Indian Journal of Experimental Biology
Vol. 63, July 2025, pp. 539-548
DOI: 10.56042/ijeb.v63i07.16066

Rutin alleviates liver damage caused by cold stress through the signaling pathways of PI3K-AKT-mTOR and Nrf2-Keap1 in mice

Hao Yu, Shenao Wang, Jing Sun, Chunting Yu, Xueyan Qi, Xuehong Chai, Xue Sun, Peiyue Guan, Yihan Jiao, Xingjun Feng* & Xiao Liu*

College of Animal Science and Technology, Northeast Agricultural University, NO.600 Changjiang Street, Xiangfang District, Harbin 150030, China

Received 30 December 2024; revised 13 April 2025

Chronic cold exposure induces oxidative damage and inflammatory responses in animals. While rutin demonstrates established health benefits, its therapeutic mechanisms against cold stress remain incompletely characterized. The aim of this study was to investigate the effect and mechanism of oral rutin on the liver of mice under cold conditions. A total of 48 6-week-old male Balb/c mice with similar weight (23±1g) were randomly divided into four experimental groups: Control group (CON), Rutin group (RUT), Cold stress with rutin group (CS+RUT) and Cold stress group (CS). The results showed there was obvious oxidative stress, inflammation and liver damage of mice in the CS group, and oral rutin, significantly increased the activities of various antioxidant enzymes ($P < 0.05$) and the contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in liver ($P < 0.05$). By mediating the PI3K-AKT-mTOR and Nrf2-Keap1 signaling pathways and the symptoms of cold stress injury were significantly alleviated by rutin. This study demonstrated that the recipe rutin can significantly alleviate liver damage caused by cold stress through PI3K-AKT-mTOR and Nrf2-Keap1 signaling pathways, and lays a theoretical foundation for the application of rutin in cold-stressed animals.

Keywords: *In vivo* test, Antioxidant, Plant extracts, Production applications, Stress response

Cold stress in high-latitude regions impairs animal survival by disrupting multiple physiological systems (nervous, endocrine, reproductive, cardiovascular)[1,2] and compromising antioxidant capacity[3], energy metabolism, and immune function[4,5]. It reduces growth/reproductive performance and induces oxidative stress and inflammation, causing tissue damage[6,7]. Thermoregulatory adaptations enhance activity and heat production[8,9], cold stress dysregulates hepatic enzymes, promotes lipid peroxidation and glycogen breakdown, suppresses immunity, and causes liver damage[10,11], evidenced by elevated MDA in rats[12] and peroxidation in fish[13].

Mitigating cold-induced damage in modern animal production requires both improved environments and effective nutritional strategies, such as anti-cold stress feed additives. Studies demonstrate that dietary plant extracts including purslane (replacing oxytetracycline in broilers)[14], memantine (preventing hepatic lipid depletion in rats)[15], and aconite (increasing thermogenesis in mice)[16] significantly benefit the health and nutritional status of cold-stressed animals.

Rutin, also known as rutin glycoside or vitamin P, has the molecular formula $C_{27}H_{30}O_{16}$. It is a naturally occurring flavonoid glycoside widely found in various plants, such as buckwheat, asparagus, and apples. Rutin exhibits various pharmacological properties, including anticancer, anti-inflammatory, neuroprotective, antioxidant effect, *etc*[17]. It demonstrates anti-inflammatory and anti-liver fibrosis effects in rats, potentially via antioxidant mechanisms: enhancing endogenous enzymes, modulating Nrf2 (a negative inflammatory regulator), and antagonizing inflammatory factors[18]. Rutin may prevent animal stress by acting as an anti-peroxidant[19] and improves hepatic antioxidant capacity by mediating oxidative stress pathways[20]. Like other flavonoids, it attenuates oxidative injury via the PI3K-AKT-mediated Nrf2 pathway[21]; as a PI3K-AKT-mTOR activator, rutin protects against neurotoxicity[22].

Despite extensive study of rutin's health benefits, its role in mitigating cold stress-induced liver injury and the underlying mechanisms remain unclear. This study investigates the mechanisms of cold stress-mediated liver injury and protective effects of rutin under cold stress, providing a theoretical basis for application of rutin in animal production and strategies to enhance cold resistance.

*Correspondence:
Phone: +86-451-55191395
E-mail: fengxingjun@neau.edu.cn (XF); liuxiao@neau.edu.cn (XL)

Materials and Methods

Ethical statement

All experimental animal procedures were performed in accordance with the Ethical and Animal Welfare Committee of the Northeast Agricultural University (Protocol number: NEAU [2013]-9). The mice were housed and fed under standard conditions at the experimental base of Northeast Agricultural University.

Experimental design

Forty-eight six-week-old female Balb/c mice with an average initial body weight (bw) of 23±1 g were selected for the experiment. Mice were housed in a climatic chamber with a room temperature of 23±2°C and a relative humidity of 40% with a daily free diet. After the prefeed period of one week, mice were randomly divided into four groups (n = 12): Control group (CON); Rutin group (RUT); Cold stress group (CS) and CS+ RUT group. The conditions for inducing cold stress in mice were exposure to 4°C from 9:00-12:00 daily. The mice in the RUT and CS+RUT groups were gavaged with rutin (100mg/kg bw) dissolved in normal saline at 7:00 am every day, and the mice in the control and CS groups were gavaged with the same volume of rutin aqueous solution. Rutin with a purity of more than 97% (HPLC) were purchased from Meilun Biotech (cas: 250249-75-3). The basal diet was formulated according to the National Research Council and provided by Synergy Pharmaceutical Bioengineering (Number: 1010001). The test lasted for 21 days. Body weight and feed intake of mice were monitored during the trial.

Sample collection

On the 21-day of the experiment, mice were euthanized by ether dizziness, and blood and liver tissues were collected. The collected blood was centrifuged at 1000×g for 5 min at 4°C, and the plasma was separated immediately and stored at -80°C. Part of the liver tissue was stored in 4% paraformaldehyde for histological analysis, and the remaining liver tissue was stored at -80°C until use.

