Telomeric DNA quantity, DNA damage, and heat shock protein gene expression as physiological stress markers in chickens

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ABSTRACT In this longitudinal study with Single Comb White Leghorn chickens, we investigated the effects of stress conditions in birds that were subjected to a high stocking density with feed restrictions on the quantity of telomeric DNA, the rate of DNA damage, and the expression levels of heat shock proteins (HSP) and hydroxyl-3-methyl-glutaryl coenzyme A reductase (HMGCR) genes. The telomere length and telomere-shortening rates were analyzed by quantitative fluorescence in situ hybridization on the nuclei of lymphocytes. The DNA damage rate of lymphocytes was quantified by the comet assay. The expression levels of *HSP70*, *HSP90*, and *HMGCR* genes were measured by quantitative real-time PCR in lymphocytes. The telomere-shortening rate of the lymphocytes was significantly higher in the stress group than in the control. The DNA damage also increased in birds raised under stress conditions, as compared with the control group. The stress conditions had a significant effect on the expressions of *HMGCR* and *HSP90α* in lymphocytes but had no significance on *HSP70* and *HSP90β* in blood. We conclude that the telomere length, especially the telomere-shortening rates, the quantification of total DNA damage, and the expression levels of the *HMGCR* and *HSP90α* genes can be used as sensitive physiological stress markers in chickens.

Key words: chicken, telomere, DNA damage, heat shock protein, stress marker

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INTRODUCTION

In general, chickens are exposed to a variety of external and internal stressors, including stocking density, temperature, transportation, feed restriction, feed contamination, fear, and disease (Thaxton et al., 2006; Zimmerman et al., 2006; Delezie et al., 2007; Keles et al., 2010). These stressors have adverse effects on the growth and production performances of chickens. Indeed, it has been reported that a high stocking density of broilers affects the BW, feed consumption, and leg health (Shanawany, 1988). Many studies have been conducted to identify biological markers for assessing the physiological and immunological responses of birds under stress conditions. Some of the immune parameters, such as the hematological values, and plasma corticosterone level are well known as physiological indicators (Mashaly et al., 1984; Hocking et al., 1994; Puvadolpirod and Thaxton, 2000; de Jong et al., 2002). In addition, the expression of cytokines, such as interleukin-4 (IL-4), IL-6, lipopolysaccharide-induced tumor necrosis factor-α, and inducible nitric oxide synthase, were also introduced as immunological parameters for assessing stress responses in birds (Mashaly et al., 2004; Fassbinder-Orth and Karasov, 2006; Kang et al., 2011). However, these parameters are less reliable for assessing individual stress responses because the values are easily influenced by various environmental conditions (Cook, 1991).

Telomeric DNA quantity, DNA damage, and expression profiles of stress-related genes are good candidates for physiological stress indicators. Telomeres are nucleoprotein structures at the ends of eukaryotic chromosomes that consist of tandem repeats of TTAGGG sequences and are conserved in all vertebrates (Blackburn, 1991). Telomeres shorten with the age of the organism in most of the somatic cells of vertebrates (Harley et al., 1990, 1992; Kim et al., 2011). However, telomere attrition is usually aggravated by exposure to stress conditions (von Zglinicki, 2002; Richter and Proctor, 2007). Damage to DNA naturally occurs under normal physiological conditions, but the extent of the damage to DNA is accelerated under various stressors (Chen et al., 2007). The heat shock proteins (**HSP**) are a group of conserved proteins that are expressed under various environmental stresses (Schlesinger, 1986; Zulkifli et al., 2002). Hydroxyl-3-methyl-glutaryl coenzyme A reductase (**HMGCR**) is the rate-controlling enzyme in cholesterol biosynthesis and is related to oxidative

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stress markers (Gornati et al., 2004). In our previous study, we investigated the effect of stocking density on stress-related HSP genes and telomere length in broiler chickens. We found that the expression levels of *HSP70* and *HMGCR* elevated with the increase of stocking density, and the quantity of telomeric DNA of the birds housed in high density was reduced significantly (Beloor et al., 2010). Although many stress indicators have been introduced, there have been only a few studies on identifying stress markers through the investigation of birds under direct exposure to stress conditions. To explore the effects of stressors on physiological parameters, in the present study, we applied a combination of 2 stressors, high stocking density with feed restriction, to chickens and then analyzed the quantity of telomeric DNA, quantified total DNA damage, and determined the gene expression levels of *HSP* and *HMGCR* in blood.

