Acute cold stress induces intestinal injury via CIRP-TLR4-IRE1 signaling pathway in pre-starter broilers: short communication

Juanjuan Wu[1] · Guiyao Li[1] · Haoneng Guo[1] · Bo Huang[2] · Guanhong Li[1] · and Sifa Dai[2,3]

✉ Sifa Dai
daisifa-004@163.com

[1] College of Animal Science and Technology, Jiangxi Agriculture University, Nanchang City, Jiangxi Province, 330045, China

[2] Department of Pharmaceutical and Life Sciences, Jiujiang University, Jiujiang City, Jiangxi Province, 332005, China

[3] Jiujiang Bozheng Institute of Biotechnology Industry, Jiujiang City, Jiangxi Province, 332005, China

Abstract

Background Cold stress is a common environmental stress in broiler chicks. Cold-inducible RNA-binding protein (**CIRP**) is a conserved cold shock protein that can regulate inflammatory response through Toll-like receptor 4 (**TLR4**). The mechanism that how CIRP involves in the regulation of cold stress in broilers remains unclear.

Methods and Results In this study, 360 7-day-old healthy male SZ901 chicks were selected and randomly allocated to 4 groups, and then subjected to acute cold exposure at the ambient temperature of 12 ± 1℃ for 0 h, 4 h, 8 h, and 12 h, respectively. After cold exposure, abdominal temperature, gene expression of CIRP-TLR4-IRE1 signaling pathway in ileum mucosa, and small intestinal structure were measured. The results showed that cold exposure decreased abdominal temperature, upregulated the gene expression of endoplasmic reticulum stress (ERS) markers IRE1, inflammatory factors IL-1β, IL-6, IL-10, TNF-α, and tight junction proteins ZO-1 and Occludin in ileum of chicks compared with the control group with no (0 h) cold exposure. Compared with the control group, a long time cold exposure upregulated the gene expression of CIRP, TLR4, GRP78, NF-κB in ileum mucosa, and decreased the villus height and V/C of small intestine.

Conclusions The above results suggest that acute cold stress induces endoplasmic reticulum stress via upregulating the gene expression of CIRP-TLR4-IRE1 signaling pathway, and results in the structural damage of chick intestine.

Key words **C**old stress · CIRP · Gene expression · ERS · Chick intestinal structure

Abbreviations

CIRP Cold-inducible RNA-binding protein

TLR4 Toll-like receptor 4

SZ901 Shengze 901, a new breed of white feather broiler

IRE1 Inositol-requiring kinase 1

GRP78 Glucose-regulated protein 78

XBP1 X-box binding protein-1

NF-κB Nuclear factor-kappa B

ERS Endoplasmic reticulum stress

IL-1β Interleukin-1 beta

IL-6 Interleukin-6

IL-10 Interleukin-10

TNF-α Tumor necrosis factor-α

ZO-1 Zonula occludens-1

V/C Villus height to crypt depth ratio

Introduction

Temperature will be seen as one of the most important environmental factors that affect animal growth and health. Within the background of global warming, extreme weather occurs frequently. Physiological balance is broken under extreme environmental conditions, and animals become more sensitive and vulnerable to stress [1]. Cold stress is a common environmental stress in broilers at the brooding stage. The thermoregulation capability of broilers increases in an age-dependent manner. Chicks at the first week after hatching have underdeveloped feathers and weak thermoregulatory system, which makes them susceptible to low temperatures. Once cold stress occurs, chicks allocate more energy to maintain body temperature, resulting in decreased growth rate and disease resistance, but high morbidity and mortality [2].

Cold-inducible RNA-binding protein (**CIRP**) is a conserved cold shock protein that was firstly identified in animals in 1997 [3], and also an inflammatory regulator that regulate inflammatory response through Toll-like receptor 4 (**TLR4**). CIRP is ubiquitously expressed in various tissues, and is upregulated by hypothermia, hypoxia, and oxidative stress, which leads to the migration of CIRP from the nucleus to cytoplasm, and then to the circulatory system, where it participates in cellular stress regulation [4]. As a crucial mediator of inflammatory and immune response, nuclear factor-kappa B (**NF-κB**) can be rapidly activated by environmental stress. Studies

