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Effect of individual or combination of dietary betaine and glycine on productive performance, stress response, liver health, and intestinal barrier function in broiler chickens raised under heat stress conditions

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ABSTRACT The current experiment was conducted to investigate the effect of individual or combination of dietary betaine (**Bet**) and glycine (**Gly**) on productive performance, stress response, liver health, and intestinal barrier function in broiler chickens raised under heat stress (**HS**) conditions. A total of four hundred twenty 21-d-old Ross 308 broiler chickens were randomly allotted to 1 of 5 dietary treatments with 7 replicates. Birds in treatment 1 were raised under the thermoneutral condition (**TN**; 23 ± 0.6°C). Birds in other 4 treatment groups were subjected to a cyclic HS by exposing them to 32 ± 0.9°C for 8 h/d (from 09:00 to 17:00 h) and 28 ± 1.2°C for the remaining time for 14 d. Birds were fed a basal diet in TN condition (**TN-C**) and one group in HS conditions (**HS-C**), whereas other birds raised under HS conditions were fed the basal diet supplemented with 0.20% Bet (**HS-Bet**), 0.79% Gly (**HS-Gly**), or their combination (0.20% Bet + 0.79% Gly; **HS-Bet+Gly**). Results indicated that birds in HS-Bet, HS-Gly, or HS-Bet+Gly treatment had higher ($P < 0.05$) final BW and BW gain, but lower ($P < 0.05$) feed conversion ratio (**FCR**) than those in HS-C treatment. However, values for improved final BW, BW gain, and FCR by dietary treatments were lower ($P < 0.05$) than those measured in TN-C treatment. Under HS conditions, birds in HS-Bet, HS-Gly, or HS-Bet+Gly treatment had lower ($P < 0.05$) heterophil to lymphocyte ratio than those in HS-C treatment. Birds in HS-Gly or HS-Bet+Gly treatment had higher ($P < 0.05$) villus height and goblet cell number than birds in HS-C treatment. Intestinal permeability was higher ($P < 0.05$) in all HS-treatment groups than in TN-C treatment, but it was not affected by dietary treatment. In conclusion, individual supplementation of 0.20% Bet or 0.79% Gly in diets alleviates the negative effect of HS in broiler chickens. However, the synergistic effect of the combination of 0.20% Bet and 0.79% Gly in broiler diets seems lower than expected.

Key words: betaine, broiler chicken, glycine, growth performance, heat stress

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INTRODUCTION

The current poultry industry encounters various problems because global warming exposes birds to more frequent and prolonged heat stress (**HS**; Chowdhury et al., 2021). Moreover, the recent poultry breeds are intensively selected for high productivity, which makes poultry more difficult to adapt HS than previous breeds (Quinteiro-Filho et al., 2012). Poultry exposed to HS are well-known to exhibit a variety of physiological dysfunctions, resulting in compromised performance, product quality, and health (Lara and Rostagno, 2013). Therefore, various methods have been developed and applied to mitigate HS in poultry (He et al., 2018). Dietary management with several functional nutrients has been studied to ameliorate the negative effects of HS in poultry (Kim et al., 2021).

Betaine (**Bet** or trimethylglycine) is a zwitterion at neutral pH with a negatively charged carboxyl group and a positively charged trimethyl-ammonium group (Ratriyanto and Mosenthin, 2018). The primary role of Bet in body functions is the donation of the methyl group to other metabolites in various transmethylation processes (Eklund et al., 2005). Bet is also known to play a role as an osmoprotectant, which aids in regulating body temperature by increasing water retention and reducing dehydration (Zulkifli et al., 2004; Eklund et al., 2005). Therefore, dietary supplementation of Bet has been widely reported to alleviate HS in poultry (Metzler-Zebeli et al., 2009).

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The glycine (**Gly**) is the simplest amino acid (**AA**), but plays an important role as an essential molecule of serine, creatine, heme, glutathione, bile acids, nucleic acids, and uric acid productions (Wang et al., 2013). In particular for birds, toxic ammonia produced by AA oxidation is detoxified and excreted as uric acid, which is the main excretion product as the end product of N metabolism. The formation of each uric acid molecule requires 1 molecule of Gly to build the purine ring (Siegert and Rodehutscord, 2019). Therefore, various external or internal factors may increase the requirement of Gly, resulting in a possible deficiency of Gly (Siegert and Rodehutscord, 2019; Kim et al., 2022). Our previous study reported increased AA oxidation of broiler chickens during heat stress (Kim et al., 2022), which may support a hypothesis that birds exposed to HS have increased Gly requirements because of increased uric acid synthesis. Moreover, the synergistic effect of Bet and Gly in broiler chickens exposed to HS can also be expected because of various HS-reducing effects of Bet on poultry and its possible action as a Gly supplier by virtue of its chemical structure after the complete transmethylation in the body. However, this hypothesis has not been tested previously.

Therefore, the present experiment was conducted to investigate the effect of individual or combination of dietary supplementation of Bet and Gly on growth performance, meat quality, stress response, liver health, and intestinal barrier function in broiler chickens raised under HS conditions.

MATERIALS AND METHODS

Birds, Diets, and Experimental Design

The protocol for the present experiment was approved by the Institutional Animal Care and the Use Committee at Chung-Ang University. A total of four hundred twenty 21-d-old Ross 308 growing broiler chickens with similar BW (initial BW = 984 ± 24.5 g) were used and raised in battery cages (76 × 78 × 45 cm, width × length × height for each cage) for 14 d of the feeding trial. The growing chickens were used in this experiment because growing chickens are more sensitive and vulnerable to HS conditions than young chicks (Chowdhury et al., 2021).

