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ORIGINAL PAPER

Naringenin and caffeic acid supplementation alleviates heat stress-induced liver damage by maintaining endoplasmic reticulum homeostasis in broilers

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ABSTRACT. Heat stress (HS) is a major challenge to poultry production, with significant implications for animal health and performance. This study explored the protective role of naringenin (NAR) and caffeic acid (CA) on liver health in HS-exposed broilers. First, an *in vitro* screening experiment was performed in chicken embryo fibroblast (DF-1) cells. Our results demonstrated that NAR and CA significantly reduced the expression of heat shock protein 70 (*HSP70*) and heat shock transcription factor 1 (*HSF1*), while restoring superoxide dismutase (SOD) activity and total antioxidant capacity (T-AOC) compared to the HS group ($P < 0.05$). For the *in vivo* trial, broilers subjected to chronic HS (37 ± 2 °C for 6 weeks) were supplemented with 0.1% NAR, 0.1% CA, or their combination. Dietary NAR and CA significantly improved broiler growth performance under HS. Both compounds markedly reduced serum and liver levels of *HSP70* and *HSF-1*, while enhancing antioxidant capacity (SOD and T-AOC; $P < 0.05$). Additionally, NAR and CA protected against HS-induced liver damage, as evidenced by reduced serum aspartate transaminase and alanine transaminase activity ($P < 0.05$). Mechanistically, NAR and CA preserved liver homeostasis by modulating the endoplasmic reticulum stress response signalling involving PKR-like ER kinase, activating transcription factor 4, activating transcription factor 6, eukaryotic translation initiation factor 2 alpha, C/EBP homologous protein, X-box-binding protein 1, and glucose-regulated protein 78.

Introduction

Heat stress (HS) presents a significant problem to the poultry industry (Oluwagbenga and Fraley, 2023), compromising animal health and productivity through chronic inflammation and tissue damage (Andretta et al., 2021). The liver, a central metabolic organ, is crucial for the metabolism of glucose and lipids in broilers. HS induces the production of reactive oxygen species (ROS), which disrupts

antioxidant activity, ultimately leading to liver damage (Habashy et al., 2019; Iwai et al., 2023). Additionally, HS triggers pro-inflammatory responses in the liver characterised by elevated levels of cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (Liu et al., 2022). Considering these detrimental effects, elucidating the molecular mechanisms underlying HS-induced liver injury remains a crucial research priority.

The endoplasmic reticulum (ER) plays a central role in protein folding, maturation, lipid synthesis, and transport. HS impairs ER function, leading to ER stress and liver damage. Excessive reactive oxygen species (ROS) and unfolded proteins activate the unfolded protein response (UPR) via inositol-requiring enzyme type 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Hetz, 2012; Lemmer et al., 2021; Zeng et al., 2022). Although UPR initially restores homeostasis, prolonged ER stress induces apoptosis through C/EBP homologous protein (CHOP) and caspase-12 signalling pathways (Szegezdi et al., 2006). HS further exacerbates oxidative stress and activates NF-κB-mediated inflammation, thereby worsening liver injury. ER stress and UPR also contribute to hepatic damage. This dual pathology mirrors effects observed in chemical hepatotoxicity models (e.g., CCl_4-induced toxicity) where ER stress initiates UPR signalling and hepatocyte apoptosis (Borkham-Kamphorst et al., 2020). In addition, nickel chloride ($NiCl_2$) has been shown to induce apoptosis via ER stress-mediated pathway while simultaneously activating inflammatory responses through nuclear factor kappa-B (NF-κB) signalling (Guo et al., 2016). These findings strongly suggest that therapeutic targeting of ER stress may represent an effective strategy for alleviating HS-induced hepatic damage.

Heat stress impairs antioxidant capacity, highlighting the potential of antioxidant drugs to mitigate its adverse effects. Studies have shown that compounds such as hydroxytyrosol (HA), resveratrol (RES), curcumin (CUR), cyanidin 3-O-glucoside chloride (C3OG), ursolic acid (ULA), and L-theanine (LTA) can effectively reduce oxidative stress (Bigagli et al., 2017; Yilmaz, 2019; Petrella et al., 2020; Xu et al., 2023). Among them, naringenin (NAR), a flavonoid abundant in citrus fruits such as grapefruits and oranges, is recognised for its anti-inflammatory and antioxidant properties. Research indicates that NAR exposure reduces the production of pro-inflammatory cytokines and inhibits the phosphorylation of key transcription factors in macrophages (Bodet et al., 2008). Moreover, another study has found that NAR treatment mitigates mitochondrial oxidative stress while preserving mitochondrial biogenesis by controlling the AMP-activated protein kinase (AMPK)-sirtuin 3 (SIRT3) signalling pathway during ischemia-reperfusion injury (Yu et al., 2019).

Caffeic acid (CA) is a phenolic compound commonly found in coffee, blueberries, apples, pears, and medicinal plants such as echinacea and artichokes (Nakamura and Watanabe, 2003). It exerts strong antioxidant properties, effectively scavenging ROS and reducing lipid peroxidation. Additionally, CA enhances the activity of endogenous antioxidant enzymes, including superoxide dismutase (SOD) and catalase, providing further protection against oxidative stress (Kampa et al., 2004). Moreover, it has been found to improve growth, immune response, and antioxidant capacity in fish (Yilmaz, 2019). CA also alleviates oxidative stress by modulating the nuclear factor erythroid 2-related factor 2 (Nrf2)-heme oxygenase 1 (HO1) pathway and reduces inflammation through NF-κB inhibition (Stähli et al., 2019). In this context, we explored the potential protective effects of CA and NAR in broilers subjected to heat stress.

