Effects of Dietary Iodine and Selenium on the Activities of Blood Lymphocytes in Laying Hens

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ABSTRACT : The effect of dietary iodine and selenium supplementation, alone or in combination, on peripheral blood lymphocyte function was determined in laying hens. Eight-hundred-and-sixty-four New-Loman laying hens were randomly allotted into 12 dietary treatments with different inclusion levels of iodine (0, 0.1 and 0.2 mg/kg), selenium (0, 0.05, 0.1 and 0.2 mg/kg) or their combinations for 24 weeks. The lipopolysaccharide (LPS) stimulation index, concanavalin A (ConA) stimulation index, peroxide enzyme activity and phagocytosis to neutral red particles were tested. There were significant differences in LPS stimulation index, ConA stimulation index, peroxide enzyme activity and phagocytosis to neutral red particles in different iodine or selenium supplementation levels ($p<0.05$). The highest iodine and selenium supplementation both resulted in highest LPS-/ConA-stimulation indices ($p<0.05$). However, when iodine was lower than 0.2 mg/kg, the additional effect of different levels of selenium did not always result in significant differences in these indices. The results indicated that iodine and selenium may affect immunity in laying hens and, when the iodine level in the laying hen is lower than 0.2 mg/kg, a selenium allowance higher than 0.1 mg/kg may be necessary to improve immunity. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 5 : 713-719)***

Key Words : Laying Hens, Iodine, Selenium, Lymphocyte, Immune Function

INTRODUCTION

The importance of iodine as an essential element in animal's diet arises from the fact that it is a major component of the thyroid hormone 3,5,3' tri-iodothyronine (T_3) and 3,5,3',5'tetra-iodothyronine (T_4) or thyroxine (Ganong, 2001). These hormones are essential for normal growth (and physical) as well as mental development throughout the lives of animals and human, and they also set the basal metabolic rate (BMR) and play active roles in digestion (Miller et al., 1974), thermoregulation, intermediary metabolism, growth, muscle function, circulation, the seasonality of reproduction and immune defense (Follett and Potts, 1990). It is well-known that the thyroid hormones have both gross and fine metabolic effects on lymphoid cells (Fabris, 1973; Lundell and Blomgren, 1976; Keast and Taylor, 1982; Li et al., 1993; Philip et al., 1995). Iodine deficiency or in excess has an effect on thyroid hormone synthesis and, consequently, may influence the function of the animal's lymphocytes.

Selenium is an essential component of the antioxidant enzyme glutathione peroxidase (GPx) which removed potentially damaging lipid hydroperoxides and hydrogen peroxide (Anita et al., 2004). Selenium can act as an antioxidant in the extracellular space, the cell cytosol, in association with cell membranes and specifically in gastrointestinal tract, all with potential to influence immune processes (Arthur et al., 2003). In addition to its known essentiality, selenium plays an important role in the control of thyroid hormone metabolism. The thyroid gland's major hormone product, T_4, is converted by 5' (outer ring)-deiodination to the more biologically active hormone, T_3, by the type I and type II iodothyronine deiodinases (ID-I, ID-II). T_4 can also be converted to reverse triiodothyronine (3, 5', 3'-triiodothyronine, γT_3), by 5 (inner ring) deiodination (Leonard and Visser, 1986). In each of the cases, free iodine is released upon conversion of these hormones. ID-I has been shown to be a seleno-enzyme (DePalo et al., 1994). More recently it was discovered that the ID-II and type III (ID-III, the inner ring deiodinase responsible for deactivating T_4 to γT_3 and T_3 to T_2) are also selenocysteine enzymes (Davey et al., 1995; Ramauge et al., 1996). Therefore, selenium may play a crucial role in the maintenance of normal thyroid physiology. For example, in a selenium- and iodine-deficient animal model, iodine supplementation alone caused irreversible thyroid gland fibrosis (Hotz et al., 1996). In human subjects with both selenium and iodine deficiencies, selenium supplementation alone caused an aggravation of iodine deficiency and hypothyroidism (Contempre et al., 1991). In rat studies, iodine deficiency or excess had reverse effect on lymphocytes' function (Fabris, 1973), and selenium modulated the thyroxine's metabolism. These studies demonstrated a complex interaction between iodine and selenium.

