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PI3K/AKT pathway modulation and cold acclimation alleviation concerning apoptosis and necroptosis in broiler thymus

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ABSTRACT Moderate cold stimulation regulates the thymus's growth and function and facilitates cold acclimatization in broilers. However, the underlying mechanism remains unknown. To explore the possible mechanism of the thymus in cold-acclimated broilers against cold stress, 240 one-day-old Arbor Acres (**AA**) broilers were assigned to 2 groups randomly. The control group (**C**) was housed at conventional temperatures. The temperature during the first week was 33°C to 34°C. Between the ages of 8 and 32 d, the temperature was lowered by 1°C every 2 d, i.e., gradually from 32°C to 20°C, and then maintained at 20°C until 42 d of age. The cold-acclimated group (**C-3**) was housed at the same temperature as C from 1 to 7 d after birth. Between 8 and 42 d, the temperature of C-3 was 3°C colder than C. After 24 h exposure to acute cold stress (**ACS**) at 42 d, C and C-3 were named as **S** and **S-3**. The results showed that ACS was able to induce oxidation stress, modulate PI3K/AKT signal, and cause necroptosis and apoptosis in broiler thymus. By contrast, cold acclimation could alleviate apoptosis and necroptosis induced by cold stress via alleviating oxidative stress, efficiently activating the PI3K/AKT signal, as well as decreasing apoptotic and necrotic genes' levels. This study offers a novel theoretical basis for cold acclimation to improve the body's cold tolerance.

Key words: apoptosis, broiler, cold acclimation, necroptosis, oxidative stress

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INTRODUCTION

Cold stress usually refers to a range of physiological and functional side effects caused by the disruption of the body's thermostasis when the ambient temperature falls below the animal's critical temperature (Abo-Al-Ela et al., 2021). Animal productivity and well-being are negatively affected by cold stress. Ipek and Sahan (2006) reared 1-day-old broiler chicks in a cold-stressed environment for 3 wk before returning to conventional temperature feeding and found that cold stress dramatically reduced weight gain and body weight in broilers. Rahmani et al. (2017) kept 14-day-old Ross 308 broilers in a cold environment at 13°C to 15°C until 42 d of age, and found that cold stress decreased weight gain as well as feed conversion ratio, and triggered ascites syndrome in broilers. Cold stress can cause tissue and organ damage. According to Zhao et al. (2013), cold stress at 12°C considerably decreased the intestinal villus height, resulting in the congestion of intestinal mucosa in broilers. Cold stress caused unclear lymphoid structures in the quail spleen, with lymphocytes loosely arranged around the central artery, which could induce inflammatory injury (Ren et al., 2018). Cold stress can also disrupt various physiological processes (Qu et al., 2017; Spencer and Meyer, 2017). Su et al. (2016) found that apoptosis can be triggered by activating the caspase pathway under cold stress. According to Chen and Chen (2017), cold stress caused lymphocyte apoptosis in mice by activating Caspase-3 and Caspase-9. Mice exposed to cold stress show liver damage and autophagy due to activation of the SIRT2/FoxO1 pathway (Guo et al., 2022). Cold stress can induce oxidative stress, which can damage critical biomolecules, including DNA and proteins (Stadtman and Levine, 2003). Cheng et al. (2018) found that cold stress promoted ROS production in pufferfish and induced oxidative stress, which further exacerbated cellular DNA damage. Cold stress in livestock production often results in financial losses (Nguyen et al., 2016). Currently, cold stress is mainly mitigated by

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adding some supplements to the diet or improving feeding management conditions, but the effect is limited. For example, dietary supplements such as vitamin E (Sahin et al., 2003a), trace minerals (Sahin et al., 2003b), or soapwort extract (Dalkilic et al., 2017) can enhance cold-stressed quails' productivity and antioxidant function to varying degrees and alleviate the adverse effects of cold stress, but the effects are not very satisfactory.

