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The Effect of Stocking Density on Stress Related Genes and Telomeric Length in Broiler Chickens*

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ABSTRACT : To be economically profitable, the poultry industry demands an increase in stocking density, which could adversely affect chicken welfare. The current study was performed to investigate the effect of stocking density on stress-related, heat shock protein genes (HSP70 and HSP90), 3-hydroxyl-3-methyl-glutaryl coenzyme A reductase (HMGCR) gene and telomere length in broiler chickens. Seven-day-old broiler chickens were housed at High (0.0578 m^2/bird), Standard (0.077 m^2/bird) and Low (0.116 m^2/bird) stocking densities with 8 replicates each until 35 d of age. The growth performance, such as body weight gain and average daily feed intake, was found to be significantly ($p<0.05$) higher in the Low density group, but these parameters did not show any difference between the High and Standard groups. Other growth performance, such as feed conversion ratio and final feed intake, showed no difference among the treated groups. The expression levels of HSP70 and HMGCR were found to be elevated with the increase of stocking density. The expression level of these genes was significantly ($p<0.05$) higher in the High density stocked group compared with the other groups, whereas the expression levels were not significantly different between the Low and Standard groups. The expression levels of HSP90 did not show any significant changes among the treated groups. The telomeric length of the birds housed in High density was reduced significantly ($p<0.05$) when compared to that of the birds in Low density. These results clearly indicate that birds stocked at high density show physiological adaptive changes indicative of stress at gene transcriptional and telomere levels. (**Key Words** : Chicken, Stress, Heat Shock Proteins, Telomere)

INTRODUCTION

Stocking density plays an important role in broiler production. The poultry industry always opts for higher density stocking because increasing space allowances in production systems can have a major negative economic impact for industry as revenue per unit of space increases linearly with density (Tomhave and Seeger, 1945; Proudfoot et al., 1979). In broilers, high densities have been associated with a decline in body weight (BW), feed consumption and conversion, flock uniformity, leg health, and increased frequencies of tibial dischondroplasia, gait scores, carcass bruising and scratching, disturbances or exacerbated mortality related to heat stress (Bilgili and Hess, 1995; Ekstrand et al., 1997; Estevez et al., 1997; Pettit-Riley and Estevez, 2001; Sanotra et al., 2001). Zulkufli et al. (2003) reported that the heterophil to lymphocyte ratio (H:L) is a reliable indicator of avian stress, but some studies show that stocking density has a significant effect on heterophil to lymphocyte ratio (Cravener et al., 1992; Martrenchar et al., 1997; Feddes et al., 2002) while other studies show that H: L ratio is independent of stocking density at 15, 20 and 25 birds/m^2 (Turkyilmaz, 2006). Genes coding for proteins related to stress are certainly good candidates and, with this perspective, we have focused our study on heat shock protein 70, heat shock protein 90 and hydroxyl-3-methyl-glutaryl coenzyme A reductase (HMGCR) in broiler chickens. Heat shock proteins (HSPs) are a wide family of conserved proteins which are synthesized under environmental stress. HSPs help the stressed cells to cope with the stressors, especially those which affect the protein machinery (Schlesinger, 1986; Basu and Todgham, 2002). The heat shock proteins and HMGCR mRNA levels are sensitive to heat stress (Zager and Johnson, 2001). HMGCR is a rate limiting enzyme in cholesterol biosynthesis, which in turn is a precursor of cortisol, a universal stress marker (Gornati et al., 2004). Telomeres are nucleoprotein structures, located at the ends

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of chromosomes, consisting of stretches of repetitive DNA with a high G-C content and are reported to be highly sensitive to damage induced by oxidative stress. Telomere length is a promising genetic marker for oxidative stress (Houben et al., 2007). Though these parameters (HSP70, HSP90, HMGCR and telomeric length) are influenced by stress, nothing is known about their response to stocking density. Therefore, the present study was carried out to investigate the effect of stocking density on HSPs and HMGCR expression levels and telomere length as biomarkers to monitor chicken stress conditions.

MATERIALS AND METHODS

Experimental animals and maintenance

A total of 144 2-d old male broiler chicks (ROSS 308) were obtained from Orpum (Co.), Korea.

