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Effects of dietary supplementation of vitamin C on productive performance, egg quality, antioxidant status, and tibia characteristics in laying hens at different production stages

산란계 생산 단계별 사료내 비타민 C 첨가가 생산성, 난품질, 항산화 및 경골 특성에 미치는 영향

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by

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Abstract

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The productive performance of laying hens decreases as they age, in particular after 60 wks of age. Additional supplementation of vitamin C is not required in laying hens' diets because laying hens can endogenously synthesize vitamin C in their kidney and liver. Generally, vitamin C is known to be an antioxidant, immunostimulant, and as a cofactor in collagen formation and calcium regulation, which may contribute to the improvements in bone and eggshell quality. Several studies have proven that vitamin C supplementation in the diets fed to laying hens could improve egg production and quality under normal or stressful condition. However, the results from the different previous studies were variable. Therefore, a deeper investigation on the mechanisms involving the endogenous synthesis of vitamin C and possible contribution of different levels of vitamin C from exogenous source under normal condition at different ages of laying hens is required. Therefore, the objectives of this study were to investigate the effects of dietary supplementation of vitamin C on laying performance, egg quality, antioxidant status, and tibia characteristics in laying hens at different production stages (i.e., 46 to 51 wk of age and 65 to 70 wk of age) under normal condition. Two experimental setups of 6-wk period were conducted. A total of 504 46-wk-old for Exp. 1, whereas

420 65-wk-old for Exp. 2 Hy-Line Brown laying hens were allotted to 1 of 6 dietary treatments with 7 replicates in a completely randomized design. Exp. 1 had 12 hens per replicate whereas Exp. 2 had 10 hens per replicate. Hens were fed basal diet supplemented with 0 (basal), 250, 500, 1,000, 2,000, or 3,000 mg/kg vitamin C. Results from Exp. 1 indicated that increasing supplementation of vitamin C increased hen-day egg production and egg mass (quadratic, $P < 0.05$), but decreased the incidence of broken and shell-less eggs (linear and quadratic, $P < 0.01$) and feed conversion ratio (quadratic, $P < 0.05$). These positive effects were observable at 250 mg/kg vitamin C supplementation level, and no further benefits at the greater levels were observed. Quadratic responses of increasing vitamin C supplementation in diets reveal that supplementation of 250 mg/kg vitamin C is recommended for diets fed to laying hens at 46–51 wks of age. In Exp. 2, increasing supplementation of vitamin C induced quadratic responses in the incidence of broken and shell-less eggs ($P < 0.01$) and egg weight and yolk color, both at $P < 0.05$. Numerical improvements in eggshell thickness were also observed. These observations at older laying hens indicate that vitamin C supplementation at older hens may not positively lead to the improvement of laying performance but may be beneficial in the egg quality. Investigation in the relative organ weights used in this experiment showed significant effects only in Exp. 2 where liver was heaviest at the treatment 1,000 mg/kg vitamin C inclusion and the least at the control group ($P < 0.05$), whereas spleen weight in vitamin C-supplemented groups was found to be linearly less in terms of relative organ weight ($P < 0.05$). These observations indicate the functioning of liver in lipoprotein synthesis to compensate for the increased

production during their peak production age. On the other hand, larger spleen in control group indicates that without vitamin C supplementation, older laying hens are more sensitive to oxidative stress and induce more challenges to the spleen. Antioxidant status in Exp. 1 showed that TAC in the liver of laying hens numerically increased up to 1,000 mg/kg. This observation in antioxidant status shows that vitamin C could improve antioxidant capacity of the liver of laying hens. GLO gene expression of both experiments showed greater numerical expressions in the kidney than in the liver, indicating that more vitamin C could be synthesized in the kidney of the laying hens than in the liver. Exp.1 and 2 showed numerical increases in GLO gene expression but it was only significant in Exp. 1 where improved productive performance was observed. The age of laying hens and internal feedback mechanism could be the reasons why supplemental vitamin C did not result in any improvement.

Keywords: laying hens, vitamin C, productive performance, egg quality, tibia characteristics, antioxidant status

Contents

List of Abbreviations

AT : ambient temperature

BW : body weight

Ca : Calcium

cDNA : complementary DNA

CIE : Commission internationale de l'éclairage

CO_2 : Carbon dioxide

DHA : dehydroascorbic acid

EDTA : ethylenediamine tetraacetic acid

FCR : feed conversion ratio

FLHS : fatty liver haemorrhagic syndrome

GAPDH : glyceraldehyde 3-phosphate dehydrogenase

GLO : L-gulonolactone oxidase

GLUT : glucose transporter

GPx : glutathione peroxidase

HPA : hypothalamic-pituitary adrenal axis

HU : Haugh unit

H_2O_2 : hydrogen peroxide

ICP : inductively coupled plasma spectrometer

MDA : malondialdehyde

NADPH : nicotinamide adenine dinucleotide phosphate hydrogen

OD : optical density

PCR : Polymerase Chain Reaction

RH : relative humidity

RNA : ribonucleic acid

ROS : reactive oxygen species

SMP30 : senescence marker protein 30

SOD : superoxide dismutase

SVCT : sodium-vitamin C co-transporter

TAC : total antioxidant capacity

TBARS : thiobarbituric acid reactive substances

UDP : uridine diphosphate

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Literature review

1. Vitamin C

1.1 Sources of vitamin C

Vitamin C, also known as ascorbate or ascorbic acid, is an essential micronutrient required for normal metabolic functions in the living organism (Abdalla, 2003). Like other primates, humans lost the ability to synthesize ascorbic acid, because of a mutated gene coding for L-gulonolactone oxidase, which is required for the biosynthesis of ascorbic acid in the glucuronic acid pathway (Abdalla, 2003). The precursors for ascorbic acid mainly include sugars like mannose, fructose, and glucose (Abidin and Khatoon, 2017). In addition to vitamin C synthesis in the body, vitamin C can be obtained from diets such as vegetables and fruits (Haytowitz, 1995). It is present in fruits like orange, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, pineapple, raspberries, and cherries. It is also found in green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower, and cabbage (Naidu, 2003). Synthetic vitamin C as a dietary source is also a simple white crystalline compound and was firstly isolated from the adrenal glands of mammalian origin (Abidin and Khatoon, 2017).

1.2 Chemical structure of vitamin C

L-ascorbic acid ($C_6H_8O_6$) is the trivial name of Vitamin C. It is a dibasic acid containing an enediol group composed into a five membered heterocyclic lactone ring (Hacişevki, 2009). Therefore, the chemical name for vitamin C is 2-oxo-L-

threo-hexono-1,4-lactone-2,3-enediol. L-ascorbic and dehydroascorbic acid are the major dietary forms of vitamin C (Moser and Bendich, 1990). It is a water-soluble ketolactone and has two pK_a's, with pK_1 as 4.2 and pK_2 as 11.6; thus, the ascorbate monoanion, is the dominant form at physiological pH with two ionizable hydroxyl groups (Du et al., 2012). Ascorbate works as an excellent reducing-agent and readily undergoes two consecutive and one-electron oxidations to form ascorbate radical and dehydroascorbic acid (DHA; Du et al., 2012). In some cases, ascorbic acid can donate two electrons, as observed by voltammetry (Deakin et al., 1986). However, it commonly functions as a one-electron reducing-agent because the ascorbate radical, the one-electron oxidation product of ascorbic acid, relatively does not work with non-radical species and reacts preferentially with itself and other radicals (Bielski et al., 1981; Cabelli and Bielski, 1983; Liu et al., 1985). Dehydroascorbic acid (DHA) is the fully oxidized form of ascorbic acid and can be formed by two-electron oxidation of ascorbic acid, by one-electron oxidation of ascorbate radical or by a disproportionation reaction between two ascorbate radicals (Tu et al., 2017). Electrons from ascorbate can reduce metals such as copper and iron, leading to formation of superoxide and hydrogen peroxide, and subsequently may produce reactive oxidant species (ROS), indicating that, under some conditions, ascorbate can act as pro-oxidant (Padayatty and Levine, 2016). The availability of vitamin C either in the feed or water has not been widely investigated, and thus, sometimes true vitamin C levels are likely to be greater if its degradation rates are not considered (Lohakare et al., 2004).

1.3 Absorption and transport of vitamin C

All animal cells have functional vitamin C transporters, which determine the movement of extra- and intra-cellular fluids (Savini et al., 2008). The absorption of vitamin C in the body is mediated by both simple diffusion and active transport mechanism (Ahmadu et al., 2016). Vitamin C is transported and absorbed in mammalian cells by two different types of transporter proteins: sodium-ascorbate co-transporters (SVCTs) and hexose transporters (GLUTs); in particular, SVCTs is known to be associated with importing ascorbate, the reduced form of this vitamin (Savini et al., 2008). The SVCTs appear to be the predominant system for AA transport as well in the body (Ahmadu et al., 2016).

There are two known pathways of vitamin C uptake into organs and tissues (Gess et al., 2010). Vitamin C can be transported in its oxidized form (dehydroascorbate) through glucose transporters (Liang et al., 2001; Wilson, 2005). However, low concentration of dehydroascorbate can be transported into the organs and tissues because of its competition with glucose for the glucose transporters (Dhariwal et al., 1991; Liang et al., 2001). The second pathway through which vitamin C can be transported is through the SVCTs (Gess et al., 2010). There are two forms of SVCTs: SVCT1 and SVCT2, encoded by the genes Slc23a1 and Slc23a2 (Takanaga et al., 2004). These transporters carry vitamin C in its reduced form-ascorbic acid (Gess et al., 2010). These are highly specific for ascorbic acid and are dependent on the extracellular sodium concentrations (Tsukaguchi et al., 1999; Savini et al., 2008).

The SVCT1 and SVCT2 belong to a family of nucleobase transporters, which include *Aspergillus nidulans* uric acid xanthine permease A (UapA) and general purine permease (UapC), bacterial xanthine transporter (PbuX), uracil transporter (UraA), and membrane-bound uracil permease (PyrP; Faaland et al., 1998; Meintanis et al., 2000). The SVCT1 expression and activity are thought to be limited to tissues controlling whole body homeostasis of ascorbic acid like intestine, kidney, and liver, whereas SVCT2 is thought to transport ascorbic acid mainly into tissues and organs that functionally require ascorbic acid like the neuroendocrine glands, brain, lung, and endothelium (Sotiriou et al., 2002; Bornstein et al., 2003; Best et al., 2005; Karaczyn et al., 2006). In a study by Savini et al. (2008), distribution and kinetic parameters on vitamin C uptake suggest that the primary role of SVCT1 is to maintain the whole-body homeostasis of vitamin C through dietary absorption and renal reabsorption, whereas SVCT2 plays a critical role for ascorbate uptake for metabolically active and specialized tissues, thus leading to a protection from various oxidative stress.

1.4 Biosynthesis and metabolic pathway of vitamin C

The biosynthesis of ascorbic acid in animals occurs in the glucuronic acid metabolic pathway, which is involved in the sugar metabolism under normal and various disease conditions as well as in regulation of physiological functions (Hacişevki, 2009). It is also considered an important pathway for body's detoxification processes (Hacişevki, 2009).

In poultry, vitamin C is synthesized from glucose in the kidney (Ahmadu et al., 2016). However, Drouin et al. (2011) also reported that both kidney and liver are involved in the synthesis of vitamin C.

