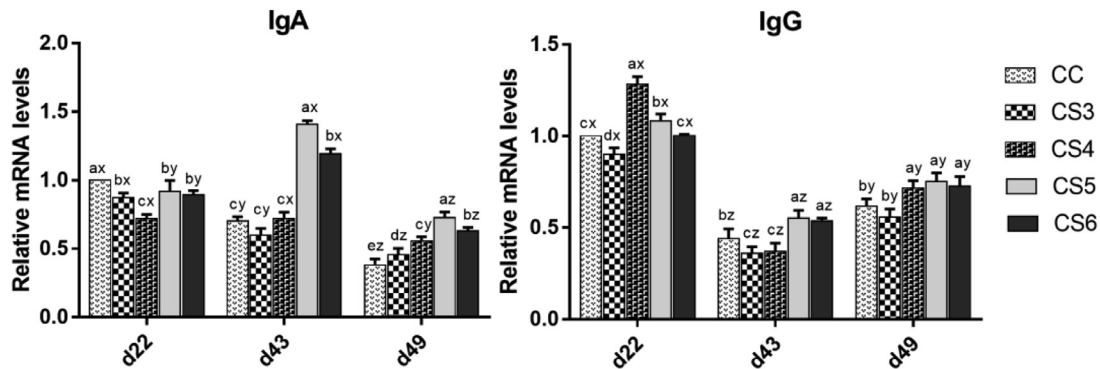


Figure 4. The mRNA levels of Immunoglobulin (IgA and IgG) in spleens of Ross-308 broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (A, B, C, D) and d of age (x, y, z).

ended for 7 and 14 d (at 43 and 49 d), upregulated IgA and IgG activities of broilers in CS5 and CS6 were noticed compared with those of untrained broilers ($P < 0.05$), besides, a remarkably higher mRNA level of IgA was attained in CS5 in comparison with CS6 ($P < 0.05$).

Relative Expression Levels of Heat Shock Protein

To ascertain whether HSPs levels after ACS were activated in spleens of Ross-308 broilers experienced IMCS, the protein and mRNA levels of HSP60, HSP70, and HSP90 were assessed and showed in Figures 5 and 6, respectively. Compared to pre-ACS, the mRNA level of HSP60 and HSP70 in CC and HSP60, HSP70, and HSP90 in CS were markedly upregulated after ACS ($P < 0.05$). Except for HSP90 mRNA and HSP70 protein in CS6, HSP90 protein in CS3, the mRNA and

protein levels of HSPs after ACS in CS were higher than those in CC ($P < 0.05$). Besides, the highest mRNA and protein levels of HSPs after ACS were found in CS5.

DISCUSSION

An organism is capable of adapting to environment including temperature alterations. Environmental adaptability can be facilitated if body accepts various environmental stimuli during the early growth period (Shahir et al., 2012; Shamma et al., 2014). Adverse environmental conditions including cold stress can reduce immunity of animals (Fu et al., 2014; Chen et al., 2020; Shah et al., 2020a,b; Hu and Cheng, 2021), while proper cold exposure can enhance immune function and disease resistance of animals. Our previous studies indicated that housing broilers at discontinuous cold stimulation could strengthen their immune-modulatory and function of small intestine and bursa and confer protection against injury of ACS (Li et al.,