The experimental facility was solid-sided and light and temperature controlled. Ventilation consisted of a single fan producing positive pressure in the house. Birds were kept in wire cages at the university experimental farm in a room equipped with control of temperature (23-33°C) and relative humidity (around 50%), and under a continuous light system until 5 wk of age. After a 5 d adjustment period, all birds were randomly assigned to 3 treatments: high (0.0578 m^2/bird), standard (0.077 m^2/bird) and low (0.116 m^2/bird) densities each with 8 replicates (Estevez, 2007). The birds were allowed free feeding and water consumption which met or exceeded the recommendations in commercial practice. The experimental duration was from d-7 to d-35 of age. The formula and chemical composition of the basal diet are shown in the Table 1. Growth performance parameters were monitored on a daily and cumulative total basis.

Table 1. Formula and chemical composition of basal diets

Items	Diets	
	Starter	Finisher
Ingredients (%)		
Corn	38.26	44.28
Wheat	20.00	20.00
Wheat bran	5.00	4.00
Animal fat	2.20	3.00
Corn gluten	4.00	4.00
Soybean meal (44% CP)	23.00	16.50
Rapeseed meal	1.50	2.00
Fish meal	1.00	1.00
Meat meal	2.00	2.00
Salt	0.20	0.23
Calcium carbonate	0.40	0.20
Tricalcium phosphate	1.40	1.60
Lysine (liquid)	0.46	0.66
Methionine	0.13	0.12
Choline-HCl	-	0.01
Vitamin premix[1]	0.20	0.20
Mineral premix[2]	0.20	0.20
Maduramycin+nicarbazine	0.05	-
Antibiotics	0.0133	0.001
Chemical composition (%)		
Crude protein	21.00	19.00
Ether extract	4.80	5.20
Crude fibre	4.30	3.90
Crude ash	5.00	4.90

[1] Contained per kg: Vit. A, 5,500,000 IU; Vit. D_3, 1,500,000 IU; Vit. E, 15,000 mg; Vit. K, 800 mg; thiamine, 1,000 mg; riboflavin, 4,000 mg; niacin, 25,000 mg; biotin, 30 mg; folic acid, 500 mg; pantothenic acid, 5,000 mg; pyridoxine, 1,500 mg; Vit. B_{12}, 15 mg.

[2] Contained per kg: Cu, 12,000 mg; Fe, 35,000 mg; Zn, 25,000 mg; Co, 150 mg; Se, 120 mg; Mn, 38,000 mg.

Sample preparation for gene expression analysis

Effects of stocking density on expression of stress related genes were analyzed in blood lymphocytes and liver tissue at the end of the experimental period. Blood samples were collected in EDTA coated tubes from the jugular vein. Blood samples were stored on ice during transportation to the laboratory for isolation of lymphocytes, and the liver samples were frozen immediately after collection in liquid nitrogen and transported to the laboratory. The lymphocytes from blood were separated by Ficoll (Sigma, St. Louis, USA) using the standard manufacturer's protocol.

Total RNA extraction and Semi-Quantitative RT-PCR

Total RNA was extracted from blood lymphocytes using a QIAamp RNA blood mini kit (Qiagen) following the standard protocol. RNA from liver tissue was isolated using Trizol reagent (Invitrogen life technologies) following the protocol provided by the manufacturer.

Semi-quantitative RT-PCR analysis was performed as previously described (Hong et al., 2005) to measure the levels of HSP70, HSP90, HMGCR and beta-actin mRNAs. The gene specific primers, annealing temperature and the size of the product are shown in Table 2. PCR products were loaded on 1.2% agarose gel. The DNA was visualized by ethidium bromide staining and analyzed using gel viewer software and the EasyDoc Gel documentation system (Easy Doc, Korea). The mRNA levels of HSPs and HMGCR were corrected using the transcription level of the beta-actin gene as an internal standard.

Telomere length analysis

Telomere length was analyzed using a fluorescence *in situ* hybridization procedure on interphase nuclei (IQ-FISH) of lymphocytes. Interphase nuclei of lymphocytes were separated from whole blood cells by hypotonic treatment.