The initial precursor of vitamin C synthesis in both organs is glucose, which undergo through a sequence of reactions involving energy expenditure by the cell (Figueroa-Mendez and Rivas-Arancibia, 2015). According to Linster and Van Schaftingen (2007), the biosynthesis of vitamin C from D-glucose-1-phosphate is as follows: (1) direct hydrolysis of uridine diphosphate (UDP)-glucuronate by enzyme(s) bound to the endoplasmic reticulum membrane, sharing many properties with, and most likely identical to, UDP-glucuronosyltransferases; (2) Non-glucuronidable xenobiotics (aminopyrine, metyrapone, chloretone, and others) stimulate the enzymatic hydrolysis of UDP-glucuronate; (3) Glucuronate is converted to l-gulonate by aldehyde reductase, an enzyme of the aldo-keto reductase superfamily; (4) l-Gulonate is converted to l-gulonolactone by a lactonase identified as SMP30 or regucalcin; (5) oxidation of l-gulonolactone to l-ascorbic acid by l-gulonolactone oxidase, an enzyme associated with the endoplasmic reticulum membrane and deficient in humans, guinea pigs, and other species due to mutations in its gene. Another metabolic fate of glucuronate is its conversion to d-xylulose through a five-step pathway, the pentose pathway, involving in identified oxidoreductases and an unknown decarboxylase (Linster and Van Schaftingen, 2007).

The final step in biosynthesis of ascorbic acid is catalyzed by l-gulonolactone oxidase (EC 1.1.3.8; l-gulono-c-lactone:oxidoreductase). This enzyme is required

for the final conversion of L-gulono-γ-lactone to 2-oxo-L-gulono-γ-lactone, which is a tautomer of L-ascorbic acid and, transforms spontaneously into vitamin C (Hacişevki, 2009).

Numerous factors such as sex, age, nutrients, the presence of dietary ascorbic acid, and environmental conditions may affect and determine L-gulonolactone oxidase (GLO) activity (Hooper et al., 2000). Biosynthesis of vitamin C, as measured by GLO activity, differed between intact and gonadectomized cockerels and moreover, was reduced by increasing testosterone levels in the body (Dieter and Breitenbach, 1968). Dietary biotin and iron are reported to be related with increasing GLO activity in kidneys and several extra-renal tissues (Lechowski and Nagorna-Stasiak, 1993; Nagorna-Stasiak et al., 1994). Extra-renal GLO activity is inconsistent despite the well-established fact that kidney is the major site of vitamin C biosynthesis in poultry (Grollman and Lehninger, 1957). For more factors affecting GLO activity, supplemental ascorbic acid may decrease GLO activity in chicks (Kratzer et al., 1996). Mature birds compared with younger birds, male birds compared with female birds, and longer fasting have shown decreased GLO activity (Hooper et al., 2000). Thus, it is likely that sex, age (physiological stage), and fasting have potential regulators to determine vitamin C synthesis in poultry (Hooper et al., 2000).

2. Vitamin C and stress

Stress is known to induce the detrimental effects on animal health and performance (Ahmadu et al., 2016). Alteration in atmosphere, mismanagement,

removal or restriction of feed and water (starvation), high ambient temperature (AT), and uncomfortable relative humidity (RH) are well-known stressors imposing stress to poultry (Ahmadu et al., 2016). The birds' responses to stress are either adaptive or protective in order to normalize their homeostasis, and are on the purpose of preventing or minimizing the potentially adverse impacts on animals (Whitehead and Keller, 2007).

During heat stress, the bird reduces its feed intake to minimize heat load, resulting to decreased production. Moreover, the bird also increases panting to increase evaporative heat loss, resulting in increasing loss of carbon dioxide, thereby increasing blood pH. In laying hens, heat stress in different extents severely affects productive performance including egg production, egg size, egg-shell strength, and mortality (Sterling et al., 2003; Lin et al., 2004; Franco-Jimenez and Beck, 2007; Ajakaiye et al., 2010). Under heat stress condition, vitamin C supplementation in poultry feeds has been reported to exert positive effects on productive performance and immune responses (Puthpongsiriporn et al., 2001; Lin et al., 2003)

On the other hand, Pardue and Williams (1990) reported that plasma vitamin C levels in poultry were significantly depressed by cold stress. Lowering environmental temperature from 24.4 to 7.8 ˚C significantly reduced the body temperature of control hens, whereas not significantly reducing the temperature of vitamin C-supplemented hens (Thornton, 1962). Thornton (1962) speculated that supplemental vitamin C may aid in the maintenance of body temperature in poultry exposed to either elevated or reduced environmental temperature.

Corticosterone, a glucocorticoid steroid hormone, is the main hormone associated with stress in chickens and its concentration increases under stressful conditions (Whitehead and Keller, 2007). Short-term stressors give rise to elevated corticosterone levels in the blood (Broom and Knowles, 1989) due to the activation of hypothalamic-pituitary adrenal axis (HPA); and the secretion of corticosteroids occur in the adrenal cortical tissue (Harbuz and Lightman, 1992).

Vitamin C supplementation in poultry diets influences various physiological parameters, particularly in birds experienced with stress (Pardue and Thaxton, 2007). It is reported that vitamin C improved performance associated with the suppressed stress responses indicated by lowering of the plasma corticosterone level and adrenocorticotropic hormone (McKee and Harrison, 1995; Sahin et al., 2003; Mahmoud et al., 2004; Lin et al., 2006). Moreover, Kutlu and Forbes (1993) also found that vitamin C reduced the synthesis of corticosteroid hormones in birds and alleviated the negative effects of stress.

3. Roles of vitamin C

3.1 Antioxidant

Vitamin C plays a major role in cellular antioxidant defenses (Ahmadu et al., 2016). However, it can exhibit both antioxidative and prooxidative effects (McDowell, 1989). Vitamin C participates in several biochemical processes and functions related to reversible oxidation and reduction characteristics in the cells (Saki et al., 2010).

Ahmadu et al. (2016) noted that environmental stress causes an oxidative stress and an imbalance in antioxidant status for stressed poultry when the plasma antioxidant vitamins and minerals including vitamin C are limited.

Gecha and Fagan (1992) showed that in vitro addition of vitamin C decreased the production rate of H_2O_2 induced proteolysis and also ameliorated destruction of exogenously added superoxide dismutase (SOD).

According to Abidin and Khatoon (2017), there are two mechanisms of vitamin C for preventing oxidation of biological tissues. First, acting as a reducing-agent, vitamin C donates electrons to a number of enzymatic and non-enzymatic biochemical pathways forming semi dehydro ascorbic acid and dehydro ascorbic acid (Abidin and Khatoon, 2017). Secondly, glutathione present in extracellular fluid as well as intracellular fluid needs a nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent enzymatic returning oxidized forms of vitamin C to a reduced form (Abidin and Khatoon, 2017). This reversible reaction takes an important part in a redox system (Abidin and Khatoon, 2017). Thirdly, vitamin C can also form into an ascorbate radical that has the tendency to damage oxygen-generated free radicals like superoxide anion, monooxygen, and the hydroxyl radical (Abidin and Khatoon, 2017). Vitamin C acts as an anti-oxidant not through reaction with all oxygen species but by the formation of an inert radical dehydroascorbyl and by transferring radical equivalents from lipid phases (Seven, 2008). It serves as an important antioxidant functioning to neutralize free radicals formed within the body during lipid peroxidation (Abidin and Khatoon, 2017). Furthermore, vitamin C participates in the regeneration of reduced glutathione from

oxidized form in the cytoplasm and also aids in tocopherol regeneration through non-enzymatic reactions (Laudicina and Marnett, 1990).

Although vitamin C is mostly considered to have an antioxidant effect, there are situations in which it functions as a pro-oxidant (Whitehead and Keller, 2007). This occurs particularly in the interaction of vitamin C with transition metals, especially iron. Ferrous (Fe^{2+}) iron reduces H_2O_2 to generate the OH^- and then becomes ferric (Fe^{3+}) iron. Ascorbic acid can convert ferric iron back to ferrous iron, itself being oxidized to dehydroascorbic acid (Whitehead and Keller, 2007). As a consequence, continuous supply of ascorbic acid may stimulate ascorbic acid-driven free radical generation from iron (Herbert et al., 1996).

3.2 Immune stimulator

Vitamin C is reported to be associated with immune responses by supporting various cellular functions of both the innate and adaptive immune system (Carr and Maggini, 2017). A high concentration of vitamin C is found in immune cells, which show increase utilization of vitamin C during immune activation (Ahmadu et al., 2016).

The immune system is highly dependent on adequate cell–cell communication and any impairment in the signaling systems (e.g., oxidative stress) will lead to a reduction in immune responses (Parkin and Cohen, 2001). Immune cells are particularly sensitive to oxidative stress because of the high content of polyunsaturated fatty acids in their plasma membranes and a high production of ROS (Victor et al., 2002).

Vitamin C also supports epithelial barrier functions against pathogens. It is reported that a decrease in epithelial barrier function in the lungs by pathogenic invasion can be restored by administration of vitamin C. The reason could be attributed to enhanced expression of tight junction proteins and prevention of cytoskeletal rearrangements (Fisher et al., 2012). It is also reported that vitamin C accumulated in phagocytic cells, such as neutrophils, and promote their chemotaxis, phagocytosis, and capacity of producing ROS (Carr and Maggini, 2017). Vitamin is also required for apoptosis and clearance of the spent neutrophils from infection sites by macrophages, which thereby decreasing necrosis and potential tissue damage (Carr and Maggini, 2017).

3.3 Collagen formation and calcium regulation

Vitamin C plays a role in the biology of connective tissues because of its relation to the biosynthesis of collagen (Whitehead and Keller, 2007). Collagen is an important constituent of skin, cartilage, and bone (Whitehead and Keller, 2007). It contains unique amino acid-derivatives, hydroxyproline, and hydroxylysine, that is synthesized by a vitamin C–dependent process that provides enzymatic transfer of hydroxyl groups to selected proline and lysine residues in the nascent procollagen chains (Libby and Aikawa, 2002). Pinnell (1985) reported that vitamin C was an essential cofactor for the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine. Vitamin C is required for the enzymes to converting peptide-bound proline and lysine into hydroxyproline and hydroxylysine, respectively (Farquharson et al., 1993). Hydroxyproline is necessary for collagen

helix formation, and in its absence, the collagen is unable to be properly secreted from fibroblasts (Pinnell, 1985). Hydroxylysine is essential for formation of the intermolecular crosslinks in the collagen (Murad et al., 1981) and its absence lead the collagen to show low stable structures (Pinnell, 1985).

