

Relationship between chicken cellular immunity and endotoxin levels in dust from chicken housing environments

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Hazardous biochemical agents in animal husbandry indoor environments are known to promote the occurrence of various illnesses among workers and animals. The relationship between endotoxin levels in dust collected from chicken farms and various immunological markers was investigated. Peripheral blood was obtained from 20 broiler chickens and 20 laying hens from four different chicken farms in Korea. Concentrations of total or respirable dust in the inside the chicken farm buildings were measured using a polyvinyl chloride membrane filter and mini volume sampler. Endotoxin levels in the dust were determined by the Limulus Amebocyte Lysate Kinetic method. Interferon- γ production by peripheral blood mononuclear cells stimulated with concanavalin A was significantly lower in broilers or layers from the farms with higher endotoxin concentrations than the chickens from the farms with lower endotoxin levels. An opposite pattern was observed for plasma cortisol concentrations with higher cortisol levels found in chickens from the farms with higher endotoxin levels. When peripheral lymphocytes were examined, the percentage of CD3⁺ I α ⁺ B cells was lower in layers from farms with higher endotoxin levels than those from locations with lower endotoxin levels. Overall, these results suggest a probable negative association between dust endotoxin levels and cell-mediated immunity in chickens.

Keywords: cellular immunity, chicken, endotoxin, dust, interferon- γ

Introduction

Animal welfare has been continuously discussed since the World Organization for Animal Health convened the first global conference on animal welfare in 2004 [26]. Among the broad spectrum of animal welfare issues, the animal husbandry environment has been considered critical because inappropriate management of this environment may detrimentally affect the health of both animals and husbandry workers. Bio-aerosols generated in livestock confinement buildings could be a major risk factor for respiratory illness in both animals and humans [12,14,15]. These aerosols are composed of organic dust, microorganisms, and endotoxins. Effects of bio-aerosol exposure on the respiratory system have not been extensively investigated in livestock but have been studied in animal husbandry workers [1,6,12].

Endotoxin or lipopolysaccharide (LPS) is found in the outer membrane of Gram-negative bacteria. Endotoxin exposure is a causative agent or contributing factor for various pulmonary illnesses including asthma, organic dust toxic syndrome, and

chronic obstructive pulmonary disease that affect human subjects including animal husbandry workers [1,3,10,12,17,19,22]. Endotoxin exposure initiates type 2 helper T cell (Th2)-mediated immune reactions resulting in respiratory allergic hyper-reactivity. Few studies have been performed to assess the effects of endotoxin exposure on immunity of livestock. LPS administration through intravenous injection into 7- to 8-month old chickens was reported to rapidly reduce the number of monocytes in peripheral blood and macrophages in the spleen; these values returned to control levels at 6 to 24 h post-LPS injection [2]. In addition, 8-week old chickens intravenously injected with LPS had lower antibody titers following infectious bronchitis virus vaccination compared to the control group [20]. Up-regulated expression of pro-inflammatory cytokine genes such as interleukin (IL)-1 β , IL-6, and IL-8 was also observed in chicken heterophils in the presence of LPS *in vitro* [11]. Aside from these studies, few systemic investigations have been performed on the modulation of chicken immunity through endotoxin exposure via the indoor environment of chicken farms.

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The current study was performed to evaluate the relationship between endotoxin levels in dust collected from chicken farms and changes in immunological markers. We measured the concentrations of total or respirable dust in broiler or laying hen houses and determined level of endotoxin in the dust. Various immune parameters including plasma IgY levels, interferon- γ (IFN- γ) production from peripheral mononuclear cells (PBMC), lymphocyte subpopulation frequencies, and plasma cortisol levels were evaluated.

Materials and Methods

Chicken farms and blood collection

Two broiler chicken farms (BS and BE) in two locations (Yeoju and Eumseong) along with two laying hen farms (LK and LG) in two other locations (Ganghwa and Gimpo) in Korea were selected. Husbandry environments of the four farms are described in detail at Table 1. Since chicken farms in Korea are concentrated in these areas, regional veterinarians were asked to select chicken farms with similar stock density and agreed to assist with our field samplings. Ten heads of chicken from each farm were randomly chosen for blood collection for which 3 mL were aseptically drawn from the brachial vein into EDTA Vacutainer tubes. All of our chicken handling, blood collection, and experimental procedures were approved (no. CUD IACUC-2012-10) by the IACUC of Catholic University (Daegu). The broilers were 30 days old and the laying hens were 2 years old at the time of blood collection.

