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The beneficial effect of nanomethionine supplementation on growth performance, gene expression profile, and histopathology of heat-stressed broiler chicken

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ABSTRACT This study investigated the effects of nanomethionine (**nano-meth**) on performance, antioxidants, and gene expression of HSP70, HSP90 and Heat Shock factor-1 (**HSF-1**) from the liver, and TLR4 from the jejunum, of broiler chickens reared under normal temperatures or under heat stress. Three hundred 1-day-old chicks were randomly assigned to 5 treatment groups. Group 1 served as control. Under normal temperature, birds in group 2 received nano-meth (10 mL/L of drinking water) from d1 until the experiment ended. Group 3 birds were heat-stressed (**HS**) and did not receive any supplementation. Group 4 received nano-meth in the same dose from d1 old until experiment ended, and the birds were exposed to HS. Group 5 birds were HS and received supplementation of nano-meth during the HS period only. Nano-meth improved ($P < 0.0001$) final body weight, weight gain, feed conversion ratio, and also decreased ($P < 0.0001$) the effect of HS on growth performance. Reduction ($P < 0.0001$) in malondialdehyde and changes in antioxidant enzymes GPX and CAT activity indicated the antioxidant effect of nano-meth. Nano-meth supplementation caused an increase in the expression of HSP70 , HSP90 and HSF1, and a downregulation of TLR4 gene expression. Additionally, nano-meth-supplemented groups showed marked improvement in the histological liver structure, intestinal morphology and villus height compared to control or HS groups.

Key words: broiler, nanomethionine, growth performance, gene expression, histopathology

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INTRODUCTION

As climate conditions change, high ambient temperatures during summers increasingly impact all living organisms worldwide. High ambient temperatures stress poultry and impair their ability to maintain a homoeothermic body temperature since they lack sweat glands and instead rely on evaporative cooling (panting) to keep themselves cool (Brugaletta et al., 2022). Hyperthermia can develop in chickens when they are exposed to high ambient temperatures (Chowdhury et al., 2012; Lan et al., 2016) which results in heat stress (**HS**) when heat production exceeds the animal's heat dissipation capacity (Sejian et al., 2018).

HS is a major problem affecting worldwide poultry production and reducing producers' profits (Liu et al., 2020). HS triggers a series of negative physiological responses, such as depressed immune response,

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endocrine disorders, and reproductive problems (Waheed et al., 2020; Mirzaei et al., 2022). HS also negatively impacts poultry productivity (Abioja and Abiona, 2021; Uyanga et al., 2022). It decreases food intake and body weight (**BW**) gain, ultimately hindering production and increasing mortality (Liu et al., 2020). Other processes affected by HS include antioxidant activity and free radical generation; these free radicals, such as reactive oxygen and nitrogen species, are highly toxic substances capable of reacting with important macromolecules, such as proteins and lipids (Hu et al., 2019). Even a transient state of oxidative stress is believed to affect animal performance negatively (Surai et al., 2019). Previous studies have investigated the use of antioxidant compounds, such as vitamins A and E (Souza et al., 2011), and amino acids (Del Vesco et al., 2015), in broiler diets to mitigate the adverse effects of HS. Several reports have shown that the dietary supplementation of certain essential amino acids can mitigate the problems resulting from HS in birds (Willemsen et al., 2011; Dai et al., 2012).

Methionine (**Meth**) is a proteinogenic amino acid that is important in initiating the translation of proteins associated with body functions (e.g., muscle deposition). Its direct and indirect antioxidant properties help minimize the harmful effects of free radicals (Adedokun et al., 2014; Aledo, 2019; Rehman et al., 2019). Meth is involved in glutathione synthesis (Tamanna et al., 2019), and is one of the most readily oxidized residues in proteins (Kim et al., 2014). The beneficial effects of methionine supplementation on antioxidant activity in heat-stressed birds have been demonstrated in previous studies (Del Vesco and Gasparino, 2013; Gasparino et al., 2018). Meth is considered the first limiting amino acid in poultry due to its high protein synthesis and feather development demand. Meth requirements, on an as-fed basis during the production stage of broiler chickens, have been shown to vary 0.50 to 0.52% during the starter phase, 0.38 to 0.45% during the grower phase, and 0.32 to 0.44% during the finisher phase (NRC, 1994; Cobb-700, 2012; KFSP, 2012; Ross-308, 2014). DL-methionine is the most commonly used synthetic Meth product (Alagawany et al., 2014, 2016; Soomro et al., 2017, 2018; Abbasi et al., 2018; Kim et al., 2019). There have been no reports about the effects of nanomethionine (**nano-meth**) in chicken. Therefore, this study attempts to determine the effects of nano-meth supplementation during normal temperatures and HS on growth performance and gene expression in broiler chickens.

MATERIALS AND METHODS

Experimental Design and Feeding Program

The Institutional Animal Care and Use Committee of University of Alexandria approved the experimental protocol used in this study (Permit #2022/013/125).

A total of 300 Cobb500 broiler chicks (1-day-old) were used in this study. The chicks were distributed randomly into 5 treatment groups (60 chicks/treatment) with 3 replicates per treatment (20 chicks per replicate). The treatment groups consisted of the following, birds in the first group (G1) received drinking water without any supplementation and were reared under normal temperatures (control). In contrast, birds in group 2 (G2) were supplemented with nano-meth from 1-day old until the end of the experiment and were reared under normal temperatures. In group 3 (G3), birds were exposed to HS at 28 d of age, and did not receive supplementation to their drinking water. Group 4 (G4) was supplemented with nano-meth in their drinking water from 1-day old and experienced HS. Group 5 (G5) was supplemented with nano-meth only during HS periods, with HS induced at 28 d of age and birds exposed to 34°C for 3 d (i.e., 72 h continuously) (Abo-Samaha et al., 2021). Nano-meth was added at a concentration of 5% (10 mL/L of this concentration) to supplement the birds' drinking water. Nano-meth was prepared according to Hojo et al. (2011).