Phosphoinositide 3-kinases (**PI3Ks**) can convert signals from various growth factors and cytokines into intracellular information, ultimately activating AKT, which acts as a PI3K downstream effector (Engelman et al., 2006). Marte and Downward (1997) pointed out that AKT participates in many biological processes, such as growth, metabolism and survival. Datta et al. (1999) found that during stress, an important regulator of cell survival is the PI3K/AKT pathway. The imbalance between antioxidants and oxidants can result in oxidative stress (Sun et al., 2024), which disrupts redox signals and impairs cell structure and function (Yu and Xiao, 2021). PI3K activation phosphorylates AKT, which can protect cells from oxidative damage and increase cell survival (Kang et al., 2010). By activating PI3K/AKT signaling, hydrogen protected the human keratinocyte cell line (HaCaT) from oxidative stress injury induced by UVB radiation (Zhang et al., 2018). Through PI3K/AKT signaling, oxidative stress can lead to different ways of cellular death, including necroptosis and apoptosis (Chen et al., 2020). According to research by Povsic et al. (2003) and Gu et al. (2004), activating the PI3K/AKT signaling can suppress apoptosis and promote cell survival. Chen et al. (2021) found that tectorigenin reduced apoptosis in HUVECs through activating the PI3K/AKT pathway. Apoptosis occurs when the PI3K/AKT pathway is inhibited. According to Chen et al. (2022), in chicken kidneys, exposure to bisphenol A reduced PI3K/AKT signaling and induced apoptosis. Zhao and Zhang (2017) found that lead exposure could induce apoptosis in chicken spleen lymphocytes by inducing oxidation stress and inhibiting PI3K/AKT signaling. According to Wang et al. (2020b), activation of the necroptosis signal and the PI3K/AKT pathway are closely linked. In the tracheal tissue of selenium-deficient chickens, there was an inhibition of PI3K/AKT expression, which could activate necroptosis-related genes and cause necroptosis (Wang et al., 2020a). Exposure to bisphenol A caused necroptosis in chicken bursa by decreasing PI3K/AKT expression and increasing necroptosis pathway-related factor expression, including MLKL, RIPK1, and RIPK3 (Liu et al., 2021). The study by Wang et al. (2020b) found that chlorpyrifos could promote necroptosis by inducing oxidative stress as well as suppressing PI3K/AKT expression.

The term "cold acclimation" describes the body's adaptability to low temperatures after repeated exposure to cold, which is established within the range of physiological tolerance. Increased body resistance to cold and prevention of cold-related damage can be achieved through cold acclimation (Rintamäki, 2000; Morabito et al., 2014). The body's ability to adapt to cold can be enhanced by prolonged or intermittent repetitive exposure to low temperatures (van der Lans et al., 2013). After receiving a cold stimulus at 3°C cooler than the typical rearing temperature from 8 to 42 d of age, broilers can establish cold acclimation, which may mitigate oxidative and inflammatory damage caused by re-exposure to ACS at 7°C to some extent (Wei et al., 2018; Su et al., 2019). In chickens, the thymus is an essential immunological organ. Thymic function and status not only affect overall immune function, but also influence the resistance to environmental stress (Chen et al., 2019). Our research team suggested that intermittent moderate cold stimulation could regulate thymus growth and function, facilitate broilers cold acclimation, and improve cold tolerance of broilers (Fu et al., 2022). Nevertheless, the specific thymic mechanism in the phenomenon of enhanced cold tolerance in cold-acclimated broilers is still unknown. Therefore, based on the previous findings of Su et al. (2020), this investigation examined the histopathological alterations, TUNEL staining, antioxidant levels, and gene expression related to apoptosis, necroptosis and the PI3K/AKT pathway in AA chicken thymus, cold-acclimated or not, before and after ACS. The aim of the current study was to better understand how the thymus protects against cold stress in cold-acclimated broilers and to provide new support and evidence for the theory that cold tolerance can be enhanced by acclimation to cold.

MATERIALS AND METHODS

Ethical Approval

This work was approved by the Laboratory Animal Ethics Committee of Northeast Agricultural University (China) for all procedures used.

Design of Animals and Experiments

240 one-day-old fast-growing AA broilers were allocated to **C** (control) and **C-3** (cold acclimation) in the random (n = 120, 4 × 30 per replicate group) and housed in cages in 2 climate-controlled barns. Group C was reared at conventional temperatures, day 1 to 3 at 34°C and day 4 to 7 at 33°C. Thereafter, the temperature decreased by 1°C every 2 d until reaching 20°C on the 32th day and remained there till day 42. From day 1 to 7, the temperature in C-3 was the same as in C, and from day 8 to 42, the temperature was consistently 3°C lower than in C. On day 42, **ACS** (acute cold stress) at 7°C was applied to all broilers throughout the day. The specific temperature conditions of this experiment are given in Table 1. After ACS, C and C-3 were respectively labeled as **S** (C + ACS) and **S-3** (C-3 + ACS). Throughout the trial, the broilers had access to food and water ad libitum, as well as an early diet (12.1 MJ/kg ME and 21% CP) from 1 to 3 weeks old and a late diet (12.6 MJ/kg ME and 19% CP) from 4 to 6 weeks old. The broilers were manually fed pelleted feed 3 times a day, and tap water (public water system) was continuously supplied to nipple drinkers in each cage. The

Table 1. Temperature regime used in this study.

Age (day)	C	C-3
1−3	34℃	Same as in C
4−7	33℃	
8−31	−1℃/2 d	C-3 ℃
32−42	20℃	17℃
Acute cold stress(43)	7℃ (S)	7℃ (S-3)

From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after acute cold stress, respectively.