Dust collection and endotoxin measurement

Concentration of total indoor dust from the chicken farms was evaluated for 8 h using a PVC membrane filter (SKC, USA) with a 2-stage cassette impactor at a flow rate of 2.0 L/min. Concentration of respirable dust (PM10) was measured for 8 h using a polyvinyl chloride membrane filter with a 10-mm Dorr-Oliver nylon cyclone at a flow rate of 1.7 L/min. Dust samples were taken from two different locations (1/3 and 2/3 distance from the exit) at each farm.

Endotoxin concentration in the dust was measured as previously described [10,19]. Endotoxin was extracted from the filters by adding 3 mL endotoxin-free Limulus Amebocyte Lysate (LAL) water (LAL Kinetic-QCL set; Lonza, USA) with 5% Tween 20 followed by shaking for 1 h at 200 g. Supernatants were collected and stored in a -80°C freezer until analysis. Endotoxin concentrations in the supernatants were measured using a microplate spectrophotometer (Model Epoch; Bio-Tek, USA).

PBMC collection and lymphocyte phenotyping

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Life Sciences, UK). Three-color flow cytometry (FACScan; BD Biosciences, USA) was used to analyze the peripheral lymphocyte subpopulations. Anti-CD3-RPE and anti-Ia-FITC antibodies (SouthernBiotech, USA) were used to identify T cell and B cell populations. Anti-CD4-FITC, anti-CD8-FITC, and anti-TCR $\gamma\delta$ -FITC antibodies (SouthernBiotech) were used for sorting helper T cell, cytotoxic T cell, and $\gamma\delta$ T cell subpopulations, respectively. RPE- or FITC-conjugated isotype controls were used for subtracting non-specific background binding of the fluorescent antibodies.

ELISA for plasma IgY and cortisol quantitation

The level of IgY in plasma was determined with a sandwich ELISA using mouse anti-chicken IgG (SouthernBiotech) as the capture antibody and horseradish peroxidase (HRP)-goat anti-chicken IgG (SouthernBiotech) as the detection antibody. Plasma samples were diluted 1/100,000 with 1% bovine serum albumin (BSA) in PBS. Plasma cortisol levels were measured using an IBL Cortisol ELISA kit (Immuno-Biological Laboratories, Germany), and absorbance was measured at 450 nm with 630 nm as the reference wavelength. The lower detection limit for cortisol was 0.05 ng/mL.

T cell activation and IFN γ measurement

PBMCs (10^6 cells/mL) in complete RPMI medium (1 mM nonessential amino acids, 1 mM sodium pyruvate, 1% sodium

Table 1. General husbandry characteristics of the poultry farms investigated

	BS	BE	LK	LG
Location	Yeoju, Gyeonggi province	Eumseong, Chungbuk province	Ganghwa, Incheon metropolitan city	Gimpo, Gyeonggi province
Chicken house style	Open house with winch curtains	Open house with winch curtains	Open house with upper windows	Environmental controlled house without window
Broiler/laying hen (breed)	Broiler (Ross)	Broiler (Arbor Acres)	Laying hen (Hy-Line Brown)	Laying hen (Hy-Line Brown)
House space (m ²)	790	840	820	1,090
Number of chicken (head)	12,000	13,300	11,000	20,000
Stocking density (m ² /head)	0.066	0.063	0.075	0.055
Ventilation	Mechanical fan	Mechanical fan	Mechanical fans	Tunnel ventilation system

bicarbonate, 2 mM glutamine, 50 μ M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum) were activated with 5 μ g concanavalin A for 72 h at 41°C in a 5% CO₂ incubator. IFN γ levels in each culture supernatant were measured with a sandwich ELISA using chicken IFN γ CytoSet (Invitrogen, USA). The lower detection limit for IFN γ was 1 pg/mL.

Statistical analyses

All statistical analyses were performed with SigmaStat 3.5 (Systat Software, USA). Differences between broiler or laying hen farms were evaluated with Student's *t*-test or Mann-Whitney rank sum test. *P* values < 0.05 were considered significant.

Results

Similarity in husbandry conditions for broiler or laying hens

Husbandry conditions including chicken house style, stocking density, and ventilation systems were very similar between the two broiler farms (Table 1). The structures of the laying hen houses on the two farms were different. The house on the LK farm had several upper windows while that on the LG farm was equipped with a tunnel ventilation system and lacked windows. The LK farm had a lower stocking density than the LG farm.

Level of endotoxins in the dust from the inside of chicken houses

There was no statistical difference in total or respirable dust concentration between the two broiler houses (Table 2). Even though the mean total and respirable dust concentration was

higher for the LK laying hen farm than the LG farm, this difference was not significant. Endotoxin levels in the total or respirable dust collected from inside the chicken houses were higher for the BE broiler and LG laying hen farms than the BS broiler and LK laying hen farms, respectively.

