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Original Research Article

Zinc alleviates the heat stress of primary cultured hepatocytes of broiler embryos via enhancing the antioxidant ability and attenuating the heat shock responses

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ABSTRACT

Zinc (Zn) has been shown to attenuate the adverse effects of heat stress on broilers, but the mechanisms involving this process remain unclear. We aimed to investigate possible protective mechanisms of Zn on primary cultured hepatocytes of broiler embryos subjected to heat stress. Three experiments were conducted. In Exp. 1, hepatocytes were treated with 0, 50, 100, 200, or 400 μmol/L added Zn as inorganic Zn sulfate (iZn) for 12, 24 or 48 h. In Exp. 2, cells were exposed to 40 °C (a normal temperature [NT]) and 44 °C (a high temperature [HT]) for 1, 2, 4, 6, or 8 h. In Exp. 3, cells were preincubated with 0 or 50 μmol/L Zn as iZn or organic Zn lysine chelate (oZn) for 8 h under NT, and then incubated with the same Zn treatments under NT or HT for 4 or 6 h. The biomarkers of antioxidative status and heat stress in cells were measured. The results in Exp. 1 indicated that 50 μmol/L Zn and 12 h incubation were the optimal conditions for increasing antioxidant ability of hepatocytes. In Exp. 2, the 4 or 6 h incubation under HT was effective in inducing heat shock responses of hepatocytes. In Exp. 3, HT elevated ($P < 0.01$) malondialdehyde content and expressions of heat shock protein 70 (*HSP70*) mRNA and protein, as well as *HSP90* mRNA. However, Zn supplementation increased ($P < 0.05$) copper zinc superoxide dismutase (CuZnSOD) activity and metallothionein mRNA expression, and effectively decreased ($P < 0.05$) the expressions of *HSP70* mRNA and protein, as well as *HSP90* mRNA. Furthermore, oZn was more effective ($P < 0.05$) than iZn in enhancing CuZnSOD activity of hepatocytes under HT. It was concluded that Zn (especially oZn) could alleviate heat stress of broiler hepatocytes via enhancing their antioxidant ability and attenuating heat shock responses.

1. Introduction

Heat stress in environmental stresses is a major concern for poultry industry because of the resulting poor egg production, depressed feed intake and feed efficiency along with high mortality (Liao et al., 2018; Zhu et al., 2015b, 2017a). In addition to decreased productive performance, heat stress was also reported to disturb the redox balance and induce oxidative stress with the excessive generation of reactive oxygen species (ROS) in broilers (Lin et al., 2006; Tan et al., 2010; Xie et al., 2015) and laying hens (Lin et al., 2008; Naziroglu et al., 2000). In response to oxidative

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stress, organisms have evolved many defense mechanisms including endogenous antioxidants and stress proteins. Copper zinc superoxide dismutase (CuZnSOD) and metallothionein (MT) are considered endogenous ROS scavengers that confer protection against oxidative stress in organisms (Alscher et al., 2005; Volkova et al., 2012). The heat shock proteins (HSP) are a set of stress proteins synthesized in response to various stresses, including heat stress (Evdonin et al., 2009; Xie et al., 2014). Heat stress induces synthesis of HSP in broilers to aid in thermotolerance and antioxidant stress (Gu et al., 2012; Soleimani et al., 2012). However, this abrupt rise in HSP expression also reflects cellular potential heat stress damage (Tedeschi et al., 2015). Therefore, CuZnSOD, MT and HSP have been broadly used as molecular biomarkers to assess the potential cellular and physiological effects of heat stress or antioxidant nutrients (Cao et al., 2015; Jang et al., 2014; Sahin et al., 2012; Wang et al., 2016; Wu et al., 2011; Yin et al., 2018).

Zinc (Zn) is an essential trace element for living organisms that acts as a cofactor for over 300 enzymes (Vallee et al., 1990). Zinc can be not stored in the animal's body; hence, there is a need for daily supplementation of Zn through the diet. Dietary supplementation with Zn is beneficial, especially in heat stress condition that induces production of ROS (Sahin et al., 2005, 2009). In broiler breeder hens, we have demonstrated that supplementation with Zn in maternal diets effectively eliminated the adverse effects of maternal heat stress on embryonic development and growth performance of offspring (Zhu et al., 2017a, 2017b). The requirement of Zn increases during heat stress. Dietary supplementation of Zn from 30 to 60 mg/kg, equating to the total level of Zn in diets from 70 to120 mg/kg, could improve feed intake, body weight, feed conversation radio, and carcass traits in broilers reared under heat stress condition (Salamat et al., 2019; Chand et al., 2014; Kucuk et al., 2003; Rama Rao et al., 2016; Ramiah et al., 2019). However, the exact mechanisms of the protective effect of Zn on broilers against heat stress are little known. Recent studies in chickens have demonstrated that dietary supplementation with either inorganic or organic Zn could elevate the mRNA abundances of *MT* and *CuZnSOD*, as well as the CuZnSOD activity and MT concentration in the liver (Azad et al., 2017; Zhang et al., 2017, 2018). Furthermore, the organic Zn was more effective than the inorganic Zn in augmenting CuZnSOD activity and MT concentration in the liver (Zhang et al., 2017, 2018). Inorganic $ZnSO_4$ is the common supplemental form of Zn in diets for poultry. Nevertheless, organic Zn sources, especially Zn amino acid complexes or chelates, have been receiving more and more attention because of their better absorption and higher bioavailabilities (Kwiecień et al., 2017; Suo et al., 2015). Series of studies from our laboratory have further indicated that absorption and bioavailability of organic Zn sources were closely related to their chelation strengths, and the organic Zn with a moderate chelation strength was most available in broilers (Huang et al., 2009, 2013; Yu et al., 2010, 2017) and heat-stressed laying broiler breeders (Liao et al., 2018). The liver is not only a key organ in chickens' response to heat stress (Lin et al., 2006; Mackei et al., 2020), but also a sensitive tissue to reflect the Zn status of broilers (Azad et al., 2017). Accordingly, primary cultured broiler hepatocytes may be an ideal in vitro model for exploring the anti-heat stress mechanisms of Zn. Therefore, we hypothesized that Zn, especially the organic Zn with a moderate chelation strength, might protect primary cultured hepatocytes of broiler embryos against heat stress via enhancing the antioxidant ability and attenuating the heat shock responses. The objective of the present study was to investigate the effect of Zn, especially the organic Zn with a moderate chelation strength, on antioxidant status and expressions of HSP in primary cultured hepatocytes of broiler embryos exposed to heat stress.