climate-controlled barn had a transverse negative pressure ventilation system. During the experiment, all broilers were subjected to artificial light (23 h of light for the first 3 d at 25 lux and 16 h of light from day 4 to the end at 20 lux) and the humidity was kept at 55% to 65%. The environment inside and outside the climate-controlled barn was cleaned daily from 5 am to 6 am and from 7 pm to 8 pm, including cleaning the floor, cleaning the feeding and drinking facilities, and removing excrement, etc. A foot disinfectant basin was located at the entrance to the climate-controlled barn, and breeders were required to change clothes and sterilize themselves before entering the climate-controlled barn. During the experiment, the climate-controlled barn was spray disinfected with 0.1% benzalkonium bromide, and chickens aged 1 to 20 d were disinfected every 3 days, 21 to 40 d every other day, and once a day thereafter. Disinfection was stopped 3 d before and after vaccination. During the experiment, the broilers were inoculated with Newcastle disease vaccine and infectious bursal disease vaccine according to routine procedures. The poultry manure generated during the experiment was transported to the Manure Treatment Laboratory of Northeast Agricultural University for composting and fermentation. The broiler carcasses after sampling were collected by the Department of Energy Conservation and Environmental Protection of Northeast Agricultural University for uniform treatment. At day 42 and 43 (before and after ACS), 8 broilers (2 per replicate) were chosen randomly from each group for euthanasia by cervical dislocation. Thymus tissue was excised right away and washed with icy-cold saline. Partial thymus was preserved in a 4% paraformaldehyde solution. For subsequent analysis, the remaining samples were kept in storage at −80°C.

Histological Examination

Thymus was immersed and dehydrated under the condition of gradually increasing ethanol concentrations after being preserved for at least 24 h in 4% paraformaldehyde, following embedded in paraffin. Light microscopy (Nikon Eclipse E400) was used to examine the microstructure after hematoxylin and eosin (**H&E**) staining of 5 to 6 μm slices.

TUNEL Staining Analysis

Apoptosis in the thymus of broilers was detected with the TUNEL assay kit (Servicebio, G1501) and all procedures were carried out per manufacturer. Observed and captured images under a fluorescence microscope (Nikon Eclipse C1). In each section, 5 fields of view (×400) were chosen at random. Total cells in each field were recorded along with the number of positive cells. Apoptosis rate refers to the proportion of apoptotic cells in total cells.

Oxidation and Antioxidant Index Detection

Malondialdehyde (**MDA**) and nitric oxide (**NO**) levels as well as the activities of inducible nitric oxide synthase (**iNOS**), catalase (**CAT**), superoxide dismutase (**SOD**), and glutathione peroxidase (**GSH-Px**) in thymus homogenate were quantified using kits purchased from Nanjing Jiancheng Bioengineering Institute. The kits used were A003-1, A012, A014-1, A007-1, A001-1 and A005 in this order. All procedures were carried out precisely as instructed.

qRT-PCR Analysis

Total thymic RNA was extracted with Trizol (Takara, China). Reverse transcription was used to generate cDNA as instructed (Takara, RR047). Table 2 displays the sequences of all primers. The Agilent Mx3000P detection system (USA) was used for the qRT-PCR. The reference gene was β-actin. mRNA expression was calculated utilizing the $2^{-\Delta\Delta Ct}$ method.

Western Blot Examination

Briefly, after extraction of total protein from thymus tissue, the concentration was determined. To separate protein samples, 8 to 15% SDS−polyacrylamide gel electrophoresis was employed. Subsequently, using a semidry trans blotter (TE77 PWR,

Table 2. Primer sequences in this study.

Gene	Primer sequences (5′-3′)
Caspase-3	F: CATCTGCATCCGTGCCTGA
	R: CTCTCGGCTGTGGTGGTGAA
Caspase-8	F: ACTTACTTGGTGAAGGAGAG
	R: AGTGGTCAGTTCTTGGATAG
Bax	F: TGCTTCAGGGTTGCATCCA
	R: AGACACTCGCTCAGCTTCTTG
Bcl-2	F: ATCGTCGCCTTCTTCGAGTT
	R: ATCCCATCCTCCGTTGTCCT
Cyt-c	F: AGGCAAGCACAAGACTGGA
	R: CTGACTATCACCAAGAACCACC
RIPK1	F: TGCCTTCTGTTCCTCACTGG
	R: CCTGCGTAGGTATTGCAGCTT
RIPK3	F: ACATCCTTCGCTCACAGCAA
	R: ACCTGTGCTGCCTTCTCTCC
MLKL	F: CACCGATACCAGGAGCCAGT
	R: AGCCAGCAAGTCCACGATCT
PI3K	F: GTCCTTGAGCCACTGATG
	R: TGTTGCCTTACGGTTGTT
AKT	F: AGGAGGAAGAGATGATGGAT
	R: GAATGGATGCCGTGAGTT
β-actin	F: CCGCTCTATGAAGGCTACGC
	R: CTCTCGGCTGTGGTGGTGAA

Table 3. The antibodies utilized in this investigation.

Antibody	Dilution ratio	Source
Bcl-2	1:300	ABclonal, China
Bax,Cyt-c,RIPK1, RIPK3, MLKL	1:500	
Caspase-3, Caspase-8, PI3K, AKT, p-AKT		Wanleibio, China
β-actin	1:8,000	Zenbio, China

USA), the proteins were transferred to a nitrocellulose membrane at 4℃. After blocking the membranes for 2 h at 37°C using 5% skim milk, the membranes were incubated with diluted rabbit primary antibodies obtained for a whole night at 4°C. Table 3 lists the antibodies utilized in this investigation. The following day, the membranes were treated for 1 h (light protection) with matching secondary antibodies (1:8,000, Zenbio, Chengdu, China). Finally, protein bands and gray values were measured using Image J software and the chemiluminescence imaging system (GeneGnomeXRQ, UK). β-actin expression served as a control.