revealed that activation of TLR4 by CIRP could activate NF-κB and increased the expression of cytokines [5, 6]. Endoplasmic reticulum (**ER**) is the organelle where intracellular protein modification and transportation mainly occurance, and also regulates intracellular signaling to maintain homeostasis. A recent study reported that endoplasmic reticulum stress (**ERS**) could be activated by CIRP-TLR4, and resulted in cellular inflammatory response and tissue injury [7]. ERS activates a conserved unfolded protein response (**UPR**) and initiates adaptive and protective mechanism to maintain tissue homeostasis. It has been confirmed that ERS and UPR critically impact the development of chronic intestinal inflammation [8]. The mammalian UPR consists of three branches that are mediated by three ER transmembrane receptors, one of which named inositol-requiring kinase 1 (**IRE1**), is a conserved transmembrane protein, which can sense the misfolded proteins in ER, and regulates the activity of X-box binding protein-1 (**XBP1**) to increase the expression of genes related to ER homeostasis [9]. IRE1 is commonly coupled with a heat shock protein named glucose-regulated protein 78 (**GRP78**) which is an ER-resident chaperone. Upon ERS, GRP78 dissociates from IRE1 and translocates to ER, which will lead to the activation of IRE1 by autophosphorylation, and thereby allows the activation of the UPR and subsequent downstream NF-κB to produce proinflammatory cytokines against ERS [10]. However, the mechanism that how CIRP involves in the regulation of cold stress in broilers remains unclear.

The current study was conducted to investigate the effects of acute cold stress with different duration on the gene expression of CIRP-TLR4-IRE1 signaling pathway and intestinal structure in pre-starter broilers, and to explore the mechanism by which acute cold stress mediates intestinal inflammatory response and tissue damage.

Materials and methods

Animals and sample collection

480 One-day-old healthy SZ901 (a new breed of white feather broiler developed by

Fujian Sunner Development) male chicks were obtained from a commercial hatchery and raised under standard environmental conditions (temperature 33 ± 1℃, humidity 55%−65%) for the first week. At 7 days of age, a total of 360 chicks with similar body weight were selected and randomly divided into 4 groups (6 replicates for each group, 15 chicks per replicate) based on a univariate experimental design. Birds in 4 groups received a different duration of acute cold stress challenge. According to Liu et al. (2019) and our preliminary pre-test result, we set the acute cold stress temperature to 12 ± 1℃. Before acute cold stress challenge, all the birds fasted for 12 h, and then were subjected to acute cold exposure at the ambient temperature of 12 ± 1℃ for 0 h, 4 h, 8 h, and 12 h, respectively.

After cold exposure for specified duration, one bird was randomly grabbed from each replicate and abdomen temperature was determined, and the birds were then euthanized by cervical dislocation. Removed the small intestine from abdominal cavity, and separated the duodenum, jejunum, and ileum. About 1-2 cm segment was cut off from the midpoint of each intestinal section and fixed with 4% paraformaldehyde. The remaining sections of ileum were opened and washed with sterile physiological saline. Scraped the inner wall of ileum with a clean microscope slide to collect the mucosa. The collected mucosa was frozen with liquid nitrogen and then stored at −80℃ for RNA extraction.

RNA extraction and RT-PCR analyses

Total RNA from ileum samples was extracted using TRIzol, cDNA was then obtained by reverse transcription from total RNA. The gene sequences of chicken *CIRP*, *TLR4*, *IRE1*, *GRP78*, *XBP1*, *NF-κB*, *IL-1β*, *IL-6*, *IL-10*, *TNF-α*, *ZO-1*, *Occudin*, *Claudin-1*, and *glyceraldehyde phosphate dehydrogenase* (***GAPDH***) were obtained from GenBank, and the Primer Premier Software 5.0 was used to synthesize the specific primers. The gene-specific primers of genes listed above are shown in Table 1. *GAPDH* was selected as an internal reference gene. Real-time quantitative PCR was used to detect the expression of the genes listed above with the use of SYBRR® Premix Ex TaqTM II kit (TAKARA, Biotechnology Co., Ltd., Japan). The relative

mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1 Gene-specific primers of related genes

