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Original Article

Effects of Vitamin A and K³ on Immune Function and Intestinal Antioxidant Capacity of Aged Laying Hens

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ABSTRACT

This study was conducted to investigate effects of vitamin A (VA) and vitamin K_3 (VK_3) on immune function and intestinal antioxidant capacity of aged laying hens. In a 3 × 3 factorial arrangement, the diets of 1080 Roman Pink laying hens (87 weeks old) was formulated with deficient, adequate and excess VA and VK_3, including 0, 7000 and 14000 IU/kg VA and 0, 2.0 and 4.0 mg/kg VK_3 for 8 weeks. Interactive effects between VA and VK_3 were observed that VA and VK_3 decreased the splenetic mRNA expression of inducible nitric oxide synthase (iNOS) and tumour necrosis factor α (TNF-α), but increased the plasma immunoglobulin G (IgG) content and jejunal mRNA expression of nuclear factor-like 2 (Nrf2). Hens fed adequate or excess VA had higher spleen index, mRNA expression of interleukin-10 (IL-10) in spleen, sIgA content, catalase (CAT), glutathione peroxidase and total dismutase (T-SOD) activity, and mRNA expression of polymeric immunoglobulin receptor (pIgR) in jejunum and lower mRNA expression of IL-1β in jejunum and iNOS, TNF-α in spleen. Furthermore, adequate or excess VK_3 significantly increased plasma IgG content, the CAT, T-SOD and total antioxidant capacity activities, up-regulated the mRNA expression of pIgR, Nrf2, SOD1 and CAT in jejunum and down-regulated the mRNA expression of iNOS and TNF-α in spleen.

In conclusion, dietary addition of adequate VA (7000 IU/kg) and VK_3 (2.0 mg/kg) improved the immune function and intestine antioxidant capacity of aged laying hens and excess levels did not exhibit superior effects.

INTRODUCTION

As laying hens age over 70 weeks old, laying performance and egg quality are generally impaired, which results in a great economic loss (Roberts, 2004; Molnar *et al.*, 2017; Rattanawut *et al.*, 2018). Importantly, the main reasons for poor production of old layers are attenuated antioxidant capacity and weak immune system (Claudio *et al.*, 2000; Holmes *et al.*, 2003; Wan *et al.*, 2017). A previous study showed that digestion, absorption and immune problems were caused by reduced intestinal health in old laying hens (Jing *et al.*, 2014). Thus, it is imperative to explore solutions from the nutritive perspective to enhance intestinal health, improve laying performance and extend the laying period of old laying hens (Zhang *et al.*, 2020).

Therefore, lots of efforts are being made to improve the laying performance of aged laying hens using nutritional interventions (Gan *et al.*, 2020). Because of their beneficial effects on antioxidant activity, reproduction and other physiological mechanisms (Combs & McClung, 2016), vitamins have justifiably attracted attention of experts in the laying hen industry. Gan *et al.* (2020) reported that dietary vitamins

exert critical effects on eliminating free radicals and improving antioxidant levels and immune functions.

As essential lipid-soluble vitamin for laying hens (Guo *et al.*, 2021), vitamin A (VA) plays an important role in maintaining the normal visual function of animals and the structural integrity of epithelial tissue (Liang *et al.*, 2019) and supporting the differentiation of epithelial cells (Brody, 1993). VA ensures normal immune function (Kheirouri & Alizadeh, 2014), thus maintaining a normal intestinal environment and enhancing antioxidant function (Pedro *et al.*, 2018). It was reported that VA deficiency results in decreased immune response (Friedman & Sklan, 1989) and disturbed immunoglobulin metabolism (Davis and Sell, 1989). However, excess VA intake also has a detrimental effect on the immune function of birds (Friedman *et al.*, 1991). Vitamin K (VK) is a cofactor in glutamyl residue carboxylation during the post-translational modification of osteocalcin, a protein associated specifically with bone formation, and other bone matrix proteins (Fleming *et al.*, 2003). McDowell (2000) reported that adding VK to the broiler's diet can be beneficial in the early days of growth for strengthening their immune system, blood clotting and calcium accumulation (Abbasi *et al.*, 2017). Furturemore, VK, like VA, is a potential antioxidant (Vervoort *et al.*, 1997; Huang *et al.*, 2018).