Statistical Analysis

Data analysis was performed using IBM SPSS (version 22.0, Armonk, NY). Duncan's multiple comparisons and one-way ANOVA were used for intergroup disparities at every time point and for intragroup variances at different times. Data are presented as mean ± standard error, with significant differences indicated by P values < 0.05.

RESULTS

Histopathological Observation

Figure 1 illustrates the effect of ACS on thymus histomorphology in broilers with and without cold acclimation. On day 42, thymus tissues in C and C-3 appeared normal, with a thick and deeply stained outer cortex (C) as well as a lightly stained medial central medullary region (M). There were a large number of well-differentiated or developing T lymphocytes in thymus tissues, and only individual apoptotic cells were present in C-3. After ACS, thymus tissues from S showed obvious damage, with a disorganized arrangement of thymocytes in the medullary region and a significant percentage of necrotic or apoptotic cells. At the same time, there was extensive nuclear fragmentation and lysis as well as localized focal necrosis with local hemorrhage. A small number of apoptotic bodies and necrotic cells were still observed in S-3, but the congestion and hemolysis caused by ACS were significantly improved, and the morphology of the thymus was complete, with a clear and regular boundary between the cortex and the medulla. The above results indicated that ACS could damage the normal morphology of the thymus. Cold acclimation, however, can reduce the tissue damage brought on by ACS.

TUNEL Apoptosis Analysis and Apoptotic Gene Expression

To investigate the impacts of ACS on apoptosis in thymus tissue from broilers with and without cold acclimation, we quantified TUNEL-positive cells using

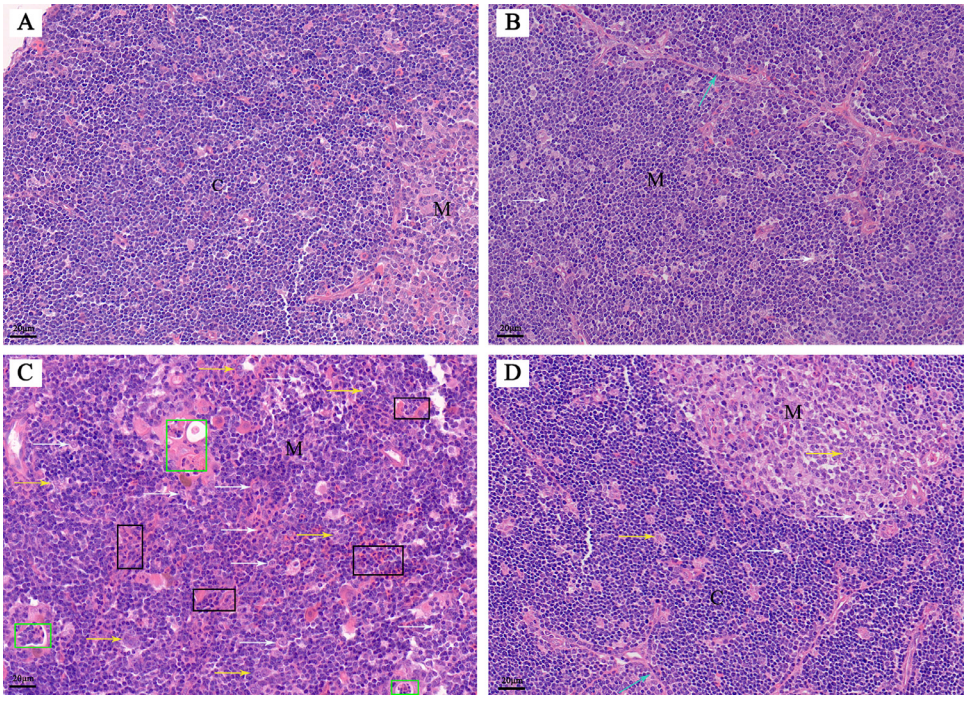

Figure 1. Thymus tissue morphology of broilers with and without cold acclimation before and after ACS. From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or at cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after ACS, respectively. (A, B) C and C-3 groups on day 42 (before ACS). (C, D) S and S-3 groups after ACS. C, cortex. M, medulla. White arrows, apoptotic bodies, or necrotic cells. Yellow arrows, nuclear fragmentation, or even dissolution. Green arrows, capsules, or septa. Black boxes, pathological congestion, or hemolysis. Green boxes, local focal necrosis. ACS, acute cold stress.