Histopathological analysis of liver tissue

Liver specimens (0.125 cm^3) were immediately collected, fixed in 4% paraformaldehyde for 72 h, dehydrated through graded ethanol series, and embedded in paraffin. Serial sections (5 μM thickness) were stained with hematoxylin and eosin (H&E) for histomorphological evaluation. Microscopic analysis was performed using a Nikon Eclipse Ci-L microscope (Tokyo, Japan) under 40-400× magnification[23].

Assay of antioxidant levels in plasma and liver

Liver tissues (0.1 g) were homogenized in 0.9 mL ice-cold physiological saline (9 g/L, pH 7.2-7.4) using a bead mill homogenizer (6.0 m/s, 30 s). The homogenate underwent centrifugation (12,000×g, 10 min, 4°C) to obtain the supernatant. Antioxidant biomarkers (T-SOD, GSH-PX, GST, MDA) in hepatic supernatant and plasma were quantified spectrophotometrically (MAPADA UV1100) using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer′s protocols: T-SOD (A001-1-2a), GSH-PX (A005-1-2), GST (A004-1-1), and MDA (A003-1-2).

Real-time quantitative PCR analysis

Total RNA was extracted from the liver samples with an RNAiso Plus kit (Takara, Japan). The gene accession numbers of the mice were obtained from the NCBI, and gene primers were synthesized by Sangon Biotech Co. Ltd (Shanghai, China) Primer sequences are shown in Table 1. RT-qPCR condition was run in ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA). After the cycles, the RT-qPCR data were evaluated using the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin expression[24].

Western blotting

The supernatant of liver samples was obtained, and protein was extracted with the lysis buffer. Then, the protein extract was mixed with an equal volume of loading buffer (catalog number: P0015, Beyotime, Shanghai, China) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (5% concentration gel and 12% separation gel). Images of the blots were obtained and adjusted by the Essential V6 virtual 2D imaging software (UVITEC, Cambridge, UK). Antibodies were obtained from Wanlei Biotechnology (Shenyang, China) including β-actin mouse monoclonal antibody (catalog number: WL01372), Nrf2 rabbit polyclonal antibody (catalog number: WL02135), Keap1 rabbit polyclonal antibody (catalog number: WL03285), p-AKT rabbit polyclonal antibody (catalog number: WLP001a), p-mTOR rabbit polyclonal antibody (catalog number: WL03694), Anti-p-PI3K rabbit monoclonal antibody (catalog number: AF5905).

Statistical analysis

The results were expressed as mean ± standard deviation (mean ± SD) and were analyzed using

Table 1 — Primer sequences for quantitative real-time PCR analysis

Transcript	Accession number		Sequence (5`–3`)	Product length (bp)
SOD1	NM_011434.2	Forward	GACTGCTGGAAAGGACGGTGTG	85
		Reverse	ACGGCCAATGATGGAATGCTCTC	
CAT	NM_009804.2	Forward	CCATAGCCAGAAGAGAAACCCACAG	123
		Reverse	GGAATCCCTCGGTCACTGAACAAG	
GSH-PX	NM_001291111.1	Forward	TGGACTTCAACATACTGGTGGATGC	134
		Reverse	GCCCTCTTTCAGGACTTGCTTGTAG	
GCLC	NM_010295.2	Forward	AGCTCCTGGAGGAAGGCATCG	123
		Reverse	AATGGTCAGACTCGTTGGCATCATC	
GCLM	NM_008129.4	Forward	ACAATGACCCGAAAGAACTGCTCTC	126
		Reverse	TCTTCACGATGACCGAGTACCTCAG	
Keap1	NM_016679.4	Forward	TGCTCAACCGCTTGCTGTATGC	99
		Reverse	TCATCCGCCACTCATTCCTCTCTG	
Nrf2	NM_001399226.1	Forward	ACTACAGTCCCAGCAGAGTGATGG	96
		Reverse	GCGTGCTCAGAAACCTCCTTCC	
PI3K	NM_001024955.2	Forward	GGAATGTCGGGAGCAGCAACC	121
		Reverse	TCTACCACTACGGAGCAGGCATAG	
AKT	NM_001165894.2	Forward	CGTGTGGCAGGATGTGTATGAGAAG	125
		Reverse	CAGGCGGCGTGATGGTGATC	
mTOR	NM_020009.2	Forward	ACCGTCCGCCTTCACAGATACC	87
		Reverse	GCAGTCCGTTCCTTCTCCTTCTTG	
PDK1	NM_001360002.1	Forward	TTAGAGGGCTACGGGACAGATGC	102
		Reverse	GTAATGCTTCCAGGCGGCTTTATTG	
PTEN	NM_008960.2	Forward	GGAAAGGGACGGACTGGTGTAATG	140
		Reverse	CGCCTCTGACTGGGAATTGTGAC	
4E-BP	NM_007918.3	Forward	CCAAAGGACCTGCCAGCCATTC	116
		Reverse	TCACCGCCTGCCCGCTTATC	
S6K	NM_001114334.2	Forward	CCTGTCAGCCCAGTCAAATTCTCTC	137
		Reverse	CCGCTCACTGTCACATCCATCTG	
β-actin		Forward	TATGCTCTCCCTCACGCCATCC	129
	NM_007393.5	Reverse	TCTACCACTACGGAGCAGGCATAG	

statistical product and service solutions (SPSS version 22.0; SPSS Inc., Chicago, IL, USA). The statistical significance of the data was evaluated using analysis of variance followed by a least significant difference test as the post hoc test, with a 5% probability of error. $P < 0.05$ was considered statistically significant. All graphs with standard deviation bars were constructed using GraphPad Prism (version 8.3.0, GraphPad Software, San Diego, CA, USA).

Results

Effect of rutin on liver morphology of cold-stressed mice

As shown in Fig. 1, the liver in the CON group and the rutin group had intact liver lobules, normal cell morphology, neat arrangement, and clearly visible nucleus. In the CS group, there was more inflammatory cell infiltration and liver cell degeneration, edema and even local necrosis, with no obvious boundary between cells. Compared with the CS group, the CS+RUT group had an orderly arrangement of liver cells, disappearance of edema, and a decrease in the number of necrotic cells.