Gene name	Accession number	Primer Sequence (5'→3')
CIRP	KC905084.1	F: CAGATGAGGGCAAGCTGTTC
		R: ATGGCATCCTTTGCATCGTC
TLR4	NM_001030693.1	F: ACCTCAATGCGATGCACTCT
		R: AGTCCGTTCTGAAATGCCGT
IRE1	NM_001285499.2	F: TGTCCATCAACATCCCTGCT
		R: CAGTCACCACCAGTCCATCT
GRP78	NM_205491.2	F: GGAATCCTTCGTGTCACAGC
		R: TAGCTCTCCAGCTCATTCCG
XPB1	NM_001006192.2	F: GTGAAGGAATCCCAGGTGGA
		R: AGACTGTCCAGAAAGCCGAA
NF-κB	NM_205129.1	F: CTCTCCCAGCCCATCTATGA
		R: CCTCAGCCCAGAAACGAAC
IL-1β	NM_204524.1	F: TGCCTGCAGAAGAAGCCTCG
		R: CTCCGCAGCAGTTTGGTCAT
IL-6	*NM_204628.1*	F: GCAGGACGAGATGTGCAAGA
		R: ATTTCTCCTCGTCGAAGCCG
IL-10	*NM_001004414.2*	F: CAGACCAGCACCAGTCATCA
		R: TCCCGTTCTCATCCATCTTCTC
TNF-α	XM_015294124.2	F: CCGCCCAGTTCAGATGAGTT
		R: GCAACAACCAGCTATGCACC
ZO-1	XM_040680632.1	F: TGAACCAATGGATAGAT
		R: GTACTGAAGGAGCAGGA
Occludin	NM_205128.1	F: ACGGCAAAGCCAACATC
		R: CTCCGAGTAGGCAAGCG
Claudin-1	NM_001013611.2	F: AGAAGATGCGGATGGCT
		R: AACGGGTGTGAAAGGGT
GAPDH	NM_204305.1	F: GACAGCCATTCCTCCACCTT
		R: TTGGATGCCATGTGGACCAT

Determination of intestinal morphology

Intestinal samples were taken out from paraformaldehyde, dehydrated and then embedded in paraffin. Consecutive sections (5 μm) were prepared, and stained with eosin-methylene blue. The prepared histological samples were observed and photographed under optical microscope. About ten intact villi were randomly selected from each sample picture, and the villus height (V, the vertical length from the top of villus to the entrance of crypt) and crypt depth (C, the vertical distance from the base

of villus to submucosa) were measured. The villus height to crypt depth ratio (V/C) was determined by the formula villus height / crypt depth.

Statistical analysis

Excel 2016 was used for the data sorting and computing, and SPSS 16.0 was used for variance analysis. Data at different time points were compared using one-way ANOVA by Duncan's multiple test. Data were presented as means ± SDs. $P < 0.05$ indicates a significant difference.

Results and discussion

Effect of acute cold stress on the body temperature in chicks

Cold stress is a common challenge in broilers. Chickens allocate more energy to maintain body temperature when a heat exchange process between the body and the ambient environment is induced by the dropping of ambient temperature. Neonatal broiler chicks are highly sensitive to low temperature because of the underdeveloped feathers and weak thermoregulatory system [11]. Acute cold exposure is more likely to cause cold stress in broiler chicks, which will lead to the decline of core body temperature. In the present study, we measured the core body temperature of 7-day-old broiler chicks after acute cold exposure. Results shown that abdominal temperatures in chicks dropped to 35.49 ± 0.77℃, 34.38 ± 0.51℃, 34.48 ± 0.84℃ from 39.26 ± 0.36℃ after receiving an acute cold exposure for 4 h, 8 h, 12 h, respectively, which was in line with the previous study that had shown acute cold exposure significantly decreased the core body temperature in mice [12].

Acute cold stress upregulated the gene expression of CIRP-TLR4-IRE1 signaling pathway

The CIRP-TLR4 signaling pathway is a key pathway in signal transduction associated with inflammatory response and cold stress. As a cold shock protein, CIRP expression will be upregulated under the condition of stress, adapting cells or tissues to

environmental changes [13]. As shown in Figure 1A, compared with the control group with no (0 h) cold exposure, acute cold exposure for 8 h and 12 h significantly increased the mRNA expression of *CIRP* and *TLR4* ($P < 0.05$), similar to those of previous studies [5, 12], which had revealed the function of CIRP-TLR4 signaling pathway in cold stress.