MATERIALS AND METHODS

Birds and Maintenance

Single Comb White Leghorn chicks produced at the experimental farm of Gyeongnam National University of Science and Technology (Jinju, Korea) were reared in pullet battery cages (8,100 cm^2/cage), with an 810 cm^2/bird stocking density in a room equipped with controlled temperature and on a light/dark cycle set. In total, 56 hens were randomized into control and stress groups at the age of 62 wk with 2 replicates. Both groups were kept in wire cages in a room equipped with controlled temperature (20–25°C) and RH (~60%) under an artificial light system until 64 wk of age. The chickens of the control group were randomly assigned to a 1,350 cm^2/bird stocking density (6 hens/cage) with feeding ad libitum for 14 d, whereas the chickens of the stress group were arranged in a 368 cm^2/bird stocking density (22 hens/cage) and were fed 75% of the voluntary intake of the control chickens for 14 d. These chickens were cared for and handled according to the procedures of the institutional animal care and use committee of the university.

Sample Preparation

At the end of the 14 d of the experimental period, all 56 birds were weighed and 12 median-weight birds from each group were sampled to obtain whole blood. Whole blood was collected from the brachial wing vein using standard blood collection procedures, and the liver and spleen were harvested by killing individual chickens. After the collection, the lymphocytes were separated from the whole blood using a Ficoll (Sigma, St. Louis, MO) treatment. The resulting lymphocyte fraction was carefully aspirated using a Pasteur pipette and washed twice with Dulbecco's (**D**)-PBS solution. After washing, the lymphocytes were separated into 3 fractions and processed separately for telomere quantification, DNA damage quantification, and *HSP* expression levels. For the telomere quantification, interphase nuclei of lymphocytes, liver, and spleen cells were prepared by treatment with a hypotonic solution (0.06 *M* KCl). After the hypotonic treatment, the cells were fixed in 3:1 methanol and acetic acid solution (Carnoy's fixative) and stored at 4°C, until they were dropped onto slides. For the quantification of the DNA damage, the lymphocytes were resuspended in D-PBS buffer, and the cell number was counted. The viability of the cells was assessed by trypan blue differential counting using a hemocytometer, and the cell density was adjusted to 1,500 viable cells/1 μL.

Quantitative Fluorescence In Situ Hybridization Using a Telomere-Specific DNA Probe

The chicken telomeric probe, containing the telomeric DNA sequence TTAGGG, was simultaneously amplified and labeled with digoxigenin (**dig**) on a dUTP by PCR, using chicken genomic DNA as the template, 5′-$(CCCTAA)_7$-3′ primer, and a dig-labeling kit (Roche, Mannheim, Germany). The procedure of quantitative fluorescence in situ hybridization (**FISH**) on the interphase nuclei was slightly modified from that of Kim et al. (2011). Briefly, the slides containing the interphase nuclei of the cells were incubated in RNase A (Sigma) and dehydrated in an increasing ethanol gradient. Hybridization solution (Roche) containing dig-labeled probes was dropped onto the slides, and the samples were denatured at 78°C for 10 min and hybridized at 37°C overnight. The slides were then incubated with an anti-dig-fluorescein isothiocyanate conjugate at 37°C for 30 min and washed with phosphate and Nonidet buffer, counter stained with propidium iodide, and examined under a fluorescence microscope (model AX-70, Olympus, Tokyo, Japan) at green (fluorescein isothiocyanate) and red (propidium iodide) dual excitation wavelengths. The images were captured using a digital camera (DP-70, Olympus) and analyzed using MetaMorph (Universal Imaging Co., West Chester, PA), an image analysis program. The telomere-specific signals in at least 100 interphase nuclei were examined for each specimen.