Before the start of the experiment, all birds were fed a commercial-type diet adequate in energy and nutrients for broiler chickens from 1 to 20 d of age. At 21 d of age, all chickens were allotted to 1 of 5 treatments with 7 replicates in a completely randomized design. Each replicate had 12 birds (6 male and 6 female birds) per battery cage. Birds in treatment 1 were raised under the recommended temperature and RH by the Ross manual (Aviagen, 2019) during the whole experimental period, which was designated as the thermoneutral condition (**TN;** 23 ± 0.6°C and 52.7 ± 2.6% RH for 14 d). Birds in other 4 treatment groups were subjected to a cyclic HS condition by exposing them to 32 ± 0.9°C for 8 h/d (from 09:00 to 17:00 h) and 28 ± 1.2°C for the remaining time with 58 ± 7.5% RH for 14 d from 21 d of age to the conclusion of the study (35 d of age). A corn-soybean meal-based basal diet was formulated to meet or exceed the requirement estimates of energy and nutrients for growing broiler chickens (Table 1). Birds in TN conditions (**TN-C**) and 1 group in HS conditions (**HS-C**) were fed the basal diet, whereas other birds raised under HS conditions were fed the basal diet supplemented with 0.20% Bet (98%, Genebiotech, Gongju-si, Republic of Korea; **HS-Bet**), 0.79% Gly (99%, Samin Chemical, Siheung-si, Republic of Korea; **HS-Gly**), or the combination of 2 supplements

Table 1. Composition and nutrient concentrations of experimental diets.

	Inclusion, %			
Item	TN-C and HS-C	HS-Bet	HS-Gly	HS-Bet+Gly
Ingredients				
Corn	59.60	59.60	59.60	59.60
Soybean meal, 45% CP	30.51	30.51	30.51	30.51
Soybean oil	4.59	4.59	4.59	4.59
MDCP	1.12	1.12	1.12	1.12
Limestone	1.05	1.05	1.05	1.05
98% DL-Met	0.29	0.29	0.29	0.29
98% L-Lys HCl	0.22	0.22	0.22	0.22
98.5% Thr	0.10	0.10	0.10	0.10
99% L-Arg	0.06	0.06	0.06	0.06
99% L-Ile	0.04	0.04	0.04	0.04
99% Gly	0.00	0.00	0.79	0.79
99% Betaine	0.00	0.20	0.00	0.20
Alanine	0.91	0.91	0.00	0.00
Cornstarch	0.00	0.00	0.45	0.45
Celite	0.56	0.36	0.23	0.03
Salt	0.20	0.20	0.20	0.20
50% Choline	0.10	0.10	0.10	0.10
$NaHCO_3$	0.20	0.20	0.20	0.20
Coccidiostats	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
Vitamin premix[1]	0.15	0.15	0.15	0.15
Mineral premix[2]	0.15	0.15	0.15	0.15
Total sum	100.00	100.00	100.00	100.00
Calculated energy and nutrient content				
AME_n, kcal/kg	3,100	3,100	3,100	3,100
CP, %	19.25	19.25	19.25	19.25
Digestible Lys, %	1.04	1.04	1.04	1.04
Digestible Met + Cys, %	0.76	0.76	0.76	0.76
Digestible Met, %	0.51	0.51	0.51	0.51
Digestible Thr, %	0.68	0.68	0.68	0.68
Digestible Trp, %	0.19	0.19	0.19	0.19
Digestible Arg, %	1.12	1.12	1.12	1.12
Digestible Ile, %	0.71	0.71	0.71	0.71
Digestible Leu, %	1.52	1.52	1.52	1.52
Digestible Val, %	0.78	0.78	0.78	0.78
Digestible Gly, %	0.63	0.63	1.41	1.41
Digestible Gly + Ser, %	1.55	1.55	2.33	2.33
Total calcium, %	0.71	0.71	0.71	0.71
Available phosphorus, %	0.33	0.33	0.33	0.33
Analyzed nutrient content				
CP, %	19.50	19.88	19.44	19.56
Total calcium, %	0.69	0.70	0.71	0.69
Total phosphorus, %	0.54	0.54	0.52	0.56
Total Gly, %	0.79	0.78	1.59	1.54
Betaine, %	1.09	1.51	1.13	1.58

Abbreviation: MDCP, monodicalcium phosphate.

[1]Provided per kilogram of the complete diet: vitamin A, 11,700 IU (retinyl acetate); vitamin D3, 3,600 IU; vitamin E, 27 IU (DL-α-tocopheryl acetate); vitamin K3, 2.7 mg (menadione dimethylpyrimidine); vitamin B1, 2.7 mg; vitamin B2, 6.3 mg; vitamin B6, 4.5 mg; vitamin B12, 18 μg; folic acid, 1.35 mg; biotin, 135 μg; niacin, 45 mg; pantothenic acid, 10.8 mg.

[2]Provided per kilogram of the complete diet: copper, 7.35 mg; iron, 46.75 mg; manganese, 87.34 mg; zinc, 75.21 mg; chromium, 100 μg; selenium, 235 μg.

(0.20% Bet + 0.79% Gly; **HS-Bet+Gly**). Supplemental level of Bet (0.20%) was determined based on previous experiments (Nofal et al., 2015; Chand et al., 2017). Supplemental level of Gly (0.79%) was designed to produce more digestible concentrations of Gly+Ser in Gly-supplemented diets (2.33%) by 50% than in the basal diet (1.55%). Analyzed concentrations of total Gly were 0.79%, 0.78%, 1.59%, and 1.54% for the basal diet and diets supplemented with Bet, Gly, and Bet+Gly, respectively (Table 1). Additional Ala was supplemented to diets containing no supplemental Gly to equalize the concentrations of CP among dietary treatments (Che et al., 2013). The diets and water were provided ad libitum throughout the experiment. A 23-h lighting schedule was applied during the experiment. The BW, BW gain (**BWG**), and feed intake (**FI**) were measured at the end of the experiment. The mortality was recorded daily. The feed conversion ratio (**FCR**) was calculated by dividing FI (g) and BWG (g) after correction for mortality (Lee et al., 2022).