Material and methods

Chemical drugs

Naringenin (≥98%; cat. no. 480-41-1) was purchased from Sichuan Kanghui Biotechnology (Chengdu, SC, China). Caffeic acid (≥98%; cat. no. 331-39-5), hydroxytyrosol (≥98%; cat. no. 10597-60-1), resveratrol (≥98%; cat. no. 501-36-0), curcumin (≥98%; cat. no. 458-37-7), cyanidin 3-O-glucoside chloride (≥98%; cat. no. 7084-24-4), ursolic acid (≥98%; cat. no. 77-52-1), and L-theanine (≥98%; 3081-61-6) were provided by Xinkang (Xi'an, SX, China).

Cell culture and treatment

The chicken embryo fibroblast DF-1 cell line was maintained in high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37 °C with 5% CO_2. All drugs were dissolved in 0.01% DMSO. Drugs were incubated with DF-1 cells for 48 h. Heat stress was induced by incubating the cells at 42 °C with 5% CO_2 for 1 h. The MTS cell viability assay kit (cat. no. G3582) was purchased from Promega (Madison, WI, USA).

Assessment of cell toxicity of various drugs

The cytotoxicity of each compound tested was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. DF-1 cells were treated with individual compounds for 48 h, followed by addition of the detection reagent and a 2-h incubation at 37 °C. Fluorescence intensity was then measured using a microplate reader at a wavelength of 490 nm.

Animal feeding and experimental design

All animal experiments were conducted in strict accordance with the Animal Ethics Procedures and Guidelines of the Ministry of Science and Technology of the People's Republic of China. The study was approved by the Animal Ethics Committee of AnKang University (Permission no. 2023-01-089). One-day-old male yellow feather broilers (200 chicks) were obtained from a commercial hatchery (LiQun Biotech, AnKang, SN, China). The broilers were housed under the same conditions as previously described, with a 23-hour light/1-hour dark cycle, relative humidity of 50 ± 15%, and ambient temperature of 24 ± 2 °C (Wang et al., 2024). After 3 weeks, the broilers were randomly assigned to 5 groups: control (CT), heat Stress (HS), naringenin supplement (NAR), caffeic acid supplement (CA), and a combined naringenin and caffeic acid supplementation (NAR + CA). Birds in the CT group were maintained at 24 ± 2 °C, while those in the HS, NAR, CA, and NAR + CA groups were exposed to 37 °C ± 2 °C from 10:00 to 18:00 daily. The CT and HS groups received only the basal diet, while the NAR, CA, and NAR + CA groups were fed basal diets supplemented with 0.1% NAR, 0.1% CA, or 0.05% NAR and 0.05% CA, respectively. The composition of the basal diet is shown in Table 1. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated by recording body weight and feed consumption, with FCR calculated as the ADFI/ADG ratio.

Table 1. Formulation of the basal diet

Ingredients	%
Soybean meal, 44%	15.55
Sunflower oil	1.50
Di-Ca-P	1.85
Ground limestone	1.95
Yellow maize	61.55
Maize gluten meal	16.25
NaCl (salt)	0.3
L-lysine-HCl	0.6
Premix	0.3
DL-methionine	0.15

Di-Ca-P – dibasic calcium phosphate

Determination of AST and ALT activity in broiler serum

The Chicken Alanine Aminotransferase (ALT) ELISA Kit (cat. no. MBS1601880) and Chicken AST (Aspartate Aminotransferase) ELISA Kit (cat. no. MBS2500223) were supplied by MyBioSource (San Diego, CA, USA). Serum AST and ALT activities were measured following the protocol described by Nam et al. (2023). Briefly, blood samples were collected from chicks via the brachial vein using a sterile 1-ml syringe with a 26G needle. The chicks were gently restrained, and the puncture site was disinfected with 70% ethanol prior to sampling. The volume of blood drawn did not exceed 1% of body weight. For serum preparation, the collected blood was allowed to clot at room temperature for 30 min., followed by centrifugation at 2500 *g* for 10 min. The resulting serum was transferred to sterile microtubes and stored at −20 °C until analysis. AST and ALT activities were quantified using the ELISA kits, following the manufacturer's protocol.

Determination of HS and antioxidant indices in DF-1 cells and broiler serum

The chicken HSF1/Heat shock factor protein 1 ELISA Kit (cat. no. E0435c) was purchased from EIAab (Wuhan, HB, China). The Chicken HSP70/Heat Shock Protein 70 (Sandwich ELISA) ELISA Kit (cat. no. LS-F22843) was purchased from LSBio (Newark, CA, USA). The Chicken Total Antioxidation (T-AOC) ELISA Kit (cat. no. MBS2611919) and Chicken Superoxide Dismutase (SOD) ELISA Kit (cat. no. MBS260216) were purchased from MyBioSource. The levels of HSP70, HSF-1, SOD activity and T-AOC were determined by using corresponding ELISA kits according to the manufacturers' protocols.