Effect of rutin on the antioxidant capacity in serum of cold-stressed mice

As shown in Table 2, the levels of MDA and H_2O_2 in the group CS were significantly higher than those in the CON, RUT and CS+RUT groups (*P<0.05*). The CON, RUT and CS+RUT groups were significantly higher in the GSH-PX activity than the CS group (*P<0.05*), and there were no significant differences between the CON group and the RUT group, and between the CON group and CS+RUT group (*P>0.05*).

Effect of rutin on the antioxidant capacity in the liver of cold-stressed mice

Effect of oral rutin on the antioxidant capacity in the liver of cold-stressed mice were as shown in

Table 3. Compared with the CON groups, the CS group showed extreme significant decrease in the activities of CAT, SOD, and GSH-PX (*P<0.01*), significant decrease in the GST activity, and extreme significant increase in the contents of H_2O_2 and MDA (*P<0.01*). In the CS group compared with these of other three groups (*P<0.05*), among which the differences of the CAT activity and the contents of H_2O_2 and MDA reached a very significant level (*P<0.01*). Compared with the CS group, the CS+RUT group showed significant improvement in all determined antioxidant indicators (*P< 0.05*), with a highly significant increase in SOD and GSH-PX activity (*P<0.01*). There was no significant difference in the activities of CAT and GST between the CS+RUT group and the CON group.

Effects of rutin on the mRNA expression levels of genes related to Nrf2-Keap1 and PI3K-AKT-mTOR signaling pathways in liver

Effect on the mRNA expression levels of genes related to Nrf2-Keap1 signaling pathway Fig. 2. showed the effect of rutin on mRNA levels of the genes related to Nrf2-Keap1 signaling pathway in the livers of cold-stressed mice. Nrf2 mRNA expression was markedly reduced in the CS group relative to other groups (*P<0.05*), whereas the CON group exhibited elevated Nrf2 levels compared to RUT and CS+RUT groups (*P<0.05*). Conversely, Keap1 mRNA abundance peaked in the CS group (*P<0.05*) but was suppressed in the RUT group versus CON and CS+RUT groups (*P<0.05*). NQO1 transcripts were significantly downregulated in the CS group (*P<0.05*), with no intergroup differences observed among CON, RUT, and CS+RUT cohorts (*P>0.05*). HO-1 mRNA levels remained comparable across all experimental conditions (*P>0.05*). GCLM mRNA level in the CS group was significantly lower than other three groups (*P<0.05*), but there was no significant difference in this indicator among the RUT, CS+RUT and CS groups (*P>0.05*). Compared with the CON group, there was no significant difference in GCLC mRNA level between the RUT+CS and RUT groups (*P>0.05*), while the GCLC mRNA level in the CS group was significantly decreased (*P<0.05*).

Effect on the mRNA expression levels of genes related to PI3K-AKT-mTOR signaling pathway

The effect of rutin on the mRNA expression levels of genes associated with the PI3K-AKT-mTOR

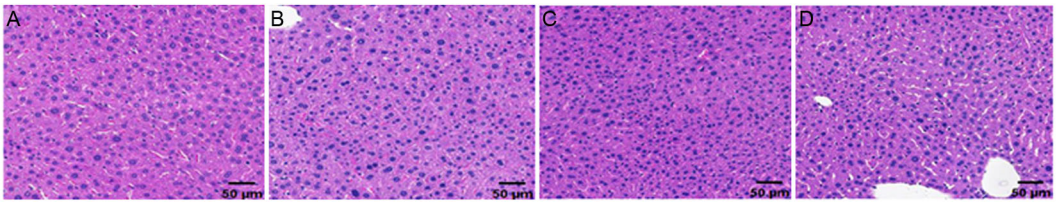

Fig. 1 — Effect of rutin on the liver microstructure of cold-stressed mice (magnification 200×). (A) control group; (B) rutin group; (C) cold stress +rutin group; (D) cold stress group.

Table 2 — Effect of rutin on the serum antioxidant capacity of cold-stressed mice

Items	CON	RUT	CS+RUT	CS
MDA	4.98±0.49^a	5.21±0.50^a	5.82±0.58^a	13.00±1.58^b
H_2O_2	72.41±6.21^a	70.47±6.56^a	89.75±6.72^a	151.41±12.52^b
GSH-PX	619.12±7.05^a	604.81±11.83^a	604.46±8.98^a	542.99±5.63^b

[Values were represented as the mean ± SEM (n = 6). $^{a, b, c}$ Mean values with different superscript letters were of significant difference (*P < 0.05*), those with same superscript letters or no letters within a row were of no significant difference (*P > 0.05*)]

Table 3 — Effect of rutin on the liver antioxidant capacity in cold-stressed mice

Items	CON	RUT	CS+RUT	CS
CAT (U/ mgmL)	265.07±3.50^a	261.90±1.78^a	263.28±2.58^a	222.86±5.89^b
T-SOD (U/ mgmL)	274.85±4.55^a	273.60±4.28^a	261.74±3.81^b	236.43±0.90^c
GSH-PX (U/ mgmL)	648.34±9.91^a	653.67±5.66^a	589.15±9.36^b	555.88±5.71^c
GST (U/ mgmL)	192.00±7.03^a	176.11±7.51^a	175.60±4.70^a	124.75±4.51^b
H_2O_2 (mmol/g)	32.19±1.37^a	33.49±1.31^a	35.32±0.53^a	40.96±0.61^b
MDA(nmol mg−1)	40.72±0.65^a	44.18±2.49^{ab}	47.27±0.84^b	68.49±0.98^c

[Values were represented as the mean ± SEM (n = 6). $^{a, b, c}$ Mean values with different superscript letters were of significant (*P<0.05*) or extremely significant difference (*P<0.01*), those with same superscript letters or no letters within a row were of no significant difference (*P>0.05*)]

pathway in the livers of cold-stressed mice was as shown in Fig. 3. There were no significant differences in mRNA levels of five genes, *PI3K*, *AKT*, *mTOR*, *4EBP*, and *PDK1*, between the four groups ($P>0.05$). S6K mRNA level was highest in the CS group ($P<0.05$) and lowest in the CON group ($P<0.05$) among four groups, and S6K mRNA level in the RUT group and CS+RUT group was significantly higher than that in the CON group and significantly lower than that in the CS group ($P<0.05$). The mRNA level of PTEN was significantly higher in the RUT group than in the other three groups ($P<0.05$), but there was no significant difference between these three groups.