Vitamin C is also required for the conversion of Vitamin D into its metabolically active form (i.e., calcitriol), which is essential for blood calcium homoeostasis and bone calcification processes (Bains, 1995; Ahmadu et al., 2016). Calcium metabolism may also be influenced by vitamin C (Thornton, 1970; Dorr and Balloun, 1976; Orban et al., 1993), and the binding capacity of calcium to its binding proteins is regulated by vitamin C (Lohakare et al., 2005). Moreover, the conversion of 25-hydroxyvitamin D_3 ($25(OH)D_3$) to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) is dependent on the supply of vitamin C (Leeson and Summers, 2001). It has been known that vitamin C specifically influences the activity of $25(OH)D_3$-1-hydroxylase, the enzyme responsible for the transformation of $25(OH)D_3$ to $1,25(OH)_2D_3$ (Lohakare et al., 2005). The concentration of $1,25(OH)D_3$ is essential for Ca uptake from the intestines as well as for the bone mineralization (Lohakare et al., 2005). Vitamin C, as a cofactor for the enzyme 1-α-hydroxylase, must be adequately present to allow for the hydroxylation of vitamin D_3 as well as 25-hydroxylcholecalciferol to synthesize the biologically active form of vitamin D (i.e., 1,25-dihydroxycholecalciferol; Weiser et al., 1988)

In relation to vitamin D metabolism, vitamin C can affect eggshell formation and quality (Dorr and Balloun, 1976). The collagen fibril network, which is required

for proper bone formation is mineralized with hydroxyapatite (Farquharson et al., 1993). Vitamin C has been reported to improve leg bone conditions in stressed birds (Lohakare et al., 2005). This observation could be supported by Farquharson et al. (1993) who reported that vitamin C influenced the developmental processes in the growth plate for bone growth and showed reduced occurrence of tibial dyschondroplasia in broiler chickens fed diets supplemented with vitamin C. Ability of hens to produce eggshells depends largely on the availability of calcium from diets and bones (Farmer et al., 1983). Vitamin C increases calcium absorption or development of bone tissue (Leeson et al., 1995) and may play some roles in improving bone properties. It has been also appreciated that vitamin C supplementation reduced calcium excretion in poultry raised under low ambient temperature (Sahin and Sahin, 2002), and thus, could reduce incidence of eggshell defects and improve eggshell quality (Newman and Leeson, 1999).

The possible role of vitamin C in ameliorating the detrimental effects of heat and cold stress on eggshell quality is likely caused by enhanced absorption of intestinal calcium and increased mobilization of calcium from bones by stimulating the synthesis of 1,25-dihydroxycalciferol, which results in an increase in blood levels of calcium (Orban et al., 1993; Kucuk et al., 2003), and increased availability of Ca for eggshells (Thornton, 1970; Dorr and Balloun, 1976).

3.4 Liver function

The liver is the central organ to regulate nutrient metabolism in animal's body. Given that vitamin C is a powerful antioxidant, vitamin C has impact on hepatic

functions in nutrient metabolism. In addition, the liver of the poultry is the main organ for lipid metabolism because de novo lipogenesis mainly takes place in the liver of the poultry (Zaefarian et al., 2019). Vitamin C is suggested to be involved in the regulation of both circulating and hepatic lipid homeostasis (Ipsen et al., 2014).

Several animal studies suggested that vitamin C effectively decreases the hepatic oxidative stress (Wei et al., 2016). The increased generation of ROS can lead to an increase in lipid peroxidation, which can result in inflammation and fibrogenesis by activating the stellate cells (Day, 2002). The ROS is also known to restrain hepatocytes to secrete the very low density lipoprotein, and lead to liver fat accumulation (Wei et al., 2016). Moreover, oxidative stress can promote insulin resistance and inflammation in hepatocyte, which are also critically involved in hepatic lipid metabolism (Gambino et al., 2011).

4. Vitamin C requirement in poultry

Domestic fowl has the innate ability to synthesize vitamin C; therefore, it is not widely practiced to add supplemental vitamin C to poultry diets (Pardue and Thaxton, 2007). However, there have been some conditions for expecting possible benefits from vitamin C supplementation. The condition may include (a) insufficient endogenous synthesis to support the needs of poultry, (b) increased requirements of vitamin C under certain circumstances like stressful conditions, and (c) high amounts of pro-oxidant presence in the diets (Whitehead and Keller, 2007).

There were several studies evaluating the required conditions for vitamin C supplementations in poultry diets. The eggs laid by hens receiving vitamin C-free

diets contained no detectable amounts of vitamin C in eggs, but the embryos developed well without problems (Hauge and Carrick, 1926). Ray (1934) reported that the non-incubated eggs contained no vitamin C, but determined that appreciable amounts were present in the avian embryo after as little as four days of incubation. The injection of vitamin C into developing embryo had no positive effects on embryonic growth (Ray, 1934). These early reports supported the general notion that poultry does not require vitamin C supplementation in diets due to their innate synthesizing capacity. However, Holst and Halbrook (1933) speculated that possible conditions exist when growing chicks are lacking to synthesize adequate amounts of vitamin C. This statement was based on the observation of a “scurvy-like” disease in chicks which was alleviated by supplementing the diet with vegetables like cabbages high in vitamin C.

4.1 Vitamin C in broiler chickens

The benefits of vitamin C supplementation in poultry diets have been investigated in the world where climatic conditions are not friendly to poultry (Whitehead and Keller, 2007). Improved body weight and feed efficiency in broiler chickens reared under hot and humid conditions was observed in Malaysia by Kassim and Norziha (1995), after addition of vitamin C to diets (400 or 600 mg/kg). McKee and Harrison (1995) also found an improvement in feed conversion ratio of broiler chickens as a result of vitamin C supplementation in diets during heat stresses. In contrast, Puron et al. (1994) found that dietary vitamin C supplementation of 200 mg/kg did not affect productive performance of broiler

chickens raised in Yucatan, Mexico. Kratzer et al. (1996) also reported that addition of vitamin C to broiler chicken diets did not improve growth performance if diets were close to practical diets, but there was a positive response when vitamin C was added to a purified form of diet. Perhaps the birds on the purified diet could be considered to be under condition of nutritional stress (Whitehead and Keller, 2007).

On a different study by Fletcher and Cason (1991), vitamin C supplementation in drinking water (973 mg/l tap water) in broiler chickens had no effect on carcass yields. The effects of increasing temperature up to 36˚C for 6-10 h/day compared with thermoneutral conditions were studied in broiler chickens fed diets supplemented with 0, 250, 500, and 1000 mg vitamin C/kg (Kutlu and Forbes, 1993). Heating depressed growth performance of chickens up to 4 weeks and this depression was accompanied by reductions in thyroid weight, and plasma concentrations of protein, potassium, and calcium but increases in glucose, cholesterol, and sodium (Whitehead and Keller, 2007). Dietary vitamin C supplementation ameliorated partly the depression in body weight and restored the metabolic parameters to more normal values, with the optimum response being occurred with 250 mg vitamin C/kg in diets (Whitehead and Keller, 2007). Moreover, Kutlu (2001) showed that supplementation with 250 mg vitamin C/kg reduced carcass lipid content and improved performance in broiler chickens exposed to 35-37˚C for 8h/day.

Dietary vitamin C supplementation has shown inconsistent results for growth stimulation in chicks fed nutritionally adequate diets (Pardue and Thaxton, 2007). Briggs et al. (1944) supplemented highly purified diets with 1,000 mg vitamin C/kg

in diets and reported improved growth rates. However, addition of vitamin C to corn-soybean-based based diets had no positive effects on growth performance. March and Biely (1953) stimulated chick growth by adding vitamin C to folic acid deficient diets. Increased growth was also noted in chicks fed a "complete" diet supplemented with aureomycin and vitamin C, but aureomycin supplementation alone was ineffective in stimulating growth performance. Sifri et al. (1977) used corn-soybean meal-based diets and did not find positive effects of vitamin C supplementation on growth performance of chicks or quail. However, Dorr and Balloun (1976) noted significant growth improvement in poultry fed diets containing 3,000 mg vitamin C/kg in diets; however, injections of ascorbic acid significantly reduced poultry weights. White Leghorn males receiving diets containing supplemental vitamin C showed significantly greater body weights than those fed no supplemental vitamin C at 3, 7, 13, and 17 wks of ages, whereas improved body weights occurred in females at 3 and 7 wks of age (Schmeling and Nockels, 1978).

4.2 *Vitamin C in laying hens*.

The effects of vitamin C supplementation on reproductive performance in laying hens have been variable (Pardue and Thaxton, 2007). Supplementation of vitamin C in diets has periodically improved egg production, egg weight, egg shell thickness, and/or interior egg quality (Pardue and Thaxton, 2007). In contrast, Peebles et al. (1992) found no effects of dietary supplementation with 100 mg vitamin C/kg over the period 47 to 67 wks of age on egg production, egg weight, or

eggshell strength of Leghorn hens. Furthermore, Bell and Marion (1990) fed diets supplemented with 0, 50, 100, 200, or 400 mg vitamin C/kg to Leghorn hens for 6 successive 4-wk periods during summer period in Southern USA where temperature raised up to 33°C. Egg production and weights were unaffected by vitamin C supplementation. Egg specific gravity was generally greatest with 200 and 400 mg/kg vitamin C, and statistically significant effects in 2 of the 6 successive 4-wk periods were observed (Bell and Marion, 1990). In contrast, Keshavarz (1996) reported that dietary vitamin C from 250 to 1,000 mg/kg had no beneficial effects on eggshell quality or other productive performance in laying hens raised under normal environmental conditions.

It seems apparent that positive responses of laying hens to vitamin C may be prevailing when laying hens are raised under stressful conditions.

1.1. Introduction

A decline in laying rate and egg quality, as well as an increase in the number of abnormal eggs and breakage rates is typically observed in laying hens when they become aged, in particular after 60 wks of age (Gan et al., 2018). These impairments cause substantial economic losses for layer producers, making it a must to develop nutritional solutions to mitigate these impairments in laying performance (Molnar et al., 2016; Gan et al., 2018). Several previous studies have suggested that dietary supplementation of vitamin C could improve egg production (Zapata and Gernat, 1995) and egg quality (Sullivan and Kingan, 1962; Orban et al., 1993; Zapata and Gernat, 1995) in laying hens raised under normal or stressful condition.

In animals' body, vitamin C plays a pivotal role in cellular antioxidant defenses by participating in several biochemical processes and functions that are related to an increase in cellular reducing power (Saki et al., 2010; Ahmadu et al., 2016). However, vitamin C may also exert prooxidative effects in some specific cases (McDowell, 1989). Vitamin C is also associated with immune responses by supporting various cellular functions of both the innate and adaptive immune systems (Carr and Maggini, 2017). The reason is that immune cells are particularly sensitive to oxidative stress because of their high concentrations of polyunsaturated fatty acids in their plasma membranes as well as high production rate of ROS (Victor et al., 2002). Vitamin C exerts oxidant-scavenging activity, and thereby, potentially protecting immune cells against a variety of environmental oxidative stress (Carr and Maggini, 2017).

Vitamin C is also known to affect calcium regulation and calcification because it aids in converting vitamin D_3 into its metabolite form as a calcitriol (Bains, 1995; Ahmadu et al., 2016). Vitamin C can also act as a cofactor in the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine which are necessary for the formation of collagen helix and intermolecular crosslinks in the collagen, respectively (Murad et al., 1981; Pinnell, 1985). In the liver, vitamin C is involved in the regulation of both circulating and hepatic lipid homeostasis as it effectively relieves the hepatic oxidative stress (Ipsen et al., 2014; Wei et al., 2016).

Vitamin C is endogenously synthesized from the glucose by poultry in their renal cells because poultry has L-gulonolactone oxidase (GLO) enzyme required for the final step in the biosynthesis of vitamin C (i.e., ascorbic acid; Nishikimi and Yagi, 1996; Ahmadu et al., 2016). Therefore, it is not widely practiced to add supplemental vitamin C to poultry diets (Pardue and Thaxton, 2007). However, there have been some particular conditions for expecting possible benefits from dietary vitamin C supplementation. The conditions may include (a) insufficient endogenous synthesis to support the needs of vitamin C for poultry, (b) increased requirements of vitamin C under certain circumstances like stressful conditions, and (c) high intake of dietary pro-oxidants (Whitehead and Keller, 2007).