The chicks were reared on the floor in an environmentally controlled room. The temperature was gradually decreased from 32°C to 24°C in 3.5°C increments weekly. A continuous lighting program was provided on the day of arrival, followed by a 23L:1D lighting schedule thereafter. All groups received the basal diet shown in Table 1 per the recommendations of the National Research Council (NRC, 1994). The formulated diets were analyzed for major nutrients. Broiler chicks were fed on starter, grower, and finisher diets during the first 2 wk, wk 3 to 4, and the last week of the experiment, respectively. The ingredient composition and chemical analyses of the basal diets used for the starter, grower, and finisher periods are presented in Table 1. The experiment used formulated diets that were isoenergetic and isonitrogenous and met the nutrient requirements of the birds.

Dry Matter and Crude Nutrients Analysis

The dry matter contents of feed samples were determined by oven-drying at 105°C for 8 h (AOAC, 1990). Ash contents were determined by incineration at 550°C overnight. Crude protein was determined using the Kjeldahl method according to Randhir and Pradhan (1981), and ether extract was determined according to Hanson and Olly (1963).

Growth Performance, Relative Organ Weight, and Rectal Temperature

Chicks were weighed individually for initial and final BW. The chicks' total weight gain (**WG**) (expressed in grams) was calculated as the difference between initial and final weights on a replicate basis. Feed intake was calculated as the difference between the weight of the offered feed and the remainder and then divided by the number of birds in each group per day and totaled per week. The feed conversion ratio was calculated by dividing the feed consumed (g) by the BW gain (g) during

Table 1. Ingredient composition and chemical analysis of the used experimental diets.

Ingredients	Starter	Grower	Finisher
Yellow corn (7.8% CP)	52.63	60.1	66.90
Soybean meal (44% CP)	34.5	27.5	24
Corn gluten (60% CP)	6.5	7	5
Vegetable oil[1]	2.5	2	1
MCP[2]	1.2	0.8	0.65
Lime-stone[3]	1.85	1.8	1.65
Lysine[4]	0.05	0.05	0.05
DL-Methionine[5]	0.07	0.05	0.05
Choline[6]	0.05	0.05	0.05
Threonine[7]	0.05	0.05	0.05
Mycotoxin adsorpant[8]	0.05	0.05	0.05
Salt	0.25	0.25	0.25
Premix (mineral and vitamin)[9]	0.3	0.3	0.3
Total	100	100	100
Chemical analysis			
Moisture %	11.7	11.9	12
Crude protein %	23.1	20.9	18.7
Ether extract %	6.2	6	5.7
Ash %	5.9	5.7	6.1
ME kcal/kg diet	3036	3053	3107

[1]Mixture of sunflower and soybean oils.
[2]Mono calcium phosphate: contain 16% calcium and 21% phosphorus.
[3]Limestone contains 38% calcium and locally produced.
[4]Lysine 87% produced by Archar Daniels method company De Caur LL. Made in USA.
[5]DL-methionine produced by Evoink Co. Guranted analysis 99.5% DL-methionine.
[6]Choline chloride 60% with vegetable carrier (corn powder) produced by Shandyuong Pharmaceutical Co. China.
[7]Crystalline L-Thr (98.5% Thr, PT. Cheil Jedang, Indonesia). [6]Choline: choline chloride 60% with vegetable carrier (corn powder) produced by Shandyuong Pharmaceutical Co. China.
[8]Beta-2-x.
[9]Each 3 kg contains: Vit A (12,000,000 IU), Vit D (2,000,000 IU), Vit E (10 g), Vit K_3 (2 g), Vit B_1 (1 g), Vit B_2 (5 g), VitB_6 (1.5 g), Vit B_{12} (10 g), nicotinic acid (30 g), pantothinic acid (10 g), folic acid (1 g), biotin (50 mg), choline chloride 50% (250 mg), iron (30 g), copper (10 g), zinc (50 g), manganese (60 g), iodine (1 g), selenium (0.1 g), cobalt (0.1 g), and carrier lime stone up to 3 kg.

the week. Broilers (9 samples/treatment) were weighed individually before slaughter (live weight). Immediately after slaughter, the liver, gizzard, proventriculus, heart, and abdominal fat were removed from the carcass and weighed to determine the relative weight as relative organ weight (%) = organ weight/live BW × 100. The rectal temperature was measured in both control and HS birds.

Lipid Peroxidation and Antioxidant Profile

Lipid peroxide was measured after the reaction with thiobarbituric acid and expressed as nmol malondialdehyde (**MDA**) per tissue weight (Ohkawa et al., 1979). Glutathione peroxidase (**GPX**) activity was analyzed according to Paglia and Valentine (1967), which is based on the reaction of hydrogen peroxide ($\mathbf{H_2O_2}$) in the presence of NADPH, GSH, and glutathione reductase. The absorbance was measured at 340 nm, and the result was expressed as IU/tissue weight. Catalase (**CAT**) activity was measured based on the rate of oxidation according to Chiang et al. (2006), and recorded as IU/tissue weight.

qRT-PCR Analysis of Stress-Related Genes

Analyses were conducted for Heat Shock protein 70 (**HSP70**), Heat Shock protein 90 (**HSP90**), and Heat Shock factor-1 (**HSF-1**) from the liver, and Toll-like receptor 4 (**TLR4**) from the jejunum. Fifty-milligram samples were collected in clean 2.0 mL microfuge tubes, snap-frozen, and stored at −80°C until analyzed. Total RNA from liver samples was extracted using easy-RED total RNA extraction kits (iNtRON Biotechnology, Inc., Seongnam-Si, South Korea) according to the manufacturer's instructions. RNA integrity was verified by agarose gel electrophoresis. A fixed concentration of RNA (2 μg) was used for cDNA synthesis using the IntronPower cDNA synthesis kit (Cat. no. 25011). For qRT-PCR assay, specific HSP70, HSP90, HSF1, and TLR4 primers were used to amplify these genes from Cobb chicken, with 18S rRNA used as an internal standard housekeeping gene. The primer sequences are displayed in Table 2. The reaction mix for each gene contained 10 μL of SensiFast SYBR Low-Rox master mix (Bioline, London, UK), 0.5 μM of each primer, and 2 μL of cDNA. Tests were conducted in duplicate, using the Stratagene MX300 P real-time PCR system (Agilent Technologies, Santa Clara, CA), with the following thermal cycling conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s, and annealing for 1 min at gene-specific annealing temperatures. The dissociation curves were analyzed by showing only 1 peak at a specific melting temperature for all tested genes, indicating specifically amplified PCR products. The relative gene expression levels were evaluated using the $2^{-\Delta\Delta ct}$ method as described by Rao et al. (2013). In this context, the fold change for each gene was normalized against the housekeeping gene (18S) and the corresponding values of the control group.

