TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 191-200

doi:10.3906/vet-1910-79

Ginkgo biloba extract EGB761 improved anti-heat stress responses in chickens in vivo via regulation of heat-shock protein expression and distribution

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Received: 23.10.2019 • **Accepted/Published Online:** 19.02.2020 • **Final Version:** 06.04.2020

Abstract: Heat stress is a lethal threat to chickens. This study investigated the protective effect of a *Ginkgo biloba* extract EGB761 against heat stress in chickens in vivo. A total of 200 one-day-old hens were separated randomly into control (Con), heat stress (HS), 0.1% EGB administration and heat stress (0.1% EGB+HS), 0.3% EGB administration and heat stress (0.3% EGB+HS), and 0.6% EGB administration and heat stress (0.6% EGB+HS) groups. After EGB761 administration for 45 days, ten chickens were selected randomly, assigned to one of the designated groups, and exposed to a one-time heat stress condition of 38 ± 1 °C for 3 h. The results showed that EGB761 administration improved the growth performance and physiological condition of the chickens, reducing feeding-related mortality. EGB761 lowered the histological scores of the heart, liver, and duodenum in the heat-stressed chickens. EGB761 significantly reduced the transcription and translation levels of *Hsp27*, *Hsp70*, and *Hsp90* in these organs. EGB761 might promote the transformation of Hsps in the heart, increase the nuclear translocation of Hsp27 and Hsp90 in hepatocytes, and regulate the accumulation of Hsp27 and Hsp70 in intestinal villi. Therefore, EGB761 administration is a potential method for protecting chickens from acute heat stress damage.

Keywords: *Ginkgo biloba* extract EGB761, heat shock proteins, expression and distribution, heat stress, chicken

1. Introduction

Heat stress (HS) causes severe disturbances in physiological homeostasis and damage to different organs, including the heart, liver, kidneys, and intestines, in farm animals, especially in broiler chickens, which are highly sensitive to heat due to the absence of sweat glands [1,2]. Heat stress provokes heat-shock responses in organisms and cells, resulting in the rapid initiation of heat-shock protein (Hsp) synthesis and dramatic changes in gene expression to generate protection [3]. The best-studied members of the Hsp family are Hsp27, Hsp70, and Hsp90, which exhibit inducible expression and different functions. Phosphorylated Hsp27 participates in the regulation of apoptosis and antioxidative stress and is also involved in the protection of the cytoskeleton [4]. Hsp70 binds to newly synthesized polypeptides to prevent aggregation and participates in protein folding [5], while Hsp90 interacts with a variety of proteins to modify their configuration [6]. Regulating Hsp expression can protect chicken tissues against heat stress [3,7].

Ginkgo biloba extract EGB761 is a compound derived from dried leaves [8] and has been used in treating age-related cognitive decline, including memory and concentration problems, and various cardiovascular diseases [9,10]. EGB761 stabilizes the mitochondrial membrane potential disturbed by excessive reactive oxygen species (ROS) and peroxynitrite generation in damaged cells and contributes to the prevention of disrupted ionic homeostasis [11,12]. Its clinical efficacy and high tolerance in patients have been confirmed in a series of clinical trials [9,10]. However, the potential effect of EGB761 against heat stress in animals has not been evaluated. The present study investigated the protective effects of EGB761 on vital organs, namely the heart, liver, and intestines, against acute heat stress in chickens and the underlying molecular mechanism of EGB761's effects.

2. Materials and methods

2.1. Animals and experimental design

A total of 200 one-day-old female chicks (Sanhuang chicken) were obtained from Wensheng Breeding Co., Ltd. (Nantong, China) and raised in an artificial climate chamber at 25 ± 1 °C and 75% humidity. All chickens

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were healthy and separated randomly into five groups with 40 chickens in each: control (Con), heat stress (HS), administration of 0.1% EGB and heat stress (0.1% EGB+HS), administration of 0.3% EGB and heat stress (0.3% EGB+HS), and administration of 0.6% EGB and heat stress (0.6% EGB+HS). After feeding for 3 days to adapt them to the conditions, the chickens in 0.1% EGB+HS, 0.3% EGB+HS, and 0.6% EGB+HS were fed daily for 45 days with commercial small pellet feed (Table 1) evenly mixed with EGB761 (components of EGb761 shown in Table 2; Angle Gene Biotechnology Co., Ltd., China), while birds in the Con and HS groups were fed only the commercial feed. The amounts fed daily, the residue of food not consumed, and the number of dead chickens were recorded. The chicken weights were recorded at the beginning and end (day 45) of the study. After administration of EGB761, blood samples from ten chickens in each group were collected. Another group of ten chickens were randomly selected from each group and exposed to a one-time acute heat stress environment created by rapidly increasing the air temperature from 25 ± 1 °C to 38 ± 1 °C for 3 h while maintaining the humidity at 80% and generating anaerobic conditions with CO_2, followed by exsanguination. Samples of heart, liver, and duodenum were collected, and the specimens intended for histopathology examination were fixed in 10% neutral buffered formalin, while those destined for biochemical analysis were frozen in liquid nitrogen. Clinical symptoms such as breathing rate, water intake, and behavioral state were recorded and the birds were allowed free access to feed and water throughout the study.

All protocols concerning the handling of the chickens were in accordance with the requirements of the experimental animal ethics guidelines of the Ethics Committee at the Laboratory Animal Center of Nanjing Agricultural University (License number: SYXK (Jiangsu) 2011-0036), and all efforts were made to minimize the suffering of the animals.