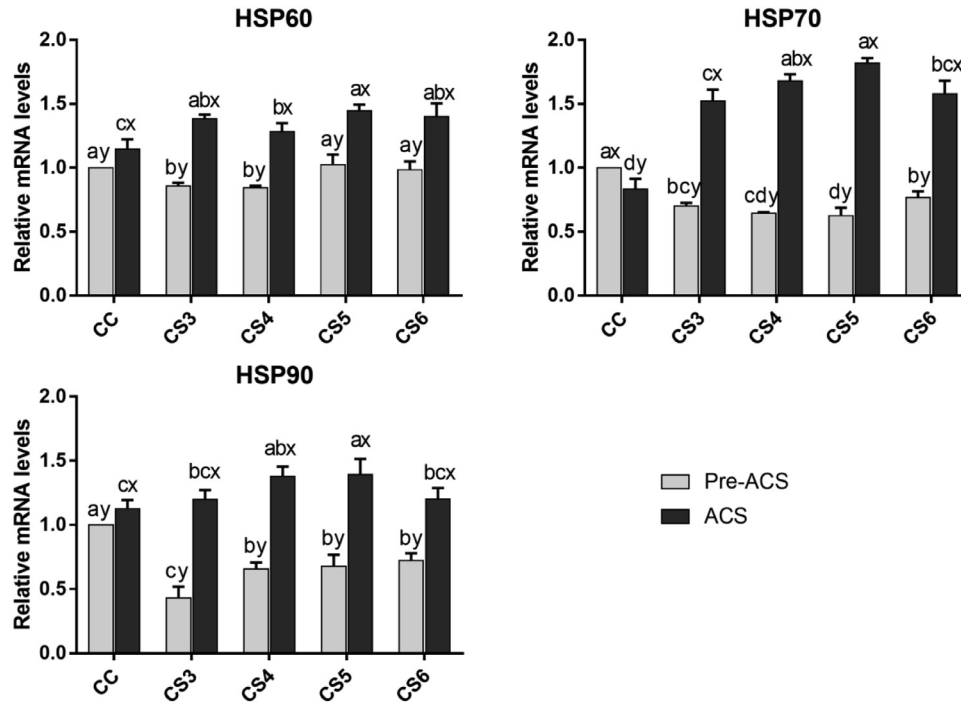


Figure 5. The mRNA levels of Heat Shock Protein (HSP60, HSP70, and HSP90) in spleens of Ross-308 broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c, d) or before and after acute cold stress (x, y).

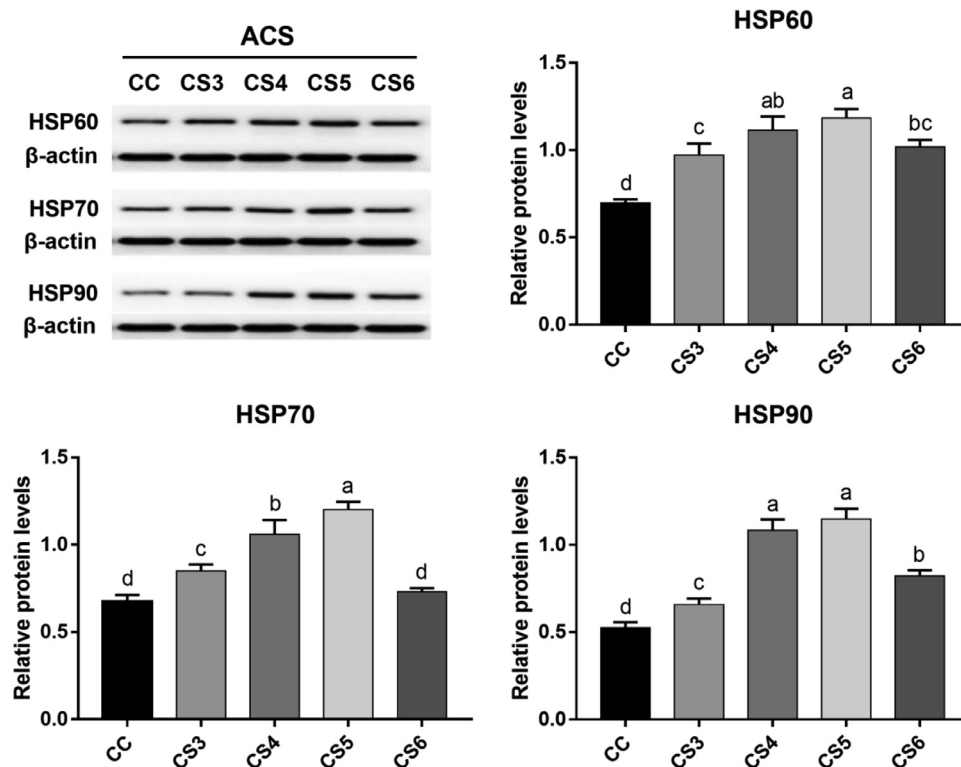


Figure 6. The protein levels of HSP60, HSP70, and HSP90 in spleens of Ross-308 broilers after the acute cold stress. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c, d).

