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RESEARCH ARTICLE

Effects of Selenium-Enriched Probiotics on Heart Lesions by Influencing the mRNA Expressions of Selenoproteins and Heat Shock Proteins in Heat Stressed Broiler Chickens

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ABSTRACT

Selenium is one of the most vital trace elements regulating various body functions. Herein, we observe the effects of selenium-enriched probiotics on heart lesion in broiler chickens under high ambient temperature and explore the underlying mechanisms. Four different groups of broiler chickens were fed a corn-soybean basal diet having no Se supplementation (Con group), basal diet with the addition of probiotics (P group), a basal diet with Se supplementation in the form of sodium selenite (SS group, 0.30mg Se/kg) and basal diet with the addition of selenium enriched probiotics (SP group, 0.30mg Se/kg). The results showed that P, SS, or SP supplementation significantly ($P<0.05$) up-regulated mRNA expression of selenoproteins (GPx1, GPx4) and down-regulated heat shock proteins (Hsp60, Hsp70 and Hsp90) in heart as compared to Con, P and SS diets. Herein, we suggest that SP product can serve as a feasible nutritive supplement, capable of protecting the heart from toxic effect of oxidative stress in summer season.

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INTRODUCTION

Heat stress is considered as a major environmental factor affecting commercial poultry production and resulting in major economic losses to the poultry industry by reducing growth rate, feed consumption and higher mortality rate (Yu *et al*., 2008). Broiler chickens produce more body heat due to higher metabolic activities. Good environmental condition is essential for poultry welfare and production (Lara and Rostagno, 2013). Therefore, it is a major concern for the poultry industry, especially in tropical regions around the globe (Hao and Gu, 2014).

Most organisms (including poultry) respond to heat stress by inducing the synthesis of a group of eternally conserved stress-modulated proteins known as heat shock proteins (Hsps). The expression of Hsps is up-regulated in response to high temperature. One of the most important functions of Hsps is to protect organisms from the toxic effects of heat stress by elevating antioxidant enzyme activities and immune system (Yu *et al*., 2008; Huang *et al*., 2011). Hsps are classified into four major groups on the basis of their molecular weight such as small Hsps, Hsp60, Hsp70, and Hsp90 (Chen *et al*., 2014). The elevations of Hsps in heart act as important biological markers and provide protection against stress-induced myocardial injuries (Yu *et al*., 2008; Yu and Bao, 2008).

Selenium (Se) is a basic micronutrient for all species of mammalian and well known for its antioxidant properties by mimicking various selenoproteins (GPx1, GPx4) and enzymes. The main biological functions of Se are in the form of selenoproteins, and involve in destroying lipid hydroperoxides and H_2O_2. Glutathione peroxidases (GPxs) are known antioxidant containing Se in the form of selenoproteins (Huang *et al*., 2012). It is well documented that selenoproteins (GPx1 and GPx4) inserts Se into the amino acid selenocysteine (organic Se) and incorporate it as a 21st amino acid that make these proteins as good candidates for prevention of tissues against oxidative injury (Bellinger *et al*., 2009a; Gan *et al*., 2014; Bai *et al*., 2014). Oxidative stress may influence the degeneration of vascular endothelial cells and exacerbate cardiovascular diseases such as congestive heart failure, hypertension and atherosclerosis, (Lum and Roebuck, 2001). Selenoproteins are crucially involved in

the cellular antioxidant defense system, thus using selenium for prevention of cardiovascular diseases (de Haan *et al*., 2006; Zhang *et al*., 2013).