Sample Collection and Analysis

At the end of the growth trial (i.e., 35 d of age), 2 male birds per replicate with a BW close to the mean BW of male birds were selected and euthanized by CO_2 asphyxiation. Jejunal mucosa samples from the first bird were immediately collected to increase cell viability in the analysis of intestinal permeability. The samples of the breast, blood, feather, liver, and jejunum from the other bird were collected for other measurements including meat quality, serum measurements, feather corticosterone (**CORT**), liver characteristics, jejunal morphology, and gene expression in jejunal mucosa.

The experimental diets were analyzed for CP (method 984.13; AOAC, 2007) using the Kjeldahl method (Kjeltec auto system 2300 Analyzer, FOSS, Hilleroed, Denmark). The diets were also analyzed for total Ca and P using an inductively coupled plasma spectrometer (Optima 5300 DV, Perkin Elmer Inc., Shelton, CT) as described by Kim et al. (2016). The Gly concentrations in the diets were analyzed using L8900 amino acid analyzer (HITACHI, Tokyo, Japan). Briefly, approximately 200 mg of diet samples put into an acid hydrolysis bottle containing 10 ml of 6N HCl. After filling with nitrogen, the mixture was hydrolyzed at 110°C for 24 h. After the hydrolysate was evaporated with a rotary evaporator, then equalized to a 50-mL volume with 0.2 M sodium citrate buffer. The solution was filtered into a 0.20-μm cellulose acetate syringe filter, and then, separated by ion-exchange chromatography (#2622PH column, 4.6 × 60 mm). Derivatives were detected at 570 and 440 nm after postcolumn derivatization with ninhydrin. In addition, Bet concentrations in the diets were also analyzed using HPLC equipped with a column (NH2P-50 4E; Shodex, Tokyo, Japan) and refractive index detector by the method of Filipčev et al. (2015) with a minor modification.

Meat Quality

The breast muscle was measured for meat quality and stored at 4°C until further analysis. For the breast muscle, the right portion was used to analyze pH, TBA-reactive substances (**TBARS**), and meat color, whereas the left portion was used to measure water holding capacity (**WHC**). The detailed procedure of meat quality analysis was reported previously (Goo et al., 2019).

Serum Measurements

Blood samples were collected via heart puncture into a 6-mL serum tubes (BD Vacutainer Serum Tube, BD, Franklin Lakes, NJ). The concentrations of aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), creatinine, and uric acid in the serum were analyzed using a Hitachi Automatic Analyzer 7020 (Hitachi Ltd., Tokyo, Japan).

Stress Indicator

Blood was used for the analysis of the heterophil to lymphocyte ratio (**H:L ratio**). Blood samples were collected via a heart puncture into a 10-mL EDTA tubes (BD Vacutainer K2E, BD, Franklin Lakes, NJ). The H:L ratio in the blood was analyzed by the method of Kim et al. (2021). The feather samples were also collected from flight feathers to analyze CORT concentrations in the feather. The concentrations of CORT in feathers were analyzed by the method of Lee et al. (2022).

Liver Characteristics and Antioxidant Status

For a measure of the occurrence of fatty liver, the liver attached to the body was pictured to measure the subjective fatty liver score using a scale from 1 to 5 (1 = dark red; 5 = yellowish red; Choi et al., 2012). In addition, the objective CIE color values for the lightness (**L***), redness (**a***), and yellowness (**b***) were determined using a colorimeter (model CR-10, Konica Minolta Optics Inc., Tokyo, Japan). The subjective liver hemorrhage was also scored from 0 to 5, with 0 indicating normal liver and 5 indicating large and massive hemorrhages (Diaz et al., 1999). The liver was weighed to measure the relative liver weight as a percentage of the total BW and collected to measure total lipid concentrations (AOAC, 2007).

A portion of the liver samples was collected to analyze the antioxidant status such as reactive oxygen species (**ROS**), malondialdehyde (**MDA**), and total antioxidant capacity (**TAC**). The antioxidant status in the liver such as ROS, MDA, and TAC was determined by using commercial kits OxiSelect In Vitro ROS/RNS Assay Kit (Green Fluorescence; STA-347, Cell Biolabs, San Diego, CA), OxiSelect TBARS Assay Kit (MDA Quantitation; STA-330, Cell Biolabs, San Diego, CA), and OxiSelect TAC Assay Kit (STA-360, Cell Biolabs,

Table 2. The sequence of the primers used in quantitative real-time PCR.

RNA target[1]	Forward	Reverse	Size for PCR product (bp)	Accession no.
GAPDH	5'- TGCTGCCCAGAACATCATCC-3'	5'-ACGGCAGGTCAGGTCAACAA -3'	142	NM_204305
ZO-1	5'-CACACTGTGACCCCAAAA-3'	5'-AAGGTCCATCTCAGTTTCAC-3'	151	XM_040680632
OCLN	5'-TCGTGCTGTGCATCGCCATC-3'	5'-CGCTGGTTCACCCCTCCGTA-3'	178	NM_205128
JAM-2	5'-AATTTACAGTTCCTCCCACT-3'	5'-GTCTTTTCCAGTAAGGCAAC-3'	151	NM_001006257

[1]*GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *ZO-1*: Zonula occludens-1; *OCLN*: Occludin; *JAM-2*: Junctional adhesion molecule B.

San Diego, CA), respectively, according to the manufacturer's protocol respectively. Protein concentration was also analyzed using a commercial kit Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The detailed procedures for the liver antioxidant status were described in our previous study (Yu et al., 2021).