2.2. Serum biochemistry analysis

Serum from the blood samples was collected and sent to Nanjing Integrated Traditional Chinese and Western Medicine Hospital for the detection of AST (aspartate aminotransferase), BUN (blood urea nitrogen), TC (total cholesterol), TG (triglyceride), CK (creatine kinase), and LDH (lactate dehydrogenase).

2.3. Histopathological analysis

The fixed specimens were embedded in paraffin, and sections were cut (5 μm thick) for staining with hematoxylin and eosin (H&E) and light microscopy analysis. Ten representative fields at a magnification of 400× were scored as follows: for the heart, enlarged myocytes (1 point), degeneration and microvascular hyperemia (2 points), and myocardial fiber fracture (3 points); for the liver, disordered and swollen hepatocytes (1 point), the presence of vacuolization and degeneration (2 points), and liver cell necrosis (3 points); for the duodenum, swelling and degeneration of epithelial cells (1 point), few necrotic villi and degenerative epithelial cells (2 points), and obvious abscission and necrosis of villi (3 points).

Table 1. The feed content and ration formulation of used commercial feed in this study.

Content	Ration
Corn	64
Soybean meal	24
Rapeseed meal	3
Bran	4
Premix	5

Table 2. Components of EGB761.

Component		Content (%)
Ginkgo Flavone Glycoside	Quercetin	11.71
	Kaempferol	10.70
	Isorhamnetin	2.20
	Total	24.31
Terpene trilactones	Bilobalide	2.65
	Ginkgolide A	1.11
	Ginkgolide B	0.78
	Ginkgolide C	0.88
	total	5.42
Proanthocyanidins		7.0
Carboxylic acid		13.0
Catechins		2.0
Nonflavone glycoside		20.0
Others (high molecules, inorganic, water, etc.)		28.0

2.4. Detection of fluorescence and quantitative real-time PCR

Total RNA was extracted from the frozen tissues using TRIzol reagent (Vazyme, China). Reverse transcription was carried out with an RT-PCR kit (Vazyme, China), and the resulting cDNA was used for RT-PCR with Power SYBR Green Master Mix (Vazyme, China) according to the manufacturer's instructions. The relative expression level of each heat-shock protein (*Hsp*) mRNA was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Primer sequences are shown in Table 3.

2.5. Western blotting

Total protein from the frozen tissues was isolated and quantified with the BCA protein assay kit (Life Technologies, USA) for western blot analysis. The methods were based on a previous study in our laboratory [13–15]. The primary antibodies (anti-Hsp27, anti-Hsp70, and anti-Hsp90) and GAPDH were purchased from ENZO (USA) and Abcam (UK), and the secondary antibodies were purchased from Boster (China). The intensity of the immunoreactive bands was analyzed using Quantity One software.

2.6. Immunohistochemistry

Tissue sections were stained with an immunohistochemical method as reported previously [13–15]. The primary antibodies against Hsp27, Hsp70, and Hsp90 were purchased from Enzo (USA), and the secondary antibodies were purchased from Dingguo (China). The immunohistochemical images were observed by light microscopy. The whole slide (400× magnification) was used for semiquantitative assessment using Image-Pro Plus 6.0 software.

2.7. Statistical analysis

Data are presented as means ± standard deviations (SDs). Differences between experimental groups were analyzed by one-way analysis of variance (ANOVA) with the least significant difference (LSD) multiple comparison test using SPSS 20.0 (IBM Corp., Armonk, NY, USA). *P < 0.05 and **P < 0.01 were considered to indicate statistical significance [16].

3. Results

3.1. Effect of EGB761 on growth performance

As shown in Table 4, compared with those of the Con group, the mean weights of the chickens in the 0.1% EGB+HS and 0.3% EGB+HS groups were increased by 2.8% (10.6 g) and 4.5% (17.3 g), respectively, while the average daily weight gain in the two groups was increased by 3.12% (P < 0.05) and 7.53% (P < 0.01), respectively. The ratio of feed-to-meat in the 0.1% EGB+HS and the 0.3% EGB+HS groups was decreased by 5.0% and 13.3%, respectively. Although the ratio of feed-to-meat was low (2.86:1, similar to that of the 0.1% EGB+HS group), the average daily weight gain in the 0.6% EGB+HS group was the lowest among the groups (particularly compared with the 0.1% EGB+HS, P < 0.05, and 0.3% EGB+HS, P < 0.01, groups).

3.2. Effect of EGB761 on physiological indexes

As shown in Table 5, increasing the concentration of the EGB761 treatment decreased the levels of serum AST, TG, and TC by 8%–11.7%, 3.2%–17.5%, and 6.4%–12.7%, respectively, in the chickens. The BUN levels in the 0.1% EGB+HS and 0.3% EGB+HS groups were significantly (P < 0.01; P < 0.05) downregulated compared with that of

Table 3. Sequences of primers for real-time PCR.

Gene	Forward primer	Reverse primer
Hsp27	5`-CCGGTGTTTCACTCGAAAATACA-3`	5`-GCTTTTCCGACTTTCCAGCTTCT-3`
Hsp70	5`-AGCGTAACA CCACCATTCC-3`	5`-TGGCTCCCACCCTATCTC-3`
Hsp90	5`-AGTCCCAGTTCATTGGCTAC-3`	5`-TCCAGTCATTGGTGAGGCT-3`
GAPDH	5`-TGAAAGTCGGAGTCAACGGAT-3`	5`-ACGCTCCTGGAAGATAGTGAT-3`

Table 4. The growth performance of chickens following EGB 761 administration (n = 40 per group).