Iodine- and/or selenium-deficiency exist in many parts of the world. In some areas of China, iodine and selenium in the base diet do not meet the poultry's requirement (Guo,

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Table 1. Composition of base diet*

Ingredients	%
Corn	63.80
Soybean meal	25.30
DL-methionine	0.11
Limestone meal	9.00
Monocalcium phosphate	1.20
Sodium chloride	0.37
Vitamin and mineral mixture[1]	0.22
Chemical composition	
Analyzed	
Iodine (mg/kg)	0.098
Selenium (mg/kg)	0.012
Calculated	
Crude protein (g/kg)	165.00
ME(MJ/kg)	11.08
Calcium (g/kg)	34.00
Available P (g/kg)	3.20

* This basal diet contains selenium, 0.012 mg/kg and iodine, 0.098 mg/kg.
[1] Vitamin and mineral mixture provided per kg diet: vitamin A, 10,800 IU; vitamin D_3, 2,160 IU; vitamin E, 6.5 mg; vitamin K_3, 1.0 mg; vitamin B_1, 0.4 mg; vitamin B_2, 5 mg; cobalamin, 6 mg; folic acid, 0.1 mg; niacin, 7 mg; Ca-pantothenate, 5 mg; Choline chloride, 1 g; Cu, 6 mg; Fe, 60 mg; Mn, 60 mg and Zn 50 mg.

1999). Laying hens, which have a long lifetime for production, need a more effective immune system to maintain its high anti-infection ability (Cheng, 2004a). In order to understand better the effect of dietary iodine and selenium, individually and interactively in laying hens, a series of experiments were conducted. The results of in particular their effect on lymphocytes, are presented in this report.

MATERIAL AND METHODS

Animals and treatments

Eight-hundred-and-sixty-four New-Loman laying hens with similar body weight (±50 g; 33 weeks old) were housed in cages (three birds per cage, stainless steel) in a climatically controlled room with auto-controlled lighting. Birds had free access to water and experimental diets (Table 2) formulated with four concentrations of supplemental Se (0, 0.05, 0.1, 0.2 mg/kg from sodium selenite) and three concentrations of iodine (0, 0.1, 0.2 mg/kg from calcium iodate) in the basal diet (Table 1) containing Se 0.012 mg/kg and iodine 0.098 mg/kg. Crude protein and metabolic energy (ME) contents of the basal diet were 165 g/kg and 11.08 MJ/kg respectively, as required by the NRC (1994) for HY-line brown variety. In a factorial randomized block designed experiment with twelve treatments, birds were distributed at random to 72 groups of twelve birds each. Each diet was offered to six replicate groups. The hens were fed those diets for 24 weeks. Blood samples from six layers in each treatment were taken from the wing vein for the lymphocytes' function test.

Determination of iodine and selenium in experimental diets

Iodine and selenium content of feed samples were determined in duplicate (Table 2) according the methods of Nutrition and Food Institute Files (No. 0016, 2000) (Nutrition and Food Safety Institute of Chinese Center for Disease Control and Prevention, Being, China) and GB/T 12399-96 (China State Standard).

Proliferation assay of peripheral blood lymphocyte

In vitro peripheral blood lymphocyte (PBL) proliferation response was determined by a previously described method (Lin, 1999; Xia, 2002) with slight modification after the experiment diets were fed for 24 weeks. The heparinized (20 U/mL) peripheral blood obtained by wing-vein puncture was added to the same volume of sterile Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The diluted blood mixture was laid over half its volume of sterile lymphocyte separation medium (density = 1.077-1.080, Academy of Military Medical Science), and separated by density-gradient centrifugation at 400×g for 30 min at 4°C to recover PBL. PBL were collected at the interface washed with HBSS three times, after which PBL were suspended in 2 mL

Table 2. Iodine and selenium contents of experimental diets

Dietary treatments	Iodine (mg/kg)		Selenium (mg/kg)	
	Supplementation	Total analyzed	Supplementation	Total analyzed
1	0	0.098	0	0.012
2	0	0.098	0.05	0.062
3	0	0.098	0.1	0.112
4	0	0.098	0.2	0.212
5	0.1	0.198	0	0.012
6	0.1	0.198	0.05	0.062
7	0.1	0.198	0.1	0.112
8	0.1	0.198	0.2	0.212
9	0.2	0.298	0	0.012
10	0.2	0.298	0.05	0.062
11	0.2	0.298	0.1	0.112
12	0.2	0.298	0.2	0.212