Effects of rutin on the protein expression levels of genes related to Nrf2-Keap1 and PI3K-AKT-mTOR signaling pathways in liver

As illustrated in Fig. 4., the CS group exhibited marked downregulation of Nrf2, p-PI3K, and p-AKT protein abundance alongside upregulated Keap1 and p-mTOR expression compared to other cohorts ($P<0.05$). Notably, the CS+RUT group displayed suppressed Nrf2, Keap1, p-PI3K, and p-AKT levels relative to the CON group ($P<0.05$), whereas p-mTOR expression was potentiated ($P<0.05$). Conversely, RUT intervention significantly restored Nrf2, p-PI3K, and p-AKT expression while attenuating Keap1 and p-mTOR levels compared to controls ($P<0.05$).

Discussion

Cold exposure, a prevalent manifestation of climate change, induces reversible or irreversible pathophysiological damage in animals. However, research elucidating the mechanisms of cold stress resistance and developing effective mitigation strategies remains limited. To address this gap and identify high-efficacy, non-toxic anti-cold formulations, this study investigates cold stress-induced hepatic oxidative damage in a murine model and the ameliorative effects of oral rutin supplementation. The underlying mechanisms are explored through the PI3K-AKT-mTOR and Nrf2-Keap1 signaling pathways, key regulators of cellular antioxidant responses.

As a primary metabolic organ, the liver exhibits significant metabolic sensitivity to temperature fluctuations. Substantial evidence indicates that stress exposure elevates metabolic rate and reactive oxygen species (ROS) generation. Excessive ROS disrupts biological system homeostasis and induces hepatocellular injury, inflammation, and fibrosis. Rutin, a flavonoid with potent antioxidant properties,

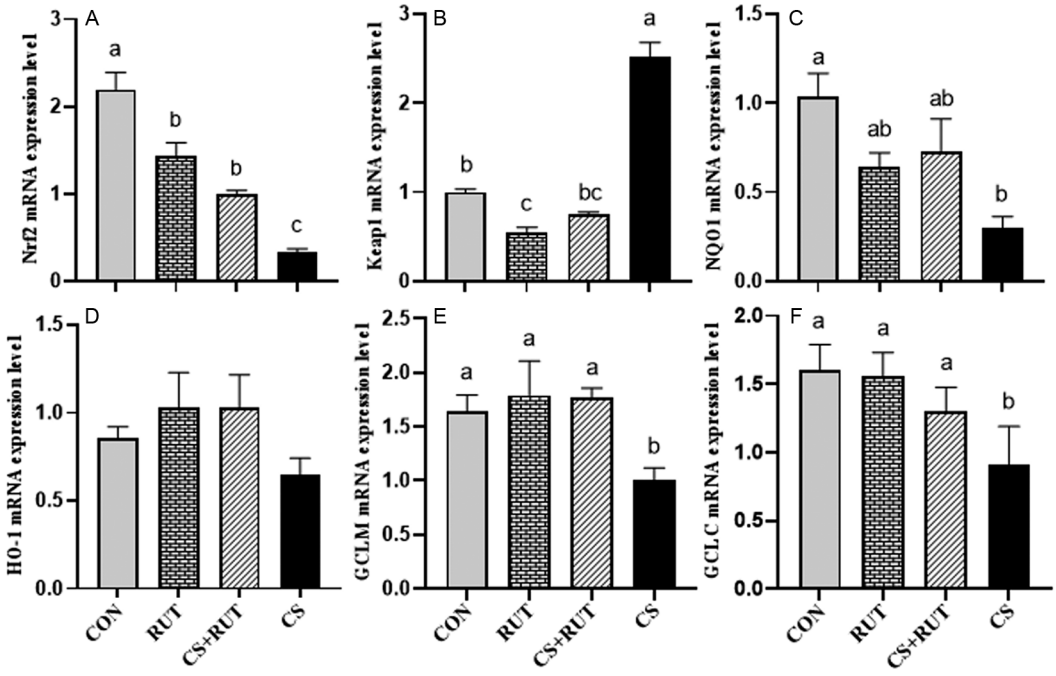

Fig. 2 — Effect of rutin on the mRNA expression levels of Nrf2-Keap1 signaling pathway-related genes in the liver of cold-stressed mice. CON: control group; RUT: rutin group; CS + RUT: cold stress + rutin group; CS: cold stress group. (A) Nrf2; (B) Keap1; (C) NQO1; (D) HO-1; (E) GCLM; (F) GCLC. Values are expressed as mean ±SEM (n =6). Different superscript letters on the same indicator differ significantly ($P < 0.05$).

demonstrates strong ROS scavenging capacity[25]. Its well-documented antioxidant efficacy mitigates oxidative stress via direct scavenging of superoxide anions, peroxyl radicals, and hydroxyl radicals[26]. Rutin's unique molecular structure facilitates direct ROS neutralization and inhibits xanthine oxidase activity, thereby suppressing ROS production at its source[26]. Consequently, rutin enhances animal health and disease prevention through these antioxidant mechanisms. This protective role, involving Nrf2 pathway modulation to reduce oxidative stress, has been demonstrated in diabetic rat models[27].

Many studies have also reported that rutin can reduce MDA levels, increase SOD, GSH-PX activity, and alleviate oxidative damage of animals[28], and the antioxidant function of rutin increased with the increase of rutin concentration[29]. The results of this study showed that compared with the cryogenic injury group, the serum and liver SOD, CAT, GPX and GST in the rutin addition group had different levels of improvement[30]. In addition, the production of H_2O_2 and MDA in serum and liver is inhibited by rutin treatment[31]. Analysis of ultrastructure of the liver showed that the hepatocytes in the cold stress group were arranged haphazardly, and the arrangement of the hepatocytesin the rutin-treated groups was obviously much more regular, which demonstrated that the cold stress caused obvious hepatocyte damage of mice, and addition of the rutin significantly improved the damage of the hepatocytes.