Our group has developed several new products that can combat heat stress (Gan *et al*., 2013). Likewise, Se-enriched probiotics (SP) were prepared by aerobic fermentation of the two probiotics strains *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* with the addition of sodium selenite (SS, inorganic Se) under appropriate conditions. The bacterial strains of both products have good ability to bind, uptake and biotransform inorganic Se (SS, sodium selenite) to organic Se (Se-methionine and Se-cystine) (Gan *et al*., 2013 and 2014; Liu *et al*., 2014). As reported previously, that SP supplementation can attenuate heat stress by scavenging free radicals in pigs through its strong antioxidant capacity as compared to other diets at high ambient temperature (Gan *et al*., 2013). It can also influence the expression of Hsps (e.g. Hsp27 and Hsp70) in liver, kidney and spleen of pigs under heat stress conditions (Gan *et al*., 2013). The SP may protect the liver from inflammation and fibrosis by ameliorating the negative effects of stress due to oxidation in rats (Liu *et al*., 2014; Nido *et al*., 2015). SP can also enhance the egg quality in layers (Pan *et al*., 2011). The toxic influence of heat stress on the health and production of broilers may be reducing by the oral supplementation of antioxidant preparations (e.g. Selenium-enriched probiotics). Till date, no reports are available on SP in relation to histopathalogical changes, mRNA expression levels of selenoproteins (GPx1 and GPx4) and heat shock proteins (Hsp60, Hsp70 and Hsp90) in heart of broiler chickens under heat stressed condition. Therefore, we investigated the effect of SP on the said parameters in broiler chickens reared under high ambient temperature in summer.

MATERIALS AND METHODS

Selenium-enriched probiotics, sodium selenite and probiotics: Both products **(**SP and P) were prepared in Institute of Nutritional and Metabolic Disorders of Domestic Animals and Fowls, Nanjing Agricultural University (China), in fermented forms. Each product contains two types of probiotic strains i.e. *L. acidophilus and S. cerevisiae*. The SP product was prepared by aerobic fermentation with the addition of sodium selenite (SS) under appropriate conditions. The total Se content in SP product was 10.0 mg/L, were determined by the AF-610A atomic fluorescence spectrometer (Ran *et al*., 2010). In this study sodium selenite (SS) used as a stock solution, having (100 mg/L) total Se.

Experimental design: Two hundred 1-day-old male broiler chicks (Ross 308) were allotted randomly to four groups, each group having five replicates, containing 10 birds per replicate and fed a corn-soybean basal diet having no Se supplementation (Con), a basal diet with the addition of Se in the form of sodium selenite (SS), supplementation probiotics (P) in basal diet and basal diet with the addition of selenium-enriched probiotics (SP), for 42 days. The basal diets were already containing 0.11 mg Se/kg feed, whereas the sodium selenite and selenium enriched probiotics increased the Se concentration from 0.11 to 0.41 mg Se/kg feed by supplementing 0.30 mg Se/kg feed as addition. The basal diets were prepared according to the guidelines of National Research Council (Sell, 1994) (Table 1). The Se concentrations in basal diet and supplement samples were detected by HG-AFS (hydride generation atomic fluorescence spectrometry) method as previously described (Gamiz-Gracia and De Castro, 1999) to confirm the calculated concentration (Table 2).

Husbandry practices: All protocols for conducting the experimental animals were confirmed by the selected committee for dealing the animals used for experimental purpose of Nanjing Agriculture University (Animal Ethical Number: SYXK (Su) 2011-0036). The experiments were carried out for 42 days (in months of June and July) of summer. The daily relative humidity inside the shed ranged between 60 to 80%. Feed and water were provided *ad libitum*.

Sample collection: At 42 days of age, one-fourth of the heart was quickly removed and kept at −70°C in liquid nitrogen, for further analysis. The heart tissue was collected and prepared for histopathology examination according to the procedure described earlier (Nido *et al*., 2015).