2. Materials and methods

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China), and experiments were performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

2.1. Isolation and cultivation of primary hepatocytes of broiler embryos

One hundred and fourteen 30-week-old female Arbor Acres broiler breeders with similar weights were purchased from a commercial hatching factory (Huadu Broiler Breeding Corp., Hebei, China) and kept in stainless-steel cages (length 50 cm × width 50 cm × height 50 cm) with 2 birds per cage. All broiler breeder hens were fed a Zn-deficient semi-purified diet containing Zn at 7.13 mg/kg (analysed value) from 30 to 38 weeks in order to obtain the low-Zn eggs so that the primary hepatocytes isolated from the embryos could be Zn-deficient, and thus more sensitive to Zn supplementation (Sun et al., 2018). During this period, lighting and feeding management followed the Arbor Acres breeder management guidelines, and birds had ad libitum access to tap water with no detectable Zn. Healthy fertilized eggs with similar weights were collected from 36- to 38-week-old broiler breeder hens. They were incubated at 38 °C and (50 ± 2)% relative humidity for 14 d. Primary cultured hepatocytes of broiler embryos were prepared according to the previous method (Zhou et al., 2005) with some modifications. Briefly, after anesthesia in cold phosphate-buffered saline (PBS), the livers of 14-d-old chick embryos were collected, washed 3 to 4 times with PBS formulated without magnesium and calcium, and then minced and digested with 0.12% collagenase type II in a shaking water bath (90 cycles/min) at 37 °C for 20 min with frequent pipetting to facilitate cell dissociation. The digested cells were then dispersed by pipettes and filtered to remove large debris using 150-μm and 70-μm meshes. Cell suspensions were centrifuged 3 times at 200 × *g* at 4 °C, each time for 5 min. The combined pellets were resuspended in the complete culture medium, consisting of L-15 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), 10 μg/mL transferrin, 10 μg/mL vitamin C, 10^{-6} mol/L dexamethasone, 10^{-6} mol/L insulin, and 1% antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Cell viability was assessed by the Trypan Blue exclusion test and was not less than 90% for each preparation. Evaluation of the cells by using light microscopy showed that about 95% of the collected cells were hepatocytes (Appendix Fig. 1). Hepatocytes were plated in 6-well cell culture plates (Corning Life Sciences, Lowell, MA, USA) at 1×10^6 cells per well in 2 mL of the complete culture medium. Cells were cultured at physiological temperature (40 °C) of chickens (Han et al., 2010) in a humidified incubator (Model 3110 series; Thermo Electron Corporation, Marietta, OH, USA) with 100% air. The medium was replaced with L-15 medium containing 5% FBS at 24 h after plating and then daily before further treatment. Seventy-two hours (100% confluence) after plating was taken as time 0, at which the different treatments were performed. In each experiment, there were 6 replicates of 6-well cells per replicate for each treatment.

2.2. Treatments of hepatocytes, sample collections and preparation

Experiment 1 was conducted to estimate the optimal dose and effective incubation time of Zn necessary to strengthen antioxidant system of hepatocytes. In this trial, the hepatocytes were

incubated in the Zn-unsupplemented fresh serum-free basal L-15 medium containing no detectable Zn (control) and the basal medium supplemented with 50, 100, 200, or 400 μmol/L Zn as inorganic Zn sulfate (iZn; reagent-grade $ZnSO_4 \cdot 7H_2O$, containing 22.5% Zn by analysis) for 12, 24 or 48 h, respectively. Zinc concentrations and incubation time points used in this study were based on previous studies (Muthuraman et al., 2014; Wang et al., 2013). At the end of each incubation time, the medium was removed and the cell monolayer was washed with ice-cold PBS 3 times. The cells in the first 3 wells from each replicate were scraped in ice-cold saline, and then pooled and sonicated at 4 °C for 2 min. Lysates were centrifuged at 1,000 × *g* at 4 °C for 10 min to harvest the supernatants for CuZnSOD activity analysis. The cells in the second 3 wells from each replicate were scraped in TRIzol reagent, and then pooled and frozen (−80 °C) for analyses of *CuZnSOD* and *MT* mRNA expressions.

Experiment 2 was conducted to measure lactate dehydrogenase (LDH) activity and HSP expressions of hepatocytes subjected to heat stress to estimate the optimal heat-stressed time range. In this experiment, hepatocytes were incubated in the fresh complete L-15 medium at 40 °C (a normal temperature [NT]) and 44 °C (a high temperature [HT]) for 1, 2, 4, 6, or 8 h, respectively. Culture temperatures and heat-stressed time points used in this study were based on previous studies (Han et al., 2010; Liu et al., 2012). At the end of each culture time, the medium from each replicate was collected, pooled and stored at −20 °C for LDH activity analysis. Meanwhile, the cells were harvested by the above mentioned method in Exp. 1 for analyses of *HSP70* and *HSP*90 mRNA expressions.

Experiment 3 was performed to investigate the effect of Zn source on the antioxidant status and HSP expressions of hepatocytes exposed to heat stress. The results in Exp. 1 showed that the cells exposed to the serum-free L-15 medium containing 50 μmol/L added Zn as Zn sulfate for 12 h had the best antioxidant ability. Additionally, the results in Exp. 2 indicated that HT affected ($P < 0.0001$) *HSP70* and *HSP*90 mRNA expressions, and the incubation time of 4 or 6 h under HT was effective in increasing the mRNA expressions of these 2 genes. Therefore, in Exp. 3, the cells were firstly preincubated with the Zn-unsupplemented fresh serum-free basal L-15 medium containing no detectable Zn (control) and the basal medium supplemented with 50 μmol/L Zn as either iZn or organic Zn lysine chelate (oZn) for 8 h at NT (40 °C). The oZn, synthesized at a 2:1 molar ratio of lysine to Zn, contained 8.28% Zn by analysis, which was the same as that used in a previous study of Sun et al. (2018). The quotient of formation (Q_f) of the oZn was 62.1, which is categorized as a moderate chelation strength (Holwerda et al., 1995). Lysine concentration in each treatment was balanced by supplementation of additional L-lysine monohydrochoride. And then, culture mediums in the all of the above preincubated cell wells were not changed, and the half of them continued to be incubated under NT, but the other half were transferred to the other incubator and incubated at HT (44 °C) for another 4 or 6 h. At the end of each incubation time, the cells in the first 2 wells from each replicate were collected and prepared by the above-mentioned method in Exp. 1 for determining CuZnSOD activity, MT and malondialdehyde (MDA) contents. The cells in the second 2 wells from each replicate were harvested and prepared by the above-mentioned method in Exp. 1 for analyses of *CuZnSOD*, *MT*, *HSP70* and *HSP*90 mRNA expressions. The cells in the third 2 wells from each replicate were lysed in radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), and then pooled and sonicated at 4 °C for 2 min. Lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min to obtain the supernatants, and then supernatants were frozen (−80 °C) for analyses of HSP70 and HSP90 protein expressions.