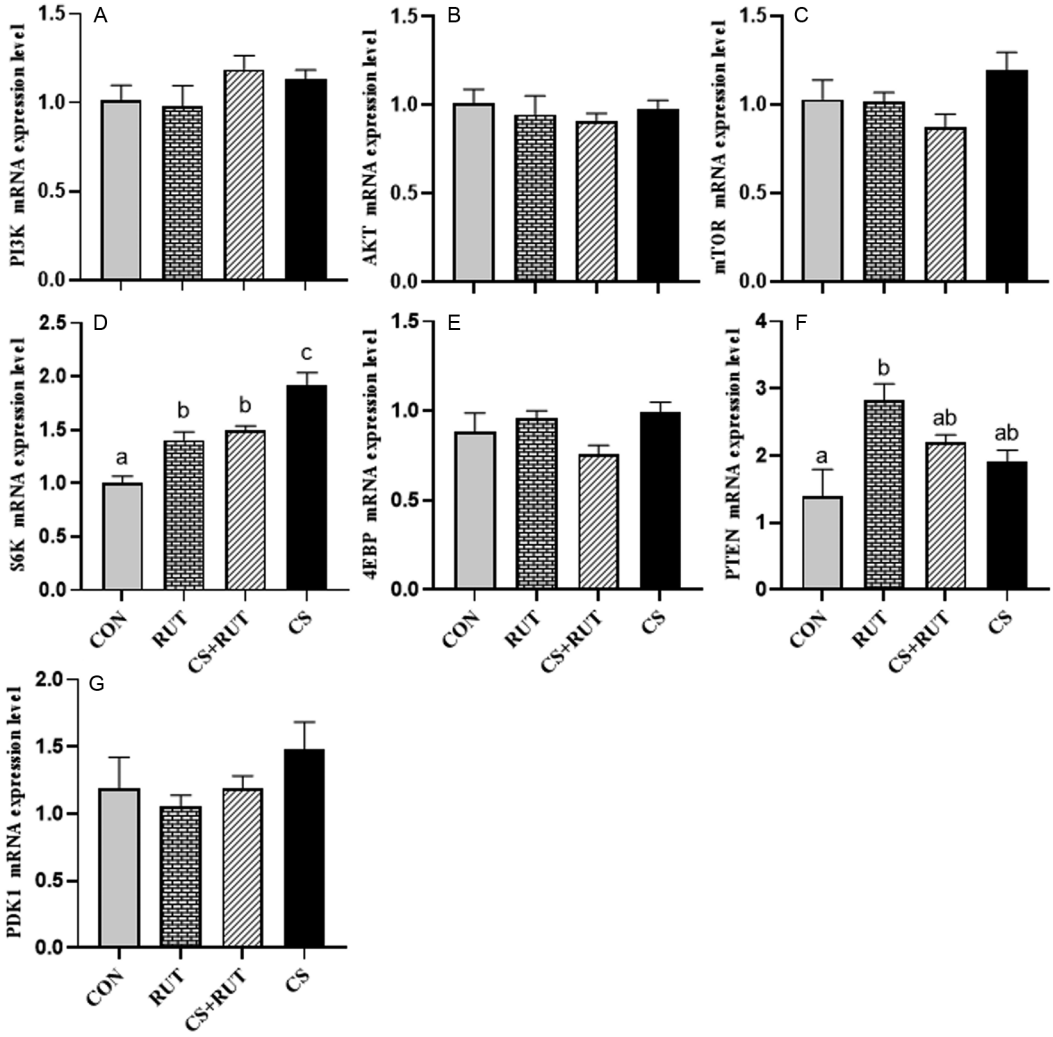

Fig. 3 — Effect of rutin on the mRNA expression levels of PI3K-AKT-mTOR signaling pathway genes in the liver of cold-exposed mice. CON: control group; RUT: rutin group; CS + RUT: cold stress +rutin group; CS: cold stress group. (A) PI3K;(B) AKT;(C) mTOR; (D) S6K; (E) 4EBP; (F) PTEN; (G) PDK2. Values are expressed as mean±SEM (n=6). Different superscript letters on the same indicator differ significantly ($P < 0.05$).