Real-time RT-PCR

Real-time PCR was conducted to assess mRNA expression levels of target genes. Briefly, RNA was extracted using RNAi Plus (TaKaRa, Kusatsu, Japan) and reverse-transcribed to cDNA using 5 × RT master mix (TaKaRa). Each PCR reaction contained 5 µl of 2 × SYBR green mix, 1 µl of primer mix, 1 µl of cDNA, and 3 µl of nuclease-free water loaded into a 96-well plate. Cycling conditions were set as follows: 94 °C for 10 min., followed by 40 cycles at 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for

Table 2. Primer sequences of target genes

Gene	Gene ID	Primer sequences (5`→3`)
HSF1	L06098.1	F: CAGGGAAGCAGTTGGTTCACTACACG R: CCTTGGGTTTGGGTTGCTCAGTC
PERK	XM_420868.6	F: GTGGATGAGCAGGAGGCAATGATG R: ATCCTTAACCAGCCATGCAGAAGC
HSP70	NM_001006685.2	F: CCAAGAACCAAGTGGCAATGAA R: CATACTTGCGGCCGATGAGA
GRP78	XM_205491.1	F: TCCTGCTCCTCGTGGTGTCC R: CTCCTCTGGTGTTAGCCGATTCTG
eIF2a	NM_001031323.2	F: GCTGCGAGTCAGTAATGGGTATAA R: CTGCCAGGAAACTTGCCACA
ATF4	NM_204880.2	F: AATTGGCTCGCTGTGGACAG R: CGGTGGCTTCCAGATGTTCC
ATF6	NM_422208	F: GATTGTGGGCGTCACTTCTCG R: TGGGATGCCAATGTTAGCCTG
IRE1	NM_001285499	F: TGAGGGCAATGAGAAATAAGAAGC R: TGTAGGAGCAGGTGAGGGAAGC
XBP1	NM_001006192	F: GCGAGTCTACGGATGTGAAGGA R: TGTGGAGGTTGTCAGGAATGGT
CHOP	NM_015273173.2	F: :CAGGAAGAAGAGCTGGCCCCACT R: TGCTGTGCTCGCCGTGCTGT
ACTB	NM_205518.1	F: ATGGCTCCGGTATGTGCAAG R: CAACCATCACACCCTGATGTC

HSF1 – heat shock transcription factor 1, *PERK* – PKR-like ER kinase, *HSP70* – *h*eat shock protein 70, *GRP78* – glucose-regulated protein 78, *eIF2a* – eukaryotic translation initiation factor 2 alpha, *ATF4* – activating transcription factor 4, *ATF6* – activating transcription factor 6, *IRE1* – inositol-requiring enzyme type 1, *XBP1* – X-box-binding protein 1, *CHOP* – C/EBP homologous protein, *ACTB* – actin beta

30 seconds. The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method (Wang et al., 2024). Primer sequences for each gene are listed in Table 2.

Liver sectioning

Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4–5 µm thick sections. The sections were deparaffinised in xylene, rehydrated through a graded ethanol series, and stained with haematoxylin for 5 min. Differentiation was carried out using 1% acid alcohol and stained blue in ammonia water. The preparations were then stained with eosin for 2 min, dehydrated in a graded ethanol series, and cleared in xylene. Finally, the slides were mounted using a resin-based medium, and histological alterations in liver tissue were examined under a microscope (Zeiss, Jena, Germany).

Statistical analysis

Data in this study are reported as means ± standard deviation (SD) from at least two replicates. Statistical analyses were performed using one-way ANOVA in GraphPad PRISM software version 10 (La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Assessment of drug cytotoxicity in DF-1 cells

The potential cytotoxic effects of drugs were assessed by exposing DF-1 cells to a range of the compounds under study. As illustrated in Figure 1, HA, RES, and NAR exerted negligible effects on cell viability at concentrations up to 100 µM. In contrast, incubating cells with CA, CUR, and C3OG at concentrations of 50 µM showed significant cytotoxicity ($P < 0.05$). Treatment with ULA at 25 µM also significantly reduced cell viability ($P < 0.05$). In addition, LTA exposure caused significantly increased cytotoxicity at 100 µM ($P < 0.05$).

Thus, the effect of drugs on antioxidative capacity was assessed at the following concentrations: HA (100 µM), NAR (100 µM), RES (100 µM), CA (25 µM), CUR (25 µM), C3OG (25 µM), ULA (10 µM), LTA (50 µM).

Antioxidative activity of drugs in heat-stressed DF-1 cells

To confirm the activation of heat stress response, mRNA expression levels of heat shock protein 70 (*HSP70*) and heat shock transcription