Comet Assay or Single-Cell Gel Electrophoresis

The conventional slides were dipped in 1% normal melting agarose. A 10-μL aliquot of the lymphocyte sample was mixed with 75 μL of 0.5% low melting-point agarose at 42°C. This mixture was layered onto the base slide and immediately covered with a coverslip. The slides were placed on a cold, steel tray on ice to solidify the agarose. The coverslip was then removed, and 1% low melting-point agarose was layered and hardened as above. The slides were carefully slipped

Table 1. Primers used for the quantitative real-time PCR

Gene[1]	Primer	Sequence (5′–3′)	Size (bp)	Temperature (°C)
HSP70	Forward	ATGCTAATGGTATCCTGAACG	145	60
	Reverse	TCCTCTGCTTTGTATTTCTCTG		
HSP90α	Forward	CAGAAGATGAAGAGAAGAAGA	133	60
	Reverse	GGAGAAGTTACCAAGCGATT		
HSP90β	Forward	TGTAGTAATGGCGAACCTAA	84	60
	Reverse	TCAGAGCGTAAGACCTAAC		
HMGCR	Forward	GAGGCAGAGCAAGATGAAG	113	60
	Reverse	GCAGGACAGTAGGTGAGT		
Actin	Forward	CCACCGCAAATGCTTCTA	96	60
	Reverse	GCCAATCTCGTCTTGTTTTATG		

[1]HSP = heat shock protein; *HMGCR* = hydroxyl-3-methyl-glutaryl coenzyme A reductase.

into freshly prepared lysis solution and maintained at 4°C for 60 min. The lysing solution was prepared by first premixing 2.5 *M* NaCl, 100 m*M* disodium EDTA, and 10 m*M* Trizma base, and the pH was adjusted to 10. The slides were removed from the lysing solution and gently transferred to the electrophoresis unit. The electrophoresis buffer (pH > 13, at 4°C) was added and then electrophoresed for 30 min at 25 V, 300 mA, with the temperature maintained at 4°C. After the completion of the electrophoresis, the slides were removed, drained, and layered 3 times with neutralization buffer, allowing each aliquot to incubate for 5 min. The slides were stained with propidium iodide and were scored using a fluorescence microscope (AX-70, Olympus) at a red excitation wavelength. The images were captured using a digital camera (DP-70, Olympus) and analyzed using the Comet Score software v. 1.5 (TriTek Corp., Sumerduck, VA). The results were calculated as the 3 following parameters: (1) the percentage of DNA in the tail, expressed as the percentage values of the total tail intensity against the total comet intensity; (2) the tail moment, expressed as the product of the percentage of DNA in the tail and the tail length; and (3) the olive moment, a summation of the tail intensity profile values multiplied by their relative distances to the center of the head, divided by the total comet intensity.

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from the lymphocytes using a QIAamp RNA blood mini kit (Qiagen GmbH, Hilden, Germany). To measure the levels of *HSP70*, *HSP90*, *HMGCR*, and β-actin mRNA, quantitative real-time PCR was performed. The gene-specific primers, annealing temperature, and the size of the product are listed in Table 1. The PCR reactions were prepared with 10 μl of SYBR Green premix Ex *Taq*TM II (TaKaRa, Tokyo, Japan), a final concentration of 400 n*M* of each primer, and 5 μL of template cDNA in a total reaction volume of 20 μL. The cycling conditions comprised an initial denaturation step of 3 min at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 40 s. Reactions were run on a MyiQ Real-Time System(Bio-Rad, Hercules, CA). The results of the real-time PCR for gene transcripts were analyzed by the $2^{-\Delta\Delta Ct}$ calculation (Livak and Schmittgen, 2001).

Statistical Analysis

The measured data were expressed as the means and SD. The data were analyzed using the GLM procedure of the Statistical Analysis System Institute (SAS Institute Inc., Cary, NC). The *t*-test procedure was used to compare the means of the 2 populations. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The physiological effect of the combined stress induced by a high stocking density with feed restriction on the BW of the chickens is shown in Table 2. The chickens assigned to the stress group had a significantly ($P < 0.01$) lower BW compared with those of the control group at the end of the experiment. In the final BW, the control and stress groups gained approximately 4.9% and −5.4%, respectively, of their initial BW.

Table 2. Effect of induced stress on BW in White Leghorns[1]

Item	Control group	Stress group	*P*-value
Initial BW at 62 wk, g	1,626.4 ± 182.7	1,610.2 ± 132.6	0.754
Final BW at 64 wk,* g	1,705.9 ± 174.8	1,523.2 ± 110.5	0.003
Gains from 62 to 64 wk, g	79.4 ± 8.3	−86.9 ± 7.8	<0.001

*Values (means ± SD) within the same row differed significantly ($P < 0.05$).