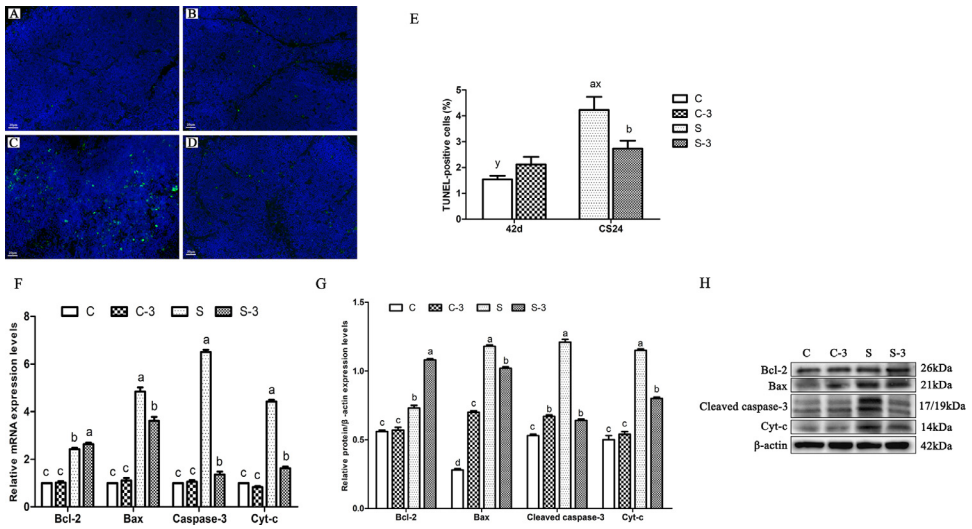

Figure 2. Effects of ACS on apoptosis in broiler thymus with and without cold acclimation. From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after ACS, respectively. (A, B) C and C-3 groups on day 42 (before ACS). (C, D) S and S-3 groups after ACS. Green fluorescence, TUNEL positive nuclei. Blue fluorescence, TUNEL negative nuclei. Magnification, 40 ×. (E) Apoptosis rate. (F) Apoptosis-related genes mRNA expression. (G, H) Apoptosis-related genes protein expression. Significant differences ($P < 0.05$) between days (x, y) and groups (a, b, c, d) are indicated by different letters. ACS, acute cold stress; CS24, 24 h after acute cold stress.

TUNEL staining and detected the expression of genes associated with apoptosis (Figure 2). Figures 2A−2E displays the TUNEL staining results. At day 42, both C and C-3 contained a small percentage of TUNEL-positive cells, but there was no significant difference between the 2 groups ($P > 0.05$). After ACS, TUNEL-positive cells dramatically increased in S (1.54−4.23%) in comparison to 42 d ($P < 0.05$). Despite an increase in TUNEL-positive cells in S-3 (from 2.12−2.73%), the increase did not reach statistical significance ($P > 0.05$). S had significantly more TUNEL-positive cells than S-3 ($P < 0.05$). We determined apoptosis-related gene expression based on TUNEL staining results (Figures 2F−2H). According to the results, at 42 d, C and C-3 did not differ significantly in Bax, Bcl-2, Cyt-c and Caspase-3 mRNA expression ($P > 0.05$). When comparing C-3 with C, Caspase-3 and Bax protein levels increased significantly ($P < 0.05$), whereas Cyt-c and Bcl-2 protein levels did not differ substantially ($P > 0.05$). In S and S-3, ACS markedly increased several apoptosis-related genes' mRNA and protein expression, including Bcl-2, Bax, Cyt-c and Caspase-3 ($P < 0.05$). Compared to S, S-3 showed significantly higher levels of Bcl-2 ($P < 0.05$) and lower levels of Bax, Caspase-3 and Cyt-c ($P < 0.05$). These findings suggested that ACS could trigger apoptosis, but that apoptosis could be alleviated by cold acclimation.

Table 4. Effects of ACS on oxidation and antioxidant indexes in thymus tissue of broilers with and without cold acclimation.

Indicator	C (S)	C-3 (S-3)	*P*-value
MDA (nmol/mg prot)			
42 d	$4.44^{y} \pm 0.11$	$4.59^{y} \pm 0.27$	0.62
CS24	$29.01^{ax} \pm 1.38$	$24.15^{bx} \pm 0.68$	0.03
P-value	<0.01	<0.01	
NO (μmol/g prot)			
42 d	$2.16^{y} \pm 0.13$	$2.30^{y} \pm 0.27$	0.66
CS24	$35.50^{ax} \pm 1.94$	$27.58^{bx} \pm 1.13$	0.02
P-value	<0.01	<0.01	
iNOS (U/mg prot)			
42 d	$7.28^{y} \pm 0.14$	$7.96^{y} \pm 0.49$	0.26
CS24	$13.53^{x} \pm 0.63$	$12.40^{x} \pm 0.08$	0.15
P-value	<0.01	<0.01	
GSH-Px (U/mg prot)			
42 d	$697.89^{bx} \pm 7.59$	$783.40^{ax} \pm 7.74$	<0.01
CS24	$408.80^{by} \pm 6.71$	$479.97^{ay} \pm 5.92$	<0.01
P-value	<0.01	<0.01	
SOD (U/mg prot)			
42 d	$89.55^{x} \pm 1.53$	$96.48^{x} \pm 2.19$	0.06
CS24	$42.16^{by} \pm 0.68$	$49.80^{ay} \pm 1.51$	0.01
P-value	<0.01	<0.01	
CAT (U/mg prot)			
42 d	$297.35^{x} \pm 4.78$	$314.67^{x} \pm 6.48$	0.10
CS24	$108.04^{by} \pm 1.86$	$120.62^{ay} \pm 1.93$	0.01
P-value	<0.01	<0.01	

From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or at cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after ACS, respectively.