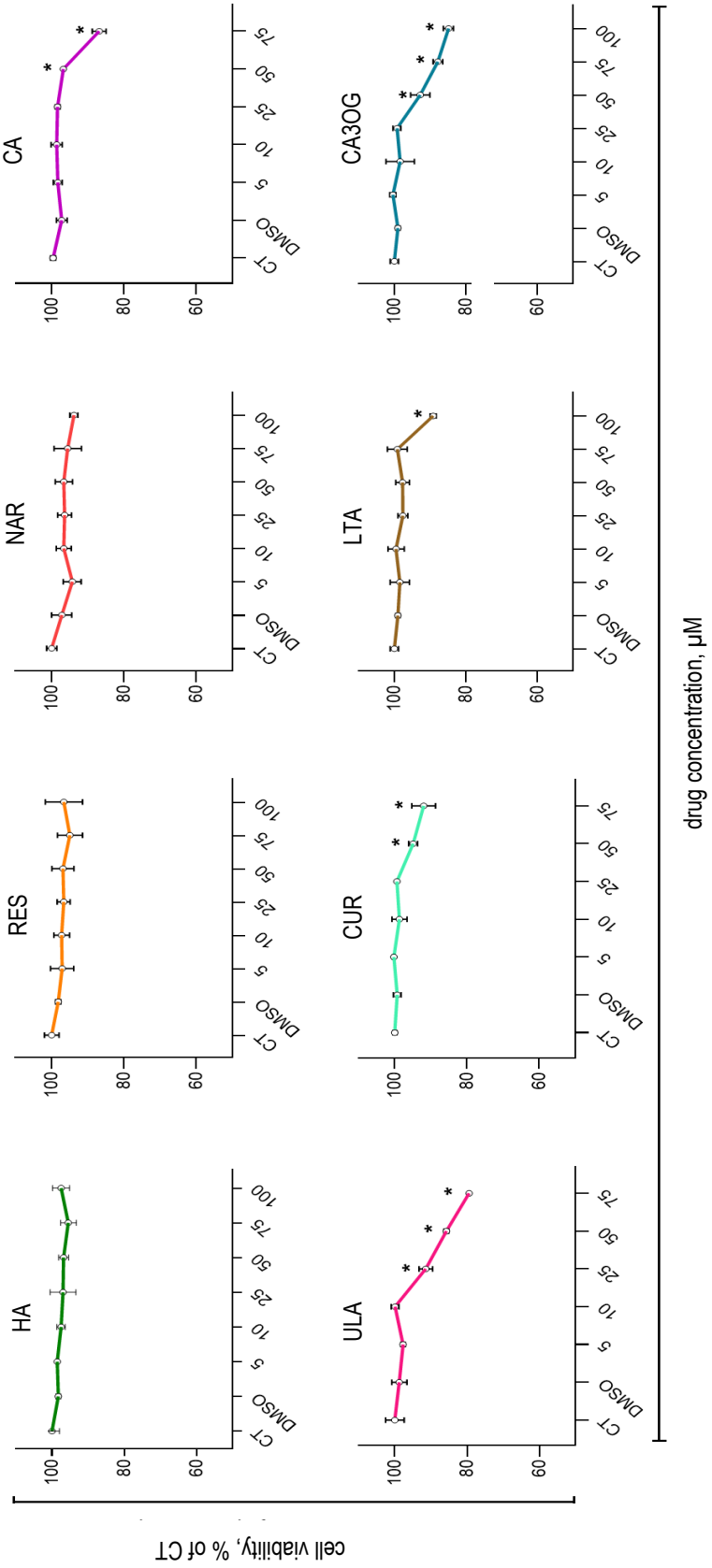

Figure 1. Cell viability of DF-1 cells exposed to various drugs. DF-1 cells were incubated with each compound at several concentrations. After 48 h, cell viability was assessed using the MTS kit. Data are presented as means ± SEM (n = 3). * $P < 0.05$ indicates significant differences

CT – control group, DF-1 cells – chicken embryo fibroblast cell line, MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, HA – hydroxytyrosol, RES – resveratrol, NAR – naringenin, CA – caffeic acid, ULA – ursolic acid, CUR – curcumin, LTA – L-theanine, C3OG – cyanidin 3-O-glucoside chloride

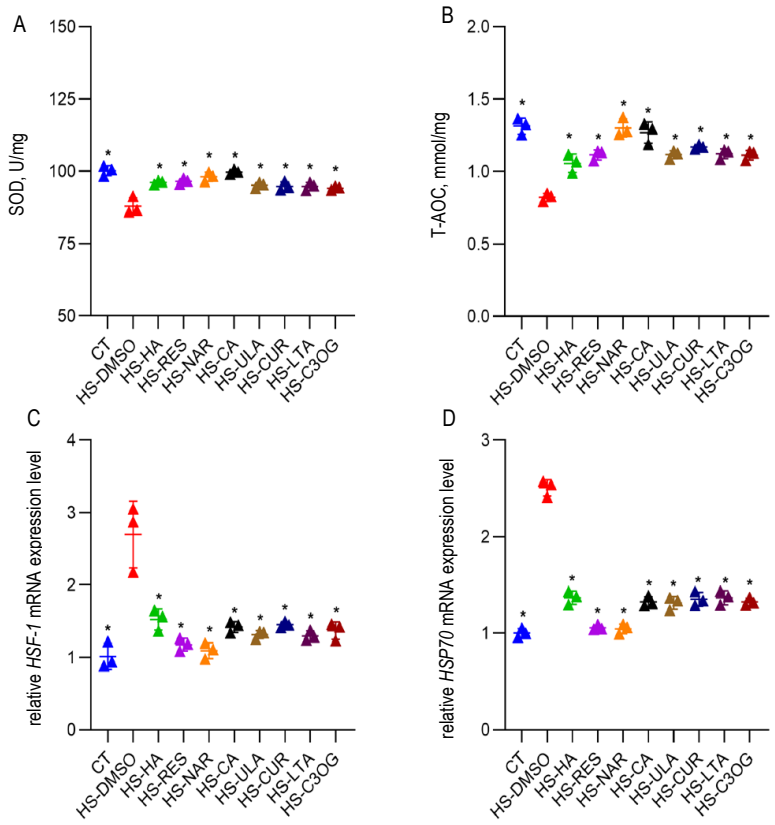

Figure 2. Antioxidative activity of drugs in heat-stressed (HS) DF-1 cells. DF-1 cells were incubated with each compound at the indicated concentrations at 42°C with 5% CO_2 for 1 h (A–B). Cell supernatant was collected and subjected to ELISA to determine SOD activity and T-AOC. (C–D) Cells were collected, and total RNA isolated to assess *HSF1* and *HSP70* mRNA levels. Data are presented as mean ± SEM (n = 3). * $P < 0.05$ indicates significant differences

DF-1 cells – chicken embryo fibroblast cell line, SOD – superoxide dismutase, T-AOC – total antioxidant capacity, *HSF1* – heat shock transcription factor 1, *HSP70* – heat shock protein 70, ELISA – enzyme-linked immunosorbent assay, HA – hydroxytyrosol, RES – resveratrol, NAR – naringenin, CA – caffeic acid, ULA – ursolic acid, CUR – curcumin, LTA – L-theanine, C3OG – cyanidin 3-O-glucoside chloride

factor 1 (*HSF1*) were measured, along with antioxidant capacity assessed by SOD activity and T-AOC. HS exposure significantly upregulated *HSP70* and *HSF1* mRNA levels ($P < 0.05$; Figure 2C–D) while markedly reducing SOD activity and T-AOC ($P < 0.05$; Figure 2A–B). Among the tested compounds, NAR treatment demonstrated the most pronounced suppression of heat shock responses, significantly lowering both *HSF1* and *HSP70* expression ($P < 0.05$). Additionally, NAR and CA treatments effectively restored antioxidant capacity, showing significant increases in SOD and T-AOC levels compared to heat-stressed controls ($P < 0.05$).