[1]The chickens of the control group were raised under full feeding (>110 g/d) and a low stocking density (1,350 cm^2/bird), but those of the stress group were raised under restricted feeding (83 g/d) and a high stocking density (368 cm^2/bird) from 62 to 64 wk.

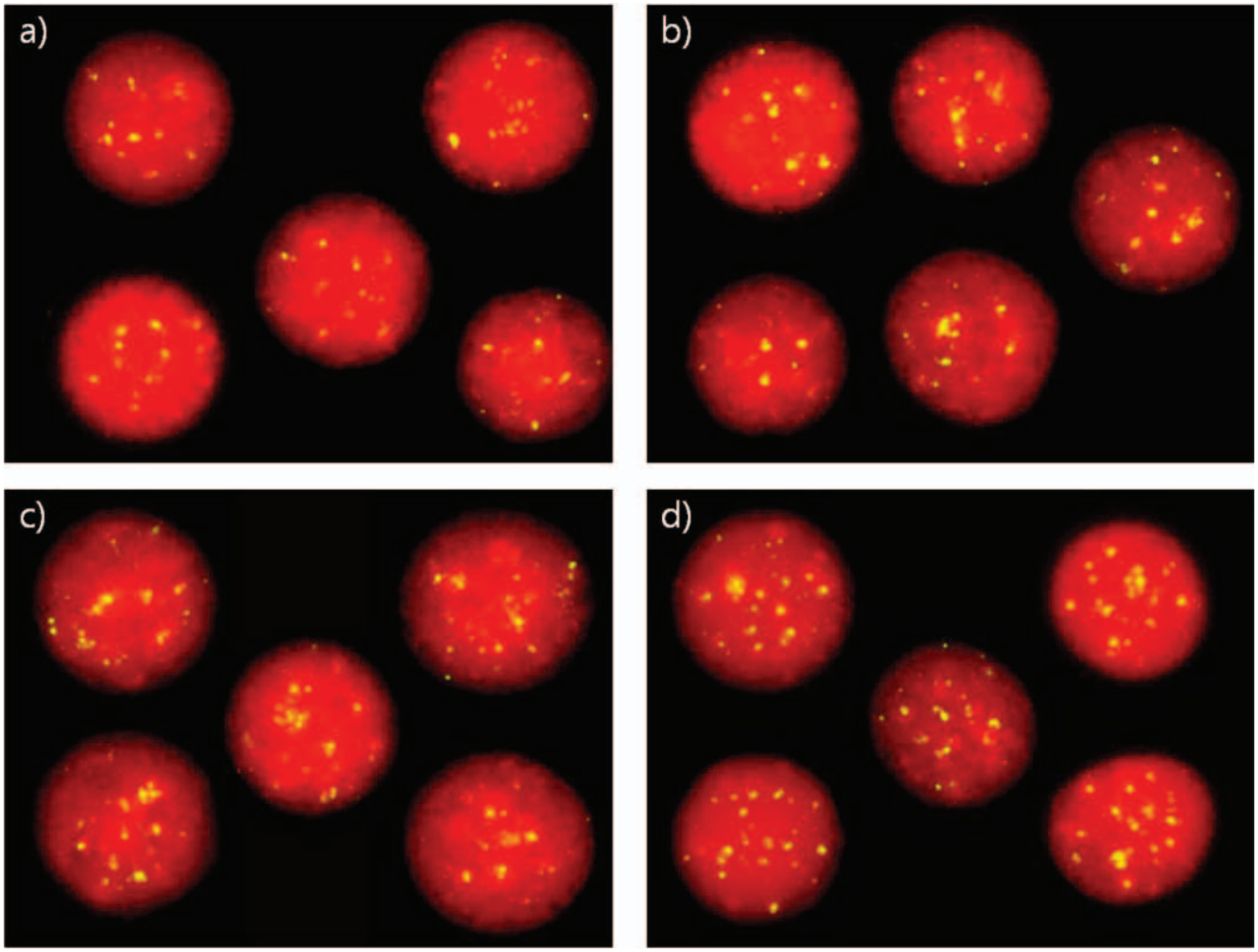

Figure 1. Chicken lymphocyte nuclei analyzed by fluorescence in situ hybridization using a chicken telomeric DNA probe. The telomeric DNA is indicated by bright (green-yellow) spots against the genomic DNA (red background). The relative quantity of telomeric DNA was determined to be 1.08 (a), 1.53 (b), 1.89 (c), and 2.30 (d). Color version available in the online PDF.