Table 2. Sequences of primers used in real-time PCR.

Gene	Primer sequence (5′−3′)	Reference	Acc. number	Annealing temp
HSP70	F:CCAAGAACCAAGTGGCAATGAA R: CATACTTGCGGCCGATGAGA	El-Kassas et al. (2019)	EU747335	60
HSP90	F: GAGTTTGACTGACCCGAGCA R: TCCCTATGCCGGTATCCACA	El-Kassas et al. (2019)	NM_206959	60
HSF1	F: 5-CAGGGAAGCAGTTGGTTCAC TACACG-3 R: 5-CCTTGGGTTTGGGTTGCTCAGTC-3	Abdo et al. (2017)	L06098.1	60
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	Iqbal et al. (2005)	AY064697	60
18s	F: 5-CGAAAGCATTTGCCAAGAAT-3 F: 5-GGCATGGTTTATGGTCGG	Primer 3	XM_288030.1	60

Histopathological Study

Representative specimens were collected from the livers and jejunums of control and treated chicks. The collected specimens were washed and soaked in a 10% neutral-buffered formalin solution for 24 h for fixation. Next, fixed specimens were prepared via the paraffin-embedding technique according to Bancroft and Gamble (2013). Briefly, the fixed specimens were dehydrated using ascending concentrations of ethyl alcohol, cleared in 3 changes of xylol, blocked in paraffin wax, and cut into 3 to 5 μm thick sections. The microtomed sections were stained with hematoxylin and eosin stain (**H&E** stain). Next, blinded examination and imaging were conducted by an experienced pathologist. The representative photomicrographs were taken using a digital camera (Leica EC3; Leica Biosystems, Wetzlar, Germany) connected to a microscope (Leica DM500).

Histomorphometric Analysis of Intestine

After blood was drawn, the chickens were cut open and the entire digestive tracts were separated. Segments of the middle portions of the digestive tracts were collected and images from the intestinal tissues of the chicks were taken using a digital camera connected to a bright field microscope (Nikon E200, Nikon Corp., Tokyo, Japan). The images were then subjected to computerized quantitative histomorphometric analysis. Light microscopic observations were used to assess the effects of nano-meth dietary supplementation on the following parameters: 1) villus height (μm, from the tip to the base of villus); 2) villus width (μm, at the tip; and the crypt/villus junction); 3) crypt depth; 4) Villus height/crypt depth ratio; and 5) tunica muscularis thickness. A computerized image analysis system was used to perform the measurements (Image J software; Bethesda, MD) (Schneider et al., 2012). The villi height, crypt depth, and villi width at the crypt/villus junction and the villi tips were measured (the mean value was determined for 15 villi/sample, × 10).

Statistical Analysis

Statistical analysis was performed using SAS software (SAS, 2014). Data were evaluated for normality before analysis using the Kolmogorov-Smirnov test. Generalized linear models were used to analyze the data with the following statistical model:

$$X_{ij} = \mu + T_i + e_{ijk};$$

where X_{ij} = the observation record; μ = overall mean; Ti = ith level effect of different treatments; e_{ij} = random error. Post hoc treatment group comparisons were conducted using Duncan's test. The overall significance level was set as $P < 0.05$. The gene expression data were analyzed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, and $P < 0.0001$****. All values are expressed as the mean ± SEM.

RESULTS

Characterization of Nanomethionine

The sizes of the Nano-Meth particles were determined by scanning electron microscopy. The solution was diluted to 50% with deionized water, then 1 drop was spread on a glass slide, and morphology (Figure 1) was observed with a JSM-IT 200 (JOEL) scanning electron microscope operated between 15 and 20 keV.

Growth Performance

Data for BW, WG, total feed intake (**TFI**), and feed conversion ratio (**FCR**) are presented in Table 3. Final BW was significantly different among groups. Specifically, the heaviest weight was recorded for G2 birds supplemented with nano-meth without exposure to HS (1,936 g). This was heavier than the control (G1) by 174 g; while the lightest weight was recorded for G3 birds (1,536 g), which had been exposed to HS without nano-meth. Supplementation of nano-meth in G4 decreased

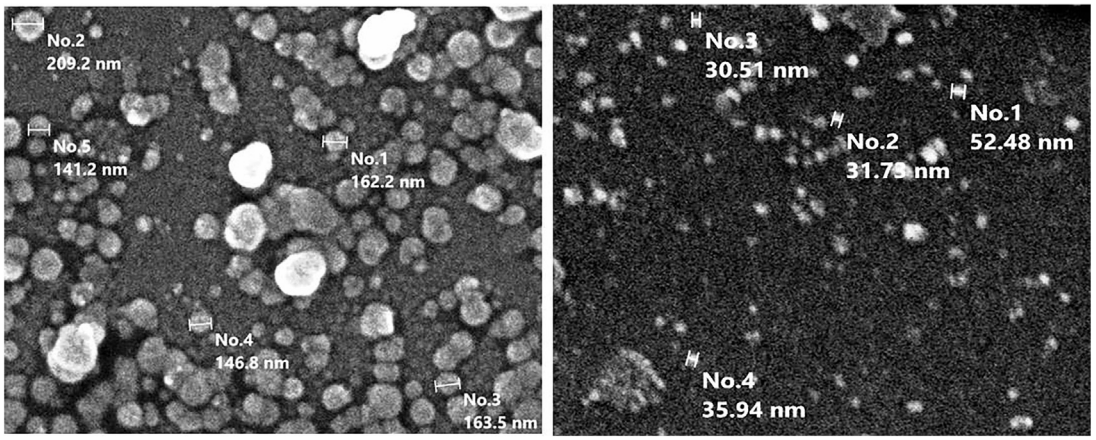

Figure 1. Characterization of nanomethionine using scanning electron microscopy, before (left) and after (right).

Table 3. Body weight, total feed intake, total weight gain, and average feed conversion ratio.