Significant differences ($P < 0.05$) between days (x, y) and groups (a, b) are indicated by different letters.

Abbreviations: CAT, catalase; ACS, acute cold stress; CS24, 24 h after acute cold stress; GSH-Px, glutathione peroxidase; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase.

Detection of Oxidation and Antioxidant Indexes

The effects of ACS on oxidation and antioxidant indexes in thymus tissue from broilers, cold-acclimated or not, are shown in Table 4. Compared to C, C-3 had a higher activity in GSH-Px at 42 d ($P < 0.05$), while the other indicators were not significantly different ($P > 0.05$). ACS considerably increased free radical levels (MDA, NO and iNOS) ($P < 0.05$), while dramatically reducing antioxidant enzyme activities (CAT, GSH-Px and SOD) in both groups ($P < 0.05$). Compared to S,

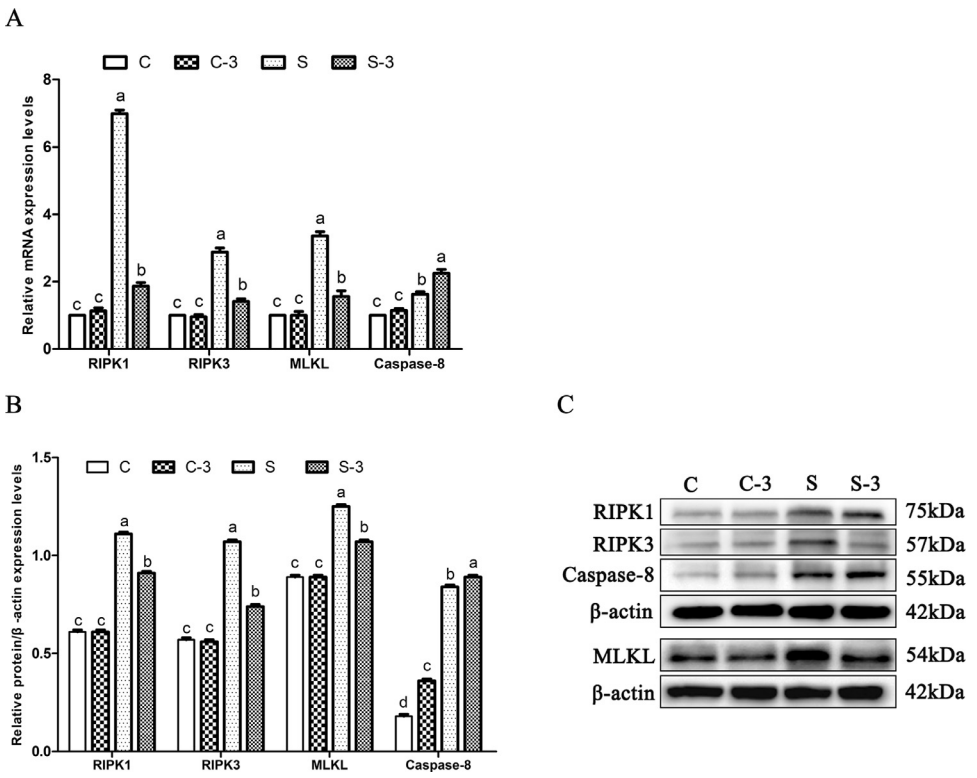

Figure 3. Necroptosis-related gene expression levels in broiler thymus with and without cold acclimation: effects of ACS. From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or at cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after ACS, respectively. (A) Necroptosis-related genes mRNA expression. (B, C) Necroptosis-related genes protein expression. Different letters (a, b, c, d) indicate significant differences ($P < 0.05$) between groups. ACS, acute cold stress.

S-3 had remarkably higher CAT, SOD, and GSH-Px activities as well as lower MDA and NO levels ($P < 0.05$). According to these results, ACS causes oxidative damage that can be alleviated by cold acclimation.

Gene Expression Associated with Necroptosis

We detected necroptosis-related gene mRNA and protein expression levels based on the HE staining results (Figure 3). The findings demonstrated that at 42 d, C and C-3 had no significant difference in MLKL, RIPK1, RIPK3 and Caspase-8 mRNA levels ($P > 0.05$). C-3 had a significantly higher Caspase-8 protein level than C ($P < 0.05$), whereas other necroptosis-associated genes showed no significant difference in protein levels ($P > 0.05$). In both groups, ACS significantly upregulated MLKL, Caspase-8, RIPK1 and RIPK3 expression at both mRNA and protein levels ($P < 0.05$). Furthermore, at both mRNA and protein levels, S-3 had significantly lower RIPK1, RIPK3 and MLKL expression and higher Caspase-8 expression than S ($P < 0.05$). The above results showed that ACS caused necroptosis, but that necroptosis could be reduced by cold acclimation.

