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Effects of the Combined Stress Induced by Stocking Density and Feed Restriction on Hematological and Cytokine Parameters as Stress Indicators in Laying Hens*

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ABSTRACT : A study was conducted to investigate the effects of the combined stressor induced by high stocking density with feed restriction on immunological parameters such as leukocyte differential counts and cytokine expression in laying hens. A total of forty White Leghorn laying hens were randomly allotted into the control (12 kg of body weight/m^2) and the stress (44 kg of body weight/m^2) groups, and then birds of the stress group were given 75% of voluntary intake of the control birds for 12-d on a daily basis. There was a significant decrease in body weight without affecting the relative weights of the liver and spleen after 12-d of the combined stressor. In hematological values, no significant difference in leukocyte differential counts including heterophils (H), lymphocytes (L), monocytes and H:L ratio was observed between the two groups. In cytokines, hepatic lipopolysaccharide-induced tumor necrosis factor-α (LITNF-α) and inducible nitric oxide synthase (iNOS) expression levels in the stress group were significantly ($p<0.05$) higher compared with those in the control group. However, the expression levels of interleukin (IL)-4 and IL-6 in the liver were not affected by the combined stressor. Splenic LITNF-α expression in the combined stressor group was significantly ($p<0.05$) up-regulated compared with that in the control birds. However, the combined stressor did not affect splenic IL-4, IL-6 and iNOS expression. In conclusion, the combined stressor caused by high stocking density with feed restriction enhanced some pro-inflammatory cytokines including LITNF-α and iNOS in lymphoid and non-lymphoid organs of birds, suggesting that altered cytokine expression to given stressors can be another parameter that can be used in assessing stress responses of birds. (**Key Words :** The Combined Stressor, Stocking Density, Feed Restriction, Hematological Values, Cytokines, Laying Hens)

INTRODUCTION

Over the past 50 years, to maximize poultry production, it has become common practice to provide the most integrated and intensified husbandry system. However, these management procedures may affect health, productivity, physiology and even behavior of birds (Nicol et al., 2006; Ozkan et al., 2006; Zimmerman et al., 2006). Thus, numerous studies have been conducted to find the appropriate way for assessing physiological and immunological parameters to monitor welfare of birds under various husbandry conditions (Puvadolpirod and Thaxton, 2000).

Routinely, birds are exposed to a variety of external and internal stressors including stocking density, temperature, transportation, feed restriction, feed contamination, fear, etc (Hangalapura et al., 2006; Thaxon et al., 2006; Delezie et al., 2007). Among various husbandry stressors, much concern is noted today, about stocking density as it relates to the welfare of chickens. In fact, stocking density has been known to affect behavioral and physiological indicators such as plasma corticosterone and heat shock protein 70 in birds (Mashaly et al., 1984; Belmore et al., 2010). However, most studies reported that stocking density did not affect hematological values such as leukocyte differential counts and heterophil (H):lymphocyte (L) ratio (Thaxton et al., 2006; Turkyilmaz, 2008). Furthermore, conflicting results showed that feed restriction alone affected (de Jong et al., 2002; Khajavi et al., 2003) or did not affect (Hocking et al., 1994; Liew et al., 2003; Fassbinder-Orth and Karasov,

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2006) immune parameters including hematological values and antibody production. It seems that a considerable lack of reliability exists in the effects of these stressors on physiological indicators, depending on the duration and extent of exposure to stressors, the sort of physiological indicators measured, and the time point at which these indicators are analyzed (Cook, 1991).

As a consequence of methodological difficulty in stress assessment, it is necessary thus to investigate more specific immunological parameters that can be applied for assessing stress responses in birds. In particular, it has been known that cytokines play a crucial role in the interplay between the immune and endocrine systems during environmental stressors (Felten et al., 1998). Previous studies with laying hens demonstrated that environmental stressors such as cold or heat stress influenced cell-mediated immunity (Mashaly et al., 2004; Hangalapura et al., 2006). However, there is still not much information available on the mechanisms of stress-induced cellular immunity as indicated by cytokine expression in birds. In our study, to investigate the effects of stressors on immunological parameters, we applied two combined stressors through stocking density with feed restriction, which can induce severe stress due to feed competition, uncomfortable laying and frustration (de Jong et al., 2003).

Therefore, the aim of our study was to assess the combined effects of stocking density with feed restriction on cytokine expression as immunological indicators, such as interleukin-4 (IL-4), IL-6, lipopolysaccharide-induced tumor necrosis factor-α (LITNF-α) and inducible nitric oxide synthase (iNOS) in birds.