	Control	HS	0.1% EGB+HS	0.3% EGB+HS	0.6% EGB+HS
Body weight at 45-days-old (g)	382.5 ± 44.4	380.5 ± 41.7	393.1 ± 24.2 †	399.8 ± 31.5 †	366.2 ± 25.4
Daily weight gain (g)	8.63 ± 0.36	8.58 ± 0.42	8.90 ± 0.48* $^{\#\dagger}$	9.28 ± 0.30 ** $^{\#\#\dagger\dagger}$	8.30 ± 0.43
Ratio of feed to meat	3.00:1	2.98:1	2.85:1	2.60:1	2.86:1

Control: Experimental group without administration of EGB761 and heat stress. HS: Experimental group with treatment of heat stress alone. 0.1% EGB+HS: Experimental group with administration of 0.1% EGB761 and heat stress. 0.3% EGB+HS: Experimental group with administration of 0.3% EGB761 and heat stress. 0.6% EGB+HS: Experimental group with administration of 0.6% EGB761 and heat stress.

Data are presented as mean ± standard deviation (SD). Comparison of values in Con with those in other groups, *P < 0.05, **P < 0.01. Values in HS were compared to those in all EGB761-treated groups, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01. Comparison of values in 0.6% EGB+HS and those in other EGB761-treated groups, †P < 0.05, ††P < 0.01. The same follows in subsequent tables.

Table 5. Effect of EGB 761 on chicken physiological indexes (n = 10 per group).

	Con	HS	0.1% EGB+HS	0.3% EGB+HS	0.6% EGB+HS
Aspartate aminotransferase (U/L)	240.0 ± 16.4	239.0 ± 13.4	220.8 ± 7.1	212.0 ± 2.5	217.3 ± 16.0
Triglycerides (mmol/L)	0.63 ± 0.41	0.59 ± 0.37	0.55 ± 0.05	0.61 ± 0.33	0.52 ± 0.07
Total cholesterol (mmol/L)	3.13 ± 0.93	3.10 ± 0.53	2.86 ± 0.12	2.93 ± 0.67	2.73 ± 0.45
Urea nitrogen (mmol/L)	0.96 ± 0.03	0.94 ± 0.03	0.76 ± 0.10 ** ## †	0.79 ± 0.08 * #	0.94 ± 0.09

Control: Experimental group without administration of EGB761 and heat stress. HS: Experimental group with treatment of heat stress alone. 0.1% EGB+HS: Experimental group with administration of 0.1% EGB761 and heat stress. 0.3% EGB+HS: Experimental group with administration of 0.3% EGB761 and heat stress. 0.6% EGB+HS: Experimental group with administration of 0.6% EGB761 and heat stress.

the Con group. The BUN in the 0.6% EGB+HS group was nearly equivalent to that in the Con group.

3.3. Effect of EGB 761 on breeding survival

In Figure 1A, the survival rate of the Con and HS group chickens is shown to be 87.5%, while the rates were 95% in the 0.1% EGB+HS and 97.5% in the 0.3% EGB+HS and 0.6% EGB+HS groups. Intake of dietary EGB761 increased the chicken's survival rate by more than 7.5% compared with the rate of chickens not administered EGB761.

3.4. Clinical behavior of the heat-stressed chickens

Chickens in the Con group remained in good condition during the study period. Birds in the HS group exhibited an increased respiratory rate and water consumption rate. After 2–3 h of heat stress, 40% of the chickens exhibited agonal respiration. Chickens in the 0.1% EGB+HS group flapped their wings and increased their water consumption, and their respiratory rates increased. They also exhibited behavior indicative of slight mental depression. Treatment with 0.3% EGB+HS showed an increase in respiratory rate and water consumption. Birds in the 0.6% EGB+HS group displayed only wing flapping and increased water consumption.

3.5. Changes in cellular damage-related enzymes

In Figure 1B, levels of serum CK and LDH in the HS group chickens are seen to be elevated ($P < 0.01$) compared to the levels in the Con group chickens. EGB761 inhibited serum CK and LDH increases to different degrees in the groups compared to the levels in the HS group, particularly the 0.6% EGB761 group.

3.6. Effects of heat stress on vital organs

As shown in Figures 1C–1H, the chicken cardiomyocytes in the HS group samples were enlarged, showing granular and fatty degeneration, and even myocardial fiber fracture and microvascular hyperemia. In the 0.1% EGB+HS group samples, cardiomyocyte degeneration and microvascular hyperemia in the heart tissues were also observed, but not myocardial fiber breakage. Samples taken from chickens treated with 0.3% EGB+HS showed moderate granular degeneration in the myocardial tissues. Microvascular hyperemia was observed definitively in the 0.6% EGB+HS group samples, with very few granular degeneration.

Hepatocytes in the HS group samples were enlarged, showing severe vacuolization and granular degeneration, accompanied by hepatocyte necrosis and severely damaged hepatocyte rings. The hepatocytes in 0.1% EGB+HS group samples were also disordered, with vacuolization and granular degeneration. In the 0.3% EGB+HS group samples, regularly arranged hepatocytes, such as in rings, and cell degeneration to different degrees could be identified. No obvious pathological changes were found in hepatocytes in the 0.6% EGB+HS group samples.

In the duodenum, obvious abscission and necrosis of the intestinal villi were observed in the HS group samples, with significant swelling and vacuolar degeneration of epithelial cells. Few abscissions or necrosis could be identified in the villi from the 0.1% EGB+HS group, while the epithelial cells in the preserved villi were well organized and showed moderate vacuolization. A greater amount of integral villi and slight cell degeneration were observed in the 0.3% EGB+HS group samples. No necrotic villi and few degenerative epithelial cells were observed in the 0.6% EGB+HS group samples.

Regarding tissue damage scores, the highest scores in all the examined organs were caused by heat stress. EGB761 administration significantly ($P < 0.01$ or $P < 0.05$) lowered the organ damage scores of the heat-stressed chickens in a dose-dependent manner.