PI3K and AKT Expression

To determine whether the mitigating effect of cold acclimation on thymocyte necroptosis and apoptosis in broilers exposed to ACS involved the PI3K/AKT pathway, we determined PI3K and AKT expression levels (Figure 4). At 42 d, there was no detectable difference in the expression of AKT and PI3K between C and C-3, either at the mRNA or protein level ($P > 0.05$). In both groups, ACS considerably increased AKT and PI3K expression at both mRNA and protein levels ($P < 0.05$). However, S-3 had considerably higher levels of both genes than S ($P < 0.05$). The aforementioned findings showed that PI3K/AKT signaling was connected to ACS-induced thymic apoptosis and necroptosis in broilers. Conversely, by efficiently activating PI3K/AKT signaling, cold acclimation may attenuate ACS-induced apoptosis and necroptosis.

DISCUSSION

Cold stress can disrupt homeostatic regulation in animals, gradually impairing antioxidant capacity, inducing apoptosis and promoting autophagy (Cong et al., 2018; Jia et al., 2019; Guo et al., 2022). Exposure to severe cold at 10℃ for up to 24 h upregulated the levels of genes involved in necroptosis, which was one of the death pathways that resulted in cold-induced death (Ren et al., 2021). However, cold acclimation can alleviate a series of adverse responses caused by cold stress. Cold acclimation was effective in alleviating cold stress-induced oxidative damage in a study on broilers by Su et al. (2019). Zebrafish cells can undergo less apoptosis after cold acclimation (Wang et al., 2022). This study investigated possible mechanisms of cold acclimation to

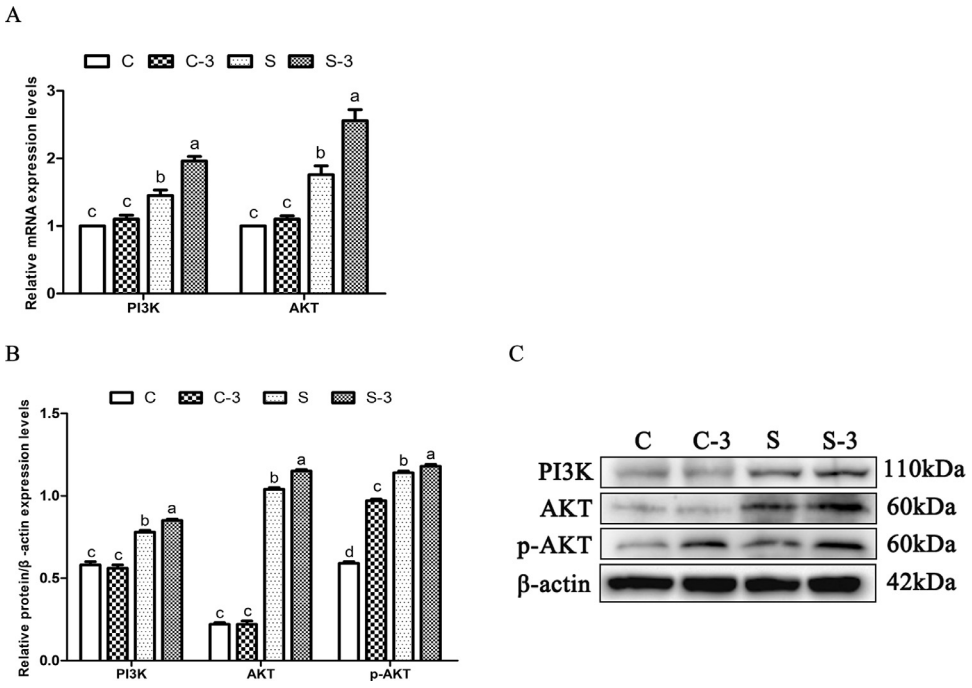

Figure 4. PI3K/AKT pathway expression levels in broiler thymus with and without cold acclimation: effects of ACS. From day 8 to day 42, the broilers were reared at either thermal conform temperatures (C) or at cold acclimation (3℃ below C, C-3). S and S-3 represent C and C-3 after ACS, respectively. (A) PI3K and AKT mRNA expression. (B, C) PI3K and AKT protein expression. Different letters (a, b, c, d) indicate significant differences ($P < 0.05$) between groups. ACS, acute cold stress.

control and eliminate cold stress-induced thymic damage. The findings demonstrated that ACS can cause oxidative stress, necroptosis, and apoptosis in the chicken thymus. It also dramatically increased the expression of necroptosis and apoptosis-related genes and suppressed PI3K/AKT signaling. In contrast, cold acclimation resulted in a significant reduction in cold stress-induced oxidative stress, apoptosis and necroptosis.