Although some previous experiments reported that dietary supplementation of vitamin C could improve laying performance and egg quality under some conditions, others (Cheng et al., 1990; Saki et al., 2010; Saki et al., 2011) have shown that dietary vitamin C supplementation has little effect on laying performance and egg quality. These inconsistent results prompted more experiments to determine the

effect of vitamin C supplementation with varying inclusion levels on the laying performance, egg quality, antioxidant status, and tibia characteristics in laying hens at different production stages (i.e., ages) of hens.

Therefore, the objectives of the current experiment were to investigate the effects of dietary supplementation of vitamin C on laying performance, egg quality, antioxidant status, and tibia characteristics in laying hens at 2 different production stages under normal condition.

1.2. Materials and Methods

1.2.1. Experiment 1

1.2.1.1. Birds, diets, and experimental design

All experimental procedures in this study were reviewed and approved by the Animal Care and Use Committee at Chung-Ang University. A total of five hundred four Hy-Line Brown laying hens of 46 wks of age were allotted to 1 of 6 dietary treatments in a completely randomized design. Each treatment had 7 replicates of 12 individually-caged (24 cm x 36 cm x 39 cm) hens per replicate. A commercial-type basal diet was prepared without addition of vitamin C in diets as control group (Table 1; calculated vitamin C concentration of basal diet = 45.32 mg/kg). Vitamin C (Vitamin C coated 97 %; Zhejiang Mingzhu Animal Health Products Co., Ltd., China) was then supplemented to basal diet at the inclusion levels of 250, 500, 1,000, 2,000 or 3,000 mg/kg. All nutrients and energy in the basal diet were formulated to meet or exceed their requirement estimates for Hy-Line Brown laying hens (Hy-Line, 2016). The experimental diets were fed to hens on an ad libitum basis for 6 wks. The temperature was maintained at 20.3 ± 0.2 and 16-hour lighting schedule was used during the entire experiment.

1.2.2. Experiment 2

1.2.2.1. Birds, diets, and experimental design

All experimental procedures in this study were reviewed and approved by the Animal Care and Use Committee at Chung-Ang University. A total of four hundred twenty Hy-Line Brown laying hens of 65 wks of age were allotted to 1 of 6 dietary

treatments in a completely randomized design. Each treatment had 7 replicates of 10 individually-caged (24 cm x 36 cm x 39 cm) hens per replicate. A commercial-type basal diet was prepared without addition of vitamin C in diets as control group (Table 2; calculated vitamin C concentration of the basal diet = 50.06 mg/kg). All management procedures were identical to those used in Exp 1.

1.2.3. Productive performance and egg quality

In both Exp. 1 and Exp. 2, productive performance including hen-day egg production, egg weight, egg mass, and broken and shell-less egg production rate were recorded daily. However, feed intake and feed conversion ratio (FCR) were recorded at the end of the 6-wk experiment. The data for productive performance were then summarized for 6 wks of the feeding trial (Shin et al., 2018).

Egg quality was assessed using 12 eggs per replicate (4 eggs randomly collected per day on the last 3 days of 3 and 6 wk) for Exp. 1, whereas 10 eggs (5 eggs randomly collected on the last 2 days of 3 and 6 wk) for Exp. 2. Eggshell strength was determined using eggshell strength tester (FHK, Fujihara Ltd., Tokyo, Japan). Egg quality was determined according to (Shin et al., 2018). In short, eggshell thickness was measured from three different regions (top, middle, bottom) using a dial pipe gauge (model 7360, Mitutoyo Co., Ltd., Kawasaki, Japan). Eggshell color was determined using the eggshell color fan (Samyangsa, Kangwon-do, Republic of Korea) and color reader (model CR-10, Konica Minolta Optics Inc., Japan). Egg yolk color estimates was determined by using the Roche color fan (Hoffman-La Roche Ltd., Basel, Switzerland). The Haugh Unit (HU) was measured using the

micrometer (model S-400, Ames, Waltham, MA, USA), and the HU values were calculated from egg weight (W) and albumen height (H), considering the following equation: HU = 100 log (H – 1.7 $W^{0.37}$ + 7.6) as demonstrated by Eisen et al. (1962).

1.2.4. Sample collection

At the conclusion of the experiment, 1 bird per replicate with a body weight (BW) close to the replicate mean BW (i.e. 7 birds per treatment) was euthanized by CO_2 asphyxiation, and then immediately dissected. The liver, kidney and spleen were collected and weighed to measure the relative organ weights as a percentage of the total BW for Exp. 1 (Shin et al., 2018). For Exp. 2, the same organs were collected and weighed but abdominal fat pad was also weighed (Shin et al., 2018). Kidney and liver samples were then stored at -80 ˚C for further analysis. A portion of the kidney and liver samples were kept for the analysis of GLO gene expression. Moreover, a bigger portion of liver in each sample was used for the analysis of liver fat and liver antioxidant status such as Malondialdehyde (MDA) and total antioxidant capacity (TAC). The right tibia was collected and stored for the analysis of ash, calcium (Ca) and phosphorus (P), whereas the left tibia was collected and analyzed for tibia breaking strength.

1.2.5. Tibia analysis

The tibia samples for Ca and P were oven-dried for 24 hrs at 100˚C, cooled at room temperature, wrapped in filter paper (Whatman filter paper, Grade 2), and

undergone Soxhlet Ether Extraction method for 48 hrs to a boiling temperature of 50˚C. Tibia samples were then dried in the fume hood and oven-dried for another 24 hrs at 100˚C. These dried samples were then ground and put to furnace at 600˚C for 24 hrs. The ash was then weighed, recorded and subsequently used for Ca and P analysis using inductively coupled plasma spectrometry (ICP) following the methods of Kurtoğlu et al. (2005). Tibia breaking strength was determined using texture analyzer (model TAHDi 500, Stable Micro System, Goldaming, UK).

1.2.6. Liver fat and antioxidant status

The liver fat was analyzed using acid-hydrolyzed ether extraction (method 996.01) by Soxhlet apparatus following the method of AOAC (2007) .

Antioxidant status in the liver such as MDA and TAC was determined by using commercially available kits OxiSelect™ TBARS Assay Kit (MDA Quantitation; STA- 330, Cell Biolabs, USA) and OxiSelect™ TAC Assay Kit (STA-360, Cell Biolabs, USA) respectively, according to the manufacturer's protocol.

1.2.7. Analysis of GLO gene expression

The expression of GLO gene was analyzed in the kidney and liver samples according to a modified method of Pitargue et al. (2019). Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the kidney and liver samples. The RNA was then diluted with 20 µL of RNase-free water. The determination of total RNA concentration was at optical density (OD) 260 nm (NanoDrop-1000, Thermo Fisher Scientific, Watham, MA). Verification of RNA

purity was done by evaluating the ratio of OD 260 nm to OD 280 nm. RNA sample (i.e., 2 μL) was then treated with DNaseI according to the manufacturer's instruction (Thermo Scientific DNase I, RNase-free, Thermo Fisher Scientific). Desalting of treated RNA was done to prevent carryover of magnesium, before cDNA synthesis using EDTA (Thermo Scientific DNase I, RNase-free, Thermo Fisher Scientific). Reverse transcription of 2 μg of RNA to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Watham, MA), was done according to the manufacturer's protocol. The cDNA was then stored at -20˚C. CFX Connect™ Real-time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA) was used to perform quantitative real-time PCR. qRT-PCR used 20 μL reaction mixture composed of 1 μL cDNA, 10 μL of 2x AMPIGENE qPCR Green Mix Lo-ROX (Enzo Life Sciences Inc., Farmingdale, NY, USA), and 10 pmol each of forward and reverse primers of GLO genes. The primer of the GLO gene was based upon sequence design by Gan et al. (2018) and was synthesized by Genotech Co. Ltd. (Daejeon, South Korea). Initial incubation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and termination by final incubation at dissociation temperatures 95°C (10 s), 65°C (60 s), 97°C (1 s), and 37°C (30 s) were the thermal conditions during the conduct of qPCR. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used to quantify relative gene expression and served as an internal control gene to normalize for RNA abundance. Each reaction was run in duplicate. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method

after normalization with the GAPDH (Livak and Schmittgen, 2001). GLO (Gan et al., 2018) and GAPDH (Hong et al., 2006) primers used are shown in Table 3.

1.2.8. Statistical analysis

ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Institute Inc., Cary, NC) was used to analyse all the data. The replicates were considered as experimental unit for productive performance and egg quality, whereas individual bird for relative organ weights, kidney and liver analyses, and tibia analyses were considered as an experimental unit. Outlier data were checked using the PROC UNIVARIATE procedure of SAS. The LSMEANS procedure was used to calculate treatment means and the PDIFF option of SAS was used to separate the means if the difference was significant. In addition, a preplanned orthogonal polynomial contrast test was performed to verify the linear and quadratic effects of increasing supplementation of vitamin C. Significance for statistical tests was set at $P < 0.05$.

1.3. Results

1.3.1. Experiment 1

1.3.1.1. Productive performance and egg quality

Increasing supplementation of vitamin C in diets fed to laying hens from 46 to 51 wk of age had significantly increased hen-day egg production, FCR, and egg mass (quadratic, $P < 0.05$; Table 4). Moreover, incidence of broken and shell-less eggs was significantly reduced with increasing vitamin C supplementation in the diet (linear and quadratic, $P < 0.01$). The hens fed diets with dietary vitamin C supplementation had a lower incidence of broken and shell-less eggs than those fed the control diet ($P < 0.01$). The significant improvement in hen-day egg production, FCR, and egg mass and reduction in incidence of broken and shell-less eggs was observed at a supplementation level of a greater than 250 mg vitamin C/kg. However, average daily feed intake and egg weight were not affected by the dietary treatments.

For the egg quality at 48 wk of age, increasing level of vitamin C supplementation in the diet did not significantly affect the eggshell thickness, eggshell strength, Haugh unit, and eggshell color based on eggshell color fan (Table 5). However, increasing vitamin C supplementation in the diet significantly increased the egg yolk color (linear, $P < 0.05$) of the laying hens. Moreover a decrease in yellowness (b) of eggshell color based on CIE Lab value was observed with increasing level of vitamin C supplementation in the diet (quadratic, $P < 0.05$).

At 51 wk of age, the eggshell thickness, eggshell strength, Haugh unit, eggshell color based on both eggshell color fan and CIE lab values were not significantly

affected by increasing supplementation of vitamin C in the diets (Table 6). However, egg yolk color of laying hens fed diets supplemented with vitamin C decreased with increasing inclusion level of vitamin C in the diet (quadratic, $P < 0.01$).

The overall egg quality of laying hens for 6 wks of feeding trial was not significantly affected by increasing supplementation of vitamin C in the diets (Table 7).

1.3.1.2. Relative organ weight

The relative weights of the kidney, liver and spleen to BW of laying hens were not significantly affected by increasing supplementation of vitamin C in the diets (Table 8).

1.3.1.3. Tibia characteristics

Neither tibia breaking strengths nor ash, Ca, and P concentrations were affected by increasing supplementation of vitamin C in the diets (Table 9). However, numerical improvements in tibia Ca and P concentrations were found for vitamin C-supplemented diets up to 2,000 mg/kg.

1.3.1.4. Liver fat and antioxidant status

The total fat concentration as percentage of liver dry matter (% DM) and gram of total fat in the liver were not affected by increasing level of vitamin C supplementation in the diets (Table 10).

Moreover, antioxidant parameters such as MDA and TAC were not influenced by increasing supplementation of vitamin C in the diets.

1.3.1.5. GLO gene expression

The expression of GLO gene in the kidney significantly increased with increasing level of vitamin C supplementation in the diets (linear, $P < 0.05$; Table 11). On the other hand, the expression of GLO gene in the liver was not affected by increasing supplementation of vitamin C in the diets.