2.3. Measurement of Zn contents

The Zn contents in Zn sources, the Zn-deficient semi-purified diet and tap water were determined by inductively coupled plasma emission spectroscope (model IRIS Intrepid II; Thermal Jarrell Ash, Waltham, MA, USA) after wet digestions with HNO_3 and $HClO_4$, as described by Huang et al. (2009).

2.4. Determinations of LDH activity, MDA and MT levels and CuZnSOD activity

The LDH activity was analyzed according to the instructions in the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MDA levels were determined using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MT concentration was determined by the method of ELISA with commercial kits (Mlbio, Shanghai, China). The CuZnSOD activity was determined using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was isolated from each sample using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. The purity, concentration, and integrity of the RNA were checked using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively. Single-strand cDNA was synthesized using a PrimeScriptTM RT Master Mix kit (Takara, Dalian, China). Real-time quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Foster, CA, USA) using SYBR-Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA). The amplification started with initial denaturation step at 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and an annealing step at 60 °C for 34 s, at which point fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 °C for 15 s followed by 60 °C for 1 min and ramped to 95 °C with acquired fluorescence. Specific primers (Table 1) for *CuZnSOD*, *MT*, *HSP70*, *HSP90*, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed based on sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Gene expression levels were normalized to those of β-actin and *GAPDH* to attain relative expression levels using the $2^{-\Delta\Delta Ct}$ method.

2.6. Western blotting

Proteins of hepatocytes were extracted using lysis buffer and the concentration determination was performed by using a bicinchninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Total proteins (30 μg) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (Merck-Millipore, Bedford, MA, USA). After blocked in 5% bovine serum albumin (BSA) blocking solution for 1 h at room temperature, the membrane was incubated overnight at 4 °C with primary antibodies against HSP70 (Abcam, diluted 1:5,000), HSP90 (Abcam, diluted 1:5,000) and GAPDH (Abcam, diluted 1:5,000). Subsequently, the membrane was developed with a horseradish peroxides conjugated goat anti-rabbit secondary antibody (Abcam; diluted 1:10,000). Protein bands were visualized using enhanced chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA) and quantified with an ImageQuant LAS4000 scanner (GE

Table 1
Primers used for the target and reference genes.

Genes	Forward	Reverse
CuZnSOD	5′-GGAGGAGTGGCAGAAGT-3′	5′-TAAACGAGGTCCAGCAT-3′
MT	5′-AAGGGCTGTGTCTGCAAGGA-3′	5′-CTTCATCGGTATGGAAGGTACAAA-3′
HSP70	5′-CGTCAGTGCTGTGGACAAGAGTA-3′	5′-CCTATCTCTGTTGGCTTCATCCT-3′
HSP90	5′-GAGTTTGACTGACCCGAGCA-3′	5′-TCCCTATGCCGGTATCCACA-3′
β-actin	5′-ACCTGAGCGCAAGTACTCTGTCT-3′	5′-CATCGTACTCCTGCTTGCTGAT-3′
GAPDH	5′-CTTTGGCATTGTGGAGGGTC-3′	5′-ACGCTGGGATGATGTTCTGG-3′

CuZnSOD = copper zinc superoxide dismutase; *MT* = metallothionein; *HSP70* = heat-shock protein 70; *HSP90* = heat-shock protein 90; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

Healthcare Life Sciences, Buckinghamshire, UK), and analyzed with TotalLab Quant Software (TotalLab, Newcastle, UK). The GAPDH protein was used to normalize the expression levels of target proteins.

2.7. Statistical analyses

Data from the Exp. 1 or 2 were subjected to two-way ANOVA using the general linear model (GLM) procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Data from the Exp. 3 were subjected to three-way ANOVA using GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The replicate served as the experimental unit. Differences among means were tested by the least significant difference (LSD) method, and the statistical significance was set at $P \leq 0.05$.

3. Results

3.1. The optimal dose and effective incubation time for strengthening the antioxidant ability of primary cultured hepatocytes of broiler embryos (Exp. 1)

There was an interaction ($P = 0.0002$) between incubation time and added Zn level in CuZnSOD activity (Table 2). At 12 h incubation, 50 μmol/L added Zn increased ($P < 0.03$) CuZnSOD activity compared with the control and all other added Zn levels, whereas

Table 2
Effects of incubation time and added Zn level on the CuZnSOD activity, and mRNA expressions of *CuZnSOD* and *MT* in primary cultured hepatocytes of broiler embryos (Exp. 1).

Item	Added Zn level, μmol/L	CuZnSOD activity, U/mg prot.	*CuZnSOD* mRNA[1], RQ	*MT* mRNA[1], RQ
Incubation time [2]				
12 h	0	53.8 ± 1.50^{b}	1.30 ± 0.11	0.280 ± 0.048
	50	59.3 ± 2.16^{a}	0.958 ± 0.097	1.85 ± 0.38
	100	48.0 ± 1.65^{c}	0.662 ± 0.045	1.72 ± 0.22
	200	45.5 ± 1.13^{c}	1.37 ± 0.39	2.00 ± 0.30
	400	51.5 ± 1.40 ^{bc}	1.65 ± 0.15	2.88 ± 0.56
24 h	0	52.3 ± 2.26 ^{bc}	1.29 ± 0.63	0.126 ± 0.028
	50	53.7 ± 1.10^{b}	1.80 ± 0.56	1.96 ± 0.77
	100	50.5 ± 2.17 ^{bc}	1.33 ± 0.46	1.55 ± 0.49
	200	43.5 ± 2.06^{c}	1.49 ± 0.47	1.42 ± 0.18
	400	45.9 ± 1.95^{c}	0.622 ± 0.215	2.84 ± 1.22
48 h	0	46.0 ± 1.40^{c}	0.682 ± 0.094	0.077 ± 0.021
	50	45.0 ± 1.84^{c}	0.773 ± 0.151	0.700 ± 0.154
	100	43.4 ± 0.92^{c}	0.643 ± 0.064	1.06 ± 0.12
	200	47.2 ± 1.27^{c}	0.770 ± 0.161	2.17 ± 0.25
	400	47.2 ± 1.58^{c}	0.757 ± 0.197	1.41 ± 0.38
Incubation time [3], h				
12		51.6 ± 1.11	1.19 ± 0.11^{a}	1.75 ± 0.21
24		49.2 ± 1.12	1.31 ± 0.21^{a}	1.63 ± 0.36
48		45.8 ± 0.65	0.725 ± 0.060^{b}	1.12 ± 0.16
Added Zn level [4], μmol/L				
0		50.7 ± 1.26	1.10 ± 0.22	0.167 ± 0.031^{b}
50		52.7 ± 1.72	1.18 ± 0.21	1.48 ± 0.32^{a}
100		47.4 ± 1.12	0.878 ± 0.166	1.44 ± 0.19^{a}
200		45.4 ± 0.91	1.24 ± 0.21	1.84 ± 0.18^{a}
400		48.2 ± 1.14	1.01 ± 0.15	2.34 ± 0.48^{a}
P-value				
Incubation time		<0.0001	0.016	0.121
Added Zn level		<0.0001	0.712	<0.0001
Interaction		0.0002	0.216	0.511