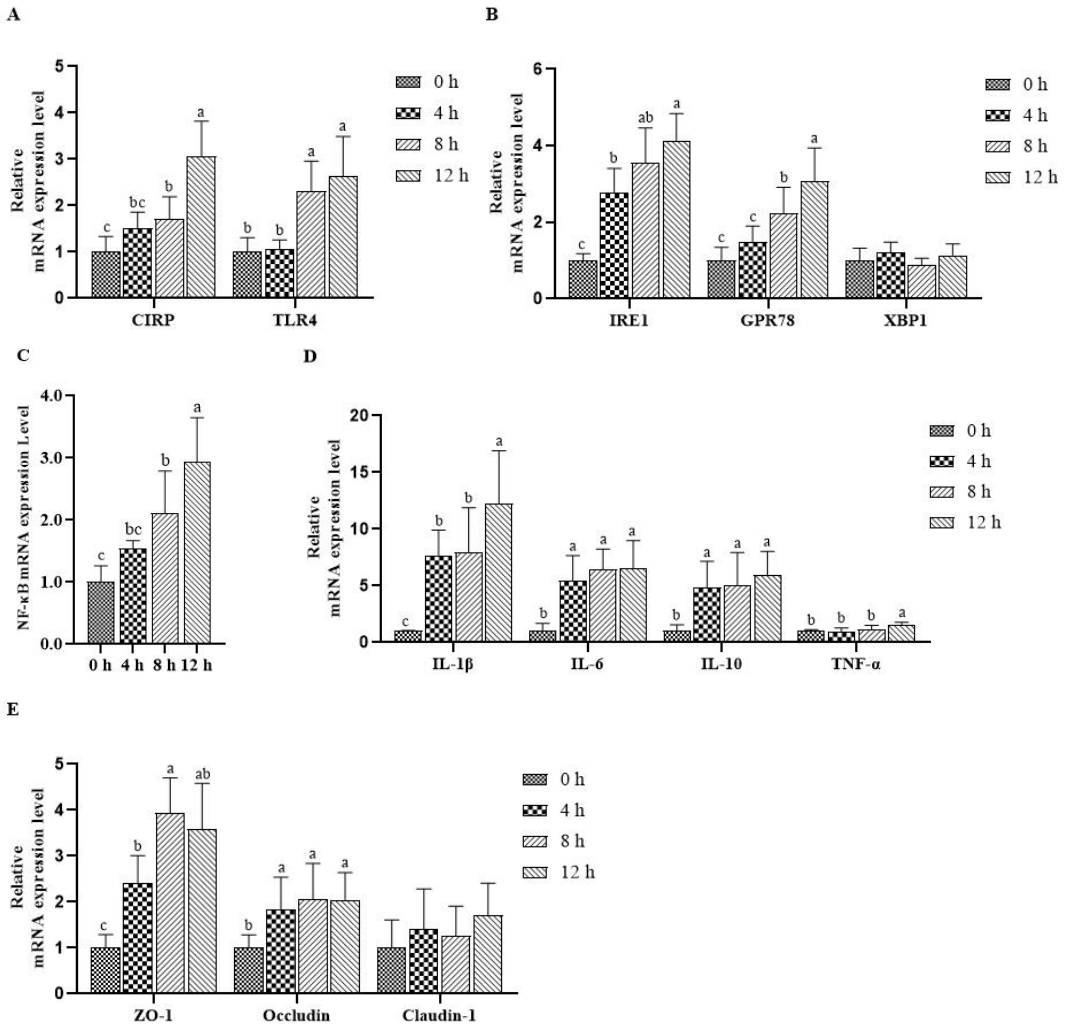

Figure 1 The effects of acute cold stress on the gene expression of CIRP-TLR4-IRE1 signaling pathway and tight junction proteins in ileum of 7-day-old broilers. The mRNA expression level of CIRP and TLR4 (A), IRE1, GRP78, and XBP1 (B), NF-κB (C), inflammatory cytokines (D), tight junction proteins (E). Data were presented as means ± SD (n = 6). Significant differences were shown by bars labeled with different lowercase letters.