Oxidative stress is known to be a major cause of apoptosis and necroptosis. Bisphenol A exposure induced necroptosis and apoptosis in chicken kidneys by causing oxidative stress, inhibiting PI3K/AKT signaling and upregulating the levels of genes critical for necroptosis and apoptosis, including RIPK1, RIPK3, MLKL, Bax, Caspase-3 and Caspase-9 (Chen et al., 2022). Oxidative stress-induced renal necroptosis in chickens was enhanced by suppressing the PI3K/AKT pathway, as reported by Zhang et al. (2021). Oxidation stress and PI3K/AKT pathway inhibition can result from cold and heat stress. For example, in human dermal microvascular endothelial cells, low temperature caused oxidative stress, reducing PI3K/AKT signal expression (Guan et al., 2019). Heat exposure could induce oxidative stress, suppress PI3K and AKT expression, and ultimately cause rainbow trout hepatocytes to undergo apoptosis, as reported by Li et al. (2023). Similarly, in S and S-3, we found that ACS could cause oxidative stress in the thymus, as it considerably raised NO, MDA and iNOS levels, while dramatically reducing GSH-Px, SOD and CAT activities. The thymus was stained with both HE and TUNEL to determine the extent of necroptosis and apoptosis. The findings demonstrated that after ACS, S had obvious damage and a high number of apoptotic cells, indicating that oxidative stress induced by ACS significantly increased necroptosis and apoptosis in the thymus. However, acclimation can alleviate oxidative stress caused by stress. According to Ou et al. (2021), oxidative stress in C57BL/6J mice heart was decreased during ischemia-reperfusion injury once hypoxic acclimation had been established. When low-temperature stress was applied to cold-acclimated broilers, the degree of oxidative damage in the trachea was dramatically decreased, as reported by Su et al. (2019). Consistent with the above findings, in this investigation, MDA and NO contents were much reduced in S-3 (cold acclimation was achieved at an early growth stage) compared to S after ACS, whereas GSH-Px, SOD and CAT activities were significantly higher, indicating that ACS-induced oxidative stress of the thymus could be mitigated by cold acclimation. After ACS, only a small number of apoptotic and necrotic cells were present in S-3 and the morphology of the thymus was intact, indicating that cold acclimation alleviated the apoptosis and necroptosis caused by ACS, and helped to maintain the normal morphology of the thymus. PI3K/AKT signaling plays a role in controlling both necroptosis and apoptosis, and is closely linked to oxidative stress, as reported by Reddy et al. (2015). However, apoptosis can be reduced by reducing oxidative stress and enhancing the PI3K/AKT pathway. In type 2 diabetic rats, curcumin prevents cardiomyocyte apoptosis by lowering oxidative stress and triggering PI3K/AKT signaling (Ren et al., 2020). Coptisine effectively suppressed oxidative stress generated by hyperuricemia, upregulated PI3K/AKT signaling as well as reduced apoptosis in KM mice (Liu et al., 2022). Furthermore, PI3K/AKT signaling can be activated by

cold stress (Liu et al., 2017). Similar to this, our findings demonstrated that after ACS, PI3K/AKT signaling was more highly expressed in S and S-3 than at 42 d, suggesting that ACS could activate PI3K/AKT signaling. Key apoptosis (Bax, Bcl-2, Caspase-3, and Cyt-c) and necroptosis (Caspase-8, MLKL, RIPK1, and RIPK3) related genes' expression was also significantly upregulated in the thymus following ACS, suggesting that ACS induced both processes. However, the establishment of acclimation responses can attenuate apoptosis caused by unfavorable factors via efficiently activating the PI3K/AKT pathway. As an example, exercise preconditioning by inducing acclimation responses in rats and increasing PI3K/AKT signaling, has been shown to reduce apoptosis and cardiac injury induced by strenuous exercise (Li et al., 2020). Thermal preconditioning at slightly higher temperatures could induce AKT activation in a PI3K-dependent manner, thereby alleviating low potassium-induced apoptosis (Cao et al., 2007). Similarly, in this study, after ACS, compared with S, S-3 had considerably higher PI3K, AKT, and p-AKT levels and greater PI3K/AKT pathway activation. Most necroptosis as well as apoptosis-related genes were expressed at much lower levels in S-3 than in S. This suggests that via activating the PI3K/AKT signal, cold-acclimated broilers may attenuate ACS-induced necroptosis and apoptosis.

CONCLUSIONS

ACS can generate oxidative stress, regulate PI3K/AKT signaling, and cause necroptosis and apoptosis in broiler thymus through RIPK3/MLKL and Bcl-2. In contrast, through decreasing oxidative stress, effectively activating the PI3K/AKT signal as well as downregulating necroptosis and apoptosis-related gene expression, cold acclimation can attenuate cold stress-induced necroptosis and apoptosis in the thymus and protect it from cold stress injury. Our results provide insight into the possible mechanisms of cold stress resistance in cold-acclimated broiler thymuses and offer a novel theoretical foundation for cold acclimation to improve the body's cold tolerance.

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DISCLOSURES

The authors declare that they have no competing interests.

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