1.3.2. Experiment 2

1.3.2.1. Productive performance and egg quality

Increasing supplementation of vitamin C in the diets showed no significant effect on hen-day egg production, egg mass, average daily feed intake, and FCR of laying hens from 65 to 70 wk of age (Table 12). However, there was a quadratic relationship ($P < 0.05$) between increasing supplementation of vitamin C in the diets and broken and shell-less eggs. A similar quadratic association was also found for egg weight ($P < 0.05$).

For the egg quality at 67 wk of age, increasing supplementation of vitamin C in the diets did not affect eggshell thickness, Haugh unit, and eggshell color based on both eggshell color fan and CIE lab value (Table 13). However, increasing supplementation of vitamin C in the diets showed significant effects on eggshell strength ($P < 0.05$) and egg yolk color ($P < 0.01$). Eggshell strength was the least at the treatment of 1,000 mg/kg vitamin C inclusion but the greatest at the treatment of

3,000 mg/kg vitamin C inclusion and control group. The egg yolk color was the greatest at the treatment of 3,000 mg/kg vitamin C inclusion but the least at the treatment of 500 mg/kg vitamin C inclusion.

For the egg quality at 70 wk of age, eggshell strength and eggshell color based on CIE Lab value were not affected by increasing level of vitamin C supplementation in the diets (Table 14). However, eggshell thickness and eggshell color based on eggshell color fan were increased by increasing supplementation of vitamin C in the diets (linear, $P < 0.05$). Haugh unit was observed to be greatest at the treatment of 250 mg/kg vitamin C inclusion but the least at the treatment of 2,000 mg/kg diet ($P < 0.05$). Egg yolk color was tended to be increased with increasing supplementation of vitamin C in the diets (linear, $P = 0.08$).

For the overall egg quality of laying hens for 6 wks of feeding trial, increasing supplementation of vitamin C in the diets did not affect the eggshell thickness, Haugh unit, and eggshell color using CIE Lab value (Table 15). However, increasing supplementation of vitamin C in the diets decreased the eggshell strength but increased the eggshell color based eggshell color fan (linear, $P < 0.05$). Lastly, increasing supplementation of vitamin C in the diets increased egg yolk color (quadratic, $P < 0.05$), with the greatest egg yolk color found in laying hens fed diets supplemented with 3,000 mg/kg vitamin C ($P < 0.01$).

1.3.2.2. Relative organ weight

The relative weight of abdominal fat pad and kidney to BW of laying hens were not significantly affected by increasing level of vitamin C supplementation in the

diets (Table 16). However, relative weights of the liver showed a tendency to increase with increasing level of vitamin C supplementation in the diets (P = 0.05). Relative liver weight was found at the greatest in the treatment of 1,000 mg/kg vitamin C inclusion but the least in the control group ($P < 0.05$). Lastly, relative spleen weight decreased with increasing level of vitamin C supplementation in the diets (linear, $P < 0.05$).

1.3.2.3. Tibia characteristics

Tibia breaking strength and tibia ash and P concentrations were not significantly affected by increasing supplementation of vitamin C in the diets (Table 17). However, tibia Ca concentrations tended to be increased (quadratic, $P < 0.05$) with increasing supplementation of vitamin C in the diets.

1.3.2.4. Liver fat and antioxidant status

The total fat concentration as percentage of the liver DM and gram of total fat in the liver were not affected by increasing level of vitamin C supplementation in the diets (Table 18).

Moreover, antioxidant parameters such as MDA and TAC were not influenced by increasing supplementation of vitamin C in the diets.

1.3.2.5. GLO gene expression

The expression of GLO gene in the liver and kidney was not affected by increasing supplementation of vitamin C in the diets (Table 19).

1.4. Discussion

1.4.1. Productive performance and egg quality

Most previous studies investigating dietary vitamin C supplementation showed significant effects on laying hens raised under stressful conditions, whereas under normal conditions, the effect on productive performance of laying hens was variable (Abidin and Khatoon, 2013). In Exp. 1, increasing supplementation of vitamin C in the diets significantly increased hen-day egg production, FCR, and egg mass, but did not affect the feed intake and egg weight. The increase in hen-day egg production as observed in this experiment agrees with the results of Peebles and Brake (1985) and Skřivan et al. (2013). According to Ciftci et al. (2005), the addition of dietary vitamin C as an antioxidant could partially inhibit adversely oxidative protein denaturation and would improve nutrients digestibility in diets, which may lead to an improvement in laying performance (Shit et al., 2012). Considering that the hen-day egg production reflects in egg mass and FCR, the obvious improvement in hen-day egg production would result in improved egg mass and FCR. Moreover, the decrease in the incidence of broken and shell-less eggs could be attributed to the role of vitamin C in Ca regulation, specifically for eggshell formation. Orban et al. (1993) reported that large doses of vitamin C in diets could influence Ca metabolism in hens. Vitamin C plays a role in the stimulation of 1,25 dihydroxycholecalciferol, and therefore increases Ca mobilization, which is highly correlated with eggshell formation and quality (Dorr and Balloun, 1976). As a consequence, vitamin C prevents shell defects and improves shell quality (Newman and Leeson, 1999). In this experiment, although

not statistically significant, numerical improvements in eggshell thickness and strength were observed in hens fed diets supplemented with vitamin C. Haugh unit and eggshell color based on eggshell color fan were not statistically different among treatments in both Exp. 1 and Exp. 2. The yellowness (b) based on CIE Lab value of eggshell color at 48 wk of age decreased with increasing supplementation of vitamin C in the diets and the reason for this observation is not clear. Moreover, egg yolk color was increased with increasing vitamin C supplementation in the diets. However, this result is in contrast with the egg yolk color measured at 51 wk of age which was observed to be increasing with increasing level of vitamin C supplementation in the diets. Saki et al. (2010) reported that dietary supplementation of vitamin C in the diets showed inconsistent effects on egg yolk color between ages of laying hens. On the other hand, Skřivan et al. (2013) observed the similar effect of reduction on egg yolk color with vitamin C supplementation in the diets. Skřivan et al. (2013) reported that dietary supplementation of vitamin C reduced oxidative stability of yolk lipids, indicating that vitamin C may act as a pro-oxidant. It was reported that vitamin C may act as pro-oxidant in some circumstances, particularly when animals have adequate vitamin E stores and vitamin C is supplemented at very high inclusion levels in diets (Chen, 1989). In this experiment, we can conclude that vitamin C possibly acted as pro-oxidant because the experimental diets contained vitamin E at an inclusion level of 648 mg/kg. Similarly, Franchini et al. (2002) reported elevated concentrations of TBARS in eggs of hens fed a diet supplemented with 500 mg/kg vitamin C and 100 mg/kg vitamin E. In a study by Sunder and Flachowsky (2001),

high vitamin E supplementation (1 g/kg) decreased the concentration of canthaxanthin, and at very high level of vitamin E in diets (10 g/kg), a significant decrease in yolk color was observed. Considering that vitamin C and E have the same antioxidative actions, the reduced deposition of carotenoids, which are responsible for the yolk color, may be the reason for decrease in the yolk color in hens fed diets supplemented with vitamin C (Skřivan et al., 2013). The significant improvement in hen-day egg production, FCR, and egg mass but reduction in incidence of broken and shell-less eggs was observed in particular at a supplementation level of 250 mg vitamin C/kg basal diet. Therefore, supplementation of vitamin C in younger hens (i.e. from 46 wk up to 60 wk of age) is beneficial to laying performance and egg quality.

Different effect of dietary vitamin C supplementation was observed in Exp. 2 where older laying hens (i.e., 65 wk to 70 wk of age) were used. In terms of productive performance, increasing supplementation of vitamin C in the diets did not affect hen-day egg production, egg mass, average daily feed intake, and FCR. There were quadratic relationships between vitamin C supplementation and both the incidence of broken and shell-less eggs and egg weight. This result is consistent with the study using old laying hens (50 wk to 62 wk of age) by Keshavarz (1996), where no significant effects on egg production, egg mass, feed intake, and FCR were observed after vitamin C supplementation in the diets up to 1,000 mg/kg. A different study by Newman and Leeson (1999) demonstrated that vitamin C supplementation at a level of 100 mg/kg in laying hens at 72 wk of age had no effects on egg production and feed intake under normal conditions. However, the

observation for the quadratic association for incidence of broken and shell-less eggs and egg weight in the current experiment is difficult to be explained by increasing vitamin C supplementation. Elaroussi et al. (1994) reported that the increase in cracked eggs was observed in aged layers and suggested that this result could be a consequence of possible disturbances of Ca homeostasis. Reduction in egg weight after feeding diets containing additional vitamin C was also observed by Newman and Leeson (1999) in laying hens supplemented with vitamin C during the start or first 5 days of vitamin C supplementation in the diets. Decrease in egg weight without a concurrent decrease in eggshell weight will likely cause an increase in shell quality (Al-Batshan et al., 1994). In this study, although eggshell weight was not measured, the decrease in egg weights was observed with the increase in eggshell thickness during egg quality analysis at 70 wk of age. The eggshell thickness at 67 wk of age was not statistically significant, but decreased numerically at the treatment of 3,000 mg/kg vitamin C inclusion. The improved eggshell thickness which is apparent on the egg quality measurement at 70 wk of age and at the overall experimental period could be a result of the fact that more Ca is deposited in the shell by high amounts of ascorbic acid in the diets (Orban et al., 1993). As indicated by El-Boushy et al. (1968), the effect of ascorbic acid seems to be expressed on the thickness of the shell as a whole. Eggshell strength at 67 wk of age was similar between control group and 3,000 mg/kg vitamin C-supplemented group, but least at 1,000 mg/kg vitamin C-supplemented group. The reason for the reduction in eggshell strength for vitamin C-supplemented group compared with the control group is not clear, but this result agrees with our observation for increasing

incidence of broken and shells less eggs. These observations could indicate as potential effects of age on eggshell ultrastructure formation. Egg yolk color in both Exp.1 and Exp. 2 was increased in vitamin C-supplemented groups as compared to the control group. Shell color using CIE lab value was not affected by increasing supplementation of vitamin C in the diets; however, eggshell color based on eggshell color fan was linearly increased with increasing level of vitamin C supplementation at 70 wk of age and during the overall experimental period. It was generally accepted that eggshell brown color gets lighter as hens become aged (Odabaşi et al., 2007). On the contrary, according to Odabaşi et al. (2006), the increase in eggshell color was found with dietary vitamin C supplementation for laying hens and the reason could be related to the fact that this water soluble vitamin is more available for enzyme-catalyzed pigmentation reactions that occur in the water phase of the shell gland cells. Lastly, the reason for the significant decrease in Haugh unit as affected by increasing level of vitamin C supplementation in the diet is not clear. However, Silversides and Scott (2001) noted that one of the major factors affecting Haugh unit is the age of laying hens.

1.4.2. Relative organ weight

The kidney, liver, and spleen are considered the immune-related organs which either increase or decrease in size by responding to certain metabolic or cytotoxic physiological process. In Exp. 1, the relative weights of kidney, liver and spleen to BW of laying hens were not significantly affected by increasing supplementation of vitamin C. This observation implies that addition of vitamin C up to 3,000 mg/kg in

the diet of laying hens at 46 wk to 51 wk of age is less than the critical level that may cause toxic effects. As stated by Turner (1948), enlargement of immune-related organs in laying hens fed diets supplemented with a certain additive indicate that the additive placed an excessive burden upon the animal, thereby leading to an enlargement in size. According to Aumailley et al. (2016), vitamin C plays important roles in reducing the accumulation of free radical compounds and maintaining the physiological function in organs such as kidney, liver and spleen. Therefore, in this experiment, increasing supplementation of vitamin C in the diets up to 3,000 mg/kg did not exert any burden in the laying hens' kidney, liver, and spleen.