Variable	Group					*P* value
	G1	G2	G3	G4	G5	
Initial weight (day old)	49.75 ± 0.54	49.09 ± 0.29	49.73 ± 0.38	50.25 ± 0.35	49.25 ± 0.50	NS
Final weight (32 d)	1762.65 ± 31.08^{b}	1936.47 ± 27.82^{a}	1536.37 ± 25.36^{d}	1767.68 ± 20.82^{b}	1688.60 ± 26.51^{c}	<0.0001
Total WG	1712.90 ± 30.56^{b}	1887.37 ± 27.54^{a}	1486.63 ± 25.01^{d}	1717.42 ± 20.50^{b}	1639.35 ± 26.06^{c}	<0.0001
TFI	2631.70 ± 0.00^{b}	2775.28 ± 0.00^{a}	2482.05 ± 0.00^{d}	2574.95 ± 0.00^{c}	2465.35 ± 0.00^{e}	<0.0001
FCR	1.54 ± 0.03^{b}	1.47 ± 0.02^{b}	1.67 ± 0.03^{a}	1.49 ± 0.02^{b}	1.50 ± 0.03^{b}	<0.0001

The data presented as mean ± standard error. Means bearing different superscript letters within the same row are significantly different ($P < 0.05$). NS: nonsignificant. WG: weight gain, TFI: total feed intake, FCR: average feed conversion ratio. G1: control, G2: Nanomethionine in drinking water, G3: heat stress (HS), G4: nanomethionine+HS, G5: nanomethionine during HS.

Table 4. Rectal temperature, dressing percentage, and some organs relative weight.

Variables	Groups					*P* value
	G1	G2	G3	G4	G5	
Rectal temp	40.50 ± 0.06^{c}	40.70 ± 0.08^{c}	41.87 ± 0.07^{a}	41.47 ± 0.15^{b}	40.90 ± 0.25^{c}	<0.0001
Dressing %	73.99 ± 0.40^{a}	74.45 ± 0.29^{a}	69.22 ± 1.52^{b}	72.58 ± 0.81^{a}	69.95 ± 0.18^{b}	<0.0001
Liver	2.33 ± 0.04^{a}	2.33 ± 0.09^{a}	1.74 ± 0.08^{c}	2.09 ± 0.10^{ab}	1.94 ± 0.09b^{c}	<0.0001
Gizzard	1.59 ± 0.03^{a}	1.67 ± 0.08^{a}	1.33 ± 0.03^{b}	1.40 ± 0.04^{b}	1.36 ± 0.01^{b}	<0.0001
Proventriculus	0.34 ± 0.01^{b}	0.40 ± 0.01^{a}	0.31 ± 0.01^{b}	0.32 ± 0.02^{b}	0.33 ± 0.02^{b}	0.0031
Heart	0.43 ± 0.01^{a}	0.41 ± 0.01^{a}	0.37 ± 0.01^{b}	0.41 ± 0.01^{a}	0.38 ± 0.01^{b}	0.0014
Abdominal fat	1.36 ± 0.01^{a}	1.03 ± 0.04^{b}	0.97 ± 0.05^{b}	1.03 ± 0.04^{b}	1.28 ± 0.04^{a}	<0.0001

The data presented as mean ± standard error. Means bearing different superscript letters within the same row are significantly different ($P < 0.05$). NS: nonsignificant. G1: control, G2: nanomethionine in drinking water, G3: heat stress (HS), G4: nanomethionine+HS, G5: nanomethionine during HS.

the effect of HS, where average BW was the same as the control (G1). Moreover, the supplementation of nano-meth only when HS began, as in G5, was also effective in improving the average BW when compared to those exposed to HS without nano-meth supplementation (G3). The WG had the same trend as BW, where the highest gain was observed in G2 and the least gain was observed in G3. TFI was significantly varied among groups, with the highest value seen in G2 (1,775 g), while the lowest value was in G5 (1,465 g). FCR also varied significantly among groups, with the best conversion ratio seen in G2 (1.49), and the worst conversion ratio observed in G3 (1.70). G4, which was exposed to HS with supplementation of nano-meth, performed much better than G3, in terms of final BW, WG, and FCR.

Rectal Temperature and Carcass Quality

Results for average rectal temperature of broilers are shown in Table 4. Body temperature was increased in G3 until reaching 41.87°C, followed by G4 (41.47°C), and then G5, G2, and G1 (40.90°C, 40.70°C, and 40.50° C, respectively). Dressing percentage (Table 4) varied significantly among groups, where the percentage was improved in G2, G1, and G4, while dressing percentage was decreased in G5 and G3. The organs relative weight (liver, gizzard, proventriculus, and heart) were also significantly different among the groups. Additionally, abdominal fat was significantly varied among groups ($P < 0.0001$), with the highest value recorded in G1 (control), and lowest value in G3.

Serum Oxidative/Antioxidative Indices

Table 5 shows that in comparison with control values, HS induced a significant ($P < 0.0001$) elevation in MDA level and a reduction in antioxidant enzymatic activities (GPX and CAT) in HS-treated groups, confirming that exposure to HS induces oxidative stress in broilers and the depletion of antioxidants in this group (G3). Compared to G3, coadministration or treatment with nano-meth in G4 and G5 alleviated the altered serum oxidative/antioxidative indices, which were evidenced by significant ($P < 0.0001$) reduction in MDA, and GPX and CAT increments, which indicate the antioxidant effect of nano-meth. Additionally, the nano-meth-treated group exhibited high antioxidant activity. However,

Table 5. Effect of nanomethionine on the lipid peroxidation biomarker (MDA) and antioxidant molecules (GPX, CAT) in the serum of broilers exposed to heat stress.

Parameter	Group					*P* value
	G1	G2	G3	G4	G5	
CAT	428.86 ± 14.26^{b}	463.29 ± 4.22^{a}	379.96 ± 12.23^{c}	437.76 ± 10.01^{ab}	433.30 ± 4.21^{b}	<0.0001
GPx	182.86 ± 8.34^{b}	219.67 ± 7.30^{a}	150.48 ± 6.47^{c}	194.60 ± 1.20^{b}	188.83 ± 12.50^{b}	<0.0001
MDA	12.95 ± 0.03^{b}	9.41 ± 0.55^{c}	15.34 ± 0.54^{a}	12.41 ± 0.10^{b}	12.66 ± 0.11^{b}	<0.0001

Data presented as mean ± SE. Means bearing different superscripts letters within the same row are significantly different ($P < 0.05$). G1: control, G2: Nanomethionine in drinking water, G3: Heat stress (HS), G4: Nanomethionine+HS, G5: Nanomethionine during HS.

nano-meth supplementation without HS in G2 caused a reduction in MDA level and elevation in the antioxidant enzymatic activities (GPX and CAT) compared with G1 (control).