Jejunal Morphology, Permeability, and Gene Expression

The jejunal segments of 1 to 2 cm in length were collected from the middle part between the end of duodenal loop and Meckel's diverticulum to analyze intestinal morphology and goblet cell counts. Jejunal segments were flushed and fixed with 10% neutral-buffered formalin solution and used for further analysis.

The villus height (**VH**), villus width (**VW**), crypt depth (**CD**), and VH:CD ratio were measured by the method of Hair bin Bejo (1990) with a minor modification. The detailed procedure was reported in the previous experiment (Thanh et al., 2009). All data were obtained from the mean values of 20 intestinal measurements. A goblet cell count in the jejunal villus was determined based on the method of Santos et al. (2015) with a minor modification. On average, 15 villi per sample were analyzed by viewing a 200-μm area from the central part of the villi.

Intestinal permeability in the jejunal mucosa as a measure of intestinal barrier function was determined using a dual channel self-contained Ussing chamber system (U2500, Warner Instruments, Holliston, MA). The detailed procedure was reported previously (Goo et al., 2019).

Total RNA samples from the jejunal mucosa were obtained from selected broiler chickens. The expression levels of the tight junction-related target genes were analyzed according to a modification by Shin et al. (2018). In short, total RNA was isolated using Trizol reagent (Invitrogen, Waltham, MA) according to the manufacturer's instructions and used for expression analysis of genes, such as Junctional adhesion molecules-B (***JAM-2***), Occludin (***OCLN***), and Zonula occludens-1 (***ZO-1***) in the jejunal mucosa. Following extraction, the RNA was diluted with 20 μL of Rnase-free water. Total RNA concentration was determined by the NanoDrop instrument (NanoPhotome-ter P 300, IMPLEN, München, Germany). Afterward, 2 μg of total RNA was reverse-transcribed to cDNA by using the RevertAid First Stand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. Synthesized cDNA was subsequently diluted with nuclease-free water to 100 ng/μL, and the cDNA was stored at −20°C. The primers for chicken glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***) used previously designed primers (Truong et al., 2015). The primers for target genes were previously tested, however, failed to find the amplification temperature. Therefore, the primers for target genes were newly designed. Except for *GAPDH*, quantitative real-time PCR primers used in this study were designed using NCBI/Primer-BLAST. The primers of the selected genes (*ZO-1, OCLN*, and *JAM2*) related to tight junction proteins were designed based on sequences available from public databases and were synthesized by Genotech Co. Ltd. (Daejeon-si, Republic of Korea). The primer sequences and amplification temperatures are listed in Table 2. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *GAPDH*.

Statistical Analysis

All data were analyzed by ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Inst. Inc., Cary, NC). The replicate was considered an experimental unit. Outlier data were confirmed by the UNIVARIATE procedure of SAS, but no outliers were identified. The LSMEANS procedure was used to calculate treatment means. In addition, if the difference was significant, the PDIFF option of SAS was used to separate the means. The statistical significance was considered at $P < 0.05$.

RESULTS

Growth Performance and Meat Quality

During 14 d of the experiment from 21 d to 35 d of age, birds in HS-C treatments had lower ($P < 0.05$) final BW (**FBW**), BWG, and FI, but higher ($P < 0.05$) FCR than those in TN-C treatments (Table 3). Under HS conditions, birds in HS-Bet, HS-Gly, or HS-Bet+Gly treatment had higher ($P < 0.05$) FBW and BWG, but lower ($P < 0.05$) FCR than those in HS-C treatment. However, values for improved FBW, BWG, and FCR by dietary treatments were lower ($P < 0.05$) than those measured in TN-C treatment. Birds in HS-Bet+Gly treatment had higher ($P < 0.05$) FI than those in HS-C treatments, but birds in HS-Bet and HS-Gly treatment did not show different FI as compared to those in HS-C. Meat quality including WHC, meat color, postmortem pH of breast meat, and TBARS values was not affected

Table 3. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on growth performance in broiler chickens raised under heat stress conditions.[1]

Treatments[2]	IBW, g	FBW, g	BWG, g	FI, g	FCR, g/g
TN-C	985	2,151[a]	1,166[a]	1,899[a]	1.63[c]
HS-C	984	1,855[c]	870[c]	1,725[c]	1.99[a]
HS-Bet	985	1,977[b]	992[b]	1,774[bc]	1.79[b]
HS-Gly	985	1,967[b]	983[b]	1,772[bc]	1.81[b]
HS-Bet+Gly	984	1,993[b]	1,008[b]	1,804[b]	1.79[b]
SEM	10.0	24.0	23.6	25.7	0.032
P-value	1.000	<0.001	<0.001	<0.001	<0.001

Abbreviations: BWG, BW gain; FBW, final BW; FCR, feed conversion ratio; FI, feed intake; IBW, initial BW.

[a–c]Means within a variable with no common superscript differ significantly ($P < 0.05$).

[1]Data are least squares means of 7 observations per treatment.

[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

by either environmental conditions (i.e., TN vs. HS conditions) or dietary treatments (Table 4).

Serum Measurements

No differences in serum concentrations of AST, ALT, creatinine, and uric acid were found between birds in HS-C treatments and birds in TN-C treatments (Table 5). Under HS conditions, birds in HS-Bet+Gly treatment had lower ($P < 0.05$) serum concentrations of AST than those in HS-Bet and HS-Gly treatment. However, birds in HS-Bet, HS-Gly, and HS-Bet+Gly treatment did not have different AST concentrations compared with those in TN-C and HS-C treatment. Other serum measurements (i.e., ALT, creatinine, and uric acid) were not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination.

Stress Indicator

Birds in HS-C treatment had a higher ($P < 0.05$) H:L ratio than those in TN-C treatments (Table 6). Under HS conditions, birds in HS-Bet, HS-Gly, and HS-Bet +Gly treatment had lower ($P < 0.05$) H:L ratio than those in HS-C treatment. However, the values for decreased H:L ratio observed by dietary supplementation of Bet, Gly, and their combination were higher ($P < 0.05$) than the values observed in TN-C treatment. Feather CORT concentrations were not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination.