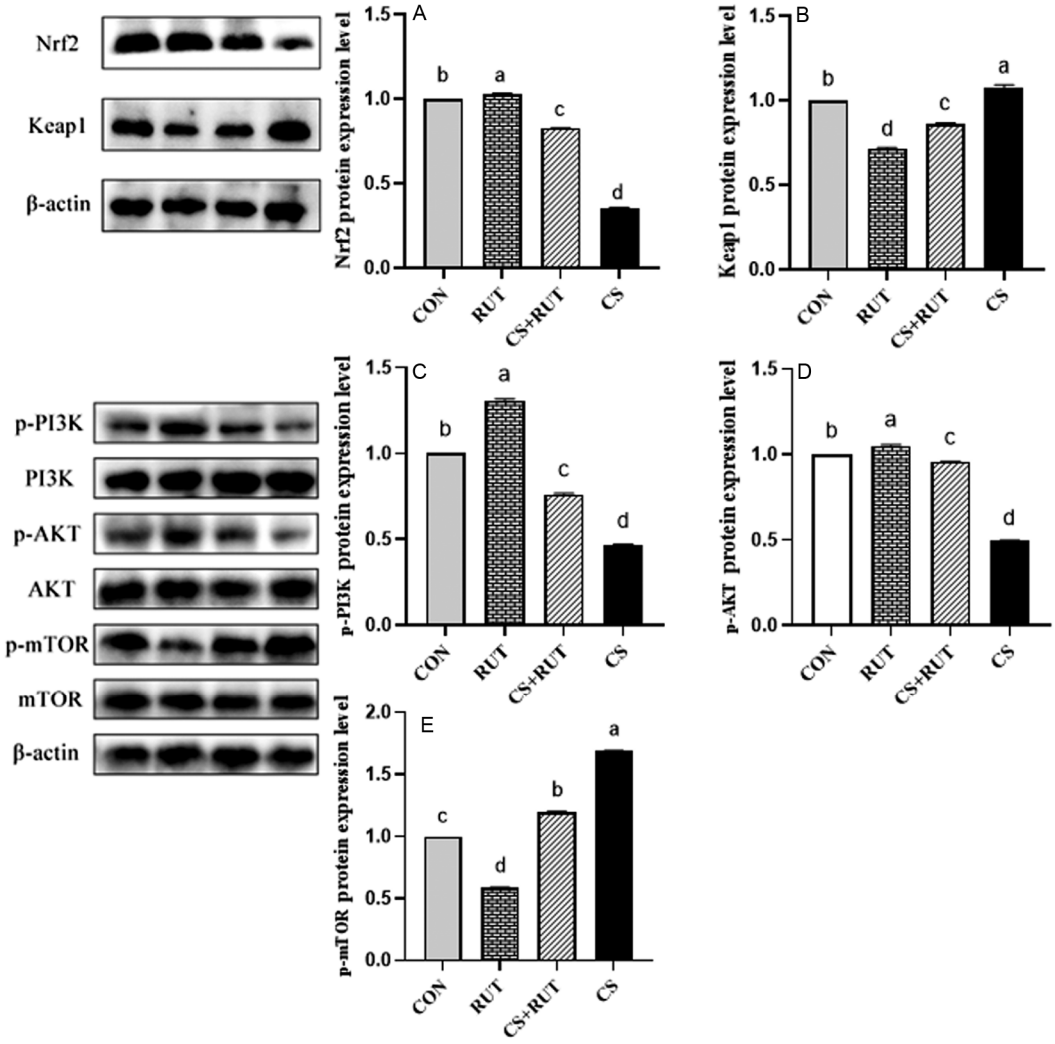

Fig. 4 —— Effect of rutin on the protein expression levels of genes related to Nrf2/Keap1 and PI3K-AKT-mTOR signaling pathway in the liver of cold-stressed mice. CON: control group; RUT: rutin group; CS + RUT: cold stress + rutin group; CS: cold stress group. (A) Nrf2; (B) Keap1; (C) p-PI3K; (D) p-AKT; (E) p-mTOR. Values are expressed as mean±SEM (n =6). Different superscript letters on the same indicator differ significantly ($P < 0.05$).

Nrf2 is an important factor that plays a vital role in the defense system of animal bodies against oxidative stress and involves in regulating the expression of many antioxidant genes such as *SOD*, *catalase* (*CAT*), *GSH-PX*, *heme oxygenase-1* (*HO-1*), *GST*, *NQO1*, *glutamate cysteine ligase catalysis* (*GLC*) and *GCLM*[32]. Keap1, as a negative regulatory factor of Nrf2, can bind to Nrf2 to form a complex, thereby inhibiting its entry into the nucleus and preventing Nrf2 from promoting the expression of antioxidant genes. Shilpi *et al*. found that rutin protected t-butyl hydroperoxide-induced oxidative impairment and improved intestinal antioxidant capacity via enhancing the Nrf2 activity[33]. In this study, rutin treatment significantly increased the expression level of Nrf2 and reduced the expression level of Keap1 compared with the cold stress group both at the protein level and at the gene level. This study also found that the expression of NQO1 was the lowest in the cold stress group, and the expression level of HO-1 increased after adding rutin, although the difference between groups was not obvious, but also basically showed the same trend[34]. The addition of rutin could activate the Nrf2 signaling pathway to enhance the activity of antioxidant enzymes such as, SOD, CAT, in mice, and inhibit oxidative damage of liver induced by low-temperature tissue[35].

The PI3K-AKT-mTOR signaling pathway plays an important role in a variety of biological functions including reducing inflammatory response and

reducing tissue damage by improving energy metabolism and oxidative stress[36]. mTOR, as a protein kinase, is closely related to various functions such as gene transcription regulation, cytoskeletal composition, cell survival, and cell metabolism[37]. Studies have shown that activation of the PI3K-AKT-mTOR signaling pathway significantly improved the oxidative stress of the placenta and relieved brain damage in mice[38]. Cellular damage induced by oxidative stress can be alleviated by regulating the PI3K-AKT-mTOR signaling pathway[39]. There is a close connection between the PI3K-AKT-mTOR signaling pathway and Nrf2 signaling pathway. It's been proven that cardiomyocyte apoptosis caused by excess ROS can be inhibited upregulating the PI3K-AKT-Nrf2 signaling pathway[40,41]. We confirmed that cold stress has a regulatory effect on the PI3K-AKT-mTOR pathway in mouse liver by finding that low temperature changed the levels of p-PI3K, p-AKT, and p-mTOR proteins, respectively. Compared with the cold stress group, the contents of MDA and H_2O_2 and the expression levels of mTOR in the rutin treatment group decreased while the expression of p-PI3K/PI3K, p-AKT/AKT, and Nrf2 increased. The results indicated that rutin had a certain protective effect on cold stress-induced liver tissue damage, and its mechanism of action is related to the modulation of Nrf2-Keap1 and PI3K-AKT-mTOR signaling pathways. The current study found that cryogenic treatment can indeed regulate the expression of p-PI3K, p-AKT, and p-mTOR proteins, confirming evidence that cold stress regulates the PI3K-AKT-mTOR pathway. S6 kinase is an important effector downstream of mTOR, Promote mRNA growth, protein translation, and cell growth[42,43]. The results in this study showed that the S6K level was increased at cold stress, which may be due to the negative feedback effect of S6K1-IRS1 that activates the PI3K-AKT-mTOR pathway.

Conclusion

In summary, this study systematically delineates the mechanisms of cold stress-induced hepatic injury in animals and elucidates rutin's protective effects against such injury. Rutin significantly enhanced hepatic antioxidant capacity and ameliorated hepatocyte morphological alterations in mice subjected to low-temperature stress. Mechanistically, rutin activated the Nrf2-Keap1 and PI3K-AKT-mTOR signaling axes, thereby reducing oxidative biomarkers while upregulating hepatic antioxidant factors including SOD and GSH-PX. These findings establish rutin as an effective hepatoprotectant against cold stress-induced liver injury and provide a molecular basis for its application in animal production systems.

Acknowledgments

This work was supported by the Heilongjiang Provincial Natural Science Foundation of China (LH2022C029) and the National Natural Science Foundation of China (32072768).

Conflict of interest

The authors declare no competing financial interests.

References

1 Kim WS, Ghassemi NJ & Lee HG, Impact of cold stress on physiological, endocrinological, immunological, metabolic, and behavioral changes of beef cattle at different stages of growth. *Animals*, 13(6), (2023).