MATERIALS AND METHODS

Animals and experimental design

A total of 40, sixty two-week-old, highly inbred, White Leghorn layers obtained from Gyeongnam National University of Science & Technology, Korea were kept in wire cages in a room equipped with temperature control and on a light/dark cycle. Immediately after a 14-d adjustment period, the combined stressor induced by stocking density with feed restriction was generated by the following procedure. Briefly, all birds were randomly assigned and reared at stocking densities of 12 kg of body weight (BW)/m^2 (control group) and 44 kg of BW/m^2 (stress group), and birds of the stress group had access to 75% of the voluntary intake of control birds for 12-d. Birds of the control group were given their daily diet *ad libitum* until the end of trial. All birds were provided the same basal diets throughout the entire experimental period (Table 1).

Table 1. Chemical composition of experimental diet fed to laying hens

Items	Basal diet
Moisture (%)	13.93
Crude protein (%)	17.98
Crude fat (%)	4.03
Crude fiber (%)	4.95
Nitrogen free extract (%)	44.34
ME (kcal/kg)	2,650
Ca (%)	4.03
P (%)	0.84

Blood and tissue harvesting

At the end of the 12-d of the combined stressor, all 40 birds were weighed and 6 median-weight birds from each group representing as many pens were sacrificed to obtain whole blood by veinpuncture into heparinized tubes for the assay of hematological values. Immediately after bleeding, the selected birds were carefully euthanized to harvest the liver and spleen. The organs were gently soaked in 0.9% ice-cold saline to remove remaining blood. Harvested tissues were rapidly frozen in liquid nitrogen and stored at -70°C until further assay.

Assays

Total leukocyte counts (WBC), red blood cells (RBC), hemoglobin (Hb), the numbers and percentage of heterophils (H), lymphocytes (L) and monocytes, and H:L ratio in whole blood were measured using an automatic blood cell counter (HEMAVET HV950FS, Drew Scientific, Inc., USA).

To extract total RNA from frozen tissue, the method of RNAsol^{TM} B (Tel-Test Inc, Friendswood, TX) was applied. Briefly, 100 mg of tissue was removed from each organ and added to 1 ml of RNAsol solution. The tissues were homogenized using a glass-glass homogenizer. The lysate was transferred to a microcentrifuge tube and added to 1/10 vol of chloroform to remove protein extract. The aqueous phase was separated by centrifugation for 15 min at 15,000 rpm. Total RNA was precipitated with the same volume of isopropanol and centrifuged for 15 min at 15,000 rpm. The precipitated total RNA was washed with 75% ethyl alcohol, dried and diluted with DEPC-treated water. The concentration of isolated total mRNA was determined by spectrophotometer (GeneQuant *pro*, Amersham, USA) and confirmed on a 1.0% agarose gel stained with ethidium bromide (EtBr).

Previously published chicken specific cytokines (Hangalapura et al., 2006; Hong et al., 2006; Jang et al., 2007) listed in Table 2 were used for the relative quantification of target gene expression. The sequences of primers were designed by the software provided by Bioneer (Daejeon, Korea).

Semiquantification of mRNA using RT-PCR was

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

Target gene	Primer sequence	Product size (bp)	Gene bank accession No.
IL-4	5'- AACATGCGTCAGCTCCTGAAT -3' 5'- TCTGCTAGGAACTTCTCCATTGAA -3'	350 bp	AJ621735
IL-6	5'- GCTCGCCGGCTTCGA -3' 5'- TGACTCATAGCAGAGACGTG -3'	188 bp	AJ250838
LITNF-α	5'- GAACTATCCTCACCCCTACC -3' 5'-TGACTCATAGCAGAGACGTG -3'	223 bp	AY765397
iNOS	5'- GCATCCAAAATATGAGTGGT -3' 5'- AAGCACAGCCACATTTATCT -3'	274 bp	U34045
β-Actin	5'- CAAAGCGCTCGATTTCATCGC -3' 5'- TCTCTTCCACGGAGATGTCCT -3'	180 bp	NM205518