CuZnSOD = copper zinc superoxide dismutase; *MT* = metallothionein.
[a, b, c] Mean values within a column with unlike superscript letters are significantly different ($P < 0.05$).
[1] The *CuZnSOD* or *MT* mRNA abundances were calculated as the relative quantity (RQ) of the *CuZnSOD* or *MT* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; RQ = $2^{-\Delta\Delta Ct}$ (Ct = threshold cycle).
[2] Each value represents the mean ± SE of 6 replicates ($n = 6$).
[3] Each value represents the mean ± SE of 30 replicates ($n = 30$).
[4] Each value represents the mean ± SE of 18 replicates ($n = 18$).

100 and 200 μmol/L added Zn decreased ($P < 0.018$) it compared with the control, and no differences ($P > 0.14$) were observed among 100, 200 and 400 μmol/L added Zn and between 0 and 400 μmol/L added Zn. At 24 h incubation, the CuZnSOD activity was higher ($P < 0.015$) at 50 μmol/L added Zn than at 200 and 400 μmol/L added Zn, and there were no differences ($P > 0.17$) among 0, 50 and 100 μmol/L added Zn and among 0, 100, 200 and 400 μmol/L added Zn. At 48 h incubation, there were no differences ($P > 0.15$) in CuZnSOD activity among all Zn levels. Incubation time affected ($P < 0.02$) *CuZnSOD* mRNA expression in hepatocytes (Table 2). Cells at either 12 or 24 h incubation had higher ($P < 0.017$) *CuZnSOD* mRNA expression than those at 48 h with no difference ($P > 0.57$) between those at 12 and 24 h. Added Zn level affected ($P < 0.0001$) *MT* mRNA expression (Table 2). Compared with the control, the addition of Zn elevated ($P < 0.0001$) *MT* mRNA expressions, but no differences ($P > 0.18$) were observed among 50, 100, 200 and 400 μmol/L of added Zn. Based on the above combined results, 50 μmol/L of added Zn induced the optimal responses of CuZnSOD and MT expressions at 12 h incubation.

3.2. The optimal heat exposure time for establishing heat stress model with primary cultured hepatocytes of broiler embryos (Exp. 2)

An interaction between culture temperature and incubation time affected ($P < 0.002$) LDH activity, *HSP70* and *HSP90* mRNA expressions (Table 3). Compared with NT, HT had no effect ($P > 0.05$) on LDH activity when cells were treated for 1 and 2 h, but increased ($P < 0.0001$) LDH activity at 4, 6 or 8 h of incubation. Under NT, incubation time had no effect ($P > 0.95$) on *HSP70* and *HSP90* mRNA expressions. However, under HT, heat exposure time increased ($P < 0.034$) cellular *HSP70* mRNA expression within 4 h with a peak at 4 h followed by a gradual decline, and it also increased ($P < 0.023$) cellular *HSP90* mRNA expression within 6 h with a peak at 6 h followed by a significant ($P < 0.0008$) decline. Overall, on the base of the consideration of continued viability of cells, the optimal heat exposure time for responses of *HSP70* and *HSP90* mRNA to HT was 4 or 6 h.

3.3. Effects of Zn source, incubation time and culture temperature on the antioxidant status and HSP expressions of primary cultured hepatocytes of broiler embryos (Exp. 3)

The MDA content and *CuZnSOD* mRNA expression were affected ($P < 0.01$) by culture temperature. Compared with NT, HT increased ($P < 0.01$) MDA content and *CuZnSOD* mRNA expression. For CuZnSOD activity, an interaction ($P < 0.04$) between culture temperature and Zn source was observed (Table 4). At NT, iZn increased ($P < 0.005$) CuZnSOD activity compared with the CON, with no differences ($P > 0.09$) between the 2 Zn sources and between the CON and oZn; however, at HT, oZn increased ($P < 0.05$) CuZnSOD activity compared with the CON and iZn, with no difference ($P > 0.47$) between the CON and iZn (Table 5). As for the *MT* mRNA expression, a three-way interaction ($P < 0.01$) among incubation time, culture temperature and Zn source were observed (Table 4). At NT and for either 4 or 6 h of incubation time, iZn increased ($P < 0.0004$) *MT* mRNA expression compared with the CON and oZn, with no difference ($P > 0.42$) between the CON and oZn. The similar results were observed in hepatocytes subjected to HT for 6 h. However, when hepatocytes were exposed to HT for 4 h, either iZn or oZn increased ($P < 0.004$) *MT* mRNA expression compared with the CON, with no difference ($P > 0.93$) between the 2 Zn sources. Overall, HT caused oxidative damage, whereas the addition of Zn as either source might enhance hepatic antioxidant ability of chick hepatocytes.

The incubation time × culture temperature interaction ($P = 0.006$) and the culture temperature × Zn source interaction ($P = 0.029$) were detected for *HSP70* mRNA expression (Table 6). Compared with NT, HT greatly increased ($P < 0.0001$) *HSP70* mRNA expression (Tables 7 and 8). Under NT, there were no differences ($P > 0.07$) between 4 and 6 h of incubation time and among the 3 Zn source treatments, however, at HT, *HSP70* mRNA expression decreased ($P < 0.03$) over time (Table 7), and iZn reduced ($P < 0.05$) *HSP70* mRNA expression compared with the CON and oZn, with no difference ($P > 0.85$) between the CON and oZn (Table 8). A three-way interaction among incubation time, culture temperature and Zn source affected ($P < 0.04$) the expressions of HSP70 protein and *HSP90* mRNA (Table 6). Compared with NT, for either 4 or 6 h of incubation time, HT largely increased ($P < 0.0001$) the expression levels of HSP70 protein (also as shown in Fig. 1) and *HSP90* mRNA. At NT, for either 4 or 6 h of incubation time, no differences ($P > 0.70$) in HSP70 protein expression were observed among the 3 Zn source treatments; however, at HT, either iZn or oZn decreased ($P < 0.0001$) HSP70 protein expression at 6 h of heat exposure, with no differences ($P > 0.33$) between the 2 Zn sources at 6 h and among the 3 Zn source treatments at 4 h. At NT, for either 4 or 6 h of incubation time, oZn decreased ($P < 0.005$) *HSP90* mRNA expression compared with the CON and iZn, with no difference ($P > 0.15$) between the CON and iZn; however, at HT, iZn decreased ($P < 0.0003$) *HSP90* mRNA expression compared with the CON and oZn at 4 h of heat exposure, with no differences ($P > 0.58$) between the CON and oZn at 4 h and among the 3 Zn source treatments at 6 h. Overall, HT largely induced heat shock responses, whereas the addition of Zn as

Table 3
Effects of culture temperature (CT) and incubation time (IT) on LDH activity in the culture medium and mRNA expressions of *HSP70* and *HSP90* in primary cultured hepatocytes of broiler embryos (Exp. 2).