Table 3. Effect of selenium and iodine on the indices of the lymphocytes' function

Tests of btween-sbjects effects	LPS stimulated index	ConA stimulated index	Peroxide enzyme activity	Phagocytosis to neutral red particles
Selenium	0.000	0.000	0.000	0.000
Iodine	0.000	0.000	0.000	0.000
Selenium* Iodine	0.000	0.000	NS	NS

* S means no significant difference (p>0.05).

sterile RPMI 1640 media with $NaHCO_3$ (24 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 10 mM), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were detected by trpan blue dye exclusion using a microscope to assure 95% livability. Cell suspensions were adjusted to a final concentration of 1×10^6 cells/ml in RPMI-1640 medium. One hundred micro-liters of cell suspension, and 100 μL RPMI 1,640 with 45 μg/mL concanavalin A (ConA) (C2613, Sigma) or 25 μg/mL lipopolysaccharide (LPS) (L3129, Sigma) were added into a 96-well plate (Costar 3599). The cultures were set up in triplicate with mitogen (stimulated with Con A or LPS).The cells were then incubated in 5% CO_2 incubator at 41°C. After 56 h incubation, 10 μL CCK-8 (Dojindo CK04-11, Japan) was added to the cell culture. The cells were incubated for four more hours, then place the culture under a 4°C environment and vibrated for at least 3 minutes to stop the reaction. Read the absorbance at 450 nm via an automated ELISA reader (Bio-RAD, Model 550).

Peroxidase activity assay of peripheral blood lymphocyte

Peroxidase activity of peripheral blood lymphocyte was assessed as described by Liu (2004). An aliquot of the above cell suspensions with a final concentration of 1×10^6 cells/ml in RPMI-1640 medium were used for this enzyme activity assay. One hundred microlitres of cell suspension were added into a 96-well plate (Costar 3599) and each with a blank control which was added only with the RPMI-1640 medium. The cells were then incubated in 5% CO_2 incubator at 40°C for 2 h. Then, wash the wells with the RPMI-1640 medium for three times. Immediately, One hundred microlitres substrate liquor (o-phenylenediamine, 10 mg; 33% H_2O_2, 10 μL; citromalic acid-phosphate buffer, pH 4.5 and 25 ml) was added to each well. Incubated in 5% CO_2 incubator at 40°C for another 30 minutes before 50 μL H_2SO_4 was added to terminate the reaction. Read the absorbance at 490 nm via an automated ELISA reader (Bio-RAD, Model 550) with the blank as the background.

Phagocytosis of neutral red particles assay of peripheral blood lymphocyte

Phagocytosis of neutral red particles was tested according to Wang (1997). One milliliter of above cell suspension per well were cultured in 24-well culture plates (Costar 3524). Blank control wells were set up by adding culture media alone. Cells were incubated in 5% CO_2 incubator at 40°C for 2 h, then were washed for 3 times with culture media to make monolaying cells. One milliliter of neutral red (0.1%, 100 mg/100 ml saline water (0.85%)) was added onto the cell monolayer in culture plate and incubated. After 15 minutes, the supernatant were discarded, excessive neutral red was washed with saline and 1 ml cell dissolving fluid (ethanol A.R.:acetic acid A.R. = 1:1 (v/v)) was added. After static placement overnight, the absorbance of each well was read via UV/VIS spectrometer (Lambda Bio20, PERKLN ELMER) at 550 nm.

Statistical analysis

The mean effects of the selenium and iodine were analyzed by the method of multivariate in the general linear model (GLM) of SPSS 10.0. The multiple comparisons of the data were analyzed by one-way ANOVA of SPSS 10.0, and reported as Means±SE. The significance of differences among different groups was evaluated by Least Significant Difference (LSD) post-hoc multiple comparisons test. Significance was $p<0.01$ unless otherwise stated.

RESULTS

The result of the tests (Table 3) of between-subjects effects showed that the selenium and iodine affected the blood lymphocyte function significantly ($p<0.01$). The interaction of selenium and iodine affected the lymphocyte's stimulation indexes by lipopolysaccharide (LPS) ($p<0.01$) and concanavalin A (ConA) ($p<0.01$) significantly, but not that of the peroxidase activity ($p>0.05$) and the phagocytic activity ($p>0.05$).