In Exp. 2, it was observed that the relative weight of abdominal fat and kidney to BW of laying hens were not significantly affected by increasing level of vitamin C supplementation in the diet. However, liver weights were increased with increasing supplementation of vitamin C, whereas spleen weights were linearly decreased with increasing level of vitamin C supplementation in the diet. Considering that laying hens used in this experiment did not experience any form of stress, the reasons for the increase in liver weight and decrease in spleen size could not be attributed to stressful condition. The liver is a major organ of phospholipid and cholesterol synthesis (Zaefarian et al., 2019). These lipids, along with proteins, are the components of lipoproteins (Zaefarian et al., 2019). Vitellogenin is a lipoprotein synthesized in the liver of laying birds, which is under the influence of estrogen during the production phase and exported directly to the ovaries for the formation of the egg yolk (Alvarenga et al., 2011). During egg production, yolk

lipoproteins synthesis by the liver is faster than their mobilization from the hepatocytes, which may increase the liver size (Zaefarian et al., 2019). A rapid decline in egg production of laying hens begins after 480 d of age (i.e. 69 wk of age; Liu et al., 2018). Considering the egg production status of laying hens at 65 to 70 wk of age was at peak, the observed increase in the liver size could be attributed to the faster yolk lipoprotein synthesis. Faster yolk lipoprotein synthesis in the liver happens when the laying hens are at the ages of peaked production. On the other hand, a smaller spleen size in laying hens fed diets supplemented with vitamin C could possibly be due to the antioxidative sparing effect of vitamin C in the diet. The spleen, as an immune-related organ is sensitive to oxidative stress (El-Senousey et al., 2017). Vitamin C, as an antioxidant, is essential for the maintenance of the structure and functions of spleen (Kim et al., 2012). Therefore, the enlargement of the spleen in laying hens without vitamin C supplementation could be attributed to the challenge of oxidative stress. Comparing with the results from Exp. 1, where younger laying hens were used, older laying hens are more sensitive to oxidative stress, and therefore, induce more challenges to the spleen.

1.4.3. Tibia characteristics

Dietary vitamin C is known to be associated with the activity of bone resorption-related enzymes (Lohakare et al., 2005), thereby affecting the integrity of bone matrix and structure. Fritts and Waldroup (2003) reported that vitamin C influences the conversion of vitamin D into its metabolic active form as a calcitriol, which is essential for Ca and P regulation and calcification processes. However, in

both Exp. 1 and 2, increasing level of vitamin C supplementation did not affect tibia breaking strength of laying hens. This observation agrees with the report of Rowland et al. (1973), who supplemented vitamin C (15 to 1,000 mg/kg) to spent hen's diets and found no effect on tibia breaking strength. The similar experiment conducted by Newman and Leeson (1999) revealed that dietary vitamin C had little effects on bone breaking strength of Leghorn hens. If bone formation is not actively occurring in the older birds, then any increase in Ca uptake may simply be used to meet the demands of shell production, thus maintaining, but not improving, the status of the skeleton (Newman and Leeson, 1999). In this study, Ca uptake demands are visible in the numerical increase in the eggshell thickness in Exp. 1 and 2 after supplementation of vitamin C in the diets.

Vitamin C has been suggested to promote mineral mobilization from bone (Thornton, 1970) and reduce bone ash (Thornton, 1970; Ramp and Thornton, 1971). In this study, tibia bone ash was not affected by increasing supplementation of vitamin C, but numerical increases in tibia Ca and P concentrations in young laying hens (i.e., 51 wk of age) and numerical increase in P concentrations and quadratic increase in Ca concentrations of tibia in older laying hens (i.e., 70 wk of age) were evident. Similarly, Keshavarz (1996) did not find any significant effect on the concentration of crude ash in the tibia after adding vitamin C (0, 125, 250, 100 or 1000 mg/kg) in the diets. The Ca and P are primary inorganic nutrients in the tibia because they form 95% of the mineral matrices, although there are several other inorganic elements that may be important for bone health and strength (Rath et al., 2000). Vitamin C plays a role in improving bone properties (Orban et al., 1993),

which may promote Ca absorption (Saki et al., 2011). The observation for increased responses in Ca and P concentrations in Exp. 1 (up to 2,000 mg/kg) and Exp. 2 (up to 3,000 mg/kg) agrees with the results of Orban et al. (1993) who reported that bone mineralization increased in White Leghorns fed diets supplemented with 2,000 or 3,000 mg/kg vitamin C, compared to the control groups during the 4-wk experimental period.

1.4.4. Liver fat and antioxidant status

Lipogenesis in the liver of chickens is high and particularly active in laying hens, because of high estrogen secretion (Hermier, 1997). During egg production, the synthesis of yolk lipoproteins by the liver is faster than their mobilization from the hepatocytes, which may increase its fat concentrations (Zaefarian et al., 2019). Fatty liver hemorrhagic syndrome (FLHS) is the most important metabolic disorder in poultry that involve the occurrence of increasing fatty deposits in the liver (Zaefarian et al., 2019). The FLHS is associated with reduced egg production and increased mortality in laying hens (Hermier, 1997). Therefore, accurate estimation for the fat concentrations in the liver of laying hens is important. In this study, both Exp. 1 and 2 did not result in any significant changes in the fat concentration in the liver, indicating that vitamin C supplementation could have little impact on fat deposition in the liver. Another reason for liver fat accumulation in laying hens is the activity of ROS which restrain hepatocytes to secrete the very low density lipoprotein (Wei et al., 2016). Laying hens can produce ROS which could attack biological membranes and cause the formation of lipid hydrogen peroxide and

damage tissues (Nie et al., 2018). Malondialdehyde (MDA) is one of the final products of lipid oxidation which is toxic to cells. The MDA concentrations, therefore, reflects the extent of lipid peroxidation, and therefore, is one of the major biochemical markers to measure oxidative stress (Zhang et al., 2008; Hamer et al., 2009). On the other hand, total antioxidant capacity (TAC) is a measurement frequently used to assess the antioxidant status of biological samples (e.g. liver) and evaluate the antioxidant responses against the free radicals (Rubio et al., 2016). Low TAC could be indicative of oxidative stress or increased susceptibility to oxidative damage (Young, 2001). Vitamin C is a powerful antioxidant which is capable of scavenging free radicals, taking part in multiple enzymatic reactions as a reducing-agent (Buettner, 1993). Several animal studies suggested that dietary vitamin C could effectively relief the hepatic oxidative stress (Wei et al., 2016). It has also been shown to decrease mitochondrial ROS formation and stimulate the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the isolated rat liver mitochondria (Valdecantos et al., 2010). In the current experiment (Exp. 1 and 2), supplementation of vitamin C had no effect on the MDA level in the liver. Moreover, the TAC of the liver of laying hens were also not significantly affected but has shown numerical increases up to 1,000 mg/kg vitamin C supplementation in Exp. 1. This result in Exp. 1 indicates a decrease in TAC at higher levels of vitamin C supplementation in the diet and agrees with the findings of Gan et al. (2018), who observed that dietary addition of 250 mg/kg vitamin C in the diet increased the TAC concentrations in the liver of laying hens. On the contrary, excessive supplementation of vitamin C in diets may have negative-feedback inhibition on the

secretion of endogenous antioxidant enzymes as reflected by the reduction in TAC at the treatment of 3,000 mg/kg vitamin C inclusion in the study of Gan et al. (2018). Nevertheless, it was observed in this study that vitamin C supplementation can enhance the antioxidant status of the liver of laying hens in Exp. 1 up to 1,000 mg/kg vitamin C inclusion level.

1.4.5. GLO gene expression

It is generally believed that poultry can synthesize vitamin C in the kidney and liver with the action of the L-gulonolactone oxidase (GLO), which leads to a partial fulfillment of vitamin C requirement (Chaudhuri and Chatterjee, 1969; Figueroa-Mendez and Rivas-Arancibia, 2015). In Exp. 1, GLO gene expression in the kidney was significantly increased with increasing level of vitamin C supplementation in the diets. In the liver, however, numerical increase in GLO gene expression was also observed. With these increases in GLO expression, improved performance and eggshell quality were also observed, indicating that supplemental vitamin C in the diets has positive contribution in the laying hens at 46 to 51 wks of age.

In Exp. 2, GLO gene expression in both kidney and liver among all treatments were also not statistically different. However, the gene expression was numerically increasing with increasing level of vitamin C supplementation in the diets. Unlike in Exp. 1, only the eggshell thickness was improved in Exp. 2. The increase in GLO gene expression indicated that exogenous vitamin C contribution in the laying hens' physiological requirement may not be enough to improve laying performance at 65 to 70 wks of age. Another possible reason is the internal feedback mechanism.

Increasing supplementation of vitamin C in the diets resulted in higher expression of GLO gene, which indicated the possibility of synthesizing more endogenous vitamin C. However, in this study, even at the increased GLO gene expression, no improvement in the productive performance was observed. Gan et al. (2018) observed that even at higher expression of GLO gene, GLO enzyme activity in synthesizing endogenous vitamin C may not successfully proceed. Gan et al. (2018) found out that GLO gene expression was higher in the liver and kidney of old laying hens (75 wk of age) than the young laying hens (35 wk of age), but the enzymatic activity of GLO in young laying hens was higher than the older laying hens. However, in some previous experiments, increasing level of vitamin C supplementation reduced the requirement for endogenous vitamin C production, thereby reducing vitamin C synthesis in vivo, possibly mediated by decreasing GLO activity (Gan et al., 2018). Similarly, in a study by Hooper et al. (2002), supplementation of vitamin C at 1,000 mg/kg in broiler diets was reported to decrease GLO activity in the kidney. More researches regarding the relationship between dietary vitamin C supplementation and GLO expression are required for poultry.

1.5. Conclusion

Dietary supplementation of vitamin C in the diets fed to hens at 46 to 51 wk of age has beneficial effects on productive performance of laying hen in terms of hen-day egg production, egg mass, and FCR. Quadratic responses of increasing supplementation of vitamin C in diets reveal that the supplementation of 250 mg/kg vitamin C is recommended for laying hens at 46 – 51 weeks of age

Dietary supplementation of vitamin C in the diets at 65 to 70 wk of age improves eggshell thickness, eggshell color, and egg yolk color. However, these positive effects are less observed in hens at 46 to 51 wk of age. More researches are required to figure out the reason why dietary supplementation of vitamn C induces different responses in young vs. old laying hens.

Table 1. Composition and nutrient content of the basal diet for laying hens (46 to 51 wk of age; Exp. 1)

Items	Amount (%)
Corn grains	52.37
Soybean meal	16.20
Limestone	10.29
Gluten feed	10.00
Rapeseed meal	5.64
Animal fat	1.66
Corn gluten	1.10
Lysine by-product	1.00
Corn carrier	0.60
Monocalcium phosphate	0.36
Sodium bicarbonate	0.23
Methionine hydroxy analogue, 88%	0.19
Salt	0.11
Mineral premix[1]	0.08
Vitamin premix[2]	0.08
Lysine sulfate, 70%	0.03
Choline, 50%	0.03
Xylanase	0.01
Phytase	0.01
Celite[3]	0.01
Total	100.00
Calculated energy and nutrient content[4]	
AME_n, kcal/kg	2,734.00
Crude Protein, %	16.46
Lysine, %	0.82
Methionine + cysteine, %	0.72
Methionine, %	0.43
Threonine, %	0.60
Tryptophan, %	0.18
Calcium, %	4.06
Available Phosphorus, %	0.20
Vitamin C, mg/kg	45.32

[1] Provided per kg of the complete diet: copper, 10 mg; iron, 50 mg; iodine, 2 mg; manganese, 120 mg; selenium, 0.3 mg; zinc, 100 mg.