Figure 1 shows representative nuclei of the chicken lymphocytes treated by FISH using a telomere probe. Table 3 shows the relative quantity of telomeric DNA in the chicken lymphocytes and liver and spleen cells subjected to the stress conditions and the control. The results clearly showed differences in the mean quantities of telomeric DNA of the lymphocytes between the 2 groups, but no significant difference in the values of the liver and spleen cells between the groups was found. The lymphocytes in the control group had a significantly higher telomeric DNA quantity than those in the stress group at 64 wk ($P < 0.01$). The telomere-shortening rates of the lymphocytes from 62 to 64 wk were also measured in individual chickens. The telomere-shortening rate was significantly high in the stress group ($P < 0.01$), which led to decreased mean quantities of telomeric DNA in this group (Table 4).

Table 3. The relative quantity of telomeric DNA (%) of the interphase nuclei in the tissues of 64-wk-old White Leghorns subjected to control and stress conditions[1]

Treatment[2]	Lymphocytes*	Liver	Spleen
Control group	1.49 ± 0.39	1.41 ± 0.25	2.31 ± 0.56
Stress group	1.21 ± 0.34	1.41 ± 0.20	2.15 ± 0.61
P-value	<0.0001	0.8813	0.0608

*Values (means ± SD) within the same column differed significantly ($P < 0.05$).

[1]Twelve hens were sampled in each group and at least 100 cells in each individual were analyzed.

[2]The chickens of the control group were raised under full feeding (>110 g/d) and a low stocking density (1,350 cm^2/bird), but those of the stress group were raised under restricted feeding (83 g/d) and a high stocking density (368 cm^2/bird) from 62 to 64 wk.

Table 4. The telomere-shortening rate calculated in terms of quantity of telomeric DNA in lymphocytes of White Leghorns subjected to control and stress conditions[1,2]

Quantity of telomeric DNA	Control group	Stress group	*P*-value
62 wk	1.47 ± 0.34	1.47 ± 0.37	0.9646
64 wk*	1.49 ± 0.39	1.21 ± 0.34	<0.0001
Telomere-shortening rate*	−0.048 ± 0.064	0.169 ± 0.083	<0.0001

*Values (means ± SD) within the same row differed significantly ($P < 0.05$).

[1]Twelve hens were sampled in each group and at least 100 cells in each individual were analyzed.

[2]The chickens of the control group were raised under full feeding (>110 g/d) and a low stocking density (1,350 cm^2/bird), but those of the stress group were raised under restricted feeding (83 g/d) and a high stocking density (368 cm^2/bird) from 62 to 64 wk.

The extent of DNA damage was quantified by the comet assay, and the comet patterns are presented in Figure 2. The DNA damage significantly increased in the chickens raised under stress conditions, as compared with the control group ($P < 0.01$). The tail moment appeared to be a more sensitive comet assay parameter than the other 2 parameters, the percentage of DNA in the tail, and the olive moment (Table 5).

The mRNA expression levels of the *HSP70*, *HSP90*, and *HMGCR* genes in the blood of 64-wk-old chickens are shown in Table 6. The stress conditions had a significant effect on the expression of *HMGCR* and *HSP90α* in the blood ($P < 0.05$), but they had no significant effect on *HSP70* and *HSP90β*. The expressions of *HMGCR* and *HSP90α* in the stress group were almost 5 and 3 times, respectively, higher in comparison with the control group.

DISCUSSION

Birds, especially chickens, have proven to be a good model for aging, senescence, and stress-related studies (Swanberg and Delany, 2005). Especially, birds are generally thought to have better defense mechanisms against oxidative insults and generate reduced oxidants and advanced glycosylation end products (Ku and Sohal, 1993; Iqbal et al., 1999). It is well known that various husbandry stressors, such as restricted feeding and a high stocking density, have adverse effects on chicken performance (de Jong et al., 2002; Zulkifli et al., 2002; Al-Aqil and Zulkifli, 2009).