Cellular immunity of laying hens fed with different levels of dietary selenium and iodine was tested (Table 4, Figures 1 and 2). At low levels of selenium (basal and 0.05 mg/kg added) and low iodine (basal), their peripheral blood lymphocyte (PBL) displayed the lowest stimulation index by LPS or ConA. At the highest levels of selenium and iodine, their PBLs displayed the highest stimulation index by LPS or ConA. When supplemented with iodine at the level of 0.2 mg/kg, their PBLs showed a higher stimulation index by LPS or ConA along with a higher selenium supplementation. But when supplemented with iodine 0.1 mg/kg or without iodine, their PBLs did not show a

Table 4. The multiple comparisons of the indices of the lymphocytes' function

Dietary treatment	LPS stimulated index	ConA stimulated index	Peroxide enzyme activity	Phagocytosis to neutral red particles
1	0.23 ± 0.036^{k}	0.29 ± 0.006	0.06 ± 0.001^{k}	0.10 ± 0.005^{k}
2	0.27 ± 0.011^{k}	0.35 ± 0.006^{hijk}	0.07 ± 0.001^{jk}	0.11 ± 0.002^{jk}
3	0.35 ± 0.005^{hij}	0.37 ± 0.005^{ghij}	0.07 ± 0.003^{i}	0.13 ± 0.002^{ij}
4	0.38 ± 0.002^{fghi}	0.39 ± 0.003^{fghi}	0.09 ± 0.002^{j}	0.15 ± 0.003^{ghi}
5	0.39 ± 0.002^{fgh}	0.40 ± 0.003^{efgh}	0.10 ± 0.001^{gh}	0.16 ± 0.002^{fgh}
6	0.41 ± 0.004^{efg}	0.41 ± 0.002^{defg}	0.10 ± 0.001^{g}	0.16 ± 0.001^{fg}
7	0.43 ± 0.004^{ef}	0.43 ± 0.004^{def}	0.11 ± 0.003^{f}	0.17 ± 0.002^{ef}
8	0.45 ± 0.006^{de}	0.45 ± 0.003^{de}	0.13 ± 0.002^{e}	0.19 ± 0.002^{de}
9	0.48 ± 0.001^{d}	0.46 ± 0.003^{d}	0.14 ± 0.002^{d}	0.20 ± 0.003^{cd}
10	0.56 ± 0.015^{c}	0.55 ± 0.003^{c}	0.16 ± 0.002^{bc}	0.22 ± 0.004^{bc}
11	0.69 ± 0.033^{b}	0.70 ± 0.003^{b}	0.16 ± 0.003^{b}	0.23 ± 0.002^{b}
12	0.83 ± 0.018^{a}	0.87 ± 0.005^{a}	0.17 ± 0.002^{a}	0.28 ± 0.003^{a}

* Data in the same column without same superscripts differ significantly ($p<0.01$).
** Data expressed as OD values.

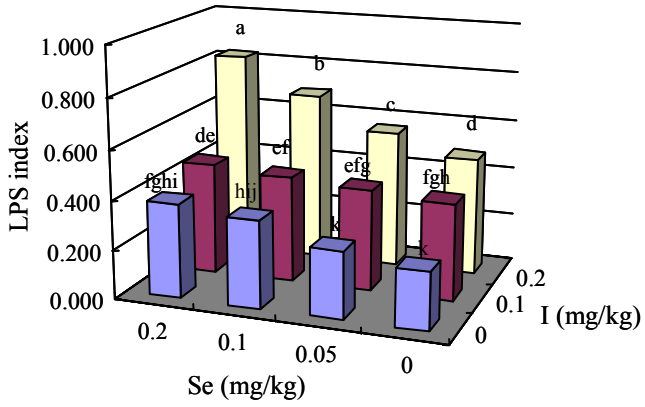

Figure 1. LPS-stimulation index of PBLs in laying hens with different dietary levels of iodine and selenium. Bars marked without same superscripts differ significantly.