The telomeric probe was made using a chicken telomeric DNA with a commercial DIG DNA labeling and Detection kit (Roche Applied Science, Germany). The Interphase Q-FISH protocol was slightly modified from that

Table 2. Primers used for semi-quantitative reverse transcription polymerase chain reaction

Gene	Primer	Sequence(5'-3')	Product size	Annealing temperature
HSP70	Forward	AATCTATCATCATGTCTGGCAAAGGGCCGG	220 bp	58°C
	Reverse	GCGGCCGATGAGACGCTTGGCATCAAAGAT		
HSP90	Forward	ATGCCGGAAGCTGTGCAAACACAGGACCAA	242 bp	55°C
	Reverse	GGAATCAGGTTAATTTTCAGGTCTTTTCCA		
HMGCR	Forward	ATGCATGGCCTTTTTGTGGCCTCTCATCCA	270 bp	52°C
	Reverse	CTTGAGAAGATTGTGAGGAGACCAGCAATA		
Beta-actin	Forward	AGGACATCTCCGTGGAAGAGA	520 bp	55°C
	Reverse	TCCTGTAGAGGCACCTTCTCT		

of Sohn et al. (2007). Briefly, the slides containing interphase nuclei of lymphocytes were incubated for 30 min at 37°C in 2XSSC solution containing 0.02% of 1 mg/ml RNase A (Sigma, St. Louis, USA) and then dehydrated with a series of increasing percentages of ethanol. Hybridization solution (Roche) containing dig-labeled probes was dropped onto the slides and followed by denaturation at 78°C for 10 min and hybridization at 37°C overnight. After hybridization, the slides were washed in 2XSSC at 72°C and immediately transferred into PN buffer containing 0.1% Nonidet P-40 (Roche) and 0.1% sodium phosphate to remove unbound probe and dried. The probes were detected using anti-dig-FITC solution followed by washing in PN buffer to remove unbound anti-dig-fluorescein isothiocyanate (FITC). This was followed by a counter staining with propidium iodide.

Images were captured by a fluorescent microscope (Olympus AX70, Tokyo, Japan) CCD camera and the suitable filter sets (CDP-70, Olympus). Quantitative analysis, the signal strengths and the relative amount of telomere (%) were calculated using the software Metamorph (Universal Imaging Corporation, USA).

Statistical analysis

Effect of stocking density on stress related genes and telomere length was analyzed using the general linear model (GLM) procedure of the SAS statistical package (SAS Institute, 1999). When the treatment effect was significant at $p<0.05$, Duncan's multiple range test was used to determine differences among treatments. The level of probability for statistical differences was established at $p<0.05$.

RESULTS AND DISCUSSION

Changes in physiological and pathological conditions, environmental modifications or exposure to chemicals can elicit responses of stress-related genes. The study of gene expression modifications at different chicken population densities may not only help the understanding of the crowding stress, but also furnish fast and reliable molecular biomarkers to predict the stress status of birds.

Published research has shown that the health and welfare of broilers is compromised if space allowances drop below 0.0625 to 0.07 m^2/bird. So this density is usually considered as a standard density for rearing broilers without any stress and at good health conditions (Estevez 2007). Thus, 3 level of stocking densities, high (0.0578 m^2/bird), standard (0.077 m^2/bird) and low (0.116 m^2/bird) density were used in our experiment.

Growth performance

Effects of stocking density on body weight gain, feed intake, and feed conversion ratio are shown in Table 3.

Table 3. Effect of stocking density on growth performance, feed intake and feed conversion ratio in broiler chickens

Items	Treatments*		
	LD	SD	HD
Initial body Wt., (g/bird)	185.08±0.87	185.88±0.53	185.93±0.18
Final body Wt., (g/bird)	2,098.38±67.63	1,970.72±53.69	1,977.75±44.43
Gain (g/bird)	1,913.29±67.92[a]	1,784.83±53.50[b]	1,791.83±44.37[b]
Feed intake (g/bird)	3,308.38±61.63	2,941.88±42.72	2,993.35±42.47
Average daily gain (g/bird)	65.98±2.35	61.55±1.85	61.79±1.53
Average daily feed intake (g/bird)	114.08±2.13[a]	101.45±1.47[b]	103.22±1.46[b]
Feed conversion ratio	1.74±0.05	1.66±0.05	1.67±0.02

Mean±SEM.