performed to quantify mRNA of the cytokines such as IL-4, IL-6, LITNF-α and iNOS. Briefly, for synthesis of first strand cDNA, 1.0 μg of total RNA was incubated at 62°C for 10 min with 1 μg of oligo dT (Invitrogen Inc, Carlsbad, Ca). Then the resulting solution was incubated at 42°C for 50 min in a reaction mixture containing 2.5 mM dNTP and 200 units reverse transcriptase (Takara Inc, Shiga, Japan). After that, 3.2 unit RNAase H was used to remove RNA hybridized with cDNA for 30 min at 37°C. The amplification of obtained RNA was performed for 32 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 10 min. The reaction mixture consisted of 10 pmol primer, 2.5 μg cDNA, 2.5 mM dNTP and 1 unit Taq polymerase (Takara Inc, Shiga, Japan). We determined the number of cycles and kept the products within the exponential phase. The density of each product in agarose gel electrophoresis (1.5%) containing EtBr was measured using a densitometer (Gel documentation system, EasyDoc, Korea). Levels of all mRNAs were expressed as the ratio of signal intensity for genes relative to that for β-actin.

Statistical analysis

Effects of the combined stressor on body weight, hematological values and cytokine expression were analyzed by Proc t-test (SAS Institute Inc., 1989). The level of probability for statistical difference was established at $p<0.05$.

RESULTS

Body weight and relative organ weight

Effects of the combined stressor induced by high stocking density with feed restriction on BW and relative organ weights are presented in Table 3. As expected, the birds assigned to the stress group had a significantly ($p<0.05$) lower BW compared with those of the control group. The control and stress groups gained about 10% and -5%, respectively, of their initial BW after 12-d of combined stressor. However, no statistical difference in relative organ weights of the liver and spleen was observed between the two groups, although the stress group tended to have numerically decreased organ weights. Overall, the combined stressor caused by high stocking density with feed restriction resulted in a significant ($p<0.05$) decrease in final BW without affecting relative organ weights of birds.

Total leukocyte (WBC) counts and leukocyte differential counts

Data on total counts of leukocyte, WBC differential counts, H:L ratio, RBC and Hb in whole blood of birds in response to the combined stressor are shown in Table 4. Total counts of leukocyte in the stress group had a tendency ($p<0.07$) to decrease compared with those in the control group. However, leukocyte differential counts including heterophils, lymphocytes and monocytes were not affected by 12-d of the combined stressor induced by stocking density with feed restriction. In addition, the ratio of H:L,

Table 3. Effects of combined stress induced by stocking density and feed restriction on body weight and relative organ weights in laying hens

Item	Treatment		p-value
	Control	Stress	
Initial BW (g)	1,626.4±57.77	1,610.2±22.08	0.754
Final BW (g)	1,705.9±55.25*	1,523.2±26.81	0.003
Liver (g/100 g BW)	2.59±0.11	2.51±0.11	0.975
Spleen (g/100g BW)	0.08±0.05	0.07±0.01	0.120

Means (means±SE). Asterisk (*) indicated significantly different between two groups at $p<0.05$.

Table 4. Effects of the combined stressor induced by high stocking density with feed restriction on hematological values in laying hens

Item	Treatment		p-value
	Control	Stress	
WBC ($\times10^3$ cells/μl)	34.11±1.29	30.79±0.93	0.07
Heterophil ($\times10^3$ cells/μl)	9.28±0.88	8.17±0.51	0.31
Lymphocyte ($\times10^3$ cells/μl)	19.84±0.98	18.03±0.47	0.13
Monocyte ($\times10^3$ cells/μl)	3.62±0.14	3.42±0.14	0.33
Heterophil/lymphocyte	0.48±0.06	0.45±0.03	0.75
RBC ($\times10^6$ cells/μl)	5.79±2.02	3.62±0.26	0.38
Hb (g/dl)	17.40±3.60	12.70±0.40	0.29

Values (mean±SE, n = 6).

an indicator of stress in birds, was below 0.5 in both groups which is the normal range from many studies. The RBC and Hb were also not affected by the combined stressor given to birds in our study.

Expression levels of cytokines in the liver and spleen

The expression levels of IL-4, IL-6, LITNF-α, and iNOS in non-lymphoid (the liver) and lymphoid (the spleen) organs of birds after 12-d of combined stressor are presented in Figure 1A and 1B. In the liver, the expression levels of LITNF-α and iNOS in the stress group were significantly ($p<0.05$) higher than those of the control group (Figure 1A). However, no significant difference in the expression levels of hepatic IL-4 and IL-6 was observed between the two groups. In the spleen, the combined stressor significantly ($p<0.05$) enhanced the level of LITNF-α, which was highly expressed in the spleen (Figure 1B), whereas the combined stressor had no significant effect on the expression levels of IL-4, IL-6 and iNOS in birds.