A mice model of acute lung injury had shown that CIRP induces ERS via TLR4

activation [7]. To explore the relationship between CIRP-TLR4 signaling and ERS in 7-day-old broiler chickens, we investigated the gene expression of ERS makers under acute cold stress. As one of the main branches of UPR, IRE1 senses the unfolded or misfolded proteins in ER, initiates the UPR, and drives the expression of genes regulating ER homeostasis [9]. GRP78 is an ER-resident Chaperone that is associated with IRE1. When high amounts of unfolded or misfolded proteins are present in ER, GRP78 binds to these proteins, dissociates from IRE1, which allows the autophosphorylation and activation of IRE1 [10]. The current study shown that the mRNA expression of *IRE1* and *GRP78* (Figure 1B) in ileum increased significantly ($P < 0.05$) in response to acute cold exposure, which indicated the occurrence of ERS. No significant difference in the gene expression of XBP1 was observed between the control and experimental groups, suggested that the signaling cascade may not depend on XBP1. NF-κB is a key transcription factor that is related to inflammatory and immune responses, and can be activated by environmental stress [14]. We know that CIRP increases NF-κB expression and inflammatory response [7]. Indeed, similar to the changes of CIRP and TLR4 gene expression, cold exposure for 8 h and 12 h significantly improved the mRNA expression level of *NF-κB* ($P < 0.05$, Figure 1C). It may provide further evidence for the link between CIRP-TLR4 and NF-κB. Similarly, the gene expression of inflammatory cytokines, *IL-1β*, *IL-6*, and *IL-10* (Figure 1D), increased significantly ($P < 0.05$) in response to acute cold exposure, which may be the result of the upregulation of *NF-κB*, suggested that acute cold exposure induced intestinal inflammatory response. In addition, a significant increase of *TNF-α* ($P < 0.05$) mRNA expression level was observed in the group exposed to cold exposure for 12 h.

Acute cold stress upregulated the gene expression of tight junction protein

Tight junction protein is crucial component of intesinal tight junction barrier. Upregulation of tight junction protein expression is a momentous cell protective mechanism against tight junction damage [15]. In the present study, we detected the gene expression of three tight junction proteins, ZO-1, Occludin, and Claudin-1 in

ileum mucosa. As shown in Figure 1E, the mRNA expression levels of *ZO-1* and *Occludin* increased after an acute cold exposure ($P < 0.05$), which might represent tight junction remodeling. Acute cold exposure for different times did not altered the gene expression of *Claudin-1* ($P > 0.05$), which was in accordance with a previous study [16].

Acute cold stress damaged intestinal structure

Small intestine is the organ where nutrients digestion and absorption mainly take place in animals, and the integrity of small intestine is the basic guarantee for intestinal function. The villi of small intestine are the first portal of nutrients digestion and absorption, while a higher villus means a stronger absorption function [17]. Crypt is a tubular structure between the base of villus and the submucosa, which is mainly composed of undifferentiated cells. The deeper the crypt, the more undifferentiated cells and the less mature the intestinal development. Because of the high turnover rate, intestinal mucosal cells are highly susceptible to various environmental and physiological stresses [18]. It has been shown that acute cold stress could cause intestinal tissue damage [19]. As shown in Table 2, cold exposure decreased the villus height, crypt depth, and V/C of small intestine. Compared with the control group, cold exposure for 8 h significantly decreased the villus height and crypt depth of duodenum ($P < 0.05$), and significantly decreased the villus height and V/C of jejunum ($P < 0.05$); A longer cold exposure for 12 h decreased the villus height, crypt depth, and V/C of duodenum ($P < 0.05$), decreased the villus height and V/C of jejunum ($P < 0.05$), and decreased the villus height of ileum ($P < 0.05$). All the results showed above suggested impaired intestinal structure caused by acute cold exposure, which were consistent with a previous study that had shown cold stress decreased the number, height and integrity of jejunum villus of broilers [20].