Gene Expression

Nano-meth in G2 had a significant upregulation of HS genes HSP70 and HSP90 genes ($P < 0.05$), increasing 1.8- and 3-fold (Figure 2), and was also associated with a recorded significant downregulation of both HSF1 and TLR4 genes ($P < 0.05$) to 0.3-fold. In contrast, G3 (exposed to HS without supplementation of nano-meth) showed increased expression of HSP70 and HSF1 by 32.2- and 16-fold, respectively. HSP90 gene expression levels significantly increased ($P < 0.05$) by 12.7-fold. TLR4 was seen to be downregulated by 0.5-fold (Figure 2).

Regarding the effect in G4 (exposed to HS with nano-meth supplementation), upregulation of HSP70, HSP90, and TLR4 to 17.7-, 21.2-, and 4-fold, respectively, was observed. Also, a mildly significant increase of HSF1 ($P < 0.05$) of 1.8-fold was observed. Supplementation of nano-meth only when HS occurred, as in G5, induced a significant upregulation of HSP70, HSP90, and HSF1 genes ($P < 0.05$) to 30.2-, 11-, and 14-fold, respectively. Meanwhile, it was associated with a somewhat significantly increased expression of TLR4 ($P < 0.05$) to 1.3-fold (Figure 2).

Histopathologic Findings

Liver Histomorphologic examination of liver tissues from G1 (control) and nano-meth-treated chicks (G2) revealed normal histopathologic structure of hepatic parenchyma with polygonal hepatocytes, round-shaped nuclei, and normally structured portal veins, hepatic arteries, central veins, and sinusoids (Figure 3A, B). However, hepatic tissues from HS chickens (G3) showed diffuse moderate to severe vacuolization of hepatocytes, mostly of the hydropic type, thickening of portal triads with infiltrated mononuclear inflammatory cells (Figure 3C), multifocal areas of lytic and coagulative necrosis, as well as vascular and sinusoidal congestion and hemorrhage (Figure 3D). In contrast, hepatic tissues from HS chickens treated with nano-meth from d 1 (G4) showed marked improvement of their histologic structures; lesions in the form of mild thickening of portal areas with congested portal veins were noted (Figure 3E). While the chickens subjected to HS and treated with nano-meth at the HS time only (G5) showed moderate improvement of their liver structure;

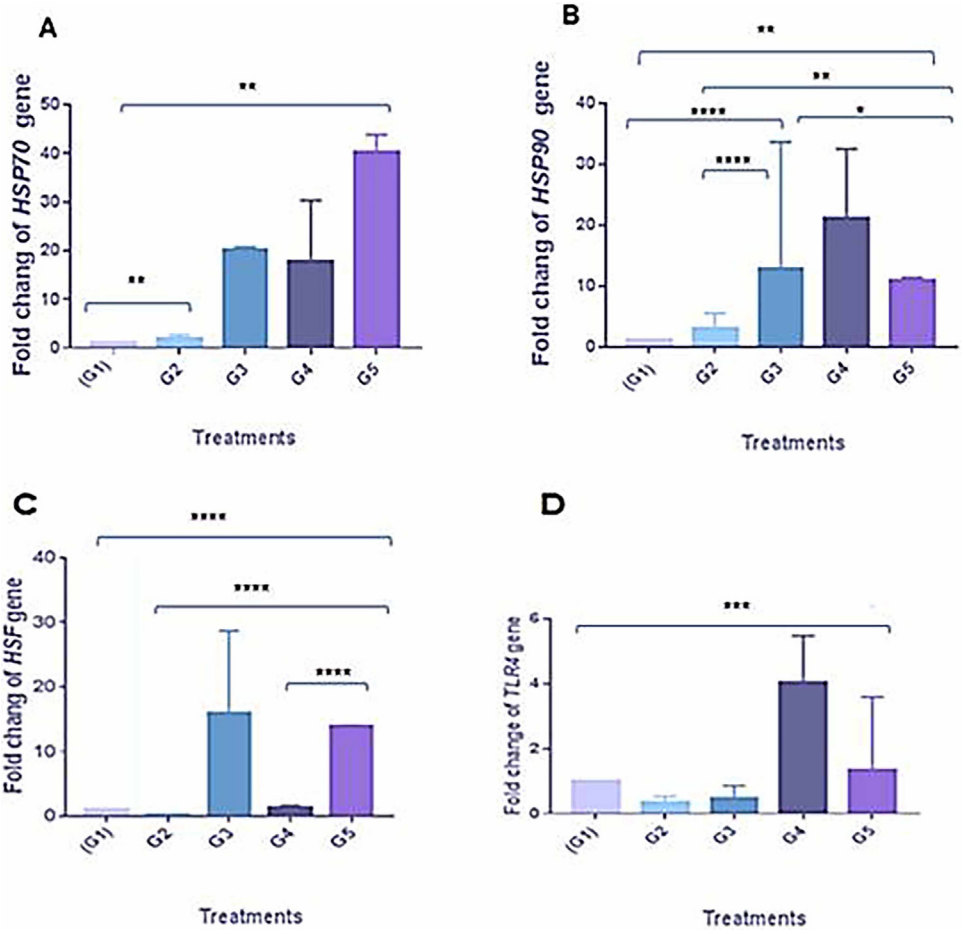

Figure 2. Relative expression of some immune response genes *HSP70* (A), *HSP90* (B), HSF1 (C), and TLR4 (D) in the chicken with different treatment. All values are expressed as mean ± SE. Asterisks on the data bars indicate when $P < 0.05$ (*), $P < 0.005$ (**), and $P < 0.0005$ (***). G1: control, G2: Nanomethionine in drinking water, G3: Heat stress (HS), G4: Nanomethionine+HS, G5: Nanomethionine during HS.