To assess the physiological and immunological parameters of birds under stress conditions, numerous studies have been undertaken. The levels of plasma corticosterone, hematological values, antibody production, and expression of various cytokines have been known as the representative physiological and immune parameters for assessing stress damage (Mashaly et al., 1984; Cook, 1991; Hocking et al., 1994; Puvadolpirod and Thaxton, 2000; de Jong et al., 2002; Mashaly et al., 2004; Fassbinder-Orth and Karasov, 2006; Kang et al., 2011). However, it seems that a considerable lack of reliability exists in the effects of these stressors on the physiological indicators, depending on the duration and extent of exposure to the stressors, the sort of physiological indicators measured, and the time point at which these indicators are analyzed. The telomere length, DNA damage, and expression of stress-related genes are the other candidates for physiological indicators to evaluate the stress responses in chickens. Telomeres are nucleoprotein structures that are located at the ends of chromosomes, consisting of stretches of repetitive DNA with high G/C sequences, and they protect chromosomes and maintain genomic integrity from recombination, exonuclease degradation, and end-to-end fusion (Blackburn et al., 2000). In the absence of any replenishing mechanisms, the replication of eukaryotic chromosomes by DNA polymerase leads to a shortening of the ends by approximately 50 to 100 bases. The loss becomes progressive until reaching a critical stage, after which the cells cease dividing and eventually die. Thus, telomeres of somatic cells tend to decrease with the age of the organism. In mitotic cells, the degree of telomere loss will depend on the levels of oxidative stress, antioxidant defenses, and telomerase activity (von Zglinicki, 2002; Richter and Proctor, 2007). A considerable number of factors, including genetic, environmental, and epigenetic factors, have been linked to interindividual variations in telomere dynamics (Delany et al., 2000; Epel et al., 2004; Kotrschal et

Table 5. The percentage of DNA in tail, olive moment, and tail moment comet assay values of lymphocytes in White Leghorns subjected to control and stress conditions

Treatment[1]	DNA in tail* (%)	Tail moment*	Olive moment*
Control group	31.37 ± 17.17	28.48 ± 29.39	28.90 ± 19.35
Stress group	54.35 ± 17.10	99.43 ± 73.10	64.78 ± 34.52
P-value	<0.0001	<0.0001	<0.0001

*Values (means ± SD) within the same column differed significantly ($P < 0.05$).

[1]The chickens of the control group were raised under full feeding (>110 g/d) and a low stocking density (1,350 cm^2/bird), but those of the stress group were raised under restricted feeding (83 g/d) and a high stocking density (368 cm^2/bird) from 62 to 64 wk.