2 Cong P, Liu Y, Liu N, Zhang Y, Tong C, Shi L, Liu X, Shi X, Liu Y, Tong Z & Hou M, Cold exposure induced oxidative stress and apoptosis in the myocardium by inhibiting the Nrf2-Keap1 signaling pathway. *BMC Cardiovasc. Disord*, 18(1), (2018) 36.

3 Deaton JW, Branton SL, Simmons JD & Lott BD, The effect of brooding temperature on broiler performance. *Poult. Sci*, 75, (1996)1217.

4 Hu Y, Liu Y & Li S, Effect of acute cold stress on neuroethology in mice and establishment of its model. *Animals*, 12(19), (2022).

5 Shinder D, Rusal M, Tanny J, Druyan S & Yahav S, Thermoregulatory responses of chicks (Gallus domesticus) to low ambient temperatures at an early age. *Poult. Sci*, 86, (2007) 2200.

6 Lyte JM, Eckenberger J, Keane J, Robinson K, Bacon T, Assumpcao ALFV, Donoghue AM, Liyanage R, Daniels KM, Caputi V & Lyte M, Cold stress initiates catecholaminergic and serotonergic responses in the chicken gut that are associated with functional shifts in the microbiome. *Poult. Sci*, 103 (3), (2024).

7 Zhang S, Liu Y, Chai Y, Xing L &Li J, Effects of intermittent cold stimulation on growth performance, meat quality, antioxidant capacity and liver lipid metabolism in broiler chickens. *Poult. Sci*,103(3), (2024).

8 Wang D, Tian Y, Wang Q, Zhang Y, Ye B, Zuo Z, He J, Pan Z, Sun D, Zou J & Xie S, Cold stress-induced autophagy and apoptosis disorders are mainly mediated by AMPK/PPAR/ PI3K/AKT/mTOR pathways. *Aquaculture*, 583, (2024).

9 Su S, Liu J, He K, Zhang M, Feng C, Peng F, Li B & Xia X, Overexpression of the long noncoding RNA TUG1 protects against cold-induced injury of mouse livers by inhibiting apoptosis and inflammation. *FEBS J*, 283, (2016) 1261.

10 Saeedi S, Hassanabadi A & Zaeemi M, Effects of cold stress on growth performance, carcass traits and tibia attributes in broiler chickens with thiram-induced dyschondroplasia. *J. Anim. Physiol. Anim. Nut*, 107 (2), (2022) 659-670.

11 Liu X, Li S, Zhao N, Xing L, Gong R, Li T, Zhang S, Li J & Bao J, Effects of acute cold stress after intermittent cold stimulation on immune-related molecules, intestinal barrier genes, and heat shock proteins in broiler ileum. *Animals*, 12(23), (2022).

12 Sun L, Wang X, Zou Y, He Y, Liang C, Li J, Li P & Zhang J, Cold stress induces colitis-like phenotypes in mice by altering gut microbiota and metabolites. *Frontiers in Microbiology*, 14, (2023).

13 Ranjan A, Alrashada YN, Hassanien HA, Abbas AO, Alkhamis SA & Alkobaby AI, Dietary propolis improves the growth performance, redox status, and immune response of nile tilapia upon a cold-stress challenge. *PloS one*, 18(11), (2023).

14 Zhang S, Gong R, Zhao N, Zhang Y, Xing L, Liu X, Bao J & Li J, Effect of intermittent mild cold stimulation on intestinal immune function and the anti-stress ability of broilers. *Poult. Sci*, 102, (2023) 102407.

15 Ferreira M, Valenti VE, Cisternas JR, Ferreira C, Meneghini A, Filho CF, Breda JR, Correa JA, de Mello Monteiro CB, Junior HM, Murad N & de Abreu LC, Memantine effects on liver and adrenal gland of rats exposed to cold stress. *Int. Arch. Med*, 4, (2011)5.

16 Liu J, Tan Y, Ao H, Feng W& Peng C, Aqueous extracts of aconite promote thermogenesis in rats with hypothermia via regulating gut microbiota and bile acid metabolism. *Chinese medicine*, 16(1), (2021).

17 Su Y, Li T, He X, Sun H & Li J, PI3K/AKT pathway modulation and cold acclimation alleviation concerning apoptosis and necroptosis in broiler thymus. *Poult. Sci*, 103(5), (2024).

18 Calabrese EJ, Pressman P, Hayes AW, Dhawan G, Kapoor R, Agathokleous E& Calabrese V, RUTIN, a widely consumed flavonoid, that commonly induces hormetic effects. *Food Chem. Toxicol*, 187, (2024).

19 Dong R, Zhang X, Liu Y, Zhao T, Sun Z, Liu P, Xiang Q, Xiong J, Du X, Yang X, Gui D & XU Y, Rutin alleviates EndMT by restoring autophagy through inhibiting HDAC1 via PI3K/AKT/mTOR pathway in diabetic kidney disease. *Phytomedicine*, 112, (2023).

20 Guan P, Yu H, Wang S, Sun J, Chai X, Sun X, Qi X, Zhang R, Jiao Y, Zhang L, Feng X & Liu X, Dietary rutin alleviated the damage by cold stress on inflammation reaction, tight junction protein and intestinal microbial flora in the mice intestine. *J. Nutr. Biochem*, 130, (2024).

21 Zhang ZF, Zhang YQ, Fan SH, Zhuang J, Zheng YL, Lu J, Wu DM, Shan Q & Hu B, Troxerutin protects against 2,2',4,4'-tetrabromodiphenyl ether (BDE-47)-induced liver inflammation by attenuating oxidative stress-mediated NAD^+-depletion. *J. Hazard. Mater*, 283, (2015)98.

22 Zhuang Y, Wu H, Wang X, He J, He S & Yin Y, Resveratrol attenuates oxidative stress-induced intestinal barrier injury through PI3K/Akt-Mediated Nrf2 signaling pathway. *Oxid. Med. Cell. Longevity*, (2019)7591840.

23 Lyu Y, Yang Y, Li P, Zhou C, Zhang L, Bi C & Shan A. The effects of antimicrobial peptide-chitosan nanoparticles on alleviating ulcerative colitis in Citrobacter rodentium infected mice. *Food Biosci*, 68 (2025) 106575.