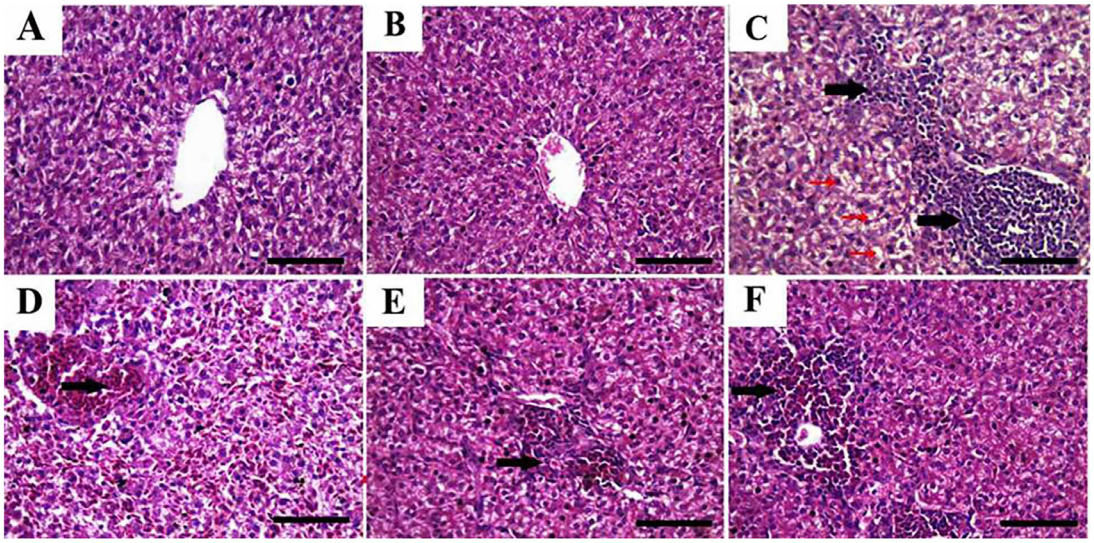

Figure 3. Representative photomicrographs (hematoxylin and eosin (H&E) staining) of liver tissues from chicken supplemented with nanomethionine and subjected to heat stress for 28 d. Livers tissues from control (A) and nanomethionine (B) revealed normal histopathologic structure of hepatic tissues. Hepatic tissues from heat stressed chickens showed diffuse moderate to severe vacuolization of hepatocytes mostly of hydropic type (red arrows), thickening of portal triads with the infiltrated mononuclear inflammatory cells (black arrows) (C), vascular and sinusoidal congestion and hemorrhage (arrow) (D). Hepatic tissues from heat stressed chicken treated with nanomethionine from d 1 showed mild thickening of portal areas with the congested portal veins (arrow) (E). Chickens in group subjected to heat stress and treated with nanomethionine at the heat stress time only showed focal areas of lytic necrosis infiltrated with mononuclear inflammatory cells and extravasated red blood cells (arrow) (F). Bar = 50 μm.

with noted lesions in the form of focal areas of lytic necrosis which were infiltrated with mononuclear inflammatory cells and extravasated red blood cells (Figure 3F).

Intestines Histologically, chicken's intestinal walls consist of 4 layers: serosa, internal and external muscular layers, mucosa, and submucosa. Figure 4 shows that the intestinal mucosa and submucosa of chickens in G1 (Figure 4A) and G2 (Figure 4B) had normal histomorphologic structures of intestinal villi, associated crypts, and tunica muscularis. Moreover, the submucosal tissues appeared free of inflammatory and/or degenerative changes and the columnar epithelium and goblet cells appeared properly arranged. In contrast, intestinal tissues from chicken subjected to HS (G3) showed blunted and necrotic tips of villi, where the columnar epithelium and goblet cells showed different degrees of necrosis and desquamation within the lumen, congestive capillaries, and mild inflammatory infiltrates were also noted in submucosal tissues (Figure 4C). On the other hand, chickens subjected to HS and treated with nano-meth, either on d 1 (G4) or during HS (G5), showed marked improvement in intestinal morphology compared to HS chickens without nano-meth supplementation (Figure 4D and E, respectively).

Concerning the histomorphometric analysis of the intestines, results in Table 6 revealed that administration of nano-meth without HS (G2) did not induce a

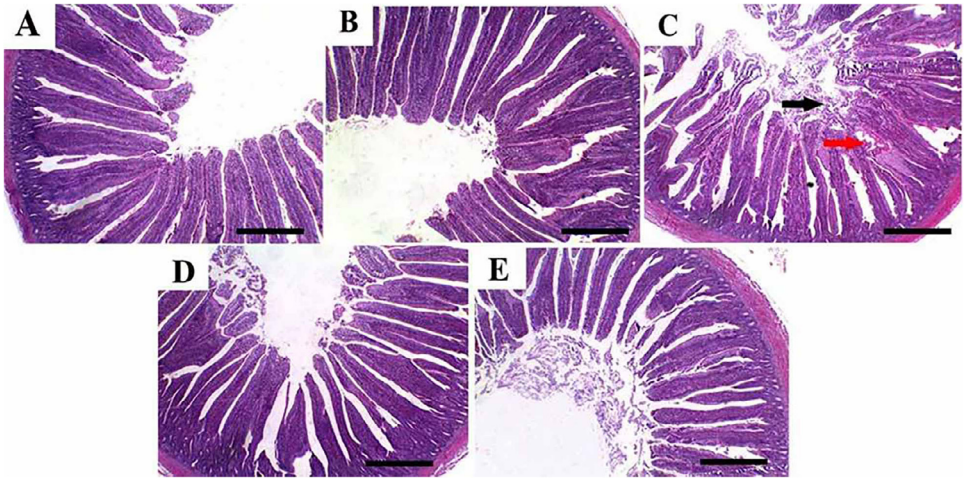

Figure 4. Representative photomicrographs (hematoxylin and eosin (H&E) staining) of intestine from chicken supplemented with nanomethionine and subjected to heat stress for 28 d. Tissues from control (A) and nanomethionine groups (B) revealed normal histomorphologic structure of intestinal villi, associated crypt, and tunica muscularis, in addition to properly arranged columnar epithelium and goblet cells. Tissues from heat stressed chickens showed necrosis of villus epithelium (red arrow) and desquamation within the lumen (black arrow). Intestinal tissues from heat stressed chicken treated with nanomethionine either from d 1 (D) or at the heat stress time only (E) showed marked improvement in the histologic structure of intestine. Bar = 200 μm.