Reverse transcription quantitative PCR (qPCR): The mRNA expression of selenoproteins (GPx1 and GPx4) and heat shock proteins (Hsp60, Hsp70 and Hsp90) were quantitatively analyzed by real-time PCR. The primers of the GAPDH (reference gene) and target genes are shown in Table 3. The total RNA was isolated from the frozen heart sample according to the protocol. The real-time PCR was performed as described previously (Gan *et al*., 2013) with some changes. Reactions were carried out in a 25μL reaction mixture containing 12.5μL of 2×SYBR Green I PCR Master Mix (TaKaRa BIO INC), 1μL of each primer (10μM), 10μL of cDNA, and 0.5μL of PCR grade water. The whole processes were conducted in an ABI Prism 7300 Detection System (Applied Biosystems, USA). Relative mRNA expression levels of the above genes were detected using the Δ^{Ct} (Δ cycle threshold) procedure (Gan *et al*., 2014). The result was applied to each gene by calculating the expression $2^{-\Delta\Delta CT}$. The reactions were performed in duplicate.

Statistical analysis: The results are expressed as the mean values with their standard errors. SPSS Statistics version 19 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Significant differences were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan post hoc test. A $P<0.05$ was considered as statistically significant.

RESULTS

Effect of SP on cardiac tissue histopathology: Histopathalogically, the cytoplasm of myocytes of broiler chickens under heat stressed condition showed slightly enlarged intracellular spaces, light pink granulation, and loss of striations in P and SS group as compared to Con. Noteworthy, no obvious histology lesions were found in the SP group (Fig. 1).

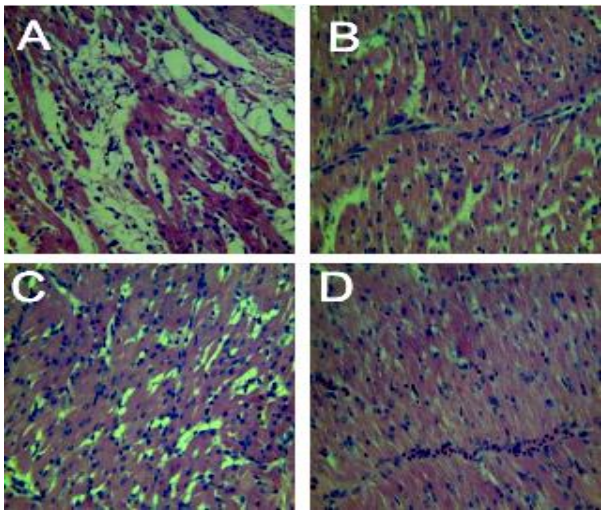

Fig. 1: Histopathalogically, the cytoplasm of myocytes of broiler chickens under heat stressed condition showed slightly enlarged intracellular spaces, light pink granulation, and loss of striations in probiotics (B) and sodium selenite (C) group as compared to control (Con). Noteworthy, no obvious histology lesions were found in the selenium enriched probiotics (D) group. It was stained with hematoxylin and eosin (H&E) and magnification Bar: 40µm (A, B, C, D).

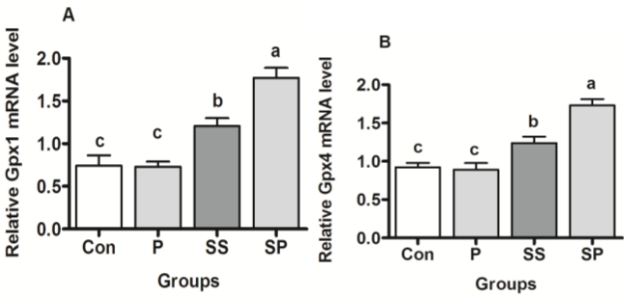

Fig. 2: Effect of P, SS and SP on mRNA levels of (A) GPx1 and (B) GPx4 in heart of heat stressed Ross male broiler chickens. The Values with unlike superscript letters (a, b, c) in the graph were different significantly (P<0.05).

Effect of SP on the mRNA expression of selenoproteins genes in heart: The selenoproteins (GPx1and GPx4) mRNA expressions were observed in the heart. The mRNA expression of GPx1and GPx4 were up-regulated (P<0.05) in SP and SS groups as compared to Con and P group. In addition, the mRNA expression of these two antioxidant-related genes showed a better effect (P<0.05) in SP group when compared with SS group. However, no significant differences were found in mRNA expression of GPx1and GPx4 between Con and P groups (Fig. 2 A, B).