Item	IT, h	LDH activity, U/L	*HSP70* mRNA[1], RQ	*HSP90* mRNA[1], RQ
CT [2]				
NT	1	471 ± 13.3 ^{cd}	0.118 ± 0.016^{c}	0.208 ± 0.040^{c}
	2	429 ± 12.8^{d}	0.115 ± 0.015^{c}	0.208 ± 0.070^{c}
	4	455 ± 33.2 ^{cd}	0.138 ± 0.012^{c}	0.325 ± 0.033^{c}
	6	460 ± 18.5 ^{cd}	0.155 ± 0.018^{c}	0.193 ± 0.060^{c}
	8	503 ± 24.6^{c}	0.150 ± 0.019^{c}	0.277 ± 0.056^{c}
HT	1	424 ± 4.49^{d}	3.12 ± 0.19^{c}	2.04 ± 0.39^{c}
	2	426 ± 16.8^{d}	11.8 ± 3.00^{b}	7.65 ± 2.64^{b}
	4	635 ± 15.6^{b}	28.3 ± 6.76^{a}	7.12 ± 1.05^{b}
	6	663 ± 14.4^{b}	27.2 ± 4.00^{a}	14.2 ± 1.88^{a}
	8	755 ± 15.7^{a}	22.6 ± 4.08^{a}	7.29 ± 2.55^{b}
CT [3]				
NT		464 ± 9.71	0.135 ± 0.007	0.242 ± 0.024
HT		581 ± 25.4	18.6 ± 2.46	7.66 ± 1.10
IT [4], h				
1		448 ± 9.25	1.62 ± 0.46	1.13 ± 0.33
2		427 ± 10.1	5.97 ± 2.27	3.93 ± 1.69
4		545 ± 31.3	14.2 ± 5.21	3.72 ± 1.10
6		561 ± 32.6	13.7 ± 4.50	7.19 ± 2.29
8		629 ± 40.5	11.3 ± 3.90	3.78 ± 1.61
P-value				
CT		<0.0001	<0.0001	<0.0001
IT		<0.0001	0.0002	0.0019
CT × IT		<0.0001	0.0001	0.0018

LDH = lactate dehydrogenase; *HSP70* = heat-shock protein 70; *HSP90* = heat-shock protein 90; NT = normal temperature, 40 °C; HT = high temperature, 44 °C.
[a, b, c, d] Mean values within a column with unlike superscript letters are significantly different ($P < 0.05$).
[1] The *HSP70* or *HSP90* mRNA abundances were calculated as the relative quantity (RQ) of the *HSP70* or *HSP90* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; $RQ = 2^{-\Delta\Delta Ct}$. (Ct = threshold cycle).
[2] Each value represents the mean ± SE of 6 replicates ($n = 6$).
[3] Each value represents the mean ± SE of 30 replicates ($n = 30$).
[4] Each value represents the mean ± SE of 12 replicates ($n = 12$).

either source might attenuate the heat shock responses of chick hepatocytes.

4. Discussion

Chickens are highly susceptible to heat stress and have a narrow thermal comfort zone. Under the hot environment, the birds are prone to heat stress which would reduce growth performance and meat quality. In broilers, we have demonstrated that dietary supplementation of Zn could alleviate the adverse effects of heat stress (Zhu et al., 2017a, 2017b). However, the exact mechanisms of the protective effects of Zn on broilers are unclear. Our hypothesis that Zn, especially the organic Zn with a moderate chelation strength, might protect primary cultured hepatocytes of broiler embryos against heat stress via enhancing the antioxidant ability and attenuating the heat shock responses has been supported by the results in the present study. The present study has demonstrated that either iZn or oZn supplementation could alleviate the adverse effects of heat stress on primary cultured hepatocytes of broiler embryos, and further indicated that Zn might exert its anti-heat stress effects via enhancing the antioxidant defense system and attenuating the heat shock responses of the above primary cultured hepatocytes. Furthermore, oZn appeared to be more effective than iZn in improving the antioxidant status of hepatocytes under high temperature. The aforementioned findings might provide a more effective nutritional strategy for protecting broilers from heat stress.

The liver is not only a key organ in chickens' response to heat stress (Lin et al., 2006; Mackei et al., 2020), but also a sensitive tissue to reflect the Zn status of broilers (Azad et al., 2017). And also, evidence suggests that liver would be the most sensitive organ synthesizing HSP in response to hyperthermia (Xie et al., 2014) and also expressing high antioxidant enzyme activities (Azad et al., 2017) in broilers. Therefore, the primary cultured broiler

Table 4
Effects of zinc source, incubation time (IT) and culture temperature (CT) on the antioxidant status of primary cultured hepatocytes of broiler embryos (Exp. 3).