3.7. Transcription of the Hsp genes

As shown in Figure 2, compared with the levels in the Con group, the levels of *Hsp27*, *Hsp70*, and *Hsp90* gene transcription in the heart, liver, and duodenum were significantly upregulated following chicken's exposure to heat stress for 3 h. All the tissues in all the groups treated with EGB761 also had significantly upregulated levels of *Hsp27*, *Hsp70*, and *Hsp90* mRNA; however, these levels were significantly decreased in a dose-dependent manner ($P < 0.05$ or $P < 0.01$) compared with levels in the HS group.

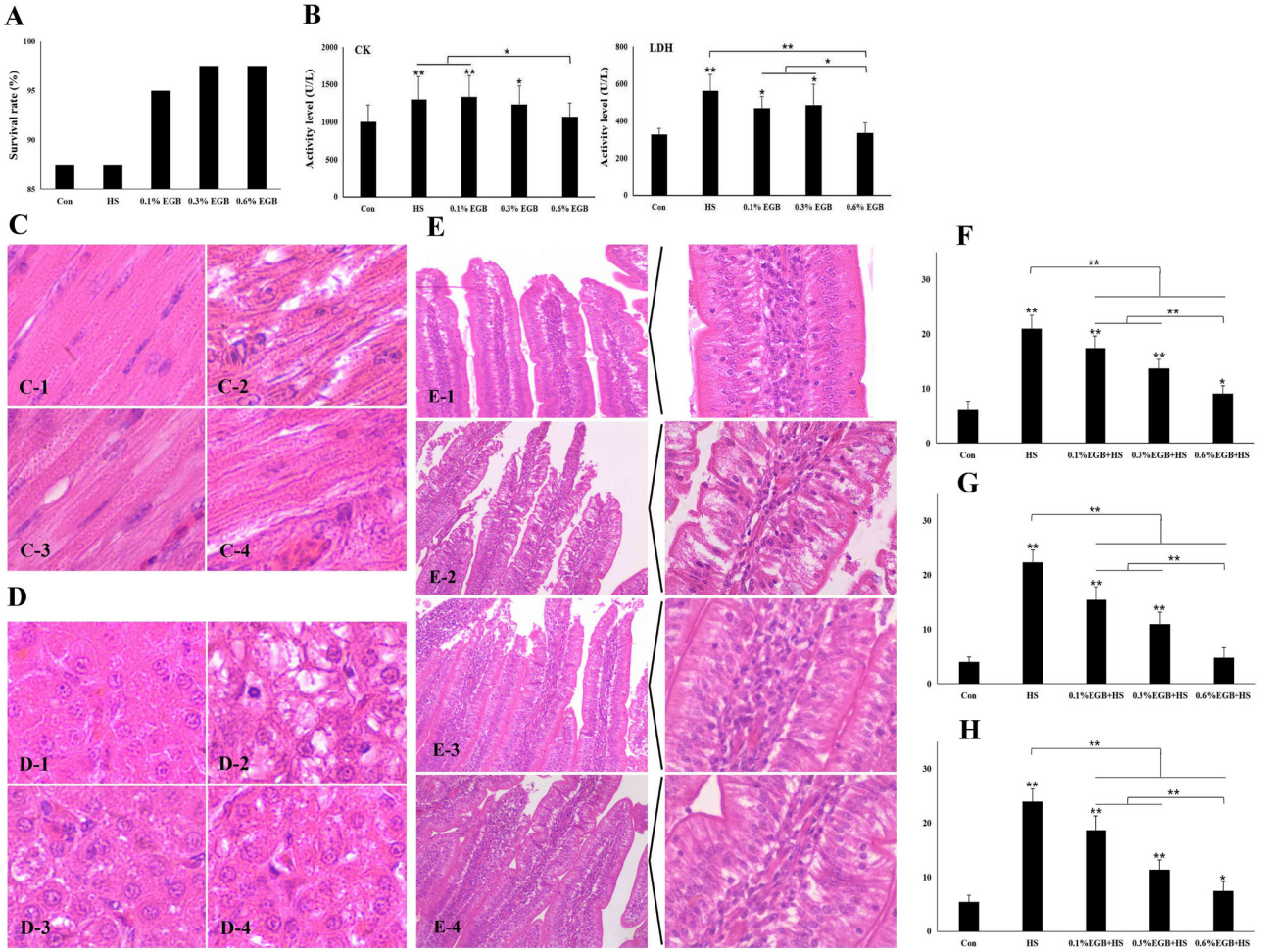

Figure 1. Chicken survival rate and serum CK and LDH levels after EGB761 administration, and the histopathological evaluation of the examined chicken tissues. A) Chicken survival rate after EGB761 administration; B) serum CK and LDH levels in the examined chickens (N = 10 per group); C) histopathological evaluation of the examined chicken tissues. H&E staining, bar = 200 μm. C, D, and E show the heart, liver, and duodenum from the Con (labeled 1), HS (labeled 2), 0.1% EGB+HS (labeled 3), and 0.6% EGB+HS (labeled 4) groups, respectively. Injury levels in the heart, liver, and duodenum were quantified by histopathological scoring and are shown in F, G, and H, respectively. *P < 0.05 or **P < 0.01.