[2] Provided per kg of the complete diet: vitamin A, 8,400 IU; vitamin D, 3,000 IU; vitamin E, 9,000 IU; vitamin K, 1,200 IU; biotin, 0.06 mg; thiamin, 1.2 mg; riboflavin, 5.28 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B6, 1.2 mg; folic acid, 0.48 mg; vitamin B12, 0.00792 mg.

[3] Vitamin C was included in the diet by replacing the same amounts of celite

[4] Calculated values from (Hy-Line, 2016)

Table 2. Composition and nutrient content of the basal diet for laying hens (65 to 70 wk of age; Exp. 2)

Items	Amount (%)
Corn grains	63.12
Soybean meal	11.90
Limestone	10.98
Wheat	5.98
Corn gluten	4.81
Monocalcium phosphate	1.39
Brown rice carrier	0.60
Lysine sulfate, 70%	0.30
Sodium bicarbonate	0.20
Methionine hydroxy analogue, 88%	0.16
Tryptophan, 20%	0.14
Salt	0.12
Mineral premix[1]	0.10
Vitamin premix[2]	0.10
Threonine, 98%	0.07
Choline, 50%	0.02
Celite[3]	0.01
Total	100.00
Calculated energy and nutrient content[4]	
AME_n, kcal/kg	2,696.00
Crude Protein, %	14.24
Lysine, %	0.76
Methionine + cysteine, %	0.65
Methionine, %	0.40
Threonine, %	0.57
Tryptophan, %	0.17
Calcium, %	4.40
Available Phosphorus, %	0.33
Vitamin C, mg/kg	50.06

[1] Provided per kg of the complete diet: copper, 10 mg; iron, 50 mg; iodine, 2 mg; manganese, 120 mg; selenium, 0.3 mg; zinc, 100 mg.

[2] Provided per kg of the complete diet: vitamin A, 8,400 IU; vitamin D, 3,000 IU; vitamin E, 9,000 IU; vitamin K, 1,200 IU; biotin, 0.06 mg; thiamin, 1.2 mg; riboflavin, 5.28 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B6, 1.2 mg; folic acid, 0.48 mg; vitamin B12, 0.00792 mg.

[3] Vitamin C was included in the diet by replacing the same amounts of celite

[4] Calculated values from Hy-Line (2016)

Table 3. Sequence of the primers used in real-time quantitative RT-PCR (Exp. 1 and 2)

RNA target	Forward	Reverse	Size for PCR product (bp)	Accession no.
GAPDH[1]	5′-GGTGGTGCTAAGCGTGTTAT-3′	5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
GLO[2]	5′-TCTCCTCTGGATCAGCACCT-3′	5′-AGCGGCACTCGTAGTTGAAG-3′	131	XM_015285218.1

[1]Glyceraldehyde 3-phosphate dehydrogenase
[2]L-gulonolactone oxidase

Table 4. Productive performance of laying hens fed diets supplemented with vitamin C (46 to 51 wk of age; Exp. 1)

Items	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
	0	250	500	1,000	2,000	3,000		T	L	Q
Hen-day egg production, %	89	92	93	91	92	93	1.4	0.40	0.39	<0.05
Broken and shell-less eggs, %	0.22[a]	0.03[b]	0.03[b]	0.04[b]	0.02[b]	0.01[b]	0.033	<0.01	<0.01	<0.01
Egg weight, g	63.5	64.0	64.3	64.3	63.8	63.7	0.41	0.60	0.53	0.94
Egg mass, g	56.7	58.9	60.0	58.5	58.6	58.9	0.82	0.15	0.24	<0.05
Average daily feed intake, g/hen	115	115	115	115	115	115	0.3	0.35	0.37	0.62
Feed conversion ratio	2.04	1.95	1.91	1.97	1.97	1.95	0.028	0.09	0.20	<0.05

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 5. Egg quality of laying hens fed diets supplemented with vitamin C at 48 wk of age (Exp. 1)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		412	417	415	416	414	415	2.8	0.84	0.26	0.78
Eggshell strength, kg/cm^2		3.76	3.71	3.83	4.01	3.74	3.86	0.076	0.09	0.36	0.70
Haugh unit		90.6	89.9	89.2	90.5	89.0	89.7	0.87	0.76	0.78	0.19
Egg yolk color (Roche color fan)		8.8	9.3	9.1	9.3	9.4	9.0	0.17	0.08	<0.05	0.99
Eggshell color (Shell color fan)		11.6	11.5	11.4	11.5	11.6	11.5	0.25	0.99	0.99	0.81
Eggshell color	L*	55.0	54.5	54.7	54.7	55.4	55.0	0.50	0.81	0.83	0.78
(CIE Lab value)	a*	22.9	23.0	23.0	23.0	22.6	22.7	0.26	0.88	0.98	0.57
	b*	28.3	28.1	27.8	28.6	28.4	28.1	0.20	0.09	0.23	<0.05

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 6. Egg quality of laying hens fed diets supplemented with vitamin C at 51 wk of age (Exp. 1)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		413	415	419	417	417	418	2.8	0.63	0.36	0.32
Eggshell strength, kg/cm^2		3.84	3.99	4.15	4.00	4.13	3.95	0.289	0.09	0.37	0.59
Haugh unit		88.4	88.5	92.0	91.6	90.7	91.8	1.30	0.20	0.50	0.10
Egg yolk color (Roche color fan)		8.0	7.7	7.6	7.8	7.7	7.5	0.17	0.08	0.14	<0.01
Eggshell color (Shell color fan)		11.6	12.0	11.6	11.3	11.5	12.0	0.21	0.12	0.79	0.13
Eggshell color	L*	55.7	56.2	55.9	56.2	55.4	56.3	0.42	0.59	0.49	0.88
(CIE Lab value)	a*	22.9	22.9	22.7	22.3	22.8	22.3	0.26	0.56	0.43	0.75
	b*	28.5	28.0	28.5	28.4	28.3	28.6	0.26	0.65	0.30	0.60

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 7. Egg quality of laying hens fed diets supplemented with vitamin C for 6 wks of feeding trial (46 to 51 wk of age; Exp. 1)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		413	416	417	417	415	416	2.6	0.75	0.23	0.70
Eggshell strength, kg/cm^2		3.84	3.86	3.93	4.00	4.00	3.91	0.076	0.52	0.23	0.85
Haugh unit		89.5	89.2	90.6	91.0	89.9	90.7	0.96	0.73	0.74	0.61
Egg yolk color (Roche color fan)		8.4	8.5	8.4	8.5	8.5	8.2	0.10	0.28	0.21	0.12
Eggshell color (Shell color fan)		11.6	11.8	11.5	11.4	11.6	11.8	0.16	0.58	0.86	0.42
Eggshell color	L*	55.3	55.3	55.3	55.4	55.4	55.6	0.36	0.99	0.80	0.78
(CIE Lab value)	a*	22.9	23.0	22.8	22.6	22.7	22.5	0.22	0.70	0.57	0.56
	b*	28.4	28.1	28.1	28.5	28.4	28.3	0.16	0.47	0.94	0.30

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 8. Relative organ weights of laying hens fed diets supplemented with vitamin C at 51wk of age (Exp. 1)

Items	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
	0	250	500	1,000	2,000	3,000		T	L	Q
Liver, % BW	2.73	2.80	2.74	2.70	2.67	2.69	0.162	0.99	0.93	0.88
Kidney, % BW	0.47	0.53	0.57	0.61	0.58	0.57	0.054	0.52	0.15	0.49
Spleen, % BW	0.10	0.10	0.10	0.10	0.09	0.11	0.011	0.86	0.70	0.68

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 9. Tibia characteristics of laying hens fed diets supplemented with vitamin C at 51wk of age (Exp. 1)

	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Tibia breaking strength, N	166.4	145.5	159.8	149.9	165.0	148.2	12.37	0.77	0.30	0.89
Ash, %	57.4	56.2	57.2	58.0	58.2	59.3	1.35	0.57	0.86	0.51
Calcium, %	32.1	33.6	33.6	33.3	32.8	30.7	2.59	0.83	0.54	0.51
Phosphorus, %	17.4	18.7	18.8	18.0	18.6	17.7	0.70	0.51	0.41	0.41

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 10. Liver fat and antioxidant status of laying hens fed diets supplemented with vitamin C at 51wk of age (Exp. 1)

Items	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
	0	250	500	1,000	2,000	3,000		T	L	Q
Amount of total fat, % DM	28.4	25.2	24.2	25.1	30.3	25.2	2.44	0.45	0.73	0.58
Total fat concentration, g	15.7	13.4	13.0	13.3	16.0	13.8	1.61	0.67	0.57	0.67
Malondialdehyde (MDA), umol/mg protein	1.17	1.29	1.17	1.05	1.54	1.20	0.149	0.30	0.61	0.48
Total Antioxidant Capacity (TAC), umol/mg protein	708.59	727.84	720.33	741.36	703.08	679.12	16.148	0.17	0.39	0.10

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 11. L-gulonolactone oxidase gene expression of laying hens fed diets supplemented with vitamin C at 51wk of age (Exp. 1) [1]

	vitamin C supplementation (mg/kg)[2]						SEM	P-value[3]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Kidney	0.86	1.42	1.14	1.53	2.20	2.12	0.338	0.05	<0.05	0.12
Liver	0.20	0.20	0.28	0.28	0.29	0.46	0.107	0.55	0.69	0.19

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Normalized by GAPDH.