In recent years, although additional vitamins in the diet of old laying hens has attracted a lot of interest in old laying hens, a few studies have reported the addition of VA and VK_3. Whether and how addition of VA and VK_3 maintained intestinal health to enhance laying performance is still uncertain. Thus, the main objective of this study was to investigate effects of VA and VK_3 on immune function and intestinal antioxidant capacity of aged laying hens to provide a theoretical basis for addition of VA and VK_3.

MATERIAL AND METHODS

All animal procedures used in the present study were performed in compliance with Hubei Provincial Regulations for Laboratory Animals (011043145-029-2013-000009), and were approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: 20190513).

Experimental design and bird management

The study was conducted using a 3 × 3 completely randomized design, and the diets of 1080 Roman Pink laying hens (87 weeks old, German Roman company) was formulated with different concentrations of VA (Retinyl acetate, providing 500000 IU/kg retinol) and VK_3 (menadione sodium bisulfite, providing 50% menadione), including 0, 7000 and 14000 IU/kg VA and 0, 2.0 and 4.0 mg/kg VK_3. The diets in nine groups, formulated using a cross-over design, were randomly assigned to hens with eight replicates, each with five adjacent cages (three birds per cage). The size of each cage (equipped with two nipple drinkers and one feeder) was 50 × 50 × 60 cm^3. An enclosed, ventilated, and conventional house (room temperature: 22 ± 2 °C, relative humidity: 60-70%) with 16 h lighting was provided for all laying hens to keep them in the same environmental conditions. Feed and water were offered *ad libitum*. The composition and nutrient levels of basal diet without addition of VA and VK_3 were shown in Table 1. The experiment lasted 10 weeks, consisting of a 2-week acclimation period with a basal diet containing 1320 IU/kg of VA and 0.5 mg/kg of VK_3 and a 8-week experimental period.

Sample collection

At the end of the experiment (8 weeks), one bird from each replicate (8 birds per group) were randomly selected and marked. After 12 h feed withdrawal (water was offered *ad libitum*), the fasting body weight of laying hens was recorded. Blood samples collected from the wing vein into ethylenediamine tetraacetic acid-coated tube were centrifuged for 10 min (3000 g, 4 °C) to obtain plasma which were aspirated by pipette, stored in 1.5-ml tubes at -80 °C until analyses and thawed at 4 °C before analysis. Then, hens were sacrificed humanely by cervical dislocation. The spleen samples drained with filter paper were weighed and quickly snap-frozen in liquid N_2 and then stored in -80 °C for the mRNA level analysis. The portion of jejunum samples were collected immediately and stored at -80 °C for the assay of immune function and antioxidant capacity.

Calculation of spleen index

Spleen index (g/kg) = 1000 × spleen weight (g) / live body weight (kg)

Detection of immunoglobulin and anti-oxidant status activity

The jejunum was homogenized in 10 % (w/v) ice-cold physiological saline and then centrifuged for 10 min at 4 °C (3000 g). The supernatant was collected to measure the content of secretory immunoglobulin A (sIgA) using the ELISA kits (Bethyl Laboratories)

Table 1 – The composition and nutrient levels of basal diet for laying hens.

Item	Value	Item	Value
Ingredients (%, unless otherwise indicated)		Calculated nutrient levels (g kg^{-1})	
Corn	55.40	Metabolic energy (MJ/kg)	11.5
Soybean meal	14.12	Crude protein	162
Soybean oil	0.60	Calcium	39.8
Corn DDGS	6.00	Available phosphorus	3.0
Rice DDGS	2.00	Lysine	74
Corn gluten meal	2.00	Methionine + Cystine	6.1
Hydrolyzed feather meal	1.50	Threonine	4.9
Sprayed corn husks	5.00		
Dicalcium phosphate	0.70		
Limestone	10.3		
Sodium chloride	0.10		
Sodium hydrogen carbonate	0.25		
L-Threonine	0.05		
L-Lysine	0.35		
Liquid DL-Methionine	0.20		
Choline chloride	0.15		
` premix[1, 3]	0.02		
Trace mineral premix[2]	0.10		
Compound enzyme preparations	0.02		
Canthaxanthin	0.01		
Betaine hydrochloride	0.02		
Encapsulated vitamin C	0.01		
Montmorillonite	0.10		
Zeolite powder	1.00		
Total	100.00		

DDGS: distiller's dried grains with solubles.