2020; Liu et al., 2020). In the present study, IMCS at an early stage could promote the immunity of spleen by increasing spleen immunity index and inducing a new homeostasis of TLRs, cytokines and immunoglobulins mRNA and enhance adaptability to acute cold challenge in broilers by activating HSPs expression faster under ACS.

The organ index reflects the development, metabolism and function of animal organ and determines the health status of a body. Cold stress can inhibit the growth and development of animal immune organs and lead to organ atrophy and reduction of peripheral lymphocytes, thus lowering the immune level (Kimura et al., 1996;

Ren et al., 2011; Shcheniavsky I., 2021). When mice were exposed to cold water at 4°C for 4 to 24 h/d for 4 d consecutively or 12-wk-old laying ducks raised in a low-temperature environment at 2°C for 42 d, the spleen tissue and thymus tissues gradually deteriorated, leading to decreased immune index and a gradual decline in immune function (Kimura et al., 1996; Ren et al., 2011). However, mild cold stimulation does not damage body but increase immune organ index and enhance immune function like Maslov et al. (2016) documented that cold exposure (4°C, 1.5 h/d, 4 wk) had no significant effect on the spleen weight of rats, 8 h/d intermittent cold stimulation resulted in increased spleen weight in rats and Wang et al. (2005) reported that thymus index of 15-day-old pheasant significantly increased and spleen index slightly increased after cold stimulation at 5°C lower than normal feeding temperature. Consistent with these results, we found that the spleen index of broilers under cold stimulation regimen was not impaired, besides the spleen index of CS5 was slightly higher than that of CC and significantly higher than that of CS3 at 49 d. The possible reason is that the cold given to the broilers was relatively short and moderate in our study, which is not enough to cause damage to spleen tissues of broilers, in contrast, allow spleen had a tendency to grow better for coping with possible cold circumstances later.

The recognition and binding of pathogen-associated molecule pattern (**PAMP**) by TLRs trigger a series of signaling cascades, activating related cytokines and play an important role in innate immune response (Kawai and Akira, 2010; Paul et al., 2013). Cytokines are characterized by mediating immune reactions and being involved in inflammatory responses (Al-Zghoul et al., 2019). Immunoglobulins are the major components of immune system, playing an important role in immune function (Zhao et al., 2013). Previous studies demonstrated that stress condition or bacteria invasion could affect the immune function of broilers, and upregulate the mRNA levels of immune factors. The mRNA levels of TLR2 and TLR4 in spleens of broilers were significantly upregulated by short-term acute heat stress with a rapid increase of 16°C for 10 h (Huang, 2017). Ji et al. (2010) reported that 4 to 8 °C cold for 12 h disrupted the immune system, first increasing IL-2, IL-4, IL-6, IL-10, and IFN- γ levels and then decreasing the levels in piglets serum, which was always higher than those before the cold stress. A proper cold stimulus was reported to regulate autoimmune function by mediating immunoglobulin production by Carr et al. (1992), who found that the levels of intestinal IgG and IgA were increased when mice were placed in a -20°C freezer for 20 min per day. In this study, the differential expression of measured immune factors in CS group compared with CC at 22 d suggested that IMCS training could regulate the levels of TLRs, cytokines and immunoglobulins mRNA. Previous 2 researches showed that under chronic environmental stimulus, animal's internal endocrine, immune, and other systems did not go into disorder, but returned to a stable state, reaching