Item	CT	Zn source	MDA content, nmol/mg prot.	MT content, pg/mg prot.	CuZnSOD activity, U/mg prot.	*CuZnSOD* mRNA[1], RQ	*MT* mRNA[1], RQ
IT [2]							
4 h	NT	CON	0.580 ± 0.040	98.2 ± 6.96	59.7 ± 1.63	0.857 ± 0.164	0.651 ± 0.161^{d}
		iZn	0.627 ± 0.051	109 ± 5.78	69.5 ± 1.96	0.840 ± 0.055	2.01 ± 0.35^{b}
		oZn	0.567 ± 0.055	102 ± 4.74	63.0 ± 3.11	1.06 ± 0.15	0.681 ± 0.046^{d}
	HT	CON	0.713 ± 0.057	96.4 ± 6.18	57.9 ± 2.92	1.26 ± 0.17	0.521 ± 0.083^{d}
		iZn	0.675 ± 0.045	89.4 ± 4.94	62.5 ± 1.12	0.956 ± 0.071	1.65 ± 0.26 ^{bc}
		oZn	0.818 ± 0.106	102 ± 4.58	70.2 ± 4.25	1.27 ± 0.15	1.68 ± 0.35 ^{bc}
6 h	NT	CON	0.827 ± 0.049	92.5 ± 7.85	57.8 ± 2.87	0.756 ± 0.083	0.497 ± 0.068^{d}
		iZn	0.636 ± 0.029	96.7 ± 7.64	63.3 ± 1.60	0.850 ± 0.122	1.75 ± 0.21^{b}
		oZn	0.570 ± 0.040	104 ± 10.7	60.9 ± 2.60	0.850 ± 0.103	0.785 ± 0.060^{d}
	HT	CON	0.850 ± 0.099	93.3 ± 5.23	60.8 ± 2.83	1.23 ± 0.21	0.624 ± 0.075^{d}
		iZn	0.813 ± 0.080	96.4 ± 3.80	60.0 ± 2.42	1.36 ± 0.09	3.06 ± 0.60^{a}
		oZn	0.855 ± 0.012	100 ± 5.33	62.8 ± 2.52	1.48 ± 0.27	1.00 ± 0.08 ^{cd}
IT [3]							
4 h			0.663 ± 0.028	99.5 ± 2.35	63.8 ± 1.28	1.04 ± 0.06	1.20 ± 0.14
6 h			0.759 ± 0.031	97.2 ± 2.72	60.9 ± 1.00	1.09 ± 0.08	1.29 ± 0.18
CT [3]							
NT			0.634 ± 0.017^{b}	100 ± 2.97	62.4 ± 1.09	0.868 ± 0.047^{b}	1.06 ± 0.12
HT			0.788 ± 0.030^{a}	96.3 ± 2.04	62.4 ± 1.25	1.26 ± 0.07^{a}	1.42 ± 0.19
Zn source [4]							
CON			0.743 ± 0.038	95.1 ± 3.07	59.1 ± 1.25	1.02 ± 0.09	0.575 ± 0.051
iZn			0.688 ± 0.030	97.9 ± 3.05	63.8 ± 1.13	1.00 ± 0.06	2.12 ± 0.21
oZn			0.703 ± 0.041	102 ± 3.19	64.2 ± 1.66	1.16 ± 0.10	1.04 ± 0.12
P-value							
IT			0.069	0.571	0.061	0.573	0.565
CT			0.006	0.250	0.998	<0.0001	0.018
Zn source			0.695	0.320	0.012	0.256	<0.0001
IT × CT			0.879	0.434	0.733	0.089	0.207
CT × Zn source			0.299	0.510	0.037	0.813	0.221
IT × CT × Zn source			0.619	0.695	0.323	0.611	0.004

MDA = malondialdehyde; MT = metallothionein; CuZnSOD = copper zinc superoxide dismutase; NT = normal temperature, 40 °C; HT = high temperature, 44 °C; CON = no Zn addition; iZn = inorganic Zn sulphate; oZn = organic Zn lysine chelate.
[a, b, c, d] Mean values within a column with unlike superscript letters are significantly different ($P < 0.05$).
[1] The *CuZnSOD* or *MT* mRNA abundances were calculated as the relative quantity (RQ) of the *CuZnSOD* or *MT* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; RQ = $2^{-\Delta\Delta Ct}$ (Ct = threshold cycle).
[2] Each value represents the mean ± SE of 6 replicates ($n = 6$).
[3] Each value represents the mean ± SE of 36 replicates ($n = 36$).
[4] Each value represents the mean ± SE of 24 replicates ($n = 24$).

Table 5
Effects of culture temperature (CT) and zinc source on CuZnSOD activity of primary cultured hepatocytes of broiler embryos (Exp. 3).[1]

Item	NT			HT			*P*-value
	CON	iZn	oZn	CON	iZn	oZn	
CuZnSOD, U/mg prot.	58.8 ± 1.59^{c}	66.4 ± 1.52 ^{ab}	62.0 ± 1.96 ^{abc}	59.4 ± 1.98^{c}	61.2 ± 1.33 ^{bc}	66.5 ± 2.61^{a}	0.037

NT = normal temperature, 40 °C; HT = high temperature, 44 °C; CON = no Zn addition; iZn = inorganic Zn sulphate; oZn = organic Zn lysine chelate; CuZnSOD = copper zinc superoxide dismutase.
[a, b, c] Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).
[1] Each value represents the mean ± SE of 12 replicates ($n = 12$).

Table 6
Effects of zinc source, incubation time (IT) and culture temperature (CT) on the expressions of heat stress proteins in primary cultured hepatocytes of broiler embryos (Exp. 3).

Item	CT	Zn source	*HSP70* mRNA[1], RQ	*HSP90* mRNA[1], RQ	HSP70 protein[2], AU	HSP90 protein[2], AU
IT[3]						
4 h	NT	CON	0.044 ± 0.012	0.255 ± 0.051^{c}	0.188 ± 0.028^{c}	0.977 ± 0.174
		iZn	0.069 ± 0.021	0.169 ± 0.020^{c}	0.192 ± 0.015^{c}	1.06 ± 0.19
		oZn	0.049 ± 0.008	0.117 ± 0.014^{d}	0.155 ± 0.020^{c}	1.11 ± 0.21
	HT	CON	27.3 ± 4.91	10.5 ± 1.78^{a}	1.22 ± 0.18^{b}	1.03 ± 0.18
		iZn	14.9 ± 1.84	4.22 ± 0.93^{b}	1.21 ± 0.15^{b}	1.20 ± 0.12
		oZn	26.8 ± 4.77	9.77 ± 1.44^{a}	1.19 ± 0.21^{b}	1.23 ± 0.14
6 h	NT	CON	0.083 ± 0.005	0.266 ± 0.043^{c}	0.168 ± 0.019^{c}	0.992 ± 0.094
		iZn	0.069 ± 0.010	0.203 ± 0.039^{c}	0.245 ± 0.063^{c}	1.23 ± 0.15
		oZn	0.045 ± 0.005	0.076 ± 0.006^{d}	0.162 ± 0.029^{c}	0.855 ± 0.114
	HT	CON	16.7 ± 2.95	6.96 ± 1.14^{a}	2.23 ± 0.40^{a}	1.31 ± 0.22
		iZn	14.1 ± 3.31	5.86 ± 0.86^{a}	1.24 ± 0.19^{b}	1.13 ± 0.08
		oZn	16.8 ± 2.32	5.89 ± 0.62^{a}	1.03 ± 0.05^{b}	1.02 ± 0.15
IT [4]						
4 h			11.5 ± 2.33	4.17 ± 0.84	0.691 ± 0.099	1.10 ± 0.07
6 h			8.04 ± 1.56	3.21 ± 0.57	0.847 ± 0.144	1.09 ± 0.05
CT[4]						
NT			0.123 ± 0.005	0.181 ± 0.017	0.185 ± 0.014	1.04 ± 0.06
HT			19.4 ± 1.62	7.20 ± 0.59	1.35 ± 0.11	1.15 ± 0.06
Zn source [5]						
CON			11.1 ± 2.78	4.49 ± 1.04	0.951 ± 0.204	1.08 ± 0.08
iZn			7.29 ± 1.75	2.61 ± 0.60	0.722 ± 0.120	1.15 ± 0.07
oZn			10.9 ± 2.68	3.96 ± 0.93	0.634 ± 0.110	1.05 ± 0.08
P-value						
IT			0.743	0.248	0.064	0.846
CT			<0.0001	<0.0001	<0.0001	0.194
Zn source			0.512	0.0001	0.016	0.671
IT × CT			0.006	0.733	0.111	0.848
CT × Zn source			0.029	<0.0001	0.013	0.771
IT × CT × Zn source			0.241	0.033	0.006	0.372