Effects of NAR and CA on growth performance in heat-stressed broilers

Growth performance was evaluated through ADG, ADFI, and FCR measurements following HS exposure and dietary NAR and CA supplementation. No significant differences in ADG, ADFI, or FCR were observed between the groups during the first 2 weeks of HS ($P > 0.05$) (Table 3). However, chronic heat stress (CHS) lasting 6 weeks led to a significant decrease in ADG and ADFI and an increase in FCR compared to the control group ($P < 0.05$). Supplementation with either NAR or CA, alone or in combination, effectively mitigated the reductions in ADG and ADFI caused by HS ($P < 0.05$). Additionally, the CHS-induced increase in FCR was significantly improved by the inclusion of these supplements ($P < 0.05$).

Effect of NAR and CA on serum levels of antioxidant indicators

Changes in antioxidant activity were evaluated by measuring serum SOD activity and T-AOC. CHS exposure significantly reduced both SOD activity and T-AOC compared to the control group ($P < 0.05$) (Figure 3A–B). Dietary supplementation with NAR, CA, or their combination effectively restored SOD activity and T-AOC compared to the HS group ($P < 0.05$).

Table 3. Effect of different HS durations on broiler growth performance (n = 10)

HS exposure ime	Groups				
	CT	HS	NAR	CA	NAR + CA
0–2 week					
ADFI, g	35.91 ± 0.31	35.95 ± 0.41	35.90 ± 0.31	36.03 ± 0.31	35.80 ± 0.28
ADG, g	16.82 ± 0.55	16.80 ± 0.39	16.76 ± 0.40	16.52 ± 0.33	16.94 ± 0.43
FCR	2.14 ± 0.08	2.14 ± 0.06	2.14 ± 0.07	2.18 ± 0.03	2.11 ± 0.07
2–6 week					
ADFI, g	71.23 ± 0.28*	68.55 ± 0.53	71.19 ± 0.58*	70.96 ± 0.43*	71.25 ± 0.31*
ADG, g	27.47 ± 0.19*	23.67 ± 0.09	27.34 ± 0.20*	27.32 ± 0.16*	27.72 ± 0.15*
FCR	2.59 ± 0.02*	2.90 ± 0.03	2.60 ± 0.04*	2.60 ± 0.03*	2.57 ± 0.02*

CT – control, HS – heat stress, NAR – naringenin supplement, CA – caffeic acid supplement, NAR+CA – combined naringenin and caffeic acid supplement; ADFI – average daily feed intake, ADG – average daily gain, FCR – feed conversion ratio; * indicates a statistically significant difference ($P < 0.05$) compared to the HS group; data are presented as means ± SEM

Effect of NAR and CA on serum levels of heat stress-related proteins

Serum concentrations of heat stress-related proteins were quantified by ELISA. Chronic HS exposure resulted in significantly elevated serum levels of HSP70 and HSF-1 compared to the control group ($P < 0.05$) (Figure 3C–D). Both NAR and CA supplementation independently demonstrated significant efficacy in reducing serum HSP70 and HSF-1 concentrations in broilers ($P < 0.05$). Importantly, the combined treatment with NAR and CA resulted in synergistic effects, achieving significantly greater suppression of both heat stress markers than either compound administered alone.