Table 2 The effect of acute cold stress on the structure of small intestine (n = 6)

Item		Acute cold exposure time				*P* value
		0 h	4 h	8 h	12 h	
Duodenum	Villus height, mm	1.30 ± 0.13[a]	1.32 ± 0.03[a]	0.97 ± 0.14[b]	0.89 ± 0.17[b]	0.010

	Crypt depth, mm	0.24 ± 0.02[a]	0.22 ± 0.01[ab]	0.18 ± 0.04[b]	0.19 ± 0.04[b]	0.048
	V/C	5.38 ± 0.66[a]	5.95 ± 0.52[a]	5.72 ± 1.45[a]	4.30 ± 0.62[b]	0.040
	Villus height, mm	1.05 ± 0.09[a]	1.04 ± 0.07[a]	0.72 ± 0.07[b]	0.72 ± 0.05[b]	0.001
Jejunum	Crypt depth, mm	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.16 ± 0.01	0.260
	V/C	6.06 ± 0.56[a]	6.10 ± 0.36[a]	4.56 ± 0.33[b]	4.57 ± 0.50[b]	0.001
	Villus height, mm	0.61 ± 0.08[a]	0.56 ± 0.03[ab]	0.62 ± 0.08[a]	0.48 ± 0.06[b]	0.037
Ileum	Crypt depth, mm	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.02	0.168
	V/C	4.31 ± 0.54	4.17 ± 0.26	4.32 ± 0.42	3.81 ± 0.15	0.236

In the same row, values with a same or no lowercase letters mean no significant difference ($P > 0.05$), whereas with different lowercase letters mean significant difference ($P < 0.05$). Abbreviation: V/C, villus height to crypt depth ratio.

Conclusion

In this study, the effects of cold stress on the gene expression of CIRP-TLR4-IRE1 signaling pathway and intestinal structure in broiler chicks were evaluated. Up-regulation of the gene expression of *CIRP* and *TLR4* suggested that CIRP-TLR4 signaling pathway participates in cold stress regulation in broiler chicks. The increased gene expression of *IRE1* and *CRP78* indicated the occurrence of ERS after acute cold exposure. Upregulation of *NF-κB* and inflammatory cytokines gene expression indicated the occurrence of intestinal inflammation, while the increased gene expression of tight junction proteins and the changes in small intestinal villus structure were seen as the results of this response. It can be concluded that acute cold exposure at 12 ± 1℃ induces ERS via upregulating gene expression of CIRP-TLR4-IRE1 signaling pathway, and results in the structural damage of pre-starter chick intestine.

Acknowledgements Not applicable

Funding This work was supported by National Natural Science Foundation of China (32160799), Jiangxi Provincial Natural Science Foundation (20224ACB205010).

Author Contributions All authors have confrmed their participation in this study.

Data availability The datasets used and/or analyzed during the present study may be obtained from the corresponding author on reasonable request.

Declarations

Competing Interests The authors have declared that they have no conflicts of interest.

Ethics approval This study was performed in line with the guidelines for the care and use of experimental animals of the Ministry of Science and Technology of the People's Republic of China. Approval was granted by the Animal Care and Use Committee of Jiangxi Agriculture University.

Informed consent None.

References

1. Monson MA, Van Goor AG, Ashwell CM, Persia ME, Rothschild MF, Schmidt CJ (2018) Immunomodulatory effects of heat stress and lipopolysaccharide on the bursal transcriptome in two distinct chicken lines. BMC Genomics 19:643. https://doi.org/10.1186/s12864-018-5033-y
2. Yang X, Luo YH, Zeng QF, Zhang KY, Ding XM, Bai SP, Wang JP (2014) Effects of low ambient temperatures and dietary vitamin C supplement on growth performance, blood parameters, and antioxidant capacity of 21-day-old broilers. Poult Sci 93:898–905. https://doi.org/10.3382/ps.2013-03438
3. Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J (1997) A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. J Cell Biol 137(4):899–908. https://doi.org/10.1083/jcb.137.4.899
4. Qiang X, Yang WL, Wu R, Zhou M, Jacob A, Dong W, Kuncewitch M, Ji Y, Yang H, Wang H, Fujita J, Nicastro J, Coppa JF, Tracey KJ, Wang P (2013) Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nat Med 19:1489–1495. https://doi.org/10.1038/nm.3368
5. Chen L, Ran D, Xie W, Xu Q, Zhou X (2016) Cold-inducible RNA-binding protein mediates cold

air inducible airway mucin production through TLR4/NF-κB signaling pathway. Int Immunopharmacolo 39: 48–56. https://doi.org/10.1016/j.intimp.2016.07.007