Liver Characteristics and Antioxidant Status

All liver characteristics including relative liver weights, liver color, hemorrhagic score, and lipid concentrations were not affected by HS conditions and dietary

Table 4. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on meat quality in broiler chickens raised under heat stress conditions.[1]

		Meat color (CIE Lab value)			Postmortem pH		
Treatments[2]	WHC, %	L*	a*	b*	1 h	24 h	TBARS
TN-C	67	46.8	2.6	14.2	6.40	5.85	0.29
HS-C	67	46.4	3.2	14.2	6.47	5.82	0.30
HS-Bet	69	45.9	2.8	14.2	6.29	5.84	0.29
HS-Gly	68	45.4	2.3	13.5	6.44	5.84	0.27
HS-Bet+Gly	68	45.8	2.4	13.7	6.45	5.80	0.31
SEM	1.4	0.55	0.41	0.71	0.064	0.025	0.012
P-value	0.894	0.444	0.595	0.929	0.278	0.685	0.262

Abbreviations: a*, redness; b*, yellowness; L*, lightness; TBARS, TBA-reactive substances; WHC, water holding capacity.

[1]Data are least squares means of 7 observations per treatment.

[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

Table 5. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on serum measurements in broiler chickens raised under heat stress conditions.[1]

Treatments[2]	AST, U/L	ALT, U/L	Creatinine, mg/dL	Uric acid, mg/dL
TN-C	296[ab]	1.80	0.21	4.78
HS-C	300[ab]	1.53	0.20	4.96
HS-Bet	346[a]	1.79	0.23	4.52
HS-Gly	348[a]	1.90	0.23	6.18
HS-Bet+Gly	236[b]	1.51	0.20	2.53
SEM	27.0	0.157	0.016	1.224
P-value	0.042	0.318	0.459	0.349

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[a,b]Means within a variable with no common superscript differ significantly ($P < 0.05$).

[1]Data are least squares means of 7 observations per treatment.

[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

Table 6. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on stress indicators in broiler chickens raised under heat stress conditions.[1]

Treatments[2]	Blood H:L ratio	Feather corticosterone (pg/mg)
TN-C	0.24[c]	1.7
HS-C	0.38[a]	2.3
HS-Bet	0.31[b]	2.0
HS-Gly	0.30[b]	1.9
HS-Bet+Gly	0.31[b]	2.0
SEM	0.022	0.25
P-value	0.002	0.517

Abbreviation: H:L ratio, heterophil to lymphocyte ratio.
[a-c]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

Table 8. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on hepatic antioxidant status in broiler chickens raised under heat stress conditions.[1]

Treatments[2]	TAC, μmol/mg protein	MDA, μmol/mg protein	ROS, mM
TN-C	808	1.9[b]	21.4
HS-C	852	5.1[a]	21.9
HS-Bet	774	3.3[ab]	20.2
HS-Gly	751	2.5[b]	20.3
HS-Bet+Gly	900	3.3[ab]	19.3
SEM	115.5	0.69	1.84
P-value	0.895	0.033	0.870

Abbreviations: MDA, malondialdehyde; ROS, reactive oxygen species; TAC, total antioxidant capacity.
[a,b]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

supplementation of Bet, Gly, or their combination (Table 7).

In the antioxidant status, birds in HS-C treatment had higher ($P < 0.05$) MDA concentrations than those in TN-C treatment (Table 8). Under HS conditions, birds in HS-Gly treatment had lower ($P < 0.05$) MDA than those in HS-C treatment. Likewise, dietary supplementation of Bet or a combination of Bet and Gly decreased MDA concentrations as compared to the control group; however, the significance was not identified. However, other measurements (i.e., TAC and ROS) were not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination.

Jejunal Morphology, Permeability, and Gene Expression

Birds in HS-C treatment had lower ($P < 0.05$) VH than birds in TN-C treatments (Table 9), but VW, CD, and VH:CD ratio did not differ between these 2 treatments. Under HS conditions, birds in HS-Gly and HS-Bet+Gly treatment had higher ($P < 0.05$) VH than those in HS-C treatment but did not differ from birds in TN-C treatment. In goblet cell counts, birds in HS-C treatments did not differ from those in TN-C treatments. Under HS conditions, however, birds in HS-Bet, HS-Gly, and HS-Bet+Gly treatment had higher ($P < 0.05$) goblet cell counts than those in HS-C treatment. In addition, dietary supplementation of 0.79% Gly resulted in the greatest ($P < 0.05$) goblet cell counts, which were higher ($P < 0.05$) than the values measured in TN-C treatment. However, the values for VW, CD, and VH:CD ratio were not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination.

All treatment groups in HS conditions had higher ($P < 0.05$) trans-epithelial voltage (**PD**) and short circuit current (**Isc**) values but lower ($P < 0.05$) trans-epithelial electrical resistance (**TER**) values in the jejunal tissue than TN conditions (Table 10). However, under HS conditions, dietary supplementation of Bet, Gly, or their combination did not affect PD, Isc, and TER values. Likewise, all expression levels of tight junction-related genes in the jejunal mucosa (*JAM-2, OCLN*, and *ZO-1*) were not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination.

Table 7. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on liver characteristics in broiler chickens raised under heat stress conditions.[1]

				Liver color (CIE Lab value)			
Treatments[2]	Relative liver weight, %	Liver color score	Liver hemorrhage score	L*	a*	b*	Lipid concentrations %
TN-C	2.2	1.0	0.3	27.2	16.5	6.8	20.3
HS-C	2.2	1.2	0.3	27.3	16.5	6.7	26.8
HS-Bet	2.3	1.2	0.6	27.4	16.0	7.6	19.3
HS-Gly	2.3	1.4	0.5	27.4	17.2	6.9	23.6
HS-Bet+Gly	2.5	1.4	0.5	26.7	15.0	6.4	18.3
SEM	0.15	0.11	0.12	0.47	0.78	0.33	2.73
P-value	0.765	0.085	0.204	0.859	0.380	0.166	0.191

Abbreviations: a*, redness; b*, yellowness; L*, lightness.
[1]Data are least squares means of 7 observations per treatment.
[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

Table 9. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on jejunal morphology in broiler chickens raised under heat stress conditions.[1]

Treatments[2]	VH (μm)	VW (μm)	CD (μm)	VH:CD ratio	Goblet cell, cells/200 μm
TN-C	1,321[a]	157	98	13.75	28.44[bc]
HS-C	1,086[b]	154	91	12.28	26.51[c]
HS-Bet	1,200[ab]	158	93	13.16	29.21[ab]
HS-Gly	1,248[a]	168	100	12.74	31.14[a]
HS-Bet+Gly	1,224[a]	155	98	12.72	29.16[ab]
SEM	44.0	6.9	3.2	0.543	0.798
P-value	0.013	0.625	0.250	0.398	0.007

Abbreviations: CD, crypt depth; VH, villus height; VH:CD ratio, villus height to crypt depth ratio; VW, villus width.
[a-c]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

Table 10. Effect of individual or combination of dietary betaine (Bet) and glycine (Gly) on jejunal permeability and tight junction-related gene expression in broiler chickens raised under heat stress conditions.[1]

	Jejunal permeability			Tight junction-related gene expression		
Treatments[2]	PD, mV	Isc, μa/cm^2	TER, Ω/cm^2	*JAM-2*	*OCLN*	*ZO-1*
TN-C	62[b]	0.33[b]	188[a]	1.29	0.87	1.21
HS-C	90[a]	0.73[a]	127[b]	1.80	1.12	1.10
HS-Bet	108[a]	0.82[a]	132[b]	1.35	0.98	1.12
HS-Gly	101[a]	0.79[a]	130[b]	1.16	0.91	1.04
HS-Bet+Gly	95[a]	0.71[a]	135[b]	1.27	0.94	1.31
SEM	9.4	0.08	4.8	0.469	0.216	0.195
P-value	0.017	0.001	<0.001	0.887	0.937	0.887

Abbreviations: Isc, short circuit current; *JAM-2*, Junctional adhesion molecules-B; *OCLN*, Occludin; PD, trans-epithelial voltage; TER, trans-epithelial electrical resistance; *ZO-1*, Zonula occludens-1.
[a,b]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]TN-C, basal diet under thermoneutral conditions; HS-C, basal diet under heat stress conditions; HS-Bet, basal diet + 0.20% betaine under HS conditions; HS-Gly, basal diet + 0.79% glycine under HS conditions; HS-Bet+Gly, basal diet + 0.20% betaine + 0.79% glycine under HS conditions.

DISCUSSION

Previous experiments consistently reported that HS impaired the productive performance of broiler chickens (Goo et al., 2019; Kim et al., 2021, 2022), which was also confirmed in this experiment. This negative impact has been associated with lowered FI, decreased utilization of energy and nutrients in diets, impaired acid-base balance, decreased intestinal barrier function, and increased oxidative stress (Wallis and Balnave, 1984; Lara and Rostagno, 2013; Goo et al., 2019).

Dietary supplementation of Bet is widely applied in poultry exposed to HS conditions because Bet plays a role as an osmoprotectant and methyl donor in animals under HS conditions (Eklund et al., 2005; Ratriyanto and Mosenthin, 2018). Many previous studies, therefore, reported that dietary supplementation of Bet from 0.10% to 0.60% ameliorated the impairment in productive performance of broiler chickens exposed to HS conditions (Chand et al., 2017; Shakeri et al., 2020). Likewise, the similar improvements in BWG and FCR of broiler chickens were also observed by dietary supplementation of 0.20% Bet in the current experiment.

The Gly was hypothesized to be a functional nutrient to mitigate decreased performance of broiler chickens under HS conditions in this experiment. The reason was that Gly is an important molecule of synthesizing creatine, heme, glutathione, bile acids, nucleic acids, and uric acid (Wang et al., 2013), and the requirements of those biomolecules may be increased by stressful environment. For instance, HS conditions accelerate AA oxidation to support increased energy requirement (Kim et al., 2022). This increase in AA oxidation enhances uric acid production in poultry, subsequently increasing the requirement of Gly as a structural molecule in uric acid. If the production of uric acid is limited by an inadequate Gly supply, toxic ammonia can be accumulated in the body, which may have a detrimental effect on poultry performance and health. However, information regarding dietary supplementation of Gly in poultry exposed to HS conditions is still lacking, because Gly can be endogenously synthesized and the relatively high amount of Gly is present in most feed ingredients (Siegert and Rodehutscord, 2019). However, dietary supplementation of Gly in low-CP diets was reported to exert positive effects on FI, BWG, and FCR in broiler chickens raised under hot environment (Awad et al., 2017). Similar improvements in productive performance were also observed by dietary supplementation of 0.79% Gly in this experiment although the diets used in this experiment contained the recommended concentrations of CP. These results indicated that dietary supplementation of Gly can be a potential means to improve productive performance in broiler chickens, particularly exposed to HS conditions.

This experiment also hypothesized that dietary supplementation of Bet and Gly combination may have a synergistic benefit on broiler chickens exposed to HS conditions. The Gly is the end product of Bet (i.e., trimethylglycine) by sequential donation of 3 methyl groups and, therefore, supplementation of Bet may act as an additional supplier of Gly after its completion of transmethylation. Moreover, it may also be postulated that additional supplementation of Gly may increase Bet availability for methyl-donation metabolisms by delaying Bet oxidation. However, dietary combination of Bet and Gly did not exert a synergistic effect on broiler performance in the current experiment, which may indicate that additional supply of dietary Gly is unlikely to affect Bet utilization or vice versa in broiler chickens exposed to HS conditions. Nevertheless, it should not be discounted because the extent of synergistical effects of dietary Bet and Gly may be insufficient to be statistically detected because the numerical improvement in FBW and BWG was observed in this experiment. Further research is required to verify the interactive effect of dietary Bet and Gly, possibly with higher supplemental levels of Bet and Gly in broiler diets.

Previous experiment reported that HS negatively affect the meat quality of broiler chickens (Lara and Rostagno, 2013). Dietary supplementation of Bet may ameliorate the negative effect of HS on meat quality, possibly due to an increase in the osmoregulatory capacity by Bet (Ratriyanto and Mosenthin, 2018; Wen et al., 2019). However, such a beneficial effect of dietary supplementation of 0.20% Bet was not observed in this experiment. It was hypothesized that dietary supplementation of Gly may aid in improving meat quality because Gly is required to synthesize creatine that is reported to improve the meat quality of broiler chickens by decreasing lactate formation and thereby pH decline postmortem (Young et al., 2004) and increasing osmotic pressure in cells (Hultman et al., 1996). As observed by dietary Bet, however, dietary supplementation of 0.79% Gly did not influence meat quality in this experiment. The experiments reporting the effects of dietary supplementation of Gly on meat quality in broiler chickens are lacking, thereby limiting our explanation. No beneficial effect of dietary supplementation of either Bet or Gly alone led to no synergistic effect on meat quality of broiler chickens. However, it should be noted that the values of meat quality measured in this experiment fell within the normal range of meat quality in broiler chickens (Chang et al., 2020). These results suggested that the current supplemental levels of Bet, Gly, or its combination under the current HS conditions may have little impact on meat quality of broiler chickens.

Increased serum concentration of transaminases, such as AST and ALT, have been reported in broiler chickens exposed to HS conditions (Hosseini-Vashan et al., 2015). Moreover, HS can promote muscle protein catabolism, thereby increasing serum levels of uric acid and creatinine of broiler chickens (Liu et al., 2016). However, in the present experiment, neither HS conditions nor dietary supplementation of Bet, Gly, or its combination influenced most of serum measurements. However, both Bet and Gly are involved in antioxidant systems via glutathione metabolism (Metzler-Zebeli et al., 2009; Wang et al., 2013), such that they may play a role in decreasing oxidative stress in the liver, which was confirmed by our observation of decreased MDA concentrations in the liver by dietary supplementation of Bet, Gly, or its combination. However, this decreased oxidative stress in the liver was not likely associated with the serum measurements in this experiment. Interestingly, birds fed diets supplemented with the combination of Bet and Gly had lower serum AST levels than those fed diets supplemented with Bet or Gly alone, and the values were the least among 5 treatments. The reason for this observation is difficult to be explained because dietary supplementation of Bet or Gly alone tended to increase serum AST levels as compared to other treatments but no differences in serum ALT levels as other indicator of liver damages were identified among all treatments.

The blood H:L ratio and feather CORT concentrations have been widely used as reliable biomarkers to determine the stress response of poultry (Lee et al., 2022) because various physiological stressors such as stock density, high temperature, water deprivation, and fasting increased the blood H:L ratio (Gross and Chickering, 1987) and feather CORT concentrations (Lee et al., 2022). The current experiment also found that birds in HS-C treatments had the greatest blood H:L ratio. In addition, dietary supplementation of Bet, Gly, or their combination lowered the blood H:L ratio in broiler chickens in this experiment. A similar result was observed by previous experiments (Awad et al., 2014; Nofal et al., 2015) reporting that dietary supplementation of Bet from 0.05 to 0.15% decreased the blood H:L ratio in broiler chickens under the HS conditions. The reason for this observation may be because Bet, known as an osmoprotectant, aids in regulating body temperature in poultry through osmotic functions such as increased fluid flow and decreased dehydration (Zulkifli et al., 2004). In addition, our results indicated that dietary supplementation of Gly decreased the blood H:L ratio in broiler chickens at the similar extent to dietary supplementation of Bet. However, to our knowledge, no previous experiments have been conducted to measure the blood H:L ratio as affected by dietary supplementation of Gly in broiler chickens raised under HS conditions. However, it is likely that the Gly is closely related to Bet metabolism, and thereby shows similar positive effects on stress responses. In addition, dietary supplementation of Gly decreased hepatic MDA production in this experiment, which represents lowered oxidative stress, such that decreased oxidative stress may be a possible reason for decreased stress responses by dietary supplementation of Gly in the current experiment. However, although dietary supplementation of either Bet or Gly alone showed an alleviating effect on stress responses, the effects of feeding diets supplemented with Bet and Gly combination were not synergistic. Moreover, although feather CORT concentrations are well-

known stress markers as blood H:L ratio (Lee et al., 2022), but they were affected by neither HS conditions nor dietary supplementation of Bet, Gly, or their combination. The reason is likely associated with the relative short period of HS conditions in this experiment. It is suggested that increasing CORT in the circulating blood by the stress response should be transferred and accumulated to the feather tissue, which requires a relatively long period of times. Therefore, the feather CORT concentrations are suggested to be a long-term stress indicator rather than a short-term stress (Lee et al., 2022).

The liver is very sensitive organ to HS because elevated blood flows from the hepato-splanchnic region to respiratory muscles and skin for increasing heat dissipation induce various problems in the liver function (Emami et al., 2021). This impaired liver function is also associated with increasing fat accumulation in the liver because poultry exposed to HS conditions often shows fatty liver symptoms (Kim et al., 2022). However, in the current experiment, the liver visual characteristics and lipid concentrations were not affected by dietary treatments and HS conditions, although numerical increase in lipid concentrations by 24% were observed in broiler chickens exposed to HS. Dietary supplementation of Bet alone or the combination of Bet and Gly decreased numerically lipid concentrations; however, the significance was not detected possibly due to large variations in hepatic lipid concentrations.

The HS is known to promote the formation of ROS, increasing MDA production in the liver of broiler chickens (Yang et al., 2010). In this experiment, MDA concentrations in the liver were also increased in the HS-C treatments than TN-C treatments. Previous experiment reported that dietary Bet had antioxidant effects by decreasing MDA concentrations in the liver (Wen et al., 2019). The antioxidant effect of Bet has been associated with restoration of S-adenosyl methionine, which is necessary for the synthesis of glutathione (Alirezaei et al., 2012). The antioxidant effect of Gly is also likely assumed to be the role of a precursor molecule for glutathione synthesis. In the current experiment, however, dietary supplementation of Gly alone showed only significant reduction in the MDA concentrations, whereas dietary supplementation of Bet alone or its combination of Bet and Gly only numerically decreased MDA concentrations in the liver.

Intestinal structure and epithelial integrity are impaired in animals exposed to HS (Nanto-Hara et al., 2020). Previous experiments have reported decreased VH and increased CD in broiler chickens exposed to HS (Hu and Guo, 2008; Quinteiro-Filho et al., 2010). The current experiment also confirmed a decrease in VH due to HS. It is reported that dietary supplementation of Bet from 0.05% to 0.20% improved the intestinal morphology of broiler chickens exposed to HS conditions possibly by its osmoregulatory and antioxidant effects (Liu et al., 2019). However, Sakomura et al. (2013) reported that dietary supplementation of 0.05% to 0.10% Bet did not influence intestinal VH and CD in broiler chickens exposed to HS. Likewise, the current study revealed that dietary supplementation of 0.20% Bet did not affect intestinal morphology despite the numerical increase in VH. However, dietary supplementation of Gly alone or its combination with Bet significantly increased VH. Interestingly, goblet cell numbers were increased by all dietary treatments under HS conditions in this experiment with dietary supplementation of Gly alone showing the greatest number among all treatments. The possibly reason may be that Gly is related to mucin production in the goblet cells (Ospina-Rojas et al., 2013) and additional supply of Gly may stimulate mucin synthesis, thereby increasing the number of goblet cells. However, such an effect was not observed by dietary supplementation of the combination of Bet and Gly.

The HS conditions impair the intestinal barrier function of broiler chickens because of intestinal hypoxia and oxidative stress, leading to an increase in intestinal permeability (Quinteiro-Filho et al., 2010; Song et al., 2013), which was also confirmed by our observation of decreased TER values in broiler chickens raised under HS conditions. Shakeri et al. (2020) reported that dietary supplementation of 0.10% Bet decreased intestinal permeability in heat-stressed broiler chickens. This positive effect is associated with increasing intracellular water, protecting intracellular enzymes against osmotic-induced inactivation, and reducing energy expenditure (Zulkifli et al., 2004; Alirezaei et al., 2012). However, dietary supplementation of 0.20% Bet did not affect intestinal permeability in this experiment. The Bet is mostly absorbed in the duodenum, where the osmotic pressure is higher, and has a greater effect on the upper intestinal cells (Egbuniwe et al., 2016). In this experiment, intestinal permeability was measured in the jejunal tissue, which may be the reason for little effect of dietary supplementation of Bet. Shakeri et al. (2018) suggested that the effect of dietary Bet on the intestinal barrier function may depend on measuring places in the intestine.

Dietary supplementation of Gly was hypothesized to improve intestinal barrier function in broiler chickens exposed to HS in this experiment because previous experiment reported that additional supply of dietary Gly may restore intestinal epithelial dysfunction induced from oxidative stress by regulating proteins involved in apoptosis and intestinal permeability in piglets (Fan et al., 2019). However, we found no positive effect on intestinal permeability by dietary supplementation of Gly. To our knowledge, no previous experiments have been conducted to determine the effects of dietary supplementation of Gly on intestinal permeability in broiler chickens raised under HS conditions.

The expression levels of some specific genes related to tight junction proteins (*JAM-2, OCLN*, and *ZO-1*; Shin et al., 2018), which are highly related to intestinal permeability, were measured in this experiment. However, the expression of *JAM-2, OCLN*, and *ZO-1* genes in the jejunal mucosa was not affected by HS conditions and dietary supplementation of Bet, Gly, or their combination. Similarly, TER values as a measure of intestinal

permeability were not influenced by dietary treatments in this experiment. Therefore, it is suggested that the current supplemental levels of Bet, Gly, or its combination under the current HS conditions may have little impact on intestinal permeability and tight junction-related gene expression in broiler chickens.

The current experiment focused on the effect of individual or combination of dietary Bet and Gly on productive performance, stress response, and intestinal health in broiler chickens specifically under HS conditions. It may be better to compare the effects of dietary treatments used in this experiment with those measured under TN conditions, which was not conducted in the current experiment. Therefore, further studies are required to compare the potential outcomes of individual or combination of dietary Bet and Gly in broiler chickens between TN and HS conditions.

CONCLUSION

Individual supplementation of 0.20% Bet or 0.79% Gly in diets improves growth performance in broiler chickens raised under HS conditions possibly by decreasing stress responses, increasing VH and goblet cell numbers without affecting intestinal permeability and tight junction-related gene expression. Dietary supplementation of the combination of 0.2% Bet and 0.79% Gly may also be effective in reducing the negative effect of HS, but the synergistic effect seems inadequate than expected.

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DISCLOSURES

The authors declare no conflict of interest for the data presented in this experiment.

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