[2] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[3] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 12. Productive performance of laying hens fed diets supplemented with vitamin C (65 to 70 wk of age; Exp. 2)

Items	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
	0	250	500	1,000	2,000	3,000		T	L	Q
Hen-day egg production, %	90	92	91	91	92	91	1.4	0.40	0.26	0.81
Broken and shell-less eggs, %	0.09	0.08	0.16	0.07	0.08	0.16	0.068	0.06	0.09	<0.01
Egg weight, g	63.9	63.7	62.4	63.6	63.1	63.4	0.40	0.12	0.89	<0.05
Egg mass, g	57.7	58.5	57.1	58.1	58.1	57.4	0.68	0.74	0.31	0.31
Average daily feed intake, g/hen	113	113	112	112	113	112	0.3	0.14	0.86	0.11
Feed conversion ratio	1.96	1.94	1.97	1.94	1.94	1.96	0.024	0.90	0.29	0.56

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 13. Egg quality of laying hens fed diets supplemented with vitamin C at 67 wk of age (Exp. 2)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		411	410	408	410	408	402	4.7	0.67	0.23	0.70
Eggshell strength, kg/cm^2		3.68^a	3.66^a	3.57^{ab}	3.32^b	3.51^{ab}	3.68^a	0.088	<0.05	0.23	0.85
Haugh unit		86.0	86.0	85.2	85.6	85.5	85.3	0.83	0.98	0.74	0.61
Egg yolk color (Roche color fan)		7.7^{bc}	7.6^c	7.6^c	7.6^c	7.9^b	8.2^{ab}	0.09	<0.01	0.21	0.12
Eggshell color (Shell color fan)		11.8	12.0	11.7	12.0	12.0	11.9	0.20	0.75	0.86	0.42
Eggshell color (CIE Lab value)	L*	55.8	55.7	56.3	55.7	55.6	54.7	0.56	0.50	0.80	0.78
	a*	22.6	22.9	22.8	22.7	23.3	23.6	0.40	0.36	0.57	0.56
	b*	29.1	29.7	29.1	28.3	29.0	28.9	0.36	0.19	0.94	0.30

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 14. Egg quality of laying hens fed diets supplemented with vitamin C at 70 wk of age (Exp. 2)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		410	414	408	421	419	421	3.8	0.06	<0.05	0.98
Eggshell strength, kg/cm^2		3.71	3.62	3.66	3.53	3.43	3.54	0.107	0.49	0.13	0.64
Haugh unit		87.3^{ab}	89.5^{a}	87.5^{ab}	87.1^{abc}	84.2^{c}	86.4^{bc}	1.05	<0.05	0.88	0.37
Egg yolk color (Roche color fan)		7.9	8.0	7.8	8.0	8.0	8.0	0.07	0.12	0.08	0.42
Eggshell color (Shell color fan)		11.7	12.0	12.0	12.0	12.2	12.3	0.18	0.13	<0.05	0.21
Eggshell color (CIE Lab value)	L*	56.6	55.9	57.4	56.4	56.5	56.0	0.58	0.48	0.27	0.66
	a*	22.2	22.6	21.6	22.0	22.2	22.1	0.32	0.39	0.40	0.39
	b*	28.3	28.8	28.4	28.1	28.3	28.4	0.21	0.22	0.75	0.35

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 15. Egg quality of laying hens fed diets supplemented vitamin C for 6 wks of feeding trial (65 to 70 wk of age; Exp. 2)

Items		vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
		0	250	500	1,000	2,000	3,000		T	L	Q
Eggshell thickness, μm		410	412	408	416	414	412	3.1	0.63	0.15	0.42
Eggshell strength, kg/cm^2		3.70	3.64	3.62	3.43	3.47	3.61	0.073	0.09	<0.05	0.73
Haugh unit		86.6	87.8	86.3	86.3	84.8	85.9	0.76	0.19	0.88	0.31
Egg yolk color (Roche color fan)		7.8^{bc}	7.8^{bc}	7.7^{c}	7.8^{bc}	7.9^{b}	8.1^{a}	0.05	<0.01	0.09	<0.05
Eggshell color (Shell color fan)		11.8	12.0	11.9	12.0	12.1	12.1	0.15	0.47	<0.05	0.65
Eggshell color	L*	56.2	55.8	56.9	56.1	56.1	55.3	0.51	0.44	0.35	0.98
(CIE Lab value)	a*	22.4	22.8	22.2	22.3	22.7	22.9	0.42	0.55	0.31	0.67
	b*	28.7	29.2	28.1	28.2	28.6	28.7	0.22	0.07	0.77	0.42

L* = lightness; a* = redness; b* = yellowness

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 16. Relative organ weights of laying hens fed diets supplemented with vitamin C at 70 wk of age (Exp. 2)

	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Abdominal fat, % BW	4.09	3.73	3.68	3.84	3.59	4.31	0.483	0.89	0.68	0.98
Liver, % BW	2.29^{b}	2.62^{ab}	2.80^{ab}	2.95^{a}	2.41^{ab}	2.79^{ab}	0.137	<0.05	0.05	0.27
Kidney, % BW	0.57	0.61	0.65	0.63	0.60	0.56	0.039	0.64	0.59	0.89
Spleen, % BW	0.13	0.11	0.11	0.11	0.11	0.10	0.009	0.24	<0.05	0.14

^{a,b} Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 17. Tibia characteristics of laying hens fed diets supplemented with vitamin C at 70 wk of age (Exp. 2)

	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Tibia breaking strength, N	174.5	197.5	210.2	182.3	187.3	166.7	15.87	0.44	0.91	0.61
Ash, %	60.4	58.8	58.5	59.8	59.8	60.9	1.00	0.54	0.79	0.80
Calcium, %	41.7	44.8	44.9	44.1	47.8	47.5	1.49	0.06	0.07	<0.05
Phosphorus, %	17.0	17.4	16.8	17.7	18.3	18.0	0.56	0.43	0.14	0.70

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 18. Liver fat and antioxidant status of laying hens fed diets supplemented with vitamin C at 70 wk of age (Exp. 2)

	vitamin C supplementation (mg/kg)[1]						SEM	P-value[2]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Amount of total fat, % DM	22.7	22.7	25.1	28.6	25.2	23.9	2.40	0.54	0.33	0.77
Total fat concentration, g	10.5	11.4	13.2	16.0	11.5	12.6	1.55	0.19	0.18	0.89
Malondialdehyde (MDA), umol/mg protein	1.29	1.27	1.17	1.17	1.11	1.30	0.119	0.81	0.62	0.88
Total Antioxidant Capacity (TAC), umol/mg protein	674.21	634.21	647.27	640.34	671.23	664.61	12.145	0.13	0.11	0.78

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[2] T = overall treatment effect; L = linear effect; Q = quadratic effect.

Table 19. L-gulonolactone oxidase gene expression of laying hens fed diets supplemented vitamin C at 70 wk of age (Exp. 2)[1]

	vitamin C supplementation (mg/kg)[2]						SEM	P-value[3]		
Items	0	250	500	1,000	2,000	3,000		T	L	Q
Kidney	1.47	1.61	1.78	1.96	1.87	1.88	0.395	0.95	0.49	0.69
Liver	0.14	0.14	0.20	0.15	0.18	0.19	0.073	0.98	0.99	0.46

[a,b] Means with different superscripts within a column differ ($P < 0.05$).

[1] Normalized by GAPDH.

[2] Dietary treatments = 0 (basal diet), 250 (basal diet + 250 mg vitamin C/kg), 500 (basal diet + 500 mg vitamin C/kg), 1,000 (basal diet + 1,000 mg vitamin C/kg), 2,000 (basal diet + 2,000 mg vitamin C/kg), 3,000 (basal diet + 3,000 mg vitamin C/kg).

[3] T = overall treatment effect; L = linear effect; Q = quadratic effect.

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국문초록

산란계 생산 단계별 사료내 비타민 C 첨가가 생산성, 난품질, 항산화 및 경골 특성에 미치는 영향

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산란계의 생산성은 산란계의 주령이 증가함에 따라, 특히 60 주령 이후에 저하된다. 산란계는 신장과 간에서 비타민 C 를 내생적으로 합성할 수 있기 때문에 산란계 사료내 추가적인 비타민 C 의 첨가는 필요하지 않다. 일반적으로, 비타민 C 는 항산화제, 면역자극제, 콜라겐 형성 및 칼슘 조절의 보조인자로 알려져 있으며, 이는 뼈와 난각의 품질 향상에 기여를 할 수 있다. 일반적인 상황 또는 스트레스 조건 하에서 산란계에게 비타민 C 를 급이하였을 때, 산란율 및 난품질이 개선되었다는 여러 연구들이 검증되었다. 그러나 이전 연구들의 결과는 매우 다양했다. 그러므로, 일반적인 상황의 서로 다른 주령을 가진 산란계에서 산란계 체내 비타민 C 의 합성(내생적 합성)과 사료내 추가적으로 첨가되는 다른 수준의 비타민 C(외인성 공급원)가 기여할 수 있는 기작에 대한 더 깊은 연구가 필요하다. 그러므로 본 연구의 목적은 일반적인 조건 하에서 2 개의 다른 생산 단계 (예, 46-51 주령 및 65-70 주령)의 산란계의 사료내 비타민 C 의 첨가가 산란계의 생산성, 난품질, 항산화 및 경골 특성에 미치는 영향을 알아보고자

실시하였다. 두 실험은 6 주간 수행되었다. 실험 1 은 총 504 수의 46 주령, 실험 2 는 총 420 수의 65 주령의 하이라인 브라운 산란계로 공시되었으며, 6 처리 7 반복으로 완전 임의 배치되었다. 실험 1 의 경우 반복당 12 마리, 실험 2 의 경우 반복당 10 마리로 구성되었다. 6 개의 처리사료로서, 산란계 사료내 비타민 C 의 첨가 수준은 0, 250, 500, 1,000, 2,000, 3,000 mg/kg 순으로 이뤄졌다. 실험 1 에서, 산란계 사료내 비타민 C 첨가수준이 증가함에 따라 헨데이산란율 및 산란양이 증가하였으나 (quadratic, $P < 0.05$), 연파란율(linear and quadratic, $P < 0.01$)과 사료요구율(quadratic, $P < 0.05$)은 감소하는 결과를 보였다. 이러한 긍정적인 효과는 사료내 비타민 C 를 250 mg/kg 첨가하였을때 나타났으며, 더 높은 수준의 비타민 C 첨가에 대한 추가적인 이점은 관찰되지 않았다. 따라서, 산란계 사료내 비타민 C 첨가 수준 증가의 쿼드라틱한 결과로서, 46-51 주령의 산란계 사료내 비타민 C 첨가 수준은 250 mg/kg 권장됨을 보였다. 실험 2 에서는 비타민 C 첨가수준이 증가함에 따라 연파란율($P < 0.01$), 난중 및 난황색 ($P < 0.05$)의 증가에 대한 쿼드라틱한 결과가 나타났다. 난각두께에서는 수치적으로 증가하는 결과를 보였다. 노령 산란계에서 이러한 관찰은 노령 산란계의 사료내 비타민 C 첨가는 생산성에는 긍정적인 영향을 미치지는 않으나, 난품질에서는 긍정적인 영향이 미쳤음을 보여준다. 실험 2 에서, 상대적인 장기 무게의 결과에서 간조직에서만 유의적인 효과가 나타났다. 실험 2 의 간 조직의 상대적인 무게는 사료내 비타민 C 를 1,000 mg/kg 첨가하였을때 가장 컸으며, 대조구에서 가장 작았다. 이러한 결과는 산란계의 산란율이 가장 최고점을 찍는 주령 동안 증가된 생산성을 보상하기 위해서 지단백질 합성하려는 간조직의 기능이라고 보여진다. 반면에, 비타민 C 첨가 처리구에서 비장의 무게가 직선적으로 감소하였다 ($P < 0.05$). 대조구의 커다란 비장에 대한 결과에 따라서, 노령 산란계에게 비타민 C 의 추가적인 첨가가 없다면, 노령 산란계는 산화 스트레스에서 더

취약하다는 것을 의미한다. 산란계의 간 조직내 총항산화능력의 경우, 실험 1 에서는 1,000 mg/kg 까지 수치적으로 증가하였다. 항산화 분석에서 이러한 결과는 비타민 C 는 산란계의 간조직내 항산화능력을 개선시킨다는 것으로 보여진다. 두 실험에서의 유전자 GLO 는 간보다는 신장에서 수치적으로 발현이 증가하였음을 보여주며, 이것은 간보다는 신장에서 비타민 C 가 더 합성된다는 것을 나타내주는 결과이다. 실험 1 과 실험 2 에서 GLO 유전자의 발현이 수치적으로 증가하는 결과를 보여주었으나, 오직 실험 1 에서만 유의적으로 생산성이 개선되는 결과를 나타냈다. 산란계의 주령과 내부 피드백 기작은 왜 비타민 C 첨가가 향상을 야기하지 않았을까에 대한 원인이 될 수 있다.

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