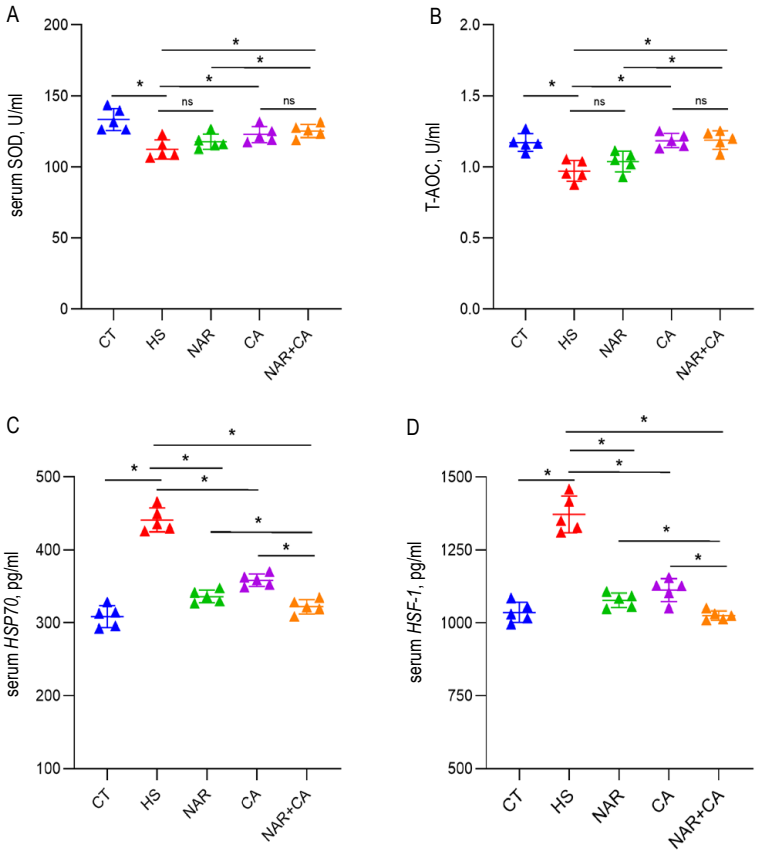

Figure 3. Effect of naringenin supplement (NAR) and caffeic acid on serum levels of heat stress (HS)-related proteins and antioxidant indicators. (A–B) Serum activity of SOD and T-AOC after 6 weeks of HS exposure were determined by ELISA. (C–D) Serum levels of *HSP70* and *HSF-1* after 6 weeks of HS exposure were determined by ELISA. Data are presented as means ± SEM (n = 5). * $P < 0.05$ indicates significant differences groups: CT – control, HS – heat stress, NAR – naringenin supplement, CA – caffeic acid supplement, NAR+CA – combined naringenin and caffeic acid supplement; NAR – naringenin, CA – caffeic acid, SOD – superoxide dismutase, T-AOC – total antioxidant capacity, ELISA – enzyme-linked immunosorbent assay, *HSP70* – heat shock protein 70, *HSF-1* – heat shock transcription factor 1

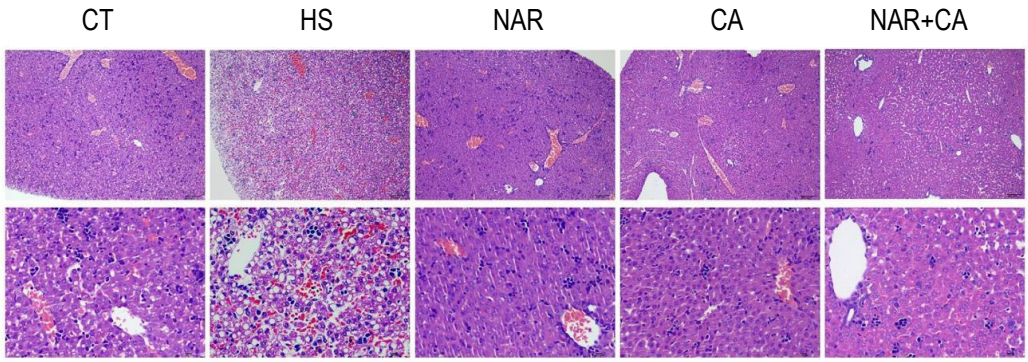

Figure 4. Effect of NAR, CA on the histology alteration of the liver. Liver from broilers in different experimental groups were subjected to H&E staining (20×)

groups: ct – control, hs – heat stress, nar – naringenin supplement, ca – caffeic Acid Supplement, NAR+CA – combined naringenin and caffeic acid supplement; NAR – naringenin, CA – caffeic acid, H&E staining – haematoxylin and eosin staining

Effect of NAR, CA on histology of liver tissue

Haematoxylin and eosin (H&E) staining revealed significant hepatic alterations following chronic heat stress. The HS group exhibited marked inflammatory responses, evidenced by substantial cell infiltration (Figure 4). In contrast, all supplemented groups (NAR, CA, and NAR + CA combination) showed significant reversal of these pathological changes (Figure 4).

Effects of NAR and CA on liver antioxidant levels

Hepatic antioxidant status was evaluated by measuring SOD activity and T-AOC. The results showed that both SOD activity and T-AOC were significantly reduced in the CHS group compared to the CT group ($P < 0.05$) (Figure 5A–B). Meanwhile, dietary supplementation with NAR, CA, or their combination effectively restored liver SOD activity and T-AOC relative to the HS group ($P < 0.05$).

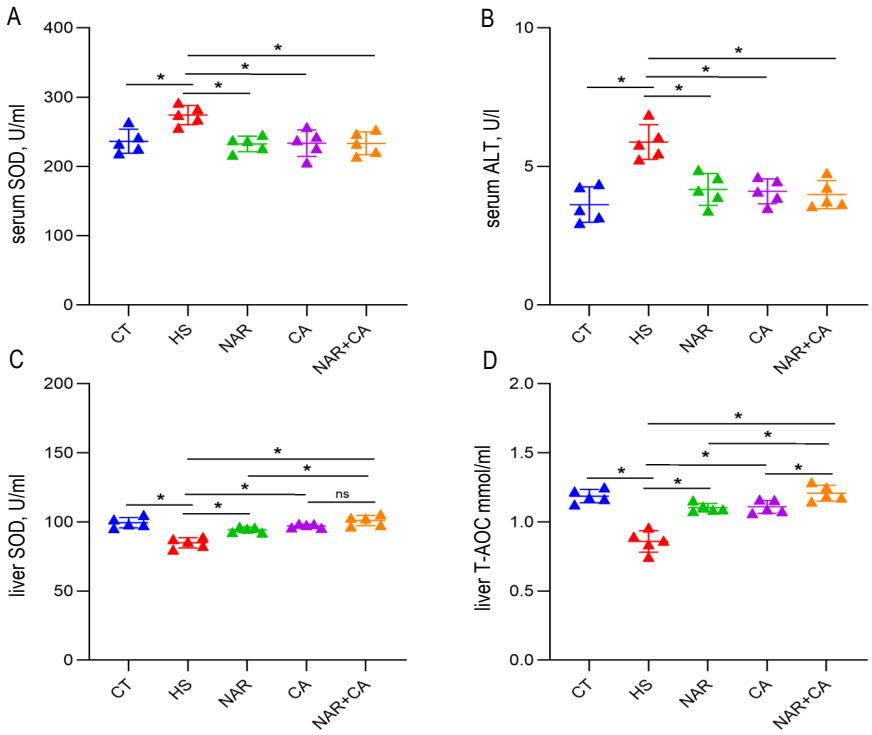

Figure 5. Effect of NAR and CA on serum activity of AST, ALT, and liver antioxidant indicators. (A–B) Serum activity of AST and ALT was determined by ELISA. (C–D) Liver activity of SOD and T-AOC were determined by ELISA. Data are presented as means ± SEM (n = 5). * $P < 0.05$ indicates significant differences