HSP70 = heat-shock protein 70; HSP90 = heat-shock protein 90; NT = normal temperature, 40 °C; HT = high temperature, 44 °C; CON = no Zn addition; iZn = inorganic Zn sulphate; oZn = organic Zn lysine chelate.
[a, b, c, d] Mean values within a column with unlike superscript letters are significantly different ($P < 0.05$).
[1] The *HSP70* or *HSP90* mRNA abundances were calculated as the relative quantity (RQ) of the *HSP70* or *HSP90* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; RQ = $2^{-\Delta\Delta Ct}$ (Ct = threshold cycle).
[2] The *GAPDH* was used to normalize the expression level of the *HSP70* or *HSP90* as an arbitrary unit (AU).
[3] Each value represents the mean ± SE of 6 replicates ($n = 6$).
[4] Each value represents the mean ± SE of 36 replicates ($n = 36$).
[5] Each value represents the mean ± SE of 24 replicates ($n = 24$).

hepatocytes may be a good stress model, which could be used to evaluate the protective effects and elucidate the mechanisms of Zn against heat stress on the liver tissue. The CuZnSOD and MT are important endogenous antioxidants that scavenge ROS, and their expression levels reflect the antioxidant ability of cells (Alscher et al., 2005; Volkova et al., 2012). In Exp. 1, we found that the optimal responses of CuZnSOD and MT occurred when hepatocytes were exposed to Zn ($ZnSO_4$; 50 μmol/L) for 12 h, suggesting that a Zn concentration of 50 μmol/L and an incubation time of 12 h could be the optimal condition necessary to strengthen the antioxidant defense system of hepatocytes. Both *HSP70* and *HSP90* are useful biomarkers for assessing cellular potential heat damages (Tedeschi et al., 2015). The results from Exp. 2 showed that HT affected *HSP70* and *HSP90* mRNA expressions of hepatocytes, and the heat exposure time of either 4 or 6 h with HT treatment was effective in increasing the mRNA expressions of the 2 genes. Taken together, primary broiler hepatocytes could be an effective model for further exploring the protective mechanisms of Zn against heat stress.

Heat stress is believed to promote the generation of ROS thereby causing oxidative stress in broilers (Lin et al., 2006; Tan et al., 2010; Xie et al., 2015) and laying hens (Lin et al., 2008; Naziroglu et al., 2000). The increased level of lipid peroxide (marked by MDA) in heat-stressed hepatocytes observed in the present study indicated the involvement of free radicals and confirmed a state of oxidative stress (Habashy et al., 2019). However, at the same time, heat stress led to up-regulation of genes that encode for CuZnSOD and MT in heat-stressed hepatocytes. According to previous studies, the up-regulation of CuZnSOD and MT is the mechanism utilized by cells to manage potential cytotoxicity induced by heat stress (Habashy et al., 2018; Wang et al., 2016). Therefore, the damage to the hepatocellular membrane resulting from free radicals may have induced the corresponding increase in its antioxidant response owing to the cellular self-protective function during heat stress. In addition to antioxidant genes, rapidly synthesized HSP are another endogenous mechanism by which living cells adapt to heat stress (Garbuz et al., 2017). Evidence suggests that HSP, especially HSP70, might be involved in the development of thermotolerance in broiler chickens (Vinoth et al., 2018; Yahav et al., 1997). Overexpression of *HSP70* obviously improved the antioxidant defense system of broilers during heat stress (Gu et al., 2012). In the present study, abrupt rises in *HSP70* mRNA and protein expression levels, as well as *HSP90* mRNA expression in hepatocytes exposed to heat stress, might favour an anti-heat stress response. However, this up-regulated response might depend on the presence of ROS and/or oxidant stress induced by heat stress (Slimen et al., 2014). Overall, the above results suggest that heat stress might interrupt the redox balance and HSP homoeostasis, thereby causing adverse effects on broilers and primary cultured hepatocytes of broiler embryos.

Zinc is an important nutritional antioxidant trace element that can help to eliminate free radicals via Zn-containing enzymes and proteins. Numerous investigators have noted that Zn is a crucial component of CuZnSOD, and the primary enzyme in cells that plays a fundamental role in the detoxification of superoxide free radicals and in the protection of cells against oxidative stress (Yuan et al.,

Table 7
Effects of culture temperature (CT) and incubation time (IT) on *HSP70* mRNA expression of primary cultured hepatocytes of broiler embryos (Exp. 3).[1]

Item	NT		HT		*P*-value
	4 h	6 h	4 h	6 h	
HSP70 mRNA[2], RQ	0.054 ± 0.008[c]	0.066 ± 0.005[c]	23.0 ± 2.47[a]	15.9 ± 1.47[b]	0.006

NT = normal temperature, 40 °C; HT = high temperature, 44 °C; *HSP70* = heat-shock protein 70.
[a, b, c] Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).
[1] Each value represents the mean ± SE of 18 replicates ($n = 18$).
[2] The *HSP70* mRNA abundances were calculated as the relative quantity (RQ) of the *HSP70* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; RQ = $2^{-\Delta\Delta Ct}$ (Ct = threshold cycle).