Table 6. Histomorphometric analysis of intestines from chickens supplemented with nanomethionine and subjected for heat stress for 28 d.

Measurement (μm)	G1	G2	G3	G4	G5	P value
Villus height	600.00 ± 10.41^{a}	615.67 ± 6.64^{a}	498.33 ± 14.24^{d}	565.00 ± 8.66^{b}	530.00 ± 5.77^{c}	<0.0001
Villus width at the tip	51.67 ± 3.76^{ab}	59.67 ± 1.45^{a}	37.00 ± 4.04^{c}	48.33 ± 4.41^{abc}	45.67 ± 2.96^{bc}	<0.0211
Villus width at the tip crypt/villus junction	55.00 ± 3.79^{a}	55.67 ± 2.33^{a}	42.67 ± 4.81^{a}	48.00 ± 4.36^{a}	45.00 ± 3.61^{a}	0.4388
Crypt depth	71.00 ± 4.73^{ab}	79.00 ± 4.73^{a}	62.67 ± 5.36^{b}	69.33 ± 3.48^{ab}	64.33 ± 2.33^{b}	0.0105
Villus height/crypt depth ratio	8.51 ± 0.42^{a}	7.84 ± 0.41^{a}	8.06 ± 0.69^{a}	8.20 ± 0.55^{a}	8.26 ± 0.34^{a}	0.6370
Tunica muscularis thickness	222.33 ± 6.74^{a}	223.33 ± 4.41^{a}	224.33 ± 5.21^{a}	218.67 ± 6.77^{a}	221.33 ± 6.98^{a}	0.5889

Data represent mean ± SE. Values with different letters in the same row indicate significant differences between different test groups at $P < 0.05$. G1: control, G2: Nanomethionine in drinking water, G3: Heat stress (HS), G4: Nanomethionine+HS, G5: Nanomethionine during HS.

significant change in any of the studied parameters. However, intestinal tissues from chicken subjected to HS (G3) showed a significant ($P < 0.05$) reduction in villus height and villus width at the tip of villi compared with the control (G1). In contrast, HS chickens in groups supplemented with nano-meth either from d 1 of treatment (G4) or during the period of HS only (G5) showed significant ($P < 0.05$) improvement in villus height compared with the untreated HS group.

DISCUSSION

It is now well known that HS compromises the health and productivity of chickens. Our study showed that nano-meth supplementation to broiler diets under normal conditions apparently benefits BW, WG, TFI, and FCR. Our results are consistent with others (Marrett and Sunde, 1965; Shen et al., 2015) who reported that young broilers fed L-Meth-supplemented diet showed better growth performance (average daily gain and FCR). The possible mechanism underlying the observed effect of nano-meth on growth is that improved utilization of meth may enhance intestinal development and thereby improve growth performance (Shen et al., 2015).

Our results showed that HS decreased BW, WG, and TFI, while it increased FCR in HS birds in G3 compared to the control (G1). Others (Attia et al., 2011; Ohtsu et al., 2015; Abo-Samaha et al., 2021) have reported similar findings, and this could be attributable to HS-induced oxidative stress, which leads to metabolic changes that subsequently decrease feed intake (Quinteiro-Filho et al., 2010; Akbarian et al., 2016). The poor growth performance observed in broilers under HS may be attributed to higher energy loss via increased respiration, reduced feed intake, and retardation of nutrient digestion (Mello et al., 2015).

Our results showed that dressing percentage and organs relative weights were enhanced by nano-meth supplementation, while abdominal fat was decreased. Similarly, zinc-methionine supplementation decreased the abdominal fat weight of broiler chickens in high ambient temperatures (Saleh et al., 2018). These results may be attributed to the stimulatory effect of methionine on the expression of genes coding for growth-related hormones and myogenic regulatory factors involved in muscular development (Del Vesco et al., 2015). However, our findings indicated that exposure to HS decreased heart, liver, and gizzard yields, and this concurred with Rosa et al. (2007).

HS is a major source of systemic oxidative stress since it decreases the body's antioxidant capacity as well as intestinal immunity (Song et al., 2018). ROS can lead to DNA damage, lipid peroxidation, and protein denaturation (Ray et al., 2012). In the present study, the serum MDA level was increased; in contrast, the GSH and catalase serum concentration was decreased when chicks were exposed to HS, whereas supplementation with nano-meth alleviated the negative effects of HS on serum MDA level, as well as GSH and catalase concentration. This finding concurred with Miao et al. (2021), who revealed that adding 0.45% methionine to the diet decreased GPX in the liver and plasma; and increased the ratio of GSH/GSSG in plasma of high stocking density birds. Moreover, supplementation with methionine decreased the MDA level in the jejunum. Additionally, Magnuson et al. (2020) showed that high methionine levels elevated ($P < 0.05$) GSH concentration in the thighs of grower and finisher chickens. Furthermore, the extra DL-Meth supplementation led to significant decreases in MDA concentrations in finishers' breast and thigh meat.

Methionine is an essential amino acid and, as a nonenzymatic antioxidant, acts through methionine sulfoxide reductase, thioredoxin reductase, and thioredoxin to prevent oxidative damage to nucleic acids, lipids, and proteins (Luo and Levine, 2009). S-adenosylmethionine is the active form of methionine and serves as the methyl donor and as a glutathione precursor (Kumar et al., 2020). In our study, the addition of methionine in nano-form showed the ability to alleviate the altered serum oxidative/antioxidative indices, which were evidenced by significant ($P < 0.0001$) reduction in MDA, and increments in GPX and CAT, which indicate the antioxidant effect of nano-meth.