groups: CT – control, HS – heat stress, NAR – naringenin supplement, CA – caffeic acid supplement, NAR+CA – combined naringenin and caffeic acid supplement; NAR – naringenin, CA – caffeic acid, AST – aspartate transaminase, ALT – alanine transaminase, SOD – superoxide dismutase, T-AOC – total antioxidant capacity, ELISA – enzyme-linked immunosorbent assay

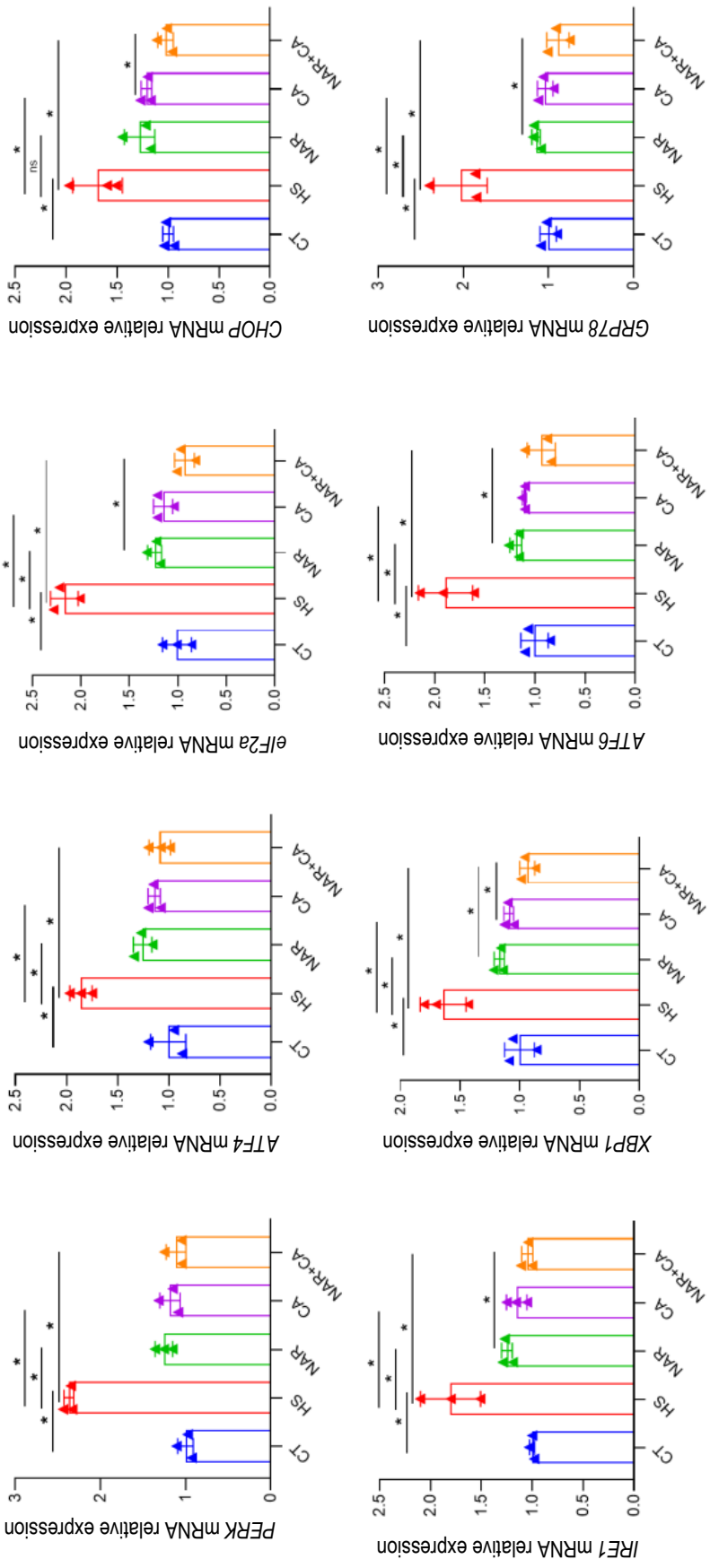

Figure 6. Effect of NAR and CA on ER stress-related signalling pathway activation in the liver. The mRNA levels of *PERK*, *ATF4*, *ATF6*, *eIF2α*, *CHOP*, *IRE1*, *XBP-1*, and *GRP78* were determined by real-time PCR. Data are presented as means ± SEM (n = 5). * $P < 0.05$ indicates significant differences

groups: CT – control, HS – heat stress, NAR – naringenin supplement, CA – caffeic acid supplement, NAR+CA – combined naringenin and caffeic acid supplement; *PERK* – PKR-like ER kinase, *GRP78* – glucose-regulated protein 78, *eIF2a* – eukaryotic translation initiation factor 2 alpha, *ATF4* – activating transcription factor 4, *ATF6* – activating transcription factor 6, *IRE1* – inositol-requiring enzyme type 1, *XBP1* – X-box-binding protein 1, CHOP – C/EBP homologous protein

Effects of NAR and CA on serum AST and ALT activity

Liver function was assessed through serum analysis of AST and ALT activities. Chronic HS exposure led to a significant increase in AST and ALT activities in the HS group compared to the CT group ($P < 0.05$) (Figure 5C–D). On the other hand, supplementation with NAR, CA, or their combination significantly lowered serum AST and ALT activities relative to the HS group ($P < 0.05$).