24 Lyu Y, Li P, Bian Y, Hou W, Li B, Zhang L & Shan A. Antimicrobial peptide-chitosan nanoparticles combat ETEC-induced bacterial infection in mice. *Int. J. Biol. Macromol*, 311 (Pt4) (2025) 144047.

25 Wu Y, Zhou S, Zhao A, Mi Y& Zhang C, Protective effect of rutin on ferroptosis-induced oxidative stress in aging laying hens through Nrf2/HO-1 signaling. *Cell Biol. Int*, 47 (3), (2022) 598-611.

26 Rahmani S, Naraki K, Roohbakhsh A, Hayes AW& Karimi G, The protective effects of rutin on the liver, kidneys, and heart by counteracting organ toxicity caused by synthetic and natural compounds. *Food Sci. Nutr*, 11(1), (2022) 39-56.

27 Chen C, Wang C, Jiang H, Wang M, Rahman SU, Chen C, Ding H, Zhao C, Huang W & Wang X, Rutin alleviates zearalenone-induced endoplasmic reticulum stress and mitochondrial pathway apoptosis in porcine endometrial stromal cells by promoting the expression of Nrf2. *Toxins*, 17(1), (2024).

28 Chen S, Liu H, Zhang J, Zhou B, Zhuang S, He X, Wang T & Wang C, Effects of different levels of rutin on growth performance, immunity, intestinal barrier and antioxidant capacity of broilers. *Ital. J. Anim. Sci*, 21(1), (2022) 1390-1401.

29 Ali YA, Soliman HA, Abdel-Gabbar M, Ahmed NA, Attia KAA, Shalaby FM, El-Nahass E-S, Ahmed OM & El-Magd M, Rutin and hesperidin revoke the hepatotoxicity induced by paclitaxel in male wistar rats via their antioxidant, anti-inflammatory, and antiapoptotic activities. *J. Evidence-Based Complementary Altern. Med*, 2023(1), (2023).

30 Ahmed OM, Elkomy MH, Fahim HI, Ashour MB, Naguib IA, Alghamdi BS, Mahmoud HUR, Ahmed NA & Hussain T, Rutin and quercetin counter doxorubicin-induced liver toxicity in wistar rats via their modulatory effects on inflammation, oxidative stress, apoptosis, and Nrf2. *Oxid. Med. Cell. Longevity*, 2022, (2022) 1-19.

31 Kamalakkannan N & Stanely Mainzen Prince P, Rutin improves the antioxidant status in streptozotocin-induced diabetic rat tissues. *Mol. Cell. Biochem*, 293, (2006) 211.

32 Korkmaz A & Dürdane K, Protective effect of rutin on the ischemia/reperfusion induced damage in rat kidney. *J. Surg. Res*, 164, (2010) 309.

33 Naseeb M, Albajri E, Almasaudi A, Alamri T, Niyazi HA, Aljaouni S, Mohamed ABO, Niyazi HA, Ali AS, Shaker Ali S, Saber SH, Abuaraki HA, Haque S & Harakeh S, Rutin promotes wound healing by inhibiting oxidative stress and inflammation in metformin-controlled diabetes in rats. *ACS omega*, (2024).

34 Zhang J, Sun X, Chai X, Jiao Y, Sun J, Wang S, Yu H & Feng X. Curcumin mitigates oxidative damage in broiler liver and ileum caused by aflatoxin B1-contaminated feed through Nrf2 signaling pathway. *Animals,* 14 (2024): 409.

35 Zhao X, Chen X & Yue C, Rutin ameliorates inflammation and oxidative stress in ulcerative colitis by inhibiting NLRP3 inflammasome signaling pathway. *Cell Biochem. Biophys*, 82(4), (2024) 3715-3726.

36 Gao L, Liu C, Wu J, Cui Y, Zhang M, Bi C, Shan A & Dou X, EGCG improve meat quality, restore lipid metabolism disorder and regulate intestinal flora in high-fat fed broilers. *Poult. Sci*, 104(3), (2025).

37 Wu X, Wang J, Li B, Gong M, Cao C, Song L, Qin L, Wang Y, Zhang Y & Li Y, Chlorogenic acid, rutin, and quercetin from lysimachia christinae alleviate triptolide-induced multi-organ injury *in vivo* by modulating immunity and AKT/mTOR signal pathway to inhibit ferroptosis and apoptosis. *Toxicol. Appl. Pharmacol*, 467, (2023).

38 Ma Z, Wen X, Zhang Y, Ai Z, Zhao X, Dong N, Dou X & Shan A, Thymol alleviates colitis by modulating intestinal barrier damage, gut microbiota, and amino acid metabolic pathways. *J Agric Food Chem*, 73(12), (2025) 7211-7227.

39 Saafan SM, Mohamed SA, Noreldin AE, El Tedawy FA, Elewa YHA, Fadly RS, Al Jaouni SK, El-Far AH & Alsenosy AA, Rutin attenuates d-galactose-induced oxidative stress in rats' brain and liver: molecular docking and experimental approaches. *Food Funct*, 14(12), (2023) 5728-5751.

40 Yang H, Wang Y, Jin S, Pang Q, Shan A & Feng X, Dietary resveratrol alleviated lipopolysaccharide-induced ileitis through Nrf2 and NF-κB signalling pathways in ducks (Anas platyrhynchos). *J. Anim. Physiol. Anim. Nutr*, 106(6), (2021) 1306-1320.

41 Chien WJ, Saputri DS & Lin HY, Valorization of Taiwan's citrus depressa hayata peels as a source of nobiletin and tangeretin using simple ultrasonic-assisted extraction. *Curr. Res. Food Sci*, 5, (2022) 278-287.

42 Acosta-Martinez M & Cabail MZ, The PI3K/Akt pathway in meta-inflammation. *Int. J. Mol. Sci*, 23(23), (2022).

43 Zhang L, Zhang J, Zang H, Yin Z, Guan P, Yu C, Shan A & Feng X. Dietary pterostilbene exerts potential protective effects by regulating lipid metabolism and enhancing antioxidant capacity on liver in broilers. J. Anim. Physiol. Anim. Nutr, 108 (2024)921-933.