Table 8
Effects of culture temperature (CT) and zinc source on *HSP70* mRNA expression of primary cultured hepatocytes of broiler embryos (Exp. 3).[1]

Item	NT			HT			*P*-value
	CON	iZn	oZn	CON	iZn	oZn	
HSP70 mRNA[2], RQ	0.062 ± 0.009[c]	0.069 ± 0.011[c]	0.047 ± 0.004[c]	22.0 ± 3.16[a]	14.5 ± 1.81[b]	21.8 ± 2.94[a]	0.029

NT = normal temperature, 40 °C; HT = high temperature, 44 °C; CON = no Zn addition; iZn = inorganic Zn sulphate; oZn = organic Zn lysine chelate; *HSP70* = heat-shock protein 70.
[a, b, c] Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).
[1] Each value represents the mean ± SE of 12 replicates ($n = 12$).
[2] The *HSP70* mRNA abundances were calculated as the relative quantity (RQ) of the *HSP70* mRNA to the geometric mean of β-actin and *GAPDH* mRNA; RQ = $2^{-\Delta\Delta Ct}$ (Ct = threshold cycle).

2011). Further evidence shows that the activity of CuZnSOD can be regulated by the availability of its component trace elements, including Zn and Cu ions (Kopeć et al., 2013). The present study showed that the addition of Zn as either iZn or oZn significantly increased CuZnSOD activity of hepatocytes. Our results are consistent with Azad et al. (2017) and Liu et al. (2015), who reported that organic or inorganic Zn addition in the feed elevated CuZnSOD activity in the liver of broilers. Moreover, our results indicated that oZn was more effective than iZn in improving antioxidant status of heat-stressed hepatocytes with elevated CuZnSOD activity, which might be attributed to the fact that the bioavailability of moderately chelated organic Zn is higher than that of inorganic $ZnSO_4$ for broilers (Huang et al., 2008; Huang et al., 2013; Yu et al., 2010, 2017). Consistent with this explanation, Zhang et al. (2017) reported that organic Zn glycinate had a better effect on hepatic antioxidant status than inorganic $ZnSO_4$ in broiler breeders, due to the increased Zn retention in the liver resulting from a higher bioavailability of Zn glycinate. Additionally, Zn is an effective inducer of MT, a cysteine-rich protein acting as a free radical scavenger (Alscher et al., 2005). In vivo, previous researches have indicated that dietary supplementation with Zn could increase *MT* mRNA expression in the liver of broilers (Azad et al., 2017; Zhang et al., 2017; Zhu et al., 2017b). In vitro, it has been reported that the induction of MT conferred protection against ROS in liver (Garla et al., 2017), kidney (Sharma et al., 2002) and islet (Li et al., 2004) cells. In the present study, either iZn or oZn increased *MT* mRNA expression in heat-stressed hepatocytes, suggesting that Zn-treated hepatocytes might be better prepared to deal with oxidative stress induced by heat stress.

Heat shock, as a promoter of oxidative stress, creates a redox imbalance by increasing the generation of ROS (Yang et al., 2010). Subsequent cellular damage caused by the accumulation of ROS has been considered as a key factor for the activation of *HSP* genes (Mahmoud et al., 2003; Sahin et al., 2003). When cells are subjected to heat shock with an increase in lipid peroxidation, HSP accumulate and might serve as a type of tissue biomarkers for potential stress damage (Tedeschi et al., 2015). Thus, HSP expression levels might be regarded as a kind of responses to damage resulting from a strong stress to the organism (Burdon et al., 1987). In addition, the increased activities of SOD and catalase likely scavenge free radicals that inhibit the expression of HSP proteins and improve cell survival (Omar et al., 1993). In the present study, the addition of Zn as either iZn or oZn could attenuate heat shock responses of hepatocytes exposed to heat stress, which was evidenced by the decrease in the expressions of *HSP70* mRNA and protein, as well as *HSP90* mRNA. The possible reasons may be that Zn enhanced CuZnSOD activity and MT expression, thereby lowering ROS levels and weakening stress-

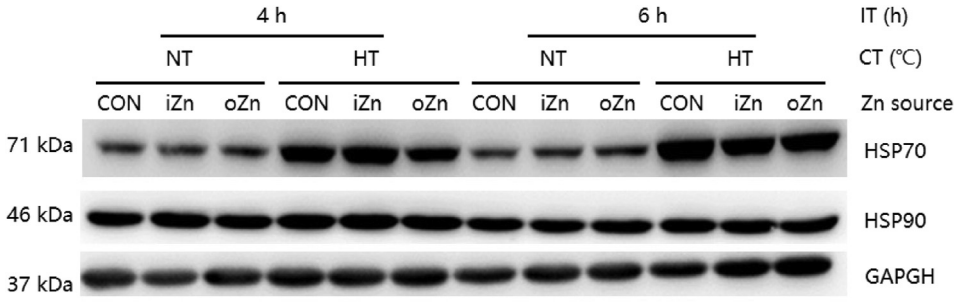

Fig. 1. Representative immunoblots demonstrating heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90) of primary cultured hepatocytes of broiler embryos subjected to varying incubation time (IT), culture temperature (CT) and Zn source (Exp. 3). NT = normal temperature, 40 °C; HT = high temperature, 44 °C; CON = no Zn addition; iZn = inorganic Zn sulphate; oZn = organic Zn lysine chelate; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

induced heat shock responses. Similarly, it has been reported that the reduced induction of HSP70 protein occurred in selenium -fed chickens because of the higher glutathione peroxidase activity in the liver (Mahmoud et al., 2003). Increased activity of manganese superoxide dismutase in manganese-fed broilers was also accompanied by a decline of HSP70 expression under high temperatures (Zhu et al., 2015a).

5. Conclusions

This study revealed that heat stress-induced oxidative damage evidenced by the elevated MDA content and the up-regulated expressions of *HSP*70 mRNA and protein, as well as *HSP*90 mRNA. Zinc supplementation enhanced antioxidant ability with increased CuZnSOD activity and up-regulated *MT* mRNA expression against oxidative damage, and effectively attenuated the heat shock responses with down-regulated expressions of *HSP*70 mRNA and protein, as well as *HSP*90 mRNA. Furthermore, oZn appeared to be more effective than iZn in improving the antioxidant status of hepatocytes at high temperatures. It is suggested that Zn, especially oZn, could alleviate the heat stress of primary cultured hepatocytes of broiler embryos via enhancing the antioxidant ability and attenuating the heat shock responses of above primary hepatocytes. Considering that the in vitro cellular model cannot completely mimic the in vivo environment, additional in vivo studies are necessary to verify the protective effect of supplemental Zn on the liver of broilers at high temperatures.

Author contributions

T. T. Li: data curation, writing - original draft preparation. **W. G. He**: investigation. **X. D. Liao**: formal analysis. **X. Lin**: conceptualization. **L. Y. Zhang**: investigation. **L. Lu**: methodology. **Y. L. Guo**: methodology. **Z. P. Liu**: resources. **X. G. Luo**: supervision, writing - review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

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