DISCUSSION

To investigate the effects of husbandry stressors on

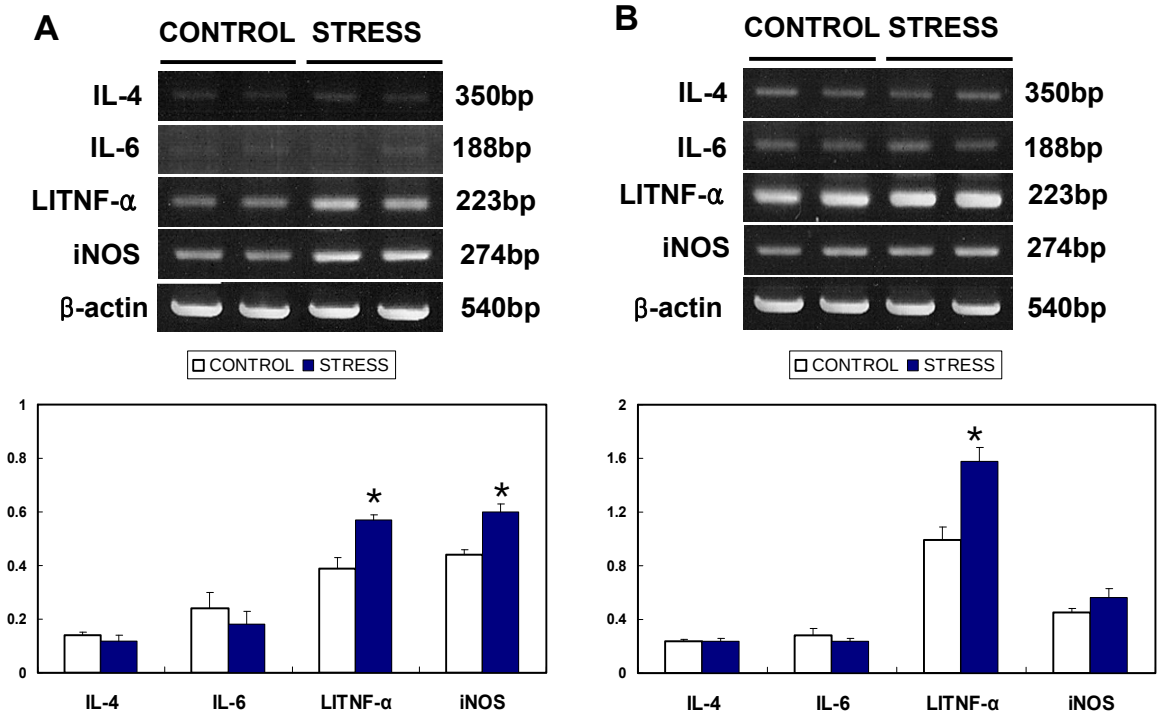

Figure 1. Semi-quantification of mRNA expressions of IL-4, IL-6, LITNF-α and iNOS in the liver (A) and spleen (B) of laying hens in the control (□) and the combined stress groups (■) induced by stocking density with feed restriction. Levels of all mRNA were expressed as the value of signal intensity for genes relative to that for β-actin. Two of five replicates from each group are pictured in the figure. Values are means per group (n = 5) with standard errors shown by vertical bars. Asterisk(*) indicated significantly different between the two groups at $p<0.05$.

immunological response as a stress indicator, we applied two simultaneously combined stressors caused by high stocking density with feed restriction for 12-d, which would give rise to severe stress due to feed competition, uncomfortable laying, frustration, etc. (de Jong et al., 2003). According to the report of Star et al. (2007), the combination of lipopolysaccharide (LPS) and heat stressors activated the natural and specific immune competence of laying hens after 8~14-d of treatment. In our study, the combined stressor group apparently showed more aggression and higher feather-pecking behavior as reported by other studies (Zimmerman et al., 2006). Also, the combined stressor group resulted in a significant decrease in BW, indicating that the combined stressor seems to induce severe stress in birds. Similar to our result, other studies also reported that there was a significant reduction of BW attributable to stressors such as increased stocking density or feed restriction in birds (Patterson and Siegal, 1998; Jang et al., 2007; Mtileni et al., 2007). However, we did not observe a difference in the weights of immune-related organs between the two groups. Our observation was in agreement with studies that a higher stocking density (Buijs et al., 2009) or 20-40% feed restriction of *ad libitum* (Liew et al., 2003; Fassbinder-Orth and Karasov, 2006) did not influence the weights of immune organs of birds.