Association of endotoxin levels with various immune parameters

Endotoxin administration can alter various immunologic markers in chickens. Therefore, we evaluated lymphocyte subpopulations in peripheral blood from laying hens that play a key role in humoral and cell-mediated immunity. The proportions of major T lymphocyte subpopulations including CD3⁺CD4⁺ helper T cells, CD3⁺CD8⁺ cytotoxic T cells, and $\gamma\delta$ T cells did not significantly differ when comparing the two laying hen farms (Table 3). However, the proportion of CD3⁺Ia⁺ B cells was significantly lower in hens from the LG farm that had higher endotoxin levels than hens from the LK farm.

Levels of plasma IgY, a typical marker for humoral immunity, were similar between the two broiler groups from the two different farms; this was also observed when the two laying hen groups were compared (Table 4). Next, we examined IFN γ production by peripheral T cells since this cytokine is critical for protection against infection by virulent microorganisms. IFN γ levels were significantly reduced in chickens from the farms with higher endotoxin levels (BE and LG farms) irrespective of chicken strain when compared to chickens from farms with lower endotoxin levels (BS and LK farms). Plasma cortisol levels were determined to estimate the intensity of chronic stress. Even though the difference was not significant, chickens

Table 2. Endotoxin levels in total or respirable dust collected from the chicken farms*

	BS	BE	LK	LG
Total dust (mg/m ³)	0.53 \pm 0.40	0.57 [†]	1.03 \pm 0.23	0.18 \pm 0.06
Respirable dust (mg/m ³)	0.08 \pm 0.05	0.06 \pm 0.04	0.23 \pm 0.10	0.09 \pm 0.12
Endotoxin in total dust (EU/m ³)	103.6 \pm 103.1	620.4	56.3 \pm 45.2	682.2 \pm 219.2
Endotoxin in respirable dust (EU/m ³)	1.75 \pm 1.75	6.83 \pm 1.22	0.21 \pm 0.12	171.8 \pm 200.8

*Data are expressed as the mean \pm SD. [†]Dust sampling was performed at two different locations for each farm. However, total dust data for the BE farm was obtained from one sampler due to mechanical problems with the other sampler.

Table 3. Proportion (%) of lymphocyte subsets among peripheral blood mononuclear cells from the laying hens*

Farm	CD3 ⁺ CD4 ⁺ T lymphocytes	CD3 ⁺ CD8 ⁺ T lymphocytes	CD3 ⁺ TCR $\gamma\delta$ ⁺ T lymphocytes	CD3 ⁻ Ia ⁺ B lymphocytes [†]
LK	34.8 \pm 11.7	9.6 \pm 3.0	9.4 \pm 1.1	10.6 \pm 2.3
LG	34.6 \pm 4.4	8.2 \pm 2.4	11.7 \pm 3.3	5.7 \pm 0.9

*Data are expressed as the mean \pm SEM. Ten chickens from each farm were evaluated. [†]Significant difference assessed by Mann-Whitney rank sum test (*p* < 0.05).

Table 4. Humoral or cellular immunity parameters for broiler chickens or laying hens*

	BS	BE	LK	LG
Plasma IgY (mg/mL)	1.47 ± 0.22	1.52 ± 0.50	5.63 ± 1.60	5.98 ± 0.93
INF- γ (pg/mL) [†]	17.15 ± 14.38 [‡]	0.0 ± 0.0 [§]	251.35 ± 143.57	47.06 ± 32.59
Plasma cortisol (ng/mL)	1.77 ± 0.51	2.17 ± 0.65	2.47 ± 0.79	5.51 ± 2.69

*Data are expressed as the mean ± SEM. [†]Peripheral mononuclear cells (10^6) were stimulated with 5 μ g concanavalin A at 41°C for 72 h in a 5% CO₂ incubator and the culture supernatants were collected to measure interferon- γ (INF- γ) production. [‡]Significant difference (*p* value): BS vs. BE (0.024). [§]INF- γ concentrations in all the culture supernatants were under the detection level.

from the farms with dust containing higher endotoxin levels (BE and LG farms) than the other farms (BS and LK farms) had higher plasma cortisol level (Table 4).