* Low stocking density (LD): 0.116 m^2/bird, Standard stocking density (SD): 0.077 m^2/bird, High stocking density (HD): 0.0578 m^2/bird.

[a, b] Values with different superscripts in the same row differ significantly ($p<0.05$) among groups.

The results showed that the stocking density had no significant effect on total feed intake and feed conversion ratio but, average daily feed intake (ADFI) and body weight gain were significantly affected. The body weight gain in the low density group was significantly higher ($p<0.05$) compared to that of the standard and high density groups. The ADFI was reduced from 114.08 g/bird (low density group) to 103.2 g/bird (high density group), which represented a 9.5% decrease in high density stocked birds. These results are similar to previous observations (Shanawany, 1988). Several early (Tomhave and Seeger, 1945; Heishman et al., 1952) and recent studies (Martrenchar et al., 2000) have shown that stocking density has a negative effect on body weight gain. Birds stocked at higher densities have a significantly decreased body weight gain compared to those stocked at lower densities. Although our results indicated an increased body weight in low density stocked birds compared to birds stocked at high density, the difference was not statistically significant. This may be due to the fact that our experiment was carried out for a period of 5 weeks only. Similarly, Bolton et al. (1972) showed that stocking density does not have a significant effect on BW up to the age of 8 weeks, but had a significant effect by the age of 10 weeks. Taken together, these data clearly indicate that stocking density has a significant effect on ADFI and body weight gain, whereas the other parameters of growth performance seems to be not affected by the stocking density up to the age of 35 days.

Expression of stress related genes

The mRNA expression levels of stress related HSP and HMGCR genes in blood and liver of broilers at 35 d are shown in Figures 1, 2 and 3. Stocking density had a significant ($p<0.05$) effect on the expression of HSP70 and HMGCR both in blood and liver tissue (Figure 1 and 2). The expression levels of HSP70 and HMGCR were significantly higher in the high density group while the low and standard groups did not show any difference. The expressions of HSP70 and HMGCR in the high density group were almost two times higher compared with that of low and standard densities. HSPs are a wide family of conserved proteins which are synthesized under environmental stress. HSPs help the stressed cells to cope with the stressors, especially those affecting the protein machinery (Schlesinger, 1986; Basu et al., 2002). Increased expressions of HSPs and HMGCR are the result of heat shock (Zager and Johnson, 2001). HMGCR enzyme influences the biosynthesis of cortisol, hence indirectly indicating that increased cortisol levels in high stocking density can cause negative correlation with the synthesis of HMGCR gene under stressed situations. Previous findings (Turkyilmaz, 2006) reported that there was an increasing trend in blood cortisol levels in response to stressed situations, but without statistical significance.

However, the expression levels of HSP90 in liver samples were higher in the high density group compared with the low and standard groups, but did not result in significant difference (Figure 3). Our results are similar to previous findings in sea bass (Hermesz et al., 2001: Gornati et al., 2004), which showed that the stocking density had an effect on HSP70 and HMGCR but not on HSP90.

Telomere length and stocking density

To the best of our knowledge, this study is the first report on the effect of stocking density stress on telomere length in chickens. Previous studies have shown that stress induces telomere shortening. Cellular environment also plays an important role in regulating telomere length and