3.8 Hsp expression levels

As shown in Figure 3, compared to the levels in the Con group, Hsp27, Hsp70, and Hsp90 expressions in all the tissues were upregulated in the HS group and were higher significantly than those in all the EGB761-treated groups (with the exception of Hsp90 in the heart). Hsp27 expression in all the examined tissues in the 0.1% EGB+HS group was upregulated, while Hsp27 expression in the 0.6% EGB+HS group was not altered. Hsp70 expression in all the examined tissues treated with EGB761 was increased significantly (with the exception of heart and liver Hsp70 in the 0.6% EGB+HS group) in a dose-dependent manner. Furthermore, 0.1% EGB761 treatment induced Hsp90 expression in the heart and duodenum, while 0.6% EGB761 treatment had no obvious effect. In the liver, Hsp90 expression in the 0.6% EGB+HS group was significantly upregulated, but not in the 0.1% EGB+HS or the 0.3% EGB+HS group.

3.9. Hsp distribution in the heart, liver, and intestine

As displayed in Figure 4 and Table 6, the Hsps were detected in the form of positively stained granules that were localized mainly in myocardial cells (Hsp27, Hsp70, and Hsp90), hepatocytes (Hsp70), and intestinal epithelial cells (Hsp27 and Hsp70). In the heart, the Hsps were distributed only in the cytoplasm and not in the nucleus regardless of exposure to heat stress, and nuclear translocation was not induced by EGB761 treatment. Heat stress exposure enhanced the signal density of these Hsps, and EGB761 preadministration weakened these signals. In the Con group, Hsp27 and Hsp90 granules in hepatocytes and

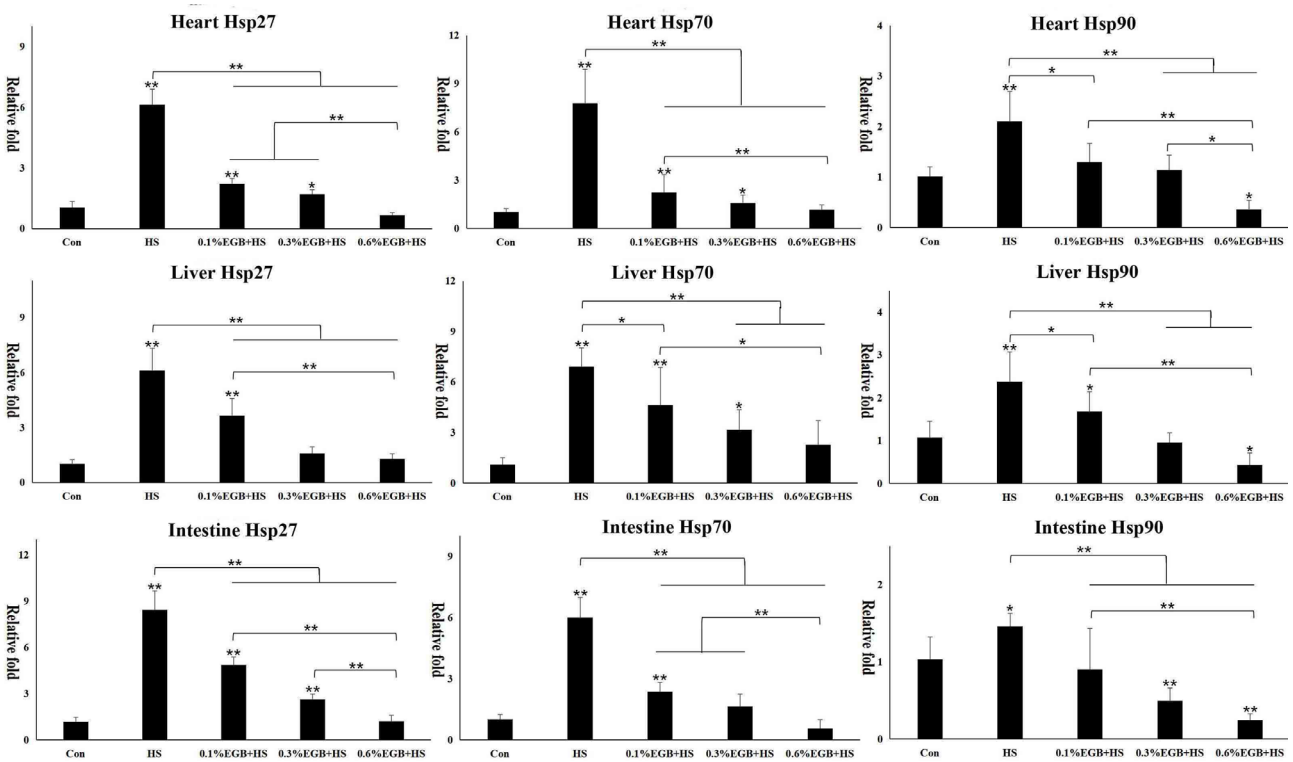

Figure 2 Transcription levels of the *Hsp27*, *Hsp70*, and *Hsp90* genes in the chicken tissues. The mRNA levels of *Hsp27*, *Hsp70*, and *Hsp90* from the heart, liver, and duodenum tissue samples were analyzed by RT-PCR. The expression levels were normalized to the level of GAPDH. *P < 0.05 or **P < 0.01.

Hsp90 in intestinal epithelial cells (IECs) were located in both the cytoplasm and nucleus, and heat stress markedly strengthened nuclear translocation. Compared with the HS group, the groups administered EGB761 pretreatment had increased nuclear translocation in liver Hsp27 and Hsp90 but decreased nuclear translocation of the IEC Hsp90. Heat stress exposure greatly increased the signal density of the IEC Hsp27 in intestinal villus tips, while EGB761 decreased the density in a dose-dependent manner.