Discussion

Growth of the poultry industry propelled the extensive production of chickens in a confined and rapid manner, leading to increased reports of various diseases due to biochemical hazards inside the poultry houses [14,15,17,20,26]. Among these biochemical hazards, dusts and endotoxin are known to cause immunologic modulations [2,5,14,15,20]. They can increase disease severity by augmenting pulmonary inflammatory responses. Even though only two broiler and two laying hen farms were investigated, our study revealed that endotoxin exposure can alter immunologic responses. Chickens reared in the farms with higher endotoxin levels had lower IFN γ production from peripheral T cells, higher plasma cortisol levels, and a reduced proportion of peripheral B cells in our study.

Our results indicated that changes in immune parameters investigated are associated with endotoxin concentrations in total or respirable dust rather than dust concentration itself. The LG laying hen farm contained lower total and respirable dust concentrations than the LK laying hen farm. However, reduced IFN γ production and B cell populations along with increased cortisol concentrations were observed in chickens from the LG farm with a higher endotoxin level compared to chickens from the LK farm with a lower endotoxin level. Various factors could affect the accumulation of endotoxin in dust particles including stocking density, ventilation efficiency, frequency of chicken house clearing, or the number of Gram-negative bacteria. When the two broiler farms were compared, general structure of the chicken houses (open house with winch curtains) and ventilation systems using mechanical fans were the same for both farms in our study. Stocking density was slightly higher at the BE farm (0.063 m²/head) with higher endotoxin levels (total dust: 620 EU/m³, respirable dust: 7 EU/m³) than the BS farm (stocking density: 0.066 m²/head, total dust endotoxin: 104 EU/m³, respirable dust: 2 EU/m³). These differences were more apparent

when comparing the laying hen farms. The stocking density was higher at the LG farm (0.055 m²/head) with a higher endotoxin level (total dust: 682 EU/m³, respirable dust: 172 EU/m³) than the LK farm (stocking density: 0.075 m²/head, total dust endotoxin: 56 EU/m³, respirable dust: 0.2 EU/m³). Stocking densities for the broilers were within the parameters recommended by Korean guidelines (0.066 m²/head), but stocking densities for the laying hens exceeded the guideline density (0.042 m²/head) [16]. Stocking density could be another factor influencing endotoxin concentration in the dust.

Leukopenia, especially decreased peripheral B cell populations, has been reported in chickens intravenously exposed to endotoxin [20,21,25]. In addition, reduction of body weight gain was also observed in chickens injected with endotoxin. These changes were resolved sooner or later following endotoxin injection. Furthermore, administration of corticosterone through drinking water was found to induce changes similar to the ones described above [20,21]. Chickens that chronically experience physical or biochemical stress including movement confinement or persistent endotoxin exposure can persistently release cortisol into the blood stream from the adrenal cortex, which results in suppression of the immune system and susceptibility to infection by pathogenic microorganisms [5,9,18,20,21]. We also observed elevated plasma cortisol levels in broiler or laying hens reared at the farms with relatively higher endotoxin levels. These chickens apparently exhibited less IFN γ production from activated peripheral T cells and a low proportion of peripheral B cells. The effect of cortisol on IFN γ production has been debated since some studies showed a negative relationship between these two factors while others did not depending on the species evaluated, whether *in vitro* or *in vivo* systems were used, source tissue of the T lymphocytes, and the inclusion of healthy or sick subjects [4,7,8,13,23,24]. Our results indicated a negative relationship between plasma cortisol levels and peripheral IFN γ production.

In summary, our investigation demonstrated that chickens reared in facilities with higher stocking density and elevated environmental endotoxin levels could be susceptible to stress resulting in lowered resistance against various pathogenic insults including infection. Since only four chicken farms were

included at our study, generalization of the results may be limited. Therefore, systemic investigations should be conducted to further elucidate the association between husbandry environment and immunity in chickens.