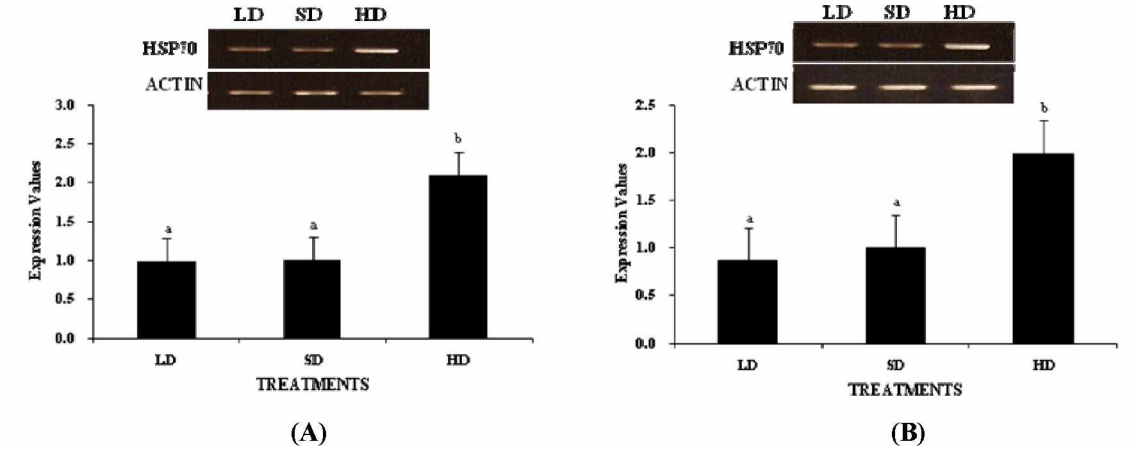

Figure 1. The mRNA expression levels of HSP70 after different stocking density treatment. Upper panel, Representative ethidium bromide stained agarose gel showing amplified HSP70 and beta-actin in blood (A) and liver (B) of broiler chickens subjected to low stocking density (LD), standard stocking density (SD) and high stocking density (HD); Lower panel, the data were normalized using beta-actin mRNA levels and expressed as arbitrary units on the y-axis. The graphical data represent mean±SEM of eight samples per group. The bars representing the treatment with different superscripts (a, b) are significantly different at $p<0.05$.

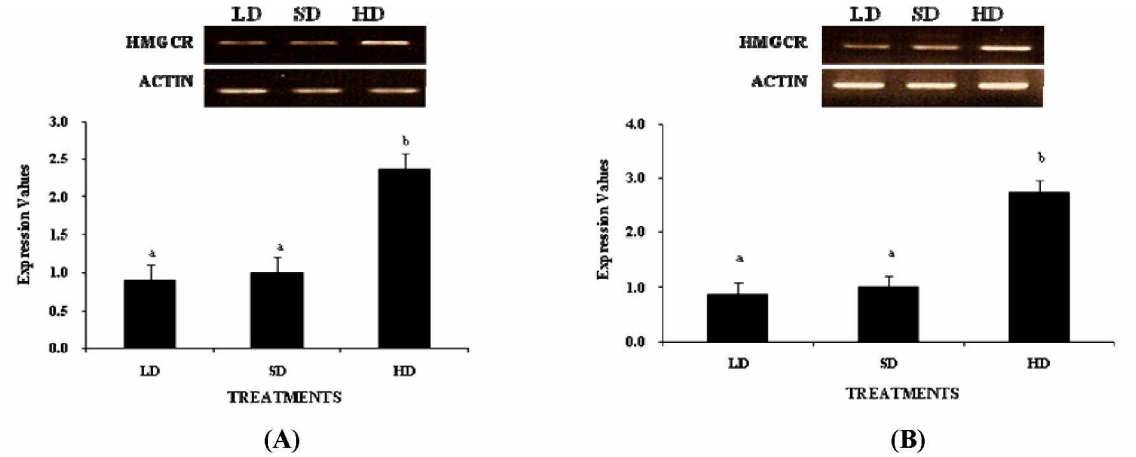

Figure 2. The mRNA expression levels of HMGCR after different stocking density treatment. Upper panel, representative ethidium bromide stained agarose gel showing amplified HMGCR and beta-actin in blood (A) and liver (B) of broiler chickens subjected to low stocking density (LD), standard stocking density (SD) and high stocking density (HD). Lower panel, the data were normalized using beta-actin mRNA levels and expressed as arbitrary units on the y-axis. The graphical data represent mean±SEM of eight samples per group. The bars representing the treatment with different superscripts (a, b) are significantly different at $p<0.05$.