4. Discussion

In this study, we first evaluated the effect of prolonged EGB761 administration on chickens using a model of intensive increase. The data showed that 0.1% and 0.3% EGB761 treatment increased the average daily weight gain by 3.12% and 7.53%, respectively, and decreased the ratio of feed-to-meat by 5.0% and 13.3%, respectively, demonstrating the ability of EGB761 to improve the feeding efficiency of chickens. This finding is consistent with a previous study showing that EGB761 administration increased duodenal and jejunal villous height, decreased jejunal crypt depth, and regulated intestinal flora [17]. EGB761 was also reported to regulate the growth of the hypothalamus-pituitary axis in Peking duck and promoted growth hormone secretion to positively impact the growth and feed conversion ratios [18]. We also found that 0.6% EGB761 induced the poorest average daily gain despite the superior ratio of feed-to-meat, which may be explained by the naturally bitter taste of the EGB761, which may have had an adverse effect on the appetite/feed intake of the chickens.

Effective physiological metastasis, which is the foundation of growth and the ability to tolerate adverse conditions, is usually characterized by clinically low levels of serum AST, TG, TC, and BUN, which are often associated with damage or dysfunction of the liver, heart, gallbladder, and kidneys and affects lipid metabolism [19–21]. In this study, treatment with EGB761 attenuated these factor levels to varying degrees, implying that EGB761 improved the cardiovascular, hepatobiliary, and urinary systems of the chickens. However, 0.6% EGB761 administration had no effect on the BUN levels, suggesting that the regulatory effect of EGB761 on organs, especially the kidneys, declines with increasing drug concentration. Combined with a reduction in breeding mortality after EGB761 treatment, these results indicated that dietary EGB761 supplementation improved the potential of the chickens to tolerate adverse conditions.

In addition to heart damage, obvious liver damage characterized by increased LDH levels and degenerative and even necrotic hepatocytes, including abscission and necrosis of villi with degenerative IECs, were also observed

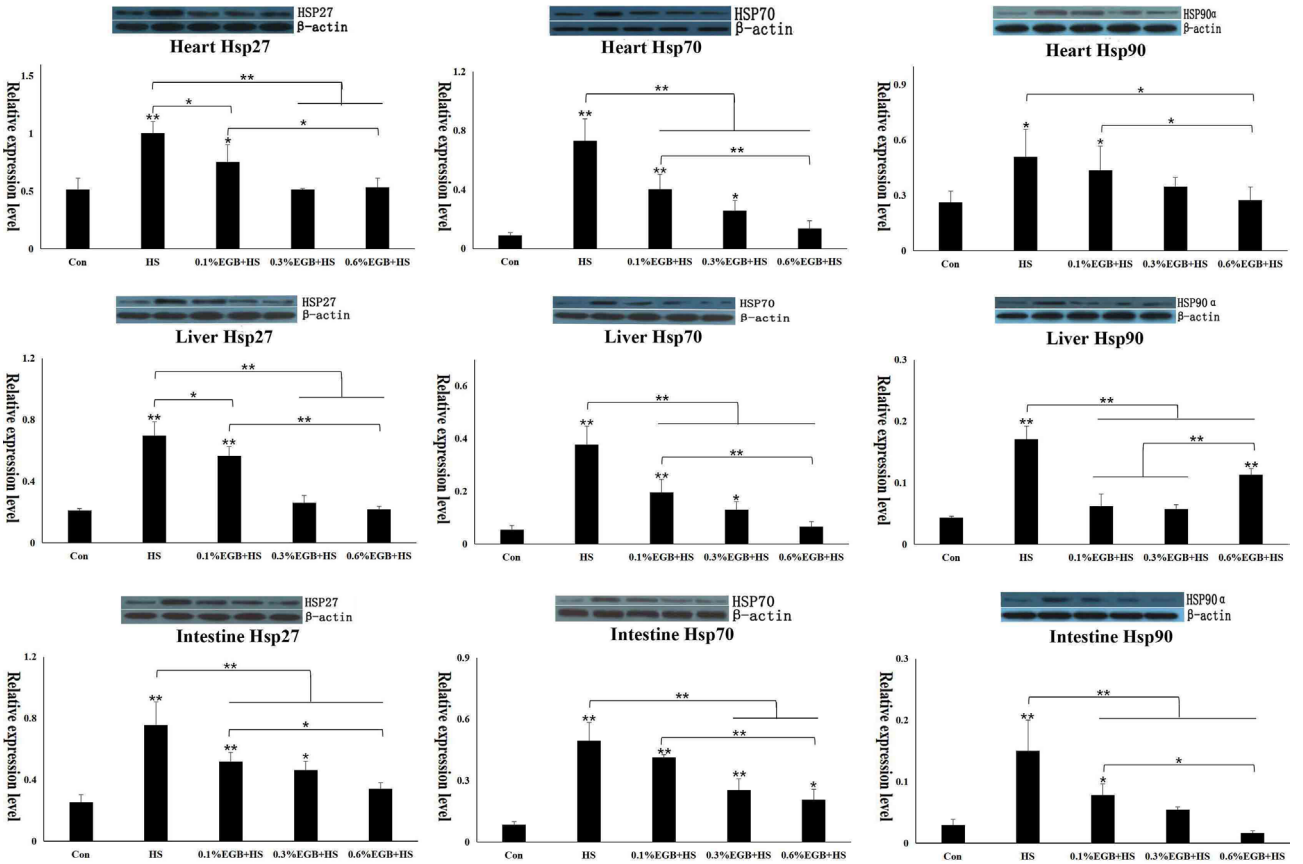

Figure 3. Heat-shock protein expression patterns in chicken tissues. Hsp27, Hsp70, and Hsp90 expression in the heart, liver, and duodenum tissues were assessed by western blotting. Representative immunoblots of the indicated proteins are shown. Protein levels were normalized to the level of GAPDH. *P < 0.05 or **P < 0.01.