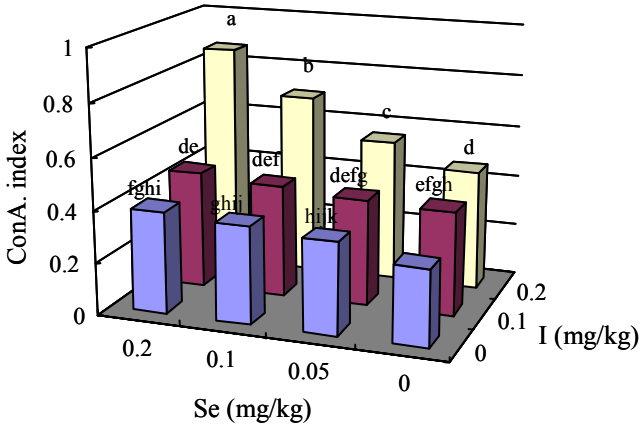

Figure 2. ConA-stimulation index of PBLs in laying hens with different dietary levels of iodine and selenium. Bars marked without same superscripts differ significantly.

corresponding higher stimulation index by LPS or ConA along with a higher selenium supplementation. When iodine was supplemented at the level of 0.1 mg/kg, the PBLs from birds without selenium supplementation showed a significantly lower stimulation index by LPS or ConA than those from 0.2 mg/kg selenium was supplemented, but no significant difference was observed among those from lower selenium supplementation (0, 0.05 and 0.1 mg/kg). The same trend existed among their PBLs from birds fed diets without iodine supplementation, in which supplementation of selenium at 0.05 mg/kg resulted in a similar LPS stimulation index but a higher ConA stimulation index than in birds without selenium supplementation.

Peroxidase activity was measured in the lymphocytes isolated from those hens within different dietary treatments (Table 4 and Figure 3). Increased iodine or selenium supplementation resulted in significant higher activity of peroxidase, and their highest combination level (0.2 mg/kg each) maintained the highest activity of peroxidase. Phagocytic activity of these cells was displayed in Table 4 and Figure 4. The higher iodine and selenium dietary supplementation caused the higher phagocytosis to neutral red particles. At the highest supplementation of iodine (0.2 mg/kg), PBLs from higher selenium supplementation displayed a significant higher phagocytic activity. At levels of low iodine supplementation (basal and 0.1 mg/kg added), although the PBLs from birds fed highest selenium supplementation (0.2 mg/kg) showed the significant higher phagocytosis activity than those from birds fed basal and with 0.05 mg/kg added, they had the similar phagocytosis with those from selenium supplementation at 0.1 mg/kg. No significant difference was observed in the PBLs between selenium supplementation at 0.1 mg/kg and 0.05 mg/kg when birds fed iodine at basal and 0.05 mg/kg.

DISCUSSION

The long production cycle of laying hens subjects them

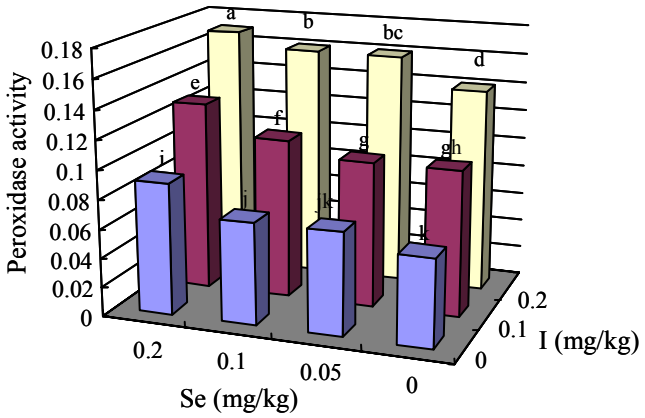

Figure 3. Lymphocyte peroxidase activity in laying hens with different dietary levels of iodine and selenium. Bars marked without same superscripts differ significantly.