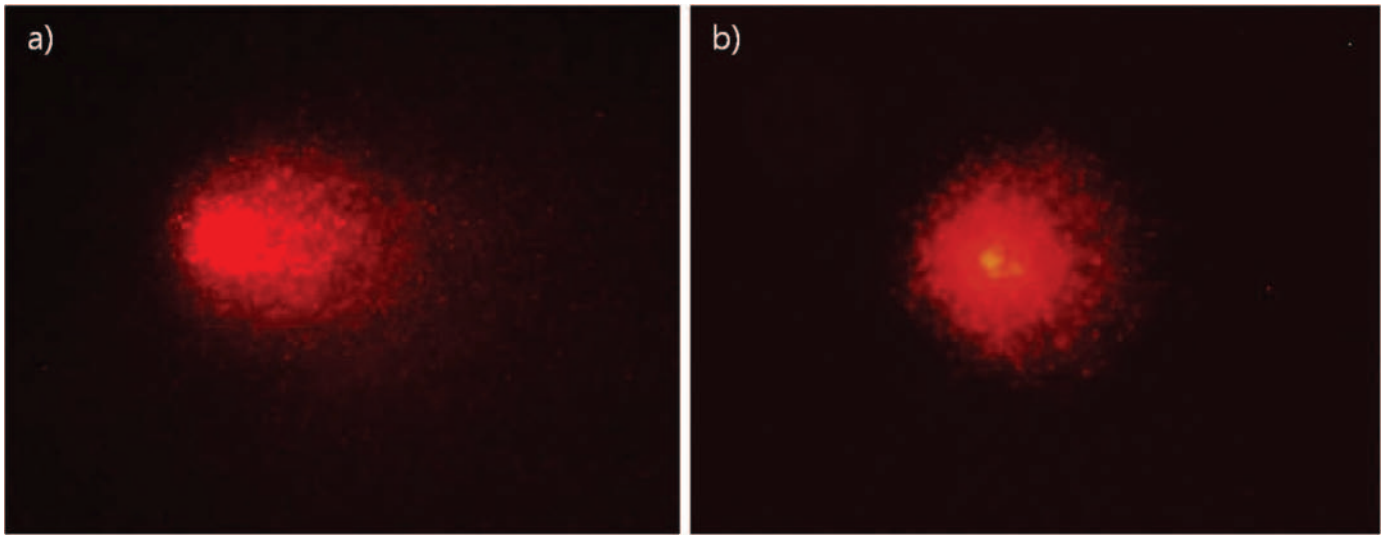

Figure 2. The representative comet assay images of chicken lymphocytes stained with propidium iodide. The DNA fragmentations display the comet-like appearance. The DNA fragmentation in the left panel (a) is greater than that in the right panel (b) (40.9% vs. 7.6% DNA in the tail). Color version available in online PDF.

al., 2007). Measuring of telomere length has become an important aspect of telomere biology. With regard to the technique employed, we must note here that absolute quantification of telomeric DNA of a given cell type is still obscure and ambiguous. Most studies are based on telomere restriction fragment (**TRF**) analysis. The traditional method of quantifying telomere quantity by TRF assay using Southern blot analysis is less sensitive over the quantitative (Q)-FISH technique, which gives a resolution of up to 200 bp (Slijepcevic, 2001; Baird and Kipling, 2004). The TRF analysis dwells in the unknown realm of the flanking DNA in the subtelomeric region and on the property and specificity of the restriction enzymes used, together with the inability to quantify the interstitial telomeric sequences. It should be noted here that the chicken genome contains ultralong telomeres with both terminal and interstitial telomeric sequences (Nanda and Schmid, 1994; Solovei et al., 1994; Delany et al., 2003). Thus, TRF analysis may be less informative in chicken species, and hence, would result in poor analysis qualitatively and less sensitive quantification in comparison to the Q-FISH analysis. Our study used the Q-FISH technique on interphase cells. Though a labor intensive and an elaborate technique needing expertise to perform, it is highly sensitive for the quantification of chicken telomeres (de Pauw et al., 1998; Slijepcevic, 2001). Another advantage of the technique is its ability to study an intact cell when compared with PCR-based techniques (Cawthon, 2002; O'Callaghan et al., 2008) and TRF analyses that depend highly on isolated DNA, which is prone to mechanical shear, damage, loss of DNA termini, and an aggregation of genomic DNA from a group of cells.

Our results showed that the telomere length of lymphocytes, indicated by the relative quantity of telomeric DNA, was significantly decreased in the stress-conditioned chickens compared with the control group chickens. However, there was no difference in the telomeric DNA in the liver and spleen cells. It seems that lymphocytes are direct immune-related cells and are more sensitive to stressors. In previous study, we investigated the effect of stocking density on stress-related HSP genes and telomere length in broiler chickens. The growth performance, such as BW gain and average

Table 6. The mRNA expression levels of *HMGCR*, *HSP70*, and *HSP90* (α and β) in the blood of White Leghorns subjected to control and stress conditions[1,2]

	*HMGCR**		*HSP70*		*HSP90α**		*HSP90β*	
Treatment[3]	ΔCt	$2^{-\Delta\Delta Ct}$	ΔCt	$2^{-\Delta\Delta Ct}$	ΔCt	$2^{-\Delta\Delta Ct}$	ΔCt	$2^{-\Delta\Delta Ct}$
Control group	7.97 ± 0.97	1	5.36 ± 0.90	1	6.50 ± 0.10	1	5.14 ± 0.97	1
Stress group	5.67 ± 0.92	4.92	4.30 ± 0.10	2.08	4.88 ± 1.09	3.08	4.11 ± 0.92	2.05
P-value	0.0018		0.1156		0.0289		0.1227	

*Values (means ± SD) within the same column differed significantly ($P < 0.05$).