Furthermore, to investigate the effect of combined stressor on hematological values, we examined WBC differential counts and H:L ratio. Despite a significant decrease in BW, the combined stressor did not affect WBC differential counts and H:L ratio. When birds were exposed to various environmental stressors, H:L ratio, widely recognized as an index of stress in birds, has been shown to increase under heat stress, transportation, electrical shock, dietary mycotoxin, etc. (Ubosi et al., 1985; McFarlane and Curtis, 1989; Mashaly et al., 2004; Huff et al., 2005). By contrast, several studies reported that stocking density alone had no significant effect on hematological values including H:L ratio (Patterson and Siegel, 1998; Nicol et al., 2006; Thaxton et al., 2006; Turkyilmaz, 2008), although various behavioral aspects of birds have been related to stocking density (Murphy et al., 1988; Andrews et al., 1997). It seems that much more severe or prolonged stressors can impair immune systems, such as immune organs and hematological values, in mature birds.

Therefore, it is necessary to investigate more specific immunological parameters that can be applied for assessing stress responses in birds. However, to date there has been little study of the influence of husbandry stressors on cell-mediated immune indicators such as cytokines in birds. In particular, it is important to examine the effect of stressor on the acquired immunity associated with two different cell types; B-lymphocytes (for humoral immunity) and T-lymphocytes (CD4 and CD8 which are associated with anti- and pro-inflammatory cytokines for cellular immunity (Janeway et al., 2001). T-helper2-cell (Th2) CD4- T lymphocytes induce the production of IL-4 and IL-6 cytokines, while T-helper1-cell (Th1) CD4- T lymphocytes produce IL-2 and IFN-γ (Jolly, 2004). IL-4 cytokine, as an anti-inflammatory cytokine, can modulate the suppression of pro-inflammatory cytokines such as IL-6, IFN-γ, etc (Jolly, 2004). LPS-induced TNF-α (LITNF-α), widely expressed in lymphoid and non-lymphoid tissues, is one of the most powerful stimulators of the pro-inflammatory process (Hong et al., 2006). Moreover, iNOS is another important gene that might be associated with the inflammatory process, since nitric oxide (NO) formed by iNOS has emerged as a multifunctional molecule that exerts modulating effects on inflammation and immune responses (Guzik et al., 2003).

In our study, hepatic LITNF-α and iNOS, pro-inflammatory cytokines, were significantly elevated in the stress group compared with those in the control group. Moreover, splenic LITNF-α expression was significantly higher in the birds treated with the combined stressor. However, IL-6 expression, anti-inflammatory cytokine, appeared to be similar between the two groups. From our results, it could be speculated that the combined stressor activated the expression of certain pro-inflammatory cytokines. Our results are supported by Khajavi et al. (2003) showing that the combined stressor including heat stress with feed restriction had higher CD4- T lymphocytes and antibody titer, but lower CD8- T lymphocytes, suggesting that induced stressors have considerable impact on the cell-mediated immune response of birds. Also, our previous study reported that feed restriction from 70 to 85% of *ad libitum* significantly affected the expression of inflammatory cytokines in birds (Jang et al., 2007). According to Shini and Kaiser's report with birds (2009), the expressions of IL-1β, IL-6 and IL-18 mRNA of blood and splenic lymphocytes were markedly enhanced during corticosterone-induced stress, indicating that a stressor can affect the immune response by modulating pro-inflammatory cytokines. Cold stress has been reported to enhance pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12β (Hangalapura et al., 2006). Even noise stress in mice activated iNOS mRNA expression and induced blood vessel wall damage (Shi and Nuttall, 2003). Judging from various studies, immune-related pro-inflammatory cytokines in birds are affected by a variety of stressors and these genes can be used as one of the indicators of stress.

Taken together, results of our study showed that high stocking density with feed restriction in birds markedly affected the cell-mediated immune responses by increasing pro-inflammatory cytokines, leading to potential modulation of immunity in birds. In fact, since high

stocking density or feed restriction itself induced more aggressive behavior in birds under conventional circumstances (Zimmerman et al., 2006), thus, it should not be ignored that an increased pro-inflammatory cytokine in response to the combined stressor could have an impact on immunity and welfare of birds.

In conclusion, the combined stressor caused by high stocking density with feed restriction enhanced expression levels of pro-inflammatory cytokines including LITNF-α and iNOS in the liver and spleen of birds. Thus, the immune response of cytokine genes to given stressors can be another indicator that can be used in monitoring stress responses of birds, although more detailed studies under various circumstances are needed to explore the ways of assessing stress in birds.

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