[1]The vitamin premix supplied the following per kilogram of diet: vitamin D_3, 2 500 IU; vitamin E, 30 IU; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; nicotinic acid, 50 mg.

[2]The trace mineral premix supplied the following per kilogram of diet: copper, 8 mg; iron, 80 mg; zinc, 75 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 0.35 mg.

[3]A 3×3 factorial randomized complete block design was employed, and all birds were acclimated on a basal diet with 1 320 IU/kg of vitamin A and 0.5 mg/kg of vitamin K_3 for 2 weeks. At week 90, deficient, adequate and excess vitamins A (0, 7 000 and 14 000 IU/kg) and K_3 (0, 2.0 and 4.0 mg/kg) were supplemented into the basal diets using cross-over design. Retinyl acetate (providing 500 000 IU/kg retinol) and menadione sodium bisulfite (providing 50% menadione) was chosen as the vitamins A and K_3 sources, respectively.

and the activities of antioxidant enzymes (, total antioxidant capacity (T-AOC), catalase (CAT), total dismutase (T-SOD), and glutathione peroxidase (GSH-Px)) and malondialdehyde (MDA) content were analyzed using analysis kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's directions. The plasma was used to determine the content of immunoglobulin A, G and M (IgA, IgG and IgM) using the ELISA kits (Bethyl Laboratories). A microplate reader (SpectraMax M5, Molecular Devices) was used in the determination.

RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted from jejunum and spleen using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of each extracted RNA sample was determined using a Nano Drop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the RNA integrity was verified by agarose gel electrophoresis. Reverse transcription was performed from 1 μg total RNA using a prime-Scripte® RT reagent Kit (TaKaRa). The primer sequences for immune function-related genes (interleukin (IL); inducible nitric oxide synthase (iNOS); Polymeric immunoglobulin receptor (pIgR); tumour necrosis factor α (TNF-α)), antioxidative genes (nuclear factor (erythroid 2)-like 2 (Nrf2); CAT; SOD1; GSH-Px) and β-actin are listed in Table 2. The quantitative real-time polymerase chain reaction (PCR) system (ABI 7500; Applied Biosystems, Foster City, CA, USA) following the protocol of SYBR Premix Ex Taq™ kit (TaKaRa) was performed with the following thermal procedure: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s, then 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. There were eight samples for each group, and each sample was performed

Table 2 – Primers used for real-time quantitative fluorescence PCR analysis.

Gene name	Accession number	Forward sequence (5'-3')	Reverse sequence (5'-3')
β-actin	NM_205518	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA
IL-1β	NM_204524.1	AAGCCTCGCCTGGATTCTGA	TCAGGTCGCTGTCAGCAAAG
IL-6	NM_204628.1	CCCAAGGTGACGGAGGAGGACGGCT	TCCAGGTAGGTCTGAAAGGCGAACA
IL-8	NM_205498.1	ATGAACGGCAAGCTTGGA	TCCAAGCACACCTCTCTTC
IL-10	NM_001004414.2	CGGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC
pIgR	XM_031689889.1	ATGACACGCTTCTTCATCTTCGTCTGCC	CTAGGCTTCTCTGGGGCCGTCCTG
iNOS	U46504	CAGCTGATTGGGTGTGGAT	TTTCTTTGGCCTACGGGTC
TNF-α	NM_204267.1	TGTGTATGTGCAGCAACCCG	AACAACCAGCTATGCACCCC
Nrf2	NM_205117.1	ATCACCTCTTCTGCACCGAA	GