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Betaine Alleviates Heat Stress-Induced Hepatic and Mitochondrial Oxidative Damage in Broilers

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The aim of this study was to evaluate the effects of dietary betaine (BET) on growth performance, redox state, and related gene expression in broilers under heat stress (HS). A total of 144 21-day-old male broiler chickens with similar body weights were assigned randomly to three treatments with six replicates (eight chickens per replicate cage). Broilers in the control (CON) group were kept at thermoneutral (TN, 22±1℃) conditions and fed a basal diet until they were 42 days of age. Broilers in the other two groups (defined as HS and HS + BET) were exposed to HS (34±1℃, 8 h/day) and fed the basal diet without or with 1000 mg/kg BET, respectively. Rectal and cockscomb temperature of broilers was increased ($P<0.05$) in HS and HS + BET groups compared with the CON group, whereas there was no difference between HS and HS + BET groups. Dietary BET supplementation restored ($P<0.05$) average daily gain (ADG) and average daily feed intake (ADFI) of broilers and reversed ($P<0.05$) the increase in serum alanine transaminase (ALT) activity and malondialdehyde (MDA) content in the liver tissue of broilers under HS. The HS + BET group had higher ($P<0.05$) activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the liver tissue and mitochondria than the HS group, and the same pattern was observed for glutathione (GSH) and GSH/glutathione disulphide (GSSG) in the liver tissue. The decreased mRNA levels of GPX1 and uncoupling protein (UCP) in the liver induced by HS were restored by BET supplementation. In conclusion, dietary BET supplementation can alleviate HS-induced hepatic and mitochondrial oxidative damage of broilers by regulating mRNA expressions of GPX1 and UCP.

Key words: betaine, broiler, heat stress, mitochondria, redox state

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Introduction

Heat stress (HS) is one of the major environmental concerns in broiler industry. High temperature conditions decrease the feed intake of broilers and disturb their normal physiological homeostasis. Then, the increase of cellular reactive oxygen species (ROS) levels occur, causing oxidative stress and mitochondrial dysfunction, which in turn, reduce nutrient digestibility and, finally, impair growth performance (Lara and Rostagno, 2013). Oxidative stress is defined as an imbalance between the production of ROS and its elimination caused by a depression in antioxidant systems (Lin *et al*., 2006). It was demonstrated that HS reduced the activity of antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPX) and the contents of glutathione (GSH), and increased lipid peroxidation reflected by malondialdehyde (MDA) content (Yang *et al*., 2010; Del Vesco *et al*., 2017).

The mitochondrial respiratory chain is the major site of ROS formation, and previous studies have revealed that increased ROS production in heat-treated broilers is associated with mitochondrial damage, such as a reduced antioxidant activity and the downregulation of uncoupling protein (UCP) (Huang *et al*., 2015). As a protein found in the inner mitochondrial membrane, UCP is related to heat production, and this uncoupling mechanism in ATP production enables a reduction of the production of ROS (Abe *et al*., 2006).

Several nutritional strategies have been proposed for alleviating the negative effects of HS in broilers, in which betaine (BET) has received increasing attention (Ratriyanto and Mosenthin, 2018). The principal physiologic role of betaine is as an osmolyte and methyl donor (transmethylation). As an osmolyte, BET protects cells, proteins, and enzymes

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from environmental stress. As a methyl donor, BET is involved in the transfer of methyl groups via the methionine cycle in vital biological processes (Kidd *et al*., 1997). The methyl group of BET can be transferred to homocysteine via betainehomocysteine methyltransferase to yield methionine; thus, BET is expected to replace the expensive synthetic methionine to reduce the feed cost (Eklund *et al*., 2005). It has been shown that BET can be used to spare about 25% of the total methionine in broiler diets without affecting growth performance (Sun *et al*., 2008), but excessive replacement may decrease meat quality and some muscle amino acid contents (Fu *et al*., 2016).

Administration of betaine has been shown to exert cellular and subcellular membrane stabilization by restoring both non-enzymic and enzymic antioxidants, and acts as a positive regulator of mitochondrial function (Lee, 2015). Dietary BET supplementation was found to reduce the negative impact of HS on growth performance of broilers (He *et al*., 2015). However, whether BET alleviates HS by regulating antioxidant systems in tissues and mitochondria of broilers has not been fully elucidated. Therefore, the present study was conducted to evaluate the effects of dietary BET on growth performance, antioxidant activity, and mRNA expression of related genes in the liver tissue and mitochondria of broiler exposed to HS.

Materials and Methods

Experimental Design, Diets, and Animal Husbandry

All experimental procedures involving animals were approved by Nanjing Agricultural University Institutional Animal Care and Use Committee.

A total of 144 21-day-old male broiler chickens with similar body weight (BW) were assigned randomly to 3 treatments with 6 replicates of 8 chickens per replicate cage (110 cm×60 cm×50 cm). Broilers in the control (CON) group were kept at thermoneutral (TN, 22±1℃) conditions and fed a basal diet until they were 42 days of age. Broilers in the other two groups (defined as HS and HS + BET) were exposed to HS (34±1℃ for 8 h from 9:00 to 17:00 and 22±1℃ the rest of time) and fed the basal diet without or with 1000 mg/kg BET (96% anhydrous, Skystone Feed Co., Ltd, Yixing, China). The betaine concentration was selected according to our previous study (Chen *et al*., 2018). Ingredient composition and calculated nutrient content of the basal diet are presented in Table 1. Broilers were allowed free access to mash feed and water with a 18L:6D lighting program. The number of birds that died during the study was recorded.

Table 1. **Ingredient composition and nutrient content of the basal diet (g/kg, unless otherwise stated)**

Items	21–42 days
Ingredient	
Corn	619
Soybean meal	256
Corn gluten meal	43
Soybean oil	38
Dicalcium phosphate	16
Limestone	12
L-Lysine, HCl	2
DL-Methionine	1
Sodium chloride	3
Choline chloride	0.6
Vitamin and mineral mix[1]	9.4
Nutrient content	
Metabolizable energy (MJ/kg)	13.10
Crude protein	197.10
Digestible lysine	9.58
Digestible methionine	4.08
Digestible methionine + cystine	7.05
Digestible isoleucine	7.10
Digestible threonine	6.94
Digestible valine	8.02
Digestible tryptophan	1.85
Calcium	9.00
Available phosphorus	4.20

[1] The premix provided per kilogram of diet: vitamin A (transretinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-α-tocopherol acetate), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 8 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc oxide), 65 mg; I (from calcium iodate), 1.1 mg; and Se (from sodium selenite), 0.3 mg.

Sample Collection

At 22, 32, and 42 days of age, the rectal and cockscomb temperature was determined at 13:00 by using a rectal thermometer and a Fluke TiR1 Thermal Imager, as previously described (Giloh *et al*., 2012). At 42 days of age, all broilers were weighed in replicates after feed deprivation for 12 h and feed intake was recorded to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Then, one broiler from each replicate was randomly selected. Blood samples (about 5 mL each) were taken from wing vein and centrifuged at 3,000×*g* for 15 min at 4℃ to separate the serum, which was frozen at −20℃ for further analysis. After blood collection, birds were killed by cervical dislocation. The liver was collected and divided into two parts: one part was immediately processed to isolate mitochondria and the other part was stored in liquid nitrogen until analysis.

Isolation of Liver Mitochondria

Liver mitochondria were isolated according to the previously described method (Zhang *et al*., 2015). Briefly, the fresh liver samples were mechanically homogenized with a Bio-Gen PRO 200 homogenizer (Pro Scientific, Inc.,Oxford, UK) in an ice-cold isolation buffer (pH=7.4, containing 10 mM Trizma hydrochloride, 250 mM sucrose, and 1 mM EDTA) and centrifuged at 800×*g* for 5 min at 4℃. The supernatant was centrifuged at 12,000×*g* for 15 min at 4℃ to obtain the mitochondrial pellet. Then, the pellet was washed twice with the isolation buffer and resuspended in the ice-cold isolation buffer, and finally stored at −80℃ until analysis.

Measurement of Serum Aminotransferase Activities

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Determination of Antioxidant Activity

The homogenate of liver tissue was prepared as previously described (Wen *et al*., 2017). The activities of SOD, glutathione peroxidase (GPX), glutathione reductase (GR), GSH, and glutathione disulphide (GSSG), and the malondialdehyde (MDA) concentration in the liver tissue and mitochondria were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute). The protein concentration of the liver homogenate and mitochondrial suspension was determined using the bicinchoninic acid assay (Bainor *et al*., 2011).

Messenger RNA Quantification

Total RNA was isolated from the liver tissue using RNAiso Reagent (TaKaRa Bio, Shiga, Japan) and diluted in diethyl pyrocarbonate treated water to an appropriate concentration, as previously described (Wen *et al*., 2012). Then, reverse transcription of total RNA was completed using PrimeScript RT Reagent Kit (TaKaRa), and the cDNA was quantified using SYBR Premix Ex Taq II Kit (TaKaRa) on ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Optimized cycling conditions of all genes were 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s, 60℃ for 31 s, and a final dissociation stage of 95℃ for 15 s, 60℃ for 1 min, 95℃ for 15 s, and 60℃ for 15 s. The sequences of primers for the genes tested (SOD1, GPX1, and UCP) were specifically designed according to the sequences located in GenBank (Table 2). The geometric means of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used to normalize the genes of interest, as recommended (Vandesompele *et al*., 2002). Relative mRNA levels (arbitrary units) were calculated on the basis of PCR efficiency and threshold cycle values, as previously described (Pfaffl, 2001). The mRNA level of each target gene for the CON group was assigned the value of one.

Statistical Analysis

All data were analyzed by one-way ANOVA using SPSS statistical software (version 22.0 for Windows, SPSS Inc., Chicago, IL). Differences among treatments were examined by Duncan's multiple range test, which were considered significant at a $P<0.05$. Data are presented as means and standard error of means (SEM).

Results

Rectal and Cockscomb Temperature

Compared with the CON group, rectal and cockscomb temperatures were increased ($P<0.05$) in HS and HS + BET groups at all days tested, but there was no difference between HS and HS + BET groups (Table 3).

Growth Performance

Mortality was low (3.1% in total) and did not differ among groups (data not shown). The HS group had a lower ($P<0.05$) final BW, ADG, and ADFI than the CON group, but there was no significant difference in FCR (Table 4). The HS + BET group resulted in a higher ($P<0.05$) final BW, ADG, and ADFI than the HS group, but did not affect the FCR.

Serum Aminotransferase Activities

The HS group had greater ($P<0.05$) activities of AST and ALT in serum than the CON group (Table 5). Betaine supplementation decreased ($P<0.05$) serum ALT activity in broilers exposed to HS, whereas this effect was not observed for AST.

Redox State in the Liver Tissue and Mitochondria

Compared with the CON group, the HS group increased ($P<0.05$) MDA content and decreased ($P<0.05$) SOD activity in the liver tissue and mitochondria (Table 6). Additionally, GSH content and GSH/GSSG of the HS group were decreased in the liver tissue ($P<0.05$). Compared with the HS group, the HS + BET group had increased ($P<0.05$) SOD and GPX activities in the liver tissue and mitochondria, and increased GSH content and GSH/GSSG ratio, and decreased MDA content in the liver tissue.

Gene Expression

Compared with the CON group, the HS group had lower ($P<0.05$) mRNA levels of GPX1 and UCP in the liver, which were restored ($P<0.05$) in the HS + BET group to the CON levels (Table 7). There was no difference in SOD1

Table 2. **Sequences for real-time PCR primers**

Gene[1]	GeneBank ID	Primer sequence, sense/antisense	Product size, bp
SOD1	NM_205064.1	AGGAGTGGCAGAAGTAGAAA	157
		TAAACGAGGTCCAGCAT	
GPX1	NM_001277853.1	GCCCGCACCTCTGTCATAC	157
		TGCTTCTCCAGGCTGTTCC	
UCP	AB088685.1	GAGAAACAGAGCGGGATTTGAT	90
		GCTCCTGGCTCACGGATAGA	
β-actin	NM_205518	TGCTGTGTTCCCATCTATCG	150
		TTGGTGACAATACCGTGTTCA	
GAPDH	NM_204305	AGAACATCATCCCAGCGTCC	133
		CGGCAGGTCAGGTCAACAAC	

[1] SOD1, superoxide dismutase 1; GPX1, glutathione peroxidase; UCP, uncoupling protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 3. **Effect of betaine on rectal and cockscomb temperature of heat-stressed broilers at different ages**

Item[1]	CON	HS	HS + BET	SEM	*P* value
Rectal temperature (℃)					
22 d	41.1[b]	41.7[a]	41.6[a]	0.11	0.028
32 d	41.3[b]	42.5[a]	42.6[a]	0.16	0.001
42 d	41.3[b]	42.1[a]	42.1[a]	0.08	0.003
Cockscomb temperature (℃)					
22 d	34.7[b]	36.5[a]	36.9[a]	0.34	0.005
32 d	34.7[b]	36.3[a]	37.2[a]	0.35	0.002
42 d	34.9[b]	36.5[a]	37.4[a]	0.37	0.002

[1] CON, broilers were kept at thermoneutral (22±1℃) conditions and fed a basal diet. HS, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet. HS + BET, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet supplemented with 1000 mg/kg betaine. SEM, standard error of means (n=6).

[a, b] Means within a row with different superscripts differ significantly at $P<0.05$. The P value is the significance level for analysis of variance F test among all groups.

Table 4. **Effect of betaine on growth performance of heat-stressed broilers from 21 to 42 days of age**

Item[1, 2]	CON	HS	HS + BET	SEM	*P* value
Initial BW	736.70	714.50	711.15	8.37	0.430
Final BW	2311.17[a]	2141.46[b]	2219.09[a]	23.83	0.004
ADG (g/day)	74.97[a]	67.95[b]	71.81[a]	1.01	0.006
ADFI (g/day)	140.98[a]	126.28[b]	137.31[a]	2.48	0.027
FCR	1.88	1.86	1.91	0.02	0.493

[1] CON, broilers were kept at thermoneutral (22±1℃) conditions and fed a basal diet. HS, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet. HS + BET, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet supplemented with 1000 mg/kg betaine. SEM, standard error of means (n=6).

[2] BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

[a, b] Means within a row with different superscripts differ significantly at $P<0.05$. The P value is the significance level for analysis of variance F test among all groups.

Table 5. **Effect of betaine on serum aminotransferase activities of heat-stressed broilers**

Item[1, 2]	CON	HS	HS + BET	SEM	*P* value
AST (U/mL)	35.35[b]	58.76[a]	57.79[a]	4.04	0.005
ALT (U/mL)	7.46[b]	8.36[a]	7.59[b]	0.14	0.006

[1] CON, broilers were kept at thermoneutral (22±1℃) conditions and fed a basal diet. HS, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet. HS + BET, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet supplemented with 1000 mg/kg betaine. SEM, standard error of means (n=6).

[2] AST, aspartate aminotransferase; ALT, alanine aminotransferase.

[a, b] Means within a row with different superscripts differ significantly at $P<0.05$. The P value is the significance level for analysis of variance F test among all groups.

mRNA expression among groups.

Discussion

Our data showed that the HS increased rectal and cockscomb temperature and decreased ADG and ADFI compared with the CON group, which was in accordance with previous research (Azad *et al*., 2010). Although BET supplementation did not affect body temperature, it resulted in higher ADG and ADFI than the HS group, which was consistent to the results of previous studies (He *et al*., 2015; Akhavan-Salamat and Ghasemi, 2016). The positive effects of BET on growth performance of broilers under HS might be partly

Table 6. **Effect of betaine on redox state of the liver tissue and mitochondria in heat-stressed broilers**

Item[1, 2]	CON	HS	HS + BET	SEM	*P* value
Liver tissue					
MDA (nmol/mg prot)	3.04[b]	4.67[a]	3.34[b]	0.27	0.016
SOD (U/mg prot)	48.27[a]	40.10[b]	47.04[a]	1.39	0.033
GPX (U/mg prot)	49.90[ab]	43.07[b]	56.90[a]	3.07	0.036
GR (U/mg prot)	8.06	7.73	7.67	0.45	0.940
GSH (mg/g prot)	9.56[a]	7.81[b]	9.14[a]	0.32	0.035
GSSG (mg/g prot)	1.79	2.03	1.93	0.13	0.777
GSH/GSSG ratio	5.25[a]	3.90[b]	4.88[a]	0.37	0.025
Mitochondria					
MDA (nmol/mg prot)	2.24[b]	3.72[a]	3.08[ab]	0.24	0.036
SOD (U/mg prot)	35.64[a]	28.37[b]	35.27[a]	1.42	0.032
GPX (U/mg prot)	31.10[ab]	25.09[b]	42.04[a]	3.69	0.003
GR (U/mg prot)	3.07	3.26	2.54	0.18	0.334
GSH (mg/g prot)	3.73	3.82	3.61	0.29	0.996
GSSG (mg/g prot)	1.65	1.54	1.46	0.11	0.777
GSH/GSSG ratio	2.5	3.3	2.9	0.08	0.545

[1] CON, broilers were kept at thermoneutral (22±1℃) conditions and fed a basal diet. HS, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet. HS + BET, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet supplemented with 1000 mg/kg betaine. SEM, standard error of means (n=6).

[2] MDA, malondialdehyde; SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide.

[a, b] Means within a row with different superscripts differ significantly at $P<0.05$. The P value is the significance level for analysis of variance F test among all groups.

Table 7. **Effect of betaine on hepatic gene expression in heat-stressed broilers**

Item[1, 2]	CON	HS	HS + BET	SEM	*P* value
SOD1	1.00	1.11	1.51	0.10	0.089
GPX1	1.00[a]	0.75[b]	1.08[a]	0.04	0.001
UCP	1.00[a]	0.71[b]	0.92[a]	0.05	0.014

[1] CON, broilers were kept at thermoneutral (22±1℃) conditions and fed a basal diet. HS, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet. HS + BET, broilers were exposed to HS (34±1℃, 8 h/day) conditions and fed a basal diet supplemented with 1000 mg/kg betaine. SEM, standard error of means (n=6).