Effect of SP on heat shock proteins mRNA expression in heart: Figs. 3 A-C show that mRNA expression of Hsp60, Hsp70 and Hsp90 in heart of broiler chickens were significantly down-regulated (P<0.05) by adding of P, SS or SP in diet as compared to Con group, respectively. In addition, no significant differences were observed in the down-regulation of Hsp60, Hsp70 and Hsp90 mRNA expression between P and SS groups. We found a significant difference in the down-regulation (P<0.05) of Hsp60, Hsp70 and Hsp90 mRNA expression in heart by the supplementation of SP diet when compared with P or SS diet.

DISCUSSION

Under heat stressed condition the cytoplasm of myocytes of broiler chickens showed enlarged intracellular spaces, loss of striations and granular and vacuolar degeneration (Yu *et al*., 2008). The SP supplementation may significantly protect the myocytes by reducing the degeneration, intracellular spaces and loss of striations in the cytoplasm. Oxidative stress may influence the degeneration of vascular endothelial cells and exacerbate cardiovascular diseases such as congestive heart failure, hypertension and atherosclerosis, (Lum and Roebuck, 2001). Selenoproteins are involved in the cellular antioxidant defense system, thus using selenium for the prevention of cardiovascular diseases. Selenium supplementation increases mRNA expression of selenoproteins (GPx1, GPx4) in vascular endothelial cells and can prevent oxidative stress, cell damage and apoptosis in human beings. Similarly, long-term selenium deficiency in rodents severely decreases the levels of selenoproteins. It can be easily reversed by supplementation of selenium. Se supplementation may enhance ischemic tolerance to prevent cardiac damages and restore cardiac functionality (Venardos *et al*., 2004; Ostadalova *et al*., 2007). GPx1 is a potential antioxidant enzyme, used for the detoxification of lipid hydroperoxides and H_2O_2. A significant down-regulation in the mRNA expression of GPx-1 may enhance the chances of cardiovascular disease (de Haan *et al*., 2006). In contrast, the up-regulation of GPx-1 mRNA expression levels in the present study by the supplementation of SP diet indicates that it may reduce the risk of cardiovascular diseases in broiler chickens under heat stress condition better as compared to P and SS diets. Furthermore, the over-expression of cellular GPx (GPx-1) is more resistant to cardiac dysfunction abnormalities in mice (Xiong *et al*., 2006). The over-expression of mitochondrial selenoproteins (GPx4) *in vitro*, protects induced ischemia in neonatal cardiac myocytes (Hollander *et al*., 2003). Previously, reported that GPx4 inhibits atherosclerosis by decreasing oxidative stress (Bellinger *et al*., 2009). We observed that SP diet significantly up-regulated the GPx1 and GPx4 mRNA expression, which are consistence with earlier studies in heat stressed piglets as compared to P or SS group (Gan *et al*., 2013; 2014). However, the current data have expanded the concept that SP could enhance the antioxidant system to protect the heart from toxic effect of oxidative stress in heat stressed broiler chickens. Moreover, it can protect from Se-deficiency disorder such as cardiovascular disease (i.e. Keshan disease in China), exudative diathesis in chicks, and white muscles disease in sheep (Bellinger *et al*., 2009; Huang *et al*., 2011; Pan *et al*., 2011). However, the resistance of broiler chickens to oxidative stress is due to an improved redox status of the Se yeast (organic Se) in a diet under high temperature (Mahmoud and Edens, 2005). The mRNA expression of both selenoproteins GPx1 and GPx4 were significantly up-regulated in the liver of male turkey poults by supplementing Se in a diet because the sequence of these two genes having >90% identity with chickens and >60% identity with rodents and human's beings (Sunde and Hadley, 2010). The transcription of these genes (GPx1 and GPx4) can be used as molecular bio-markers for assessing Se status and requirements (Huang *et al*., 2011). As reported, that in pig selenoprotein may also inhibit the oxidative stress to stop the promotion of porcine circovirus type 2 (PCV2) replication (Gan *et al*., 2016).