a new homeostasis after adaptive adjustment (Kolesnik and Derkho, 2020; Liu et al., 2020). Similarly, we found that the mRNA production of some immune molecules in CS group achieved a new balance and could sustain it from 1 to 2 wk after the ending of IMCS, in view of which we hypothesize that broilers have acquired cold adaptation, and the optimal cold stimulus program could be deduced from the number of molecules that reach a new stable state. Statistically, there were 4 immune genes (TLR5, TLR15, TLR21, and IL-2) tested in CS3 group, 7 genes (TLR3, TLR4, TLR15, TLR21, IL-2, IL-6, and IFN- γ) in CS4, and 9 genes (TLR1, TLR3, TLR4, TLR21, IL-2, IFN- α , IFN- γ , IgA, and IgG) in CS6 were stably differentially expressed in two weeks after IMCS compared with CC. What is noteworthy is that all the tested genes except IFN- α in CS5 (a total of 12) exhibited this phenomenon. Therefore, CS5 had the most stable steady-state and could be considered as the best cold adaptation scheme in terms of the effect of cold on the immune homeostasis of broiler spleen. For the establishment of the new homeostasis, the expression of genes varied between the treatment groups, but on the whole, TR1, TLR2, TLR3, TLR4, and IFN- α were downregulated, while TLR5, TLR21, IL-2, IL-6, IFN- γ , IgA, and IgG were upregulated in CS compared with CC. The high levels of immunoglobulins in CS were related to the enhancement of immune function. The increase of TLRs and cytokines were beneficial to the activation of immune function of broilers, but the increase of all TLRs and cytokines would make the body overly sensitive to environmental stimuli or viruses. So in order for broilers to remain immunologically enhanced for a long time, some TLRs and cytokines remained low levels to make broilers maintain a long-term immune enhancement status.

HSPs were key elements of the stress response system in animals. HSP60, as a molecular chaperone protein, is required for maintaining normal localization, conformation and function of proteins (Ricci et al., 2017). HSP70 assists in folding of newborn peptide chains and repairing of denatured proteins when organism encounter with a range of stressful conditions (Mayer and Bukau, 2005). HSP90 plays a crucial role in protein configuration maintenance in cells, such as cytoskeleton and enzyme configuration (Lei et al., 2009; Wernitznig et al., 2020; Uddin et al., 2021). Harmful stimuli can lead to increased expression of HSPs (Li et al., 2021; Miao et al., 2021). According to Li et al. (2006), HSP70 could be used as a hallmark of stress intensity, stronger cold stress corresponded to higher HSP70 expression and more severe deterioration of physiological status in Wistar rats. While, it had been revealed that under the same stimulus condition, overexpression of HSPs was correlated with enhanced immune and anti-stress ability of body (Puijvelde et al., 2007; Liu et al., 2009; Qian et al., 2014). In our present study, the mRNA levels of HSP60 and HSP70 in CC and all HSPs in CS increased rapidly after ACS. From here, we can see that although HSPs in CS present lower levels compared with CC after IMCS, its accelerated mobilization

reencounter cold challenge contribute to recover organism from stress and guard organism from subsequent insults. After 12 h ACS, except for HSP90 mRNA and HSP70 protein in CS6, and HSP90 protein in CS3, the mRNA and protein levels of HSPs in CS group after ACS were significantly higher than those in CC, and the highest HSPs levels were found in CS5. It indicated that CS5 had the strongest ability to adapt to cold environment and resist cold stress. Consistent with our results, Parsell and Lindquist (1993) demonstrated that when the body was subjected to more intense stress, mild stress-induced HSPs played a protective role.

CONCLUSIONS

Based on the present results, the IMCS at an early stage could slightly increase the spleen immunity index and activate the innate immune system in spleens of broilers by inducing a new homeostasis of TLRs, cytokines, and immunoglobulins. The stable state found at 43 to 49 d of age and high level of HSPs after ACS demonstrated that broilers finally established cold adaptation. Moreover, 3°C below the conventional feeding temperature for 5 h at 1-d intervals from 15 to 35 d of age (CS5) is the best cold adaptation establishment scheme.

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DISCLOSURES

The authors confirm that there are no conflicts of interest.

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