in the heat-stressed chickens. In this study, as expected, oral administration of EGB761 at doses ranging from 0.1% to 0.6% protected the heart, liver, and intestines from heat stress damage in a dose-dependent manner. To the best of our knowledge, this is the first study to demonstrate the application of EGB761 as an anti-heat stress agent in chickens. Under heat stress, granule degeneration in cells is caused by abnormal protein metabolization, leading to dysfunction of organelles such as mitochondria and finally to cellular necrosis [22,23]. Synthesis and function of protein is mainly regulated by heat shock proteins. Previous studies showed that preinduction of Hsp27, Hsp70, and Hsp90 protected chicken myocardial cells against heat stress via the PKC/MAPK and PIK3-Akt signaling pathways [14,24,25]. In this study, exposure to heat stress resulted in significantly increased expression of Hsp27, Hsp70, and Hsp90 at the mRNA and protein levels in the heart, liver, and duodenum, corresponding to the severity of tissue damage. This is in agreement with the studies of Dutta et al. [26,27], especially for Hsp70 expression. Our results also suggested that the degrees of restoring injuries were positively associated with the dosage of EGB761. Phosphorylation of Hsp27, marked consumption of Hsp70, and dimerization of Hsp90 are important for their protective functions, but all these modifications reportedly interfere negatively with quantitative detection [13,14,24,25]. Thus, the protective effects of EGB761 against heat stress may also probably be linked with the low Hsps levels.

The antistress effects of Hsp27, Hsp70, and Hsp90 correlate with their subcellular distribution, with increased nuclear translocation particularly associated with enhanced protection of proteins and DNA [13–15]. Our immunohistochemistry results showed that Hsp27, Hsp70, and Hsp90 remained in the cytoplasm of the myocardial cells despite obvious induction after exposure to heat stress, which is not consistent with previous studies reporting on 42 °C heat stress conditions [13–15]. This discrepancy may be caused by differences in the breed of chickens and the heat stress conditions critical for the nuclear translocation of Hsps [28]. In our study, Hsp70 was located only in hepatocyte cytoplasm, while Hsp27 and Hsp90 were detected in both the cytoplasm and the nucleus, and heat stress induced their marked