HS proteins are chaperones that allow biological processes to work well in a stressful environment. The usefulness of HSP70 expression to identify HS under field conditions was evaluated (Lund et al., 2006). The present findings confirmed earlier reports (Zulkifli et al., 2002, 2009; Liew et al., 2003) that heat exposure may augment HSP70 expression in chickens. The HSPs are characterized by their ability to be induced by HS and, in molecular terms, by the presence of a functional HS element in their promoter (Sharp et al., 1999). Also,

HSPs act as chaperones that bind other proteins following HS damage to preserve their structure, managing the proteins' migration across membranes or organelles, or keeping the receptor availability or specific enzyme functions under control (Kampinga and Craig, 2010).

The liver is one of the most sensitive organs that synthesize HSP70 in response to hyperthermia (King et al., 2002). In this study, HS for 3 d upregulated the expression of HSP70 in G3, and HSF1 also caused a significant increase in HSP90 gene expression levels. In agreement with our findings, Abo-Samaha et al. (2021) reported that HSP70 is upregulated in response to HS in broiler chicken. Similarly, Cedraz et al. (2017) reported that the HSP70 and HSP90 genes were highly expressed in all genetic groups under HS compared with control conditions. Mahmoud et al. (2004) also demonstrated that HS caused induction of HSP90α and HSP90ß in chicken heart, liver, and spleen. Additionally, the quantity of HSP70 was seen to increase when testis cells were exposed to high temperatures (Mezquita et al., 2021). Similarly, a relevant increment in the HSP70 expression in female broiler chicken brains after 4 d of heat treatment was observed (Zulkifli et al., 2003).

The benefits of nano-meth in inducing HSP expression have been reported in our study in both G4 and G5. In G4 the results showed upregulation of HSP70 and HSP90 to 17.7- and 21.2-fold. A mild significant increase of HSF1 ($P < 0.05$) to 1.8-fold was also seen. The addition of nano-meth when HS started in G5 induced a significant upregulation of HSP70, HSP90, and HSF1 genes ($P < 0.05$) to 30.2-, 11-, and 14-fold, respectively. Our results suggest that nano-meth supplementation may enhance the induction of HSP70 in poultry under HS conditions. Previous work (Zulkifli et al., 2003, 2009; Al-Aqil and Zulkifli, 2009) suggested that HSP70 plays a profound role in enhancing tolerance to various stressors and resistance to viral infection in broiler chicken. HS proteins are extremely potent molecules that can modify the function and destiny of other proteins and thus play critical roles in numerous physiological and immunological processes (Pockley, 2003). Recently, Han et al. (2017) found that amino acid (**AA**) levels, including leucine, were significantly decreased due to thermal treatment on embryonic brains and livers. It was also reported that HS could affect sulfur AA (including methionine) metabolism in chicks (Ito et al., 2015), so HS chickens should be supplemented with AA.

Given what has been demonstrated, it is unsurprising that chickens exposed to HS had several pathological lesions in their livers and intestines since HS usually disrupts homeostatic balance. As a result, the stress response involves essential reactions to maintain a constant state, such as increases in heart rate and blood flow to various tissues (Ewing et al., 1999). This could explain the hepatic and intestinal congestion in chickens subjected to HS. It is recognized that when body temperature rises above normal, several different types of cells typically begin to be harmed (Ma et al., 2022). Therefore, increased core body temperature mediated endothelial cell damage during HS.

Also, high blood pressure may result from an autonomic nervous system reaction, leading to circulatory rupture and subsequent organ hemorrhage and congestion, including the liver and intestine. Additionally, fluid, fat, or both accumulation in the cytoplasm (hepatic vacuolization) is a strong diagnostic indicator of liver damage. Hepatocytes with excess fluid and lipids are signs of sublethal damage (Liu et al., 2022).

Additionally, in many animal species, HS harms intestinal architecture and increases intestinal permeability (Hall et al., 2001; Dokladny et al., 2006; Yang et al., 2007; Lambert, 2009). It is widely recognized that intestinal damage, including weakened intestinal integrity and barrier function, is a primary reaction to HS (Quinteiro-Filho et al., 2010; Song et al., 2013; Varasteh et al., 2015). We assessed villus height and crypt depth, as well as the villus height to crypt depth ratio, both of which are helpful indicators of gut health in animals, in order to evaluate intestinal histological damages (Pluske et al., 1996a,b; Xu et al., 2003). HS hens' shorter intestinal villi suggest the intestinal epithelium was harmed (Fan et al., 1997). In contrast, the histopathologic image of the liver and intestine was significantly improved in chickens treated with nano-meth. This beneficial effect could mostly be ascribed to nano-meth's regulatory function of HSP expression and the resultant downregulation of oxidative stress (Nanto-Hara et al., 2019).

Toll-like receptors (**TLR**) have been extensively studied in rats, but little is known of their role in broiler chickens undergoing HS (Huang, 2017). As a result of HS, cells can adjust their metabolism to the changing environmental conditions by modifying their gene expression. These alterations offer sufficient flexibility to preserve homeostasis under stress conditions, maintain their viability and functions, as well as adapt to long-term changes (López-Maury et al., 2008). HS triggers the expression of HS-related genes, such as HSP and TLR (Zhang et al., 2015; Huang, 2017). The TLR4-mediated signal pathway is activated in response to stress, particularly to HS (Ju et al., 2014). Our study showed upregulation of TLR4 to 4-fold in G4, and a small significant increase in expression of TLR4 ($P < 0.05$) to 1.3-fold in G5. Fukata et al. (2005) reported that TLR4 is important for healing injured intestinal epithelium. Hence, the markedly increased TLR4 mRNA expression levels indicate that the expression of HSP70 was elevated and triggered TLR4 overexpression to protect the intestine from injury by HS (Huang, 2017).

CONCLUSIONS

Nanomethionine improved final BW, WG, FCR, and decreased HS's effect on growth performance. Reduction in malondialdehyde and changes in antioxidant enzymes GPX and CAT activity indicated the antioxidant effect of nano-meth. Nano-meth supplementation caused an increase in the expression of HSP70 and HSF1, HSP90, and a downregulation of TLR4 gene expression. Additionally, nano-meth treated groups showed marked

improvement of the histological liver structure compared to the control, and nano-meth supplemented groups showed marked improvement in the intestinal morphology when compared with HS groups and also showed significant improvement in villus height compared with the HS-subjected group. Based on these results, adding nano-meth amino acid appears to reduce the negative impacts of HS in chickens.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author upon reasonable request.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

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