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Conflict of Interest

There is no conflict of interest

References

1. **Astrakianakis G, Murray E.** Conflicting effects of occupational endotoxin exposure on lung health—a hypothesis-generating review of cancer and COPD risk. *J Environ Immunol Toxicol* 2014, **1**, 128-139.
2. **Bowen OT, Dienglewicz RL, Wideman RF, Erf GF.** Altered monocyte and macrophage numbers in blood and organs of chickens injected i.v. with lipopolysaccharide. *Vet Immunol Immunopathol* 2009, **131**, 200-210.
3. **Cornier Y, Israël-Assayag E, Racine G, Duchaine C.** Farming practices and the respiratory health risks of swine confinement buildings. *Eur Respir J* 2000, **15**, 560-565.
4. **Dafny N, Yang PB.** Interferon and the central nervous system. *Eur J Pharmacol* 2005, **523**, 1-15.
5. **Dickerson SS, Kemeny ME.** Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychol Bull* 2004, **130**, 355-391.
6. **Duquenne P, Marchand G, Duchaine C.** Measurement of endotoxins in bioaerosols at workplace: a critical review of literature and a standardization issue. *Ann Occup Hyg* 2013, **57**, 137-172.
7. **Goossens KE, Ward AC, Lowenthal JW, Bean AGD.** Chicken interferons, their receptors and interferon-stimulated genes. *Dev Comp Immunol* 2013, **41**, 370-376.
8. **Hickey MC, Drennan M, Earley B.** The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocytes, acute-phase proteins and in vitro interferon-gamma production. *J Anim Sci* 2003, **81**, 2847-2855.
9. **Kaiser P, Wu Z, Rothwell L, Fife M, Gibson M, Poh T-Y, Shini A, Bryden W, Shini S.** Prospects for understanding immune-endocrine interactions in the chicken. *Gen Comp Endocrinol* 2009, **163**, 83-91.
10. **Kiryuchuk SP, Dosman JA, Reynolds SJ, Willson P, Senthilselvan A, Feddes JJR, Classen HL, Guenter W.** Total dust and endotoxin in poultry operations: comparison between cage and floor housing and respiratory effects in workers. *J Occup Environ Med* 2006, **48**, 741-748.
11. **Kogut MH, Iqbal M, He H, Philbin V, Kaiser P, Smith A.** Expression and function of Toll-like receptors in chicken heterophils. *Dev Comp Immunol* 2005, **29**, 791-807.
12. **Liu AH.** Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002, **109**, 379-392.
13. **Malarkey WB, Wang J, Cheney C, Glaser R, Nagaraja H.** Human lymphocyte growth hormone stimulates interferon gamma production and is inhibited by cortisol and norepinephrine. *J Neuroimmunol* 2002, **123**, 180-187.
14. **Mani V, Weber TE, Baumgard LH, Gabler NK.** Growth and development symposium: endotoxin, inflammation, and intestinal function in livestock. *J Anim Sci* 2012, **90**, 1452-1465.
15. **Millner PD.** Bioaerosols associated with animal production operations. *Bioresour Technol* 2009, **100**, 5379-5385.
16. Ministry of Agriculture, Food and Rural Affairs (KR). Guideline on environmental friendly animal husbandry. Notice 2008-3. (Jan. 11, 2008).
17. **Mostafa E, Buescher W.** Indoor air quality improvement from particle matters for laying hen poultry houses. *Biosyst Eng* 2011, **109**, 22-36.
18. **Möstl E, Palme R.** Hormones as indicators of stress. *Domest Anim Endocrinol* 2002, **23**, 67-74.
19. **Radon K, Danuser B, Iversen M, Monso E, Weber C, Hartung J, Donham K, Palmgren U, Nowak D.** Air contaminants in different European farming environments. *Ann Agric Environ Med* 2002, **9**, 41-48.
20. **Shini S, Kaiser P, Shini A, Bryden WL.** Biological response of chickens (*Gallus gallus domesticus*) induced by corticosterone and a bacterial endotoxin. *Comp Biochem Physiol B Biochem Mol Biol* 2008, **149**, 324-333.
21. **Shini S, Kaiser P, Shini A, Bryden WL.** Differential alterations in ultrastructural morphology of chicken heterophils and lymphocytes induced by corticosterone and lipopolysaccharide. *Vet Immunol Immunopathol* 2008, **122**, 83-93.
22. **Sigsgaard T, Bonefeld-Jørgensen EC, Hoffmann HJ, Bønløkke J, Krüger T.** Microbial cell wall agents as an occupational hazard. *Toxicol Appl Pharmacol* 2005, **207** (Suppl), S310-319.
23. **Skjolaas KA, Minton JE.** Does cortisol bias cytokine production in cultured porcine splenocytes to a Th2 phenotype? *Vet Immunol Immunopathol* 2002, **87**, 451-458.
24. **Susta L, Cornax I, Diel DG, Garcia SC, Miller PJ, Liu X, Hu S, Brown CC, Afonso CL.** Expression of interferon gamma by a highly virulent strain of Newcastle disease virus decreases its pathogenicity in chickens. *Microb Pathog* 2013, **61-62**, 73-83.
25. **Wang W, Wideman RF Jr, Chapman ME, Bersi TK, Erf GF.** Effect of intravenous endotoxin on blood cell profiles of broilers housed in cages and floor litter environments. *Poult Sci* 2003, **82**, 1886-1897.
26. **World Organisation for Animal Health.** Global conference on animal welfare: an OIE initiative. pp. 121-206, OIE and the European Commission, Paris, 2004.