[2] SOD1, superoxide dismutase 1; GPX1, glutathione peroxidase 1; UCP, uncoupling protein.

[a, b] Means within a row with different superscripts differ significantly at $P<0.05$. The P value is the significance level for analysis of variance F test among all groups.

attributed to the osmoregulatory property of BET to the maintenance of protein structure, enzyme, and cell function, irrespective of its role as a methyl group donor (Craig, 2004). In addition, BET might alleviate fear response of broilers under HS by inhibiting oxidative stress, thus increasing the feed take of broilers (Ratriyanto and Mosenthin, 2018). It was reported that administration of betaine to drinking water decreased the tonic immobility in broiler chickens during the hot-dry season, which was associated with increased activities of SOD and GPX in serum (Egbuniwe *et al*., 2016). However, the improved FCR by dietary BET described in the reports above was not observed in our study. The discrepancy may be due to exposure period, temperature, genotype, and age of broilers (Attia *et al*., 2009).

As markers of hepatic damage, serum ALT and AST activities were higher in HS group than in CON group, agreeing with the previous data (Tang *et al*., 2013). This is attributed to cellular leakage and loss of the functional integrity of hepatic cell membrane induced by HS, resulting in the release of ALT and AST from the cytoplasm (Lin *et al*., 2006). The ALT activity in broilers under HS was reduced by BET supplementation, which was consistent with the previous data (Konca *et al*., 2008), suggesting that BET could alleviate HS-induced hepatic damage. This can be explained by the effect of BET in maintaining the structural and functional integrity of cell membranes (Ganesan *et al*.,

2010). No difference in serum AST activity in broilers under HS implied that it may be less responsive to BET supplementation. However, dietary BET was reported to decrease only serum AST activity in rabbits under high ambient temperature (Hassan *et al*., 2011), whereas there was no effect of BET on the activities of these two enzymes in growing pigs during HS condition (Mendoza *et al*., 2017), implying that the effect of BET may vary in different animal species.

Increased MDA content and decreased SOD activity in the liver tissue and mitochondria as well as decreased GSH content and GSH/GSSG in the liver tissue were observed in the HS group, supporting the view that oxidative stress is part of the stress response of broilers to heat exposure (Lin *et al*., 2006; Yang *et al*., 2010). The alteration of SOD activity and contents of GSH and MDA was reversed by BET supplementation, which also increased GPX activity. Our findings demonstrated that BET alleviated oxidative stress of the liver induced by HS, which might contribute to improved ADG and ADFI of broilers. Similar results were obtained by Akhavan-Salamat and Ghasemi (2016), who reported that BET supplementation increased SOD and GPX activities and reduced MDA concentration in serum of broilers under HS. Betaine may have antioxidant effects against oxidative damage by restoring S-adenosylmethionine, which contributes to an enhancement in the supply of substrate needed for the synthesis of GSH that protects the cell from ROS (Alirezaei *et al*., 2012). The increased GPX activity may be explained by the larger amount of GSH available with BET supplementation that enables the GSH antioxidant system to operate adequately.

Compared with the CON group, HS group reduced GPX1 and UCP mRNA levels in the liver. Previous reports have shown that HS stimulated ROS production possibly by downregulating UCP mRNA and protein levels (Mujahid *et al*., 2006; Del Vesco *et al*., 2015), and the increase of hydrogen peroxide production downregulated GPX1 gene expression (Habashy *et al*., 2018). Dietary BET supplementation increased mRNA expression of GPX1 and UCP genes in the liver of broilers under HS, which may contribute to the alleviation of oxidative damage induced by HS. This may be due to the methionine-sparing effect of BET, allowing more methionine to be utilized to increase the expression levels of genes related to antioxidant activity (Del Vesco *et al*., 2015). The increase of UCP mRNA expression may also be related to the positive effect of BET on mitochondrial respiration (Lee, 2015). As avian UCP is mainly expressed in the skeletal muscle (Dridi *et al*., 2004), further investigations are required to assess the role of hepatic UCP gene expression in response to HS and betaine supplementation.

In conclusion, this study indicated that dietary BET supplementation slightly alleviated the HS-induced growth repression and hepatic injury, and improved antioxidant activity in the liver tissue and mitochondria of broilers possibly by increasing the activities of SOD and GPX and GSH content, and by upregulating the transcription of GPX1 and UCP genes.

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Conflicts of Interest

The authors declare no conflicts of interest.

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