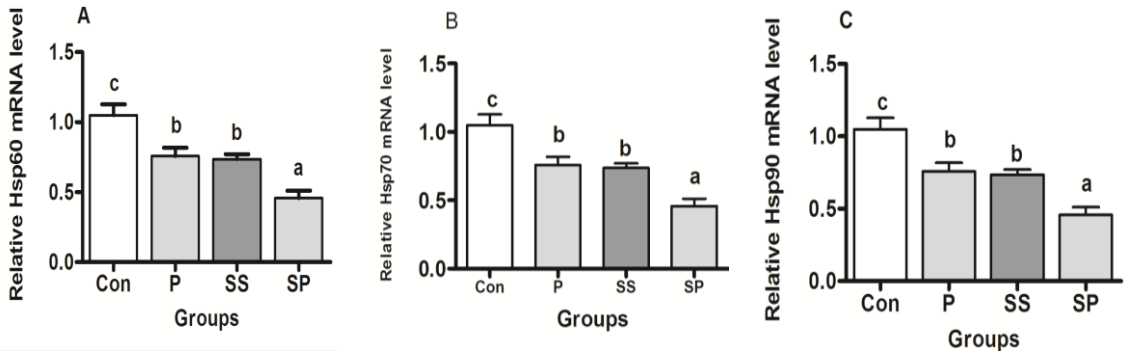

Fig. 3: Effect of P, SS and SP on mRNA levels of (A) Hsp60, (B) Hsp70 and (C) Hsp90 in heart of heat stressed Ross male broiler chickens. Mean values bearing the different alphabets differ significantly (P<0.05).

Table 1: Ingredient and basal diet composition for heat stressed broiler chickens

Ingredients (g/kg)	Starter diet	Grower diet
Corn	58.1	65.3
Soybean meal	31.6	23.3
corn gluten meal	3.9	4.5
vegetable oil	1.6	2.51
Limestone	1.32	1.22
dicalcium phosphate	1.76	1.59
sodium chloride	0.42	0.32
L-lysine	0.15	0. 16
DL-methionine	0.15	0.1
premix,[a]	1	1
Calculated Nutrient Composition		
Metabolizable energy, MJ/kg	11.27	11.77
Crude protein, %	22.2	19.3
Calcium, %	1.0	0.91
available phosphorus, %	0.44	0.37
lysine, %	1.07	0.96
methionine, %	0.51	0.44
methionine + cystine, %	0.81	0.72

[a]Provided per kg of diet: iron, 60 mg; copper, 7.5 mg; zinc, 65 mg; manganese, 110 mg; iodine, 1.1 mg; bacitracin zinc, 30 mg; vitamin A, 4500 IU; vitamin D3, 1000 IU; vitamin E, 30 IU; vitamin K, 1.3 mg; vitamin B1,2.2 mg; vitamin B2, 10 mg; vitamin B3, 10 mg; choline, 400 mg; vitamin B5, 50 mg; vitamin B6, 4 mg; biotin, 0.04 mg; vitamin B11,1 mg; vitamin B12, 1.013 mg.

Table 2: Calculated and analyzed Se concentrations in diets for heat stressed broiler chickens (mg/kg)[a]

Group	Supplemental	Calculated	Analyzed
Basal diet (Con)	0.00		0.109±0.010
Basal diet + Probiotics (P)	0.00	0.11	0.112±0.009
Basal diet + Sodium Selenide (SS)	0.30	0.41	0.414±0.013
Basal diet + Selenium-enriched Probiotics (SP)	0.30	0.41	0.420±0.018

[a] Se concentration for the basal diet and supplement samples were analyzed by hydride generation atomic fluorescence spectrometry (HG-AFS) method to confirm the calculated concentration.