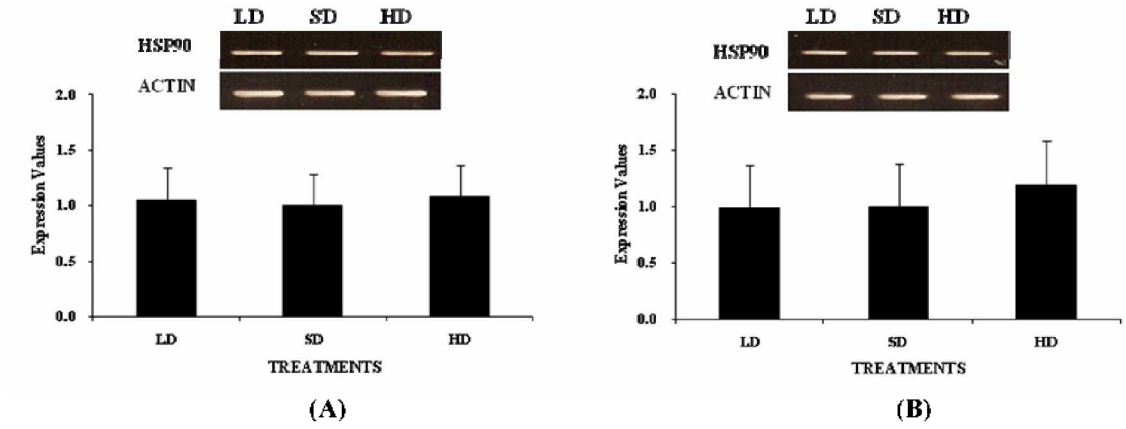

Figure 3. The mRNA expression levels of HSP90 after different stocking density treatment. Upper panel, representative ethidium bromide stained agarose gel showing amplified HSP90 and beta-actin in blood (A) and liver (B) of broiler chickens subjected to low stocking density (LD), standard stocking density (SD) and high stocking density (HD). Lower panel, the data were normalized using beta-actin mRNA levels and expressed as arbitrary units on the y-axis. The graphical data represent mean±SEM of eight samples per group.

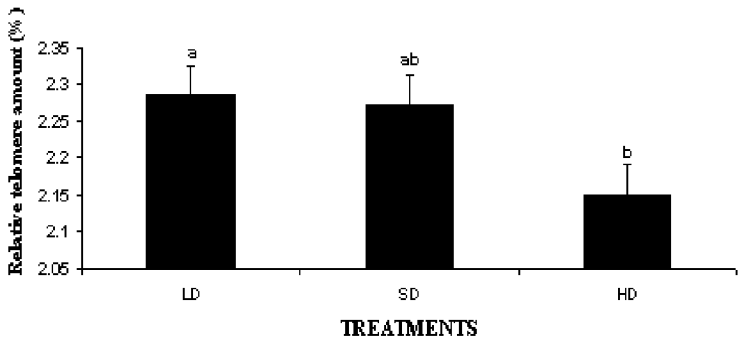

Figure 4. Relative amount of telomeric DNA in blood samples of broiler chickens subjected to low stocking density (LD), standard stocking density (SD) and high stocking density (HD). The graphical data represent mean±SEM of eight samples per group. The bars representing the treatment with different superscripts are significantly different at $p<0.05$.

telomerase activity. Oxidative stress can shorten the telomeres and antioxidants can decelerate shortening (Von Zglinicki et al., 1995; Von Zglinicki, 2002). Our results combined with other previous studies show that stocking density also induces stress in chickens. So, when the effect of stocking density on the relative amount of telomeric DNA, as telomeric length, was analysed in blood lymphocytes, the telomeres were significantly ($p<0.05$) decreased in high density stocked birds compared with the birds stocked in low density. Though the amount of telomeric DNA markedly decreased in high stocking density compared with the low stocking density group, there was no difference in telomere length between standard and low density groups. As the results clearly indicate that stocking birds at high density has a negative effect on telomere length, it is assumed that high stocking density under stressful status could be associated with shortening telomere length.

In conclusion, the stocking density showed no significant effect on performance parameters except for ADFI and body weight gain. However, the expression levels of HSP70 and HMGCR were affected by density of stocked birds. In addition, the telomeric length of high density stocked birds was significantly reduced. These observations clearly indicate that birds stocked at higher density could induce changes in specific stress related genes at transcriptional level. In order to detect stress level in chickens, the expression of HSP70 and HMGCR proteins and telomere length can be appropriate biomarkers to assess the effect of stocking density on induced stress.

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