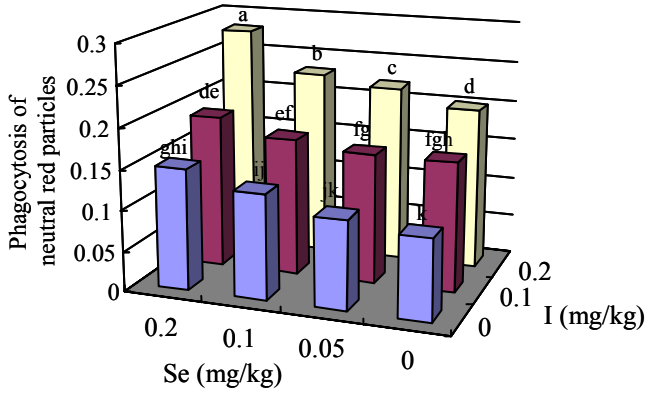

Figure 4. Lymphocyte phagocytosis of neutral red particles in laying hens with different dietary levels of iodine and selenium. Bars marked without same superscripts differ significantly.

to a number of stressful agents, including the exposure to various pathogenic microorganisms, and higher caging density (Hayirli et al., 2005). It is important for the laying hens to hold an immunocompetence to face the challenges of bacterial and viral infections. When hens were imposed with immunological challenged (such as LPS), a poor laying performance of hens might occur (Cheng, 2004b). At the cellular level, the response of lymphocytes to Con A is believed to reflect the function of the thymic-derived T lymphocytes, e.g., those associated with cell-mediated immunity (Schimizu, 1979). The response of lymphocytes to LPS mitogen stimulation is related to the function of the bone marrow-derived or B lymphocytes, e.g., those associated with antibody production and, thus, humoral immunity (Tizard, 1987). Peroxidase activity and phagocytosis activity of peripheral blood lymphocyte can be used as indication of the lymphocytes' function (Liu, 2004).

Thyroid hormones are known to influence the function and development of lymphoid organs (Paavonen, 1982). Spleen cells from thyroxine-treated mice showed an increased primary immune response *in vitro* to sheep red blood cells (SRBCs) (Chen, 1980). Treatment of dwarf mice with somatotropic hormone and/or thyroxine results in reconstitution of the immune capacity (Fabris, 1971) and prolongation of their life-span. The outflow of small lymphocytes from thymus is particularly increased during treatment with thyroxine (Ernstrom and Larsson, 1966). With regard to mitogen-induced lymphocyte transformation, thyroid hormones have been shown to have either an enhancing (Keast and Tayler, 1982) or suppressing (Gupta et al., 1983) effect on the response of T cell to phytohaemagglutinin (PHA). It is known that the iodine deficiency causes a low level of T_4 and T_3 in poultry (Guo, 1999), pigs (Schone et al., 1988), rats (Fang, 2000) and humans (Orville et al., 2000). But the effect of selenium on the thyroid hormone metabolism is controversial. Selenium depletion resulted increased T_4 but decreased T_3 in rats (Shen, 1999), increased T_3 in humans (Wayne, 2003) and increased T_4 but decreased T_3 in broilers (Guo, 1999).

In the present study, supplemented iodine or selenium alone resulted a higher levels of four indexes tested. However, the combination of iodine and selenium supplementation resulted in the highest value of all four indices. Therefore a higher iodine and/or selenium supplementation can promote the laying hens' lymphocyte functions. This observation is in agreement with that reported by Fabris (1973), who observed that iodine deficiency had a reverse effect on the rat's lymphocytes' function. The result of selenium supplementation in the present verified the conclusion of Arthur (2003), who reviewed that selenium-deficient lymphocytes were less able to proliferate in response to mitogen.

Although many studies had focus on the thyroid hormones effect on the animal's immunity, little is known regarding the importance of dietary iodine and selenium for the function of lymphocytes in laying hens. In the present study, the interactive effect between selenium and iodine on the lymphocytes' function in laying hens indicates that, in addition to its antioxidant ability which is recognized as the selenium's most important way to achieve its immune-promotion ability (Arthur, 2003), the selenium may also affect the laying hens' immunity by the way of modulating the thyroid hormone metabolism.

This research documented the importance of dietary iodine and selenium in maintaining peripheral blood lymphocyte's function in laying hens. The data implied that inadequate iodine may impair selenium nutrition. When setting selenium requirement of layers, a proper allowance of iodine should also be considered. If the iodine level in the laying hens' diet is lower than 0.2 mg/kg, higher selenium allowance than 0.1 mg/kg may be necessary to improve the laying hens' immunity. More work should be

done to determine the most optimal iodine and selenium supplemental levels to maintain the laying hens' immunity function.

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