6. Zhou K, Cui S, Duan W, Zhang J, Huang J, Wang L, Gong Z, Zhou Y (2020) Cold-inducible RNA-binding protein contributes to intracerebral hemorrhage-induced brain injury via TLR4 signaling. Brain Behav 10(6):e01618. https://doi.org/10.1002/brb3.1618
7. Khan M, Yang WL, Brenner M, Bolognese AC, Wang P (2017) Cold-inducible RNA-binding protein (CIRP) causes sepsis-associated acute lung injury via induction of endoplasmic reticulum stress. Sci Rep 7:41363. https://doi.org/10.1038/srep41363
8. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, Nieuwenhuis EES, Higgins DE, Schreiber S, Glimcher LH, Blumberg RS (2008) XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 134:743–56. https://doi.org/10.1016/j.cell.2008.07.021
9. Siwecka N, Rozpędek-Kamińska W, Wawrzynkiewicz A, Pytel D, Diehl JA, Majsterek I (2021) The Structure, Activation and Signaling of IRE1 and Its Role in Determining Cell Fate. Biomedicines 9(2):156. https://doi.org/10.3390/biomedicines9020156
10. Senft D, Ronai ZA (2015). UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends Biochem Sci 40:141–148. https://doi.org/10.1016/j.tibs.2015.01.002
11. Mujahid A, Furuse M (2009) Oxidative damage in different tissues of neonatal chicks exposed to low environmental temperature. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 152(4):604–608. https://doi.org/10.1016/j.cbpa.2009.01.011
12. Liu P, Yao R, Shi H, Liu Y, Lian S, Yang Y, Yang H, Li S (2019) Effects of cold-inducible RNA-binding protein (CIRP) on liver glycolysis during acute cold exposure in C57BL/6 mice. Int J Mol Sci 20:1470. https://doi.org/10.3390/ijms20061470
13. Zhong P, Huang H (2017) Recent progress in the research of cold-inducible RNA-binding protein. Future Sci Oa 3(4):FSO246. https://doi.org/10.4155/fsoa-2017-0077
14. Wang W, Chen MH, Jin X, Li XJ, Yang ZJ, Lin HJ, Xu SW (2018) H_2S induces Th1/Th2 imbalance with triggered NF-κB pathway to exacerbate LPS-induce chicken pneumonia response. Chemosphere 208:241–246. https://doi.org/10.1016/j.chemosphere.2018.05.152
15. Dokladny K, Ye D, Kennedy JC, Moseley PL, Ma TY (2008) Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: regulatory role of heat shock

factor-1. Am J Pathol 172(3):659–670. https://doi.org/10.2353/ajpath.2008.070522

16. Zhou HJ, Kong LL, Zhu LX, Hu XY, Busye J, Song ZG (2021) Effects of cold stress on growth performance, serum biochemistry, intestinal barrier molecules, and adenosine monophosphate-activated protein kinase in broilers. Animal 15(3):100138. https://doi.org/10.1016/J.ANIMAL.2020.100138
17. Paiva D, Walk C, Mcelroy A (2014) Dietary calcium, phosphorus, and phytase effects on bird performance, intestinal morphology, mineral digestibility, and bone ash during a natural necrotic enteritis episode. Poult Sci 93: 2752–2762. https://doi.org/10.3382/ps.2014-04148
18. Hart A, Kamm MA (2002) Mechanisms of initiation and perpetuation of gut inflammation by stress. Aliment Pharm Ther 16:2017–2028. https://doi.org/10.1046/j.1365-2036.2002.01359.x
19. Su Y, Zhang X, Xin H, Li S, Li J, Zhang R, Li X, Li J, Bao J (2018) Effects of prior cold stimulation on inflammatory and immune regulation in ileum of cold-stressed broilers. Poult Sci 97:4228–4237. https://doi.org/10.3382/ps/pey308
20. Zhao FQ, Zhang ZW, Yao HD, Wang LL, Liu T, Yu XY, Li S, Xu SW (2013) Effects of cold stress on mRNA expression of immunoglobulin and cytokine in the small intestine of broilers. Res Vet Sci 95:146–155. https://doi.org/10.1016/j.rvsc.2013.01.021