Effect of NAR and CA on activation of ER stress signalling pathway in liver

The impact of HS on ER homeostasis was evaluated by quantifying key ER stress markers using real-time RT-PCR. Chronic HS significantly upregulated hepatic mRNA expression of *PERK*, activating transcription factor 4 (*ATF4*), *ATF6*, eukaryotic translation initiation factor 2 alpha (*eIF2α*), *CHOP*, inositol-requiring enzyme type 1 (*IRE1*), X-box-binding protein 1 (*XBP-1*), and glucose-regulated protein 78 (*GRP78*) (Figure 6; $P < 0.05$). Notably, all treatment regimens (NAR, CA, and NAR + CA) effectively suppressed HS-induced activation of *PERK*, *ATF4*, *ATF6*, *eIF2α*, *GRP78*, and *XBP-1* mRNA expression in the HS group ($P < 0.05$). Additionally, CA and the combination of NAR and CA significantly reduced *CHOP* mRNA levels in the liver ($P < 0.05$).

Discussion

HS negatively impacts growth and reproduction in poultry (Zaboli et al., 2017). Dietary supplementation has emerged as a promising strategy to mitigate the adverse effects of HS. The present study investigated the protective effects of NAR and CA supplementation on broilers exposed to heat stress. CHS was shown to reduce growth performance and antioxidant activity. However, NAR and CA additives mitigated liver damage caused by HS and preserved liver function. Additionally, NAR and CA maintained liver homeostasis by modulating ER stress-related signalling pathways.

HS reduces antioxidant capacity, making antioxidant drugs an effective approach to alleviate its effects. Previous studies have demonstrated that supplementation with compounds such as HA, NAR, RES, CA, CUR, C3OG, ULA, and LTA significantly alleviates oxidative stress (Bigagli et al., 2017; Yilmaz, 2019; Petrella et al., 2020; Xu et al., 2023). In the current study, we first implemented a systematic drug screening approach using DF-1 cells to identify the most promising candidates. The *in vitro* results demonstrated that NAR and CA largely inhibited the increase of mRNA levels of HS-related proteins while restoring cellular antioxidant capacity.

HS negatively affects growth performance in poultry, often leading to reduced ADG and increased FCR (Sumanu et al., 2022; Khan et al., 2023). In the current study, the detrimental effects of extended HS exposure (6 weeks) were clearly demonstrated. However, broilers fed a diet supplemented with NAR or CA showed significant improvements in ADG and reduced FCR relative to the HS group. These findings indicate that NAR and CA supplementation can effectively enhance broiler performance under HS-stress conditions.

HSP70, a critical marker of HS, is produced in response to various stressors, protecting cells from damage (Rostagno et al., 2020), while HSF-1 is a key transcription factor regulating the expression of heat shock-related genes. In this study, chronic HS significantly elevated serum levels of HSP70 and HSF-1. Importantly, dietary supplementation with NAR and CA significantly reduced serum concentrations of both of the aforementioned proteins, demonstrating a protective effect against heat stress. HS is also associated with increased oxidative stress (Nawab et al., 2019). Antioxidant status was assessed using SOD activity, a pivotal enzymatic antioxidant, and T-AOC measurements involving nonenzymatic antioxidants. Chronic HS reduced serum SOD activity and T-AOC values, indicating elevated oxidative stress. However, dietary supplementation with NAR and CA effectively restored both SOD activity and T-AOC, demonstrating a potent enhancement of the antioxidant defence system.

Growing evidence suggests that HS contributes to liver damage, adversely affecting production performance (Zhao et al., 2021). In the present study, CHS exposure led to immune cell infiltration in liver tissue and increased serum activity of AST and ALT, two key markers of liver function. These findings collectively indicate that CHS exposure induced liver damage, although further research is required to elucidate the underlying mechanisms.

Recent studies have demonstrated that HS induces ER stress in broiler livers (Lu et al., 2019). ER stress disrupts protein folding, triggering activation of the unfolded protein response (UPR). This response detects the accumulation of misfolded proteins through three primary sensors: PERK, IRE1,

and ATF6, which initiate downstream pathways involving eIF2α, ATF4, CHOP, and XBP-1 signalling (Frakes et al., 2017). In the current study, evaluation of ER stress biomarkers in broiler liver tissue revealed that CHS exposure significantly increased mRNA expression levels of *PERK, ATF4, ATF6, eIF2α, CHOP*, *XBP-1*, and *GRP78* genes. Supplementation with NAR and CA effectively mitigated the upregulation of these ER stress markers, indicating their role in promoting ER homeostasis and alleviating liver damage in broilers under CHS conditions.

Conclusions

In summary, our findings demonstrate that heat stress induces liver dysfunction and endoplasmic reticulum stress, as evidenced by elevated liver injury markers and upregulated unfolded protein response pathway components. Importantly, dietary supplementation with naringenin (NAR) and caffeic acid (CA) attenuated these adverse effects, promoting restoration of liver homeostasis. These findings highlight the potential of NAR and CA as natural therapeutic agents to alleviate heat stress-induced liver damage in poultry production systems.

Conflict of interest

The Authors declare that there is no conflict of interest.

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