[1]Six hens were sampled in each group.

[2]ΔCt is equal to the difference in threshold cycles for target and internal control gene (β actin); $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the control. *HMGCR* = hydroxyl-3-methyl-glutaryl coenzyme A reductase. HSP = heat shock protein.

[3]The chickens of the control group were raised under full feeding (>110 g/d) and a low stocking density (1,350 cm^2/bird), but those of the stress group were raised under restricted feeding (83 g/d) and a high stocking density (368 cm^2/bird).

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. In that study, the expression levels of *HSP70* and *HMGCR* were found to be elevated with the increase of stocking density ($P < 0.05$). The telomeric DNA of the birds housed in high density was reduced significantly ($P < 0.05$) when compared with that of the birds in low density (Beloor et al., 2010). As the results clearly indicate that the stress condition had a negative effect on the quantity of telomeric DNA, it is assumed that a stressful status could be associated with telomere attrition.

Integrity of the DNA is the fundamental prerequisite for functions of life: growth and development. Deoxyribonucleic acid replication and transcription are the primary processes that are readily and directly affected by DNA damage, eventually leading to senescence, cell death, and tumorigenesis. Even in normal physiological conditions, estimates throw up at least one base modification in 130,000 bases in nuclear DNA, mostly being oxidative damage (Richter et al., 1988). The quantification of DNA damage can easily give an estimate of the physiological state of an individual organism or cell, as DNA damage is a crucial mediator for various stresses during cellular senescence regardless of whether they are telomere dependent or independent (Chen et al., 2007). There are various approaches in quantifying DNA damage by using gas chromatography-mass spectrometry with selected ion monitoring, measurement of single base products such as 8-hydroxydeoxy-guanosine, and assays involving repair endonucleases (Richter et al., 1988). However, the comet assay or single cell gel electrophoresis is a rapid and sensitive method for analyzing single cells for DNA damage in the form of DNA strand breaks, alkali-labile basic sites, or intermediates in base- or nucleotide-excision repair. The technique finds its most popular usage in testing for genotoxicity of radiation and chemicals. For the current investigation, we hypothesized that there exists stress due to living conditions the chicken are subjected to, and that for the first time, we could use the quantified amounts of total DNA damage by comet assay. The results of the comet assay demonstrated an increased DNA fragmentation in the lymphocytes of chickens subjected to the stress condition compared with the chickens under the control condition.

When some animals are exposed to thermal stress, the synthesis of most proteins is retarded, but a group of highly conserved proteins is rapidly synthesized. The HSP are a large family of conserved proteins that are synthesized in response to environmental stress. In the stressed cells, HSP may bind to heat-sensitive proteins and protect them from degradation (Schlesinger, 1986). Therefore, the increased expression of *HSP* and *HMGCR* genes are the result of heat shock. In our results, the expression levels of *HMGCR* and *HSP90α* in the blood were significantly higher in the stress group, whereas *HSP70* and *HSP90β* levels were not significantly different between the groups. According to previous report (Meng et al., 1993), different functions and regulations of HSP90α and β may exist in the avian species compared with the higher vertebrates. They reported that avian HSP90β was not inducible by thermal stress, contrary to the mouse and human HSP90α and β. As expected, HSP90β did not respond to stress conditions in chicken. These results are partially similar to a previous report in broilers that showed that the high stocking density had an effect on *HSP70* and *HMGCR* but not on *HSP90* (Beloor et al., 2010). These observations clearly indicate that stress conditions could induce changes in specific stress-related genes at the transcriptional level.

These results are in agreement with our hypothesis that harsh living conditions exert a severe physiological stress, leading to genotoxicity and a physiological decline in terms of BW in these chickens. In chickens subjected to stress conditions, the telomeric DNA quantity in the lymphocytes was significantly reduced, and the rate of DNA damage was significantly increased. In addition, the expression levels of *HMGCR* and *HSP90α* were affected by the stress conditions. Thus, we conclude that telomere quantity, especially the telomere-shortening rates, the quantification of total DNA damage, and the expression levels of *HMGCR* and *HSP90α* in lymphocytes can be used as sensitive physiological stress indicators in avian species.

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