Table 3: Primers Used for the Real-Time PCR

Target gene	Gen Bank accession no.	primer sequence (5′–3′)
GAPDH	K01458	forward: TGAAAGTCGGAGTCAACGGAT
		reverse: ACGCTCCTGGAAGATAGTGAT
GPx1	HM590226	forward: AACCAATTCGGGCACCAG
		reverse: CCGTTCACCTCGCACTTCTC
GPx4	AF498316	forward: CATCACCAACGTGGCGTCCAA
		reverse: GCAGCCCCTTCTCAGCGTATC
Hsp60	NM_001012916	forward: GAAGTTTGACCGAGGCTACATC
		reverse: ACAGCAACAACCTGAAGACCA
Hsp70	AY288298	forward: AGCGTAACACCACCATTCC
		reverse: TGGCTCCCACCCTATCTC
Hsp90	X07265	forward: AGTCCCAGTTCATTGGCTAC
		reverse: TCCAGTCATTGGTGAGGCT

Heat stress influence the synthesis of the heat shock proteins Hsp60, Hsp70, and Hsp90, which are also highly expressed and having important role in maintaining and protection of the metabolic and structural cohesion of the organ against stress-effected tissue injury (Yu *et al*., 2008; Hao and Gu, 2014; Lowman *et al*., 2014). Hsp60 plays a crucial role in preserving mitochondrial function, integrity and capacity for ATP production which is an essential for cardiac normal contraction. Similarly, Hsp70 and Hsp90 protect the cytoskeleton by its chaperone activity. High Environmental stress may influence the loss of ATP in the stress affected cells, which is danger for the structures of cytoskeleton in broilers. Similarly, the up-regulation of Hsp72 expression in heart during ischemia may indicate to repair the damaged proteins under heat stress condition **(**Lowman *et al*., 2014). The relative mRNAs expression of Hsp60, Hsp70 and Hsp90 in the heart of broiler chickens were significantly elevated after the exposure to heat stress for 2 h and then declined rapidly with further exposure. In addition, the up-regulation of these stress proteins in heart may act as important biomarkers and protective proteins at the start of heat stress. The occurrence of low signals of Hsps inside the myocardial cells indicates that myocardial cell lesions may adversely affect the function of Hsps under heat stress condition (Yu *et al*., 2008). Hsp70 might be required for thermal resistance (Hao and Gu, 2014). It has been reported, that Hsp90 having the ability to restore damage proteins into their proper position. Therefore, inside the cytoplasm and nucleus of myocardial cells, the expression of Hsp70 and Hsp90 may be related to the restoration of damage proteins under heat stress (Liu *et al*., 2007). In this study we found a down-regulation in mRNA expression of Hsp60, Hsp70 and Hsp90 in cardiac muscles that probably indicate ameliorating effects of supplementary SP against summer stress. This is in agreement with previous study that SP diet may significantly down-regulate the mRNA expression of Hsp27 and Hsp70 in liver, kidney and spleen of heat stressed pigs (Gan *et al*., 2013). Similarly, Se deficiency may increase the mRNA levels of heat shock proteins (Hsp60, Hsp70 and Hsp90) in broilers (Chen *et al*., 2014). However, the mechanism by which Hsps exerts cytoprotective effects is unclear. In this study, supplementation of SP facilitated an induction of the endogenous antioxidant defense system, and these observations indicate that an improved antioxidant status could greatly attenuate heat stress-induced Hsps expression.

Conclusions: All these findings suggest that SP is an important dietary supplement capable to reduce summer stress effects in broilers. The probiotics and selenium may enhance the function of each other after combination. It is concluded that dietary use of SP is a potential nutritive feed supplement in subtropical and tropical regions to reduce the detrimental effects of persistent summer stress in broiler production.

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Author's contribution: KH formulated the research question and designed the research. AZK, SK, HM, SA, FP, YL, HS, BMM conducted the research. AZK, SK, HM and KH analyzed the data. AZK and KH wrote the paper. Finally, the manuscript was read and approved by all authors.

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