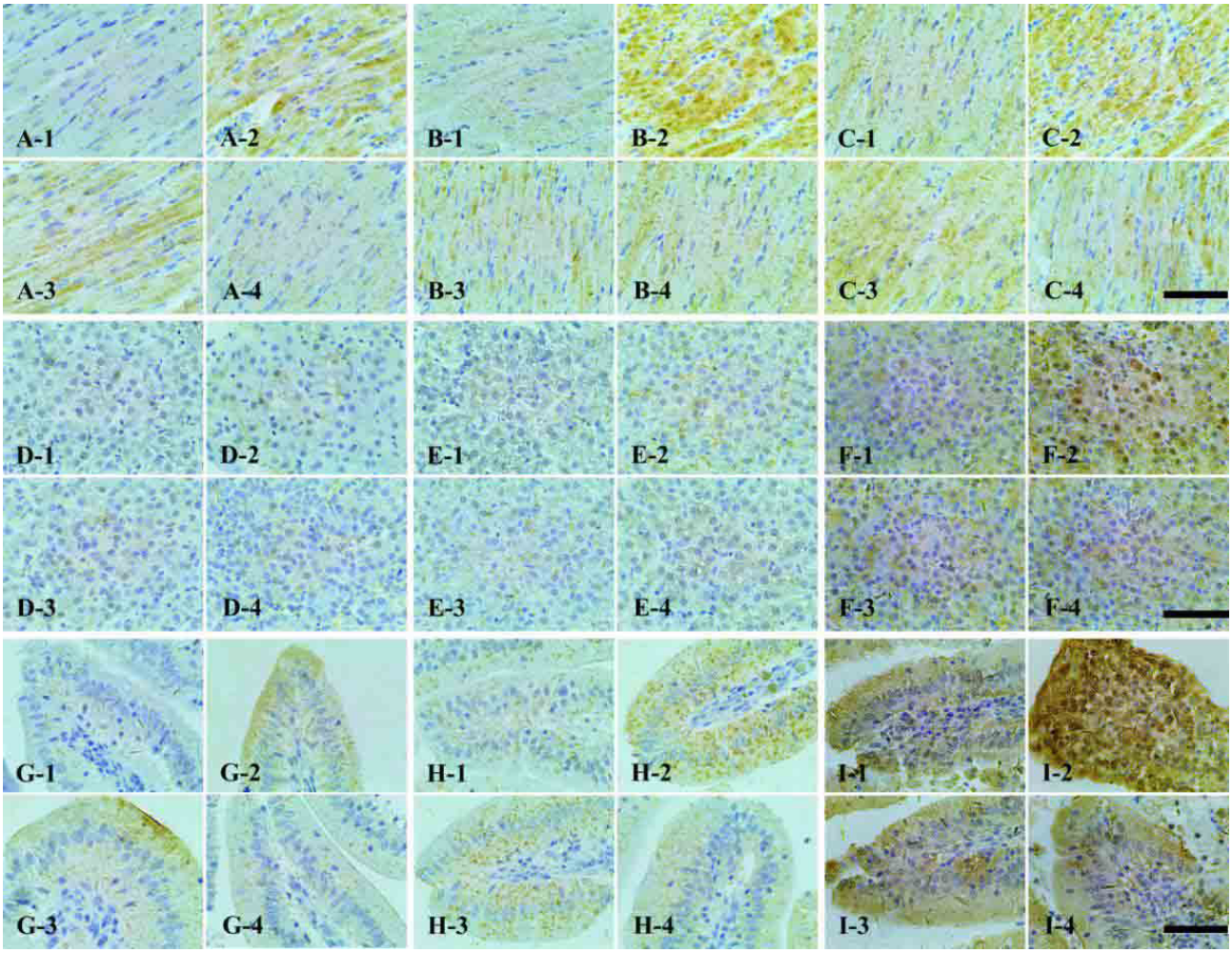

Figure 4. Positive signal distribution of Hsps in the heart, liver, and duodenum of the examined chicken tissues. Immunohistochemical staining, bar = 200 μm. A–C, D–F, and G–I show the positive signals of Hsp27, Hsp70, and Hsp90 in the heart, liver, and duodenum tissues, respectively. Meanwhile, the pictures labeled 1, 2, 3, and 4 are from the Con, HS, 0.1% EGB+HS, and 0.6% EGB+HS groups, respectively.

nuclear translocation. Hsp27 phosphorylation promotes dissociation of Hsp27 oligomers and induces nuclear translocation, where it has anti-heat stress functions [14,29,30]. The nuclear translocation of Hsp90 is essential for the protection of cellular DNA and key transcription factors [25]. Our results showed that EGB761 further increased nuclear translocation, but not the expression of Hsp27 and Hsp90 in hepatocytes, to enhance their antistress function. Hsp27 and Hsp70 were distributed only in the IEC cytoplasm, and neither heat stress nor EGB761 increased their nuclear translocation, demonstrating that the protective effects of Hsp27 and Hsp70 were mediated by the expression levels and consumption of the IECs in vulnerable sites. For instance, the IEC Hsp27 signal in villus tips was particularly enhanced by heat stress, illustrating that Hsp27 accumulation was important for resisting heat stress damage in the duodenum. Reports show that the tips of the intestine villi are sensitive to stress exposure, with a 19% reduction in villus height observed under chronic heat stress for two weeks [31]. Our observation of reduced Hsp27 expression and milder pathological injury in the villus tips indicates that EGB 761 treatment alleviates heat stress-induced damage by promoting Hsp27 phosphorylation [14]. Previous studies have shown that heat stress increased the nuclear translocation of IEC Hsp90 to protect nuclear transcription factors [32]. EGB761 decreased the nuclear translocation of IEC Hsp90 induced by heat stress, implying that EGB761 protected heat-stressed IECs by an alternative mechanism that remains to be investigated.

Therefore, EGB761 protected vital organs against acute heat stress by regulating physiological conditions and possibly promoting the transformation of Hsps in the heart, increasing the nuclear translocation of Hsp27 and

Table 6. Hsp27, Hsp70, and Hsp90 signal intensity in cytoplasm and nucleus quantified by average optical density (AOD) (n = 5 per group).

Tissue	Protein	location	Average optical density (AOD)			
			Con	HS	0.1% EGB+HS	0.6% EGB+HS
Heart	Hsp27	Cytoplasm	32.89 ± 6.94	124.00 ± 15.73**	86.61 ± 9.12** ##	42.27 ± 6.42* ## ††
		Nucleus	0	0	0	0
	Hsp70	Cytoplasm	37.99 ± 8.27	152.68 ± 17.16**	95.75 ± 7.58** ##	85.34 ± 11.45** ##
		Nucleus	0	0	0	0
	Hsp90	Cytoplasm	51.56 ± 4.44	166.27 ± 12.76**	144.76 ± 27.44**	83.23 ± 14.32** ## ††
		Nucleus	0	0	0	0
Liver	Hsp27	Cytoplasm	13.86 ± 5.45	56.78 ± 8.33**	8.22 ± 1.97##	12.35 ± 5.85##
		Nucleus	4.56 ± 2.54	43.74 ± 8.10**	83.79 ± 9.64** ##	55.64 ± 4.04** # ††
	Hsp70	Cytoplasm	13.69 ± 8.23	59.80 ± 7.48**	40.93 ± 7.56** #	12.24 ± 8.25## ††
		Nucleus	0	0	0	0
	Hsp90	Cytoplasm	33.28 ± 7.43	132.68 ± 21.71**	39.93 ± 7.56##	43.37 ± 4.74* ##
		Nucleus	7.55 ± 5.32	80.65 ± 4.58**	30.47 ± 5.78* ##	27.12 ± 3.22** ##
Duodenum	Hsp27	Cytoplasm	16.24 ± 5.95	110.63 ± 19.73**	88.80 ± 13.17** #	25.54 ± 6.58## ††
		Nucleus	0	0	0	0
	Hsp70	Cytoplasm	44.35 ± 6.20	141.55 ± 28.41**	96.07 ± 15.07** ##	71.76 ± 14.56** ## †
		Nucleus	0	0	0	0
	Hsp90	Cytoplasm	50.77 ± 9.96	195.67 ± 22.53**	103.64 ± 18.75** ##	75.49 ± 8.77* ## ††
		Nucleus	31.45 ± 5.34	200.93 ± 34.67**	54.56 ± 14.33* ##	32.35 ± 6.34 ## ††

Hsp90 in hepatocytes, and regulating the expression and consumption of IEC Hsp27 and Hsp70 in vulnerable sites. This study reveals the resistance to heat stress conferred by the use of traditional Chinese medicine.

Acknowledgments

This work was supported by grants from National Natural Science Foundation Youth Funding Project of China (31802157), National Natural Science Foundation of China (31672520), Postdoctoral Science Foundation of Jiangsu Province (2018K206C), and Open Topic from Modern Animal Husbandry and New Veterinary Engineering Technology Center of Jiangsu Province (GCZXKF1403).

Conflict of interest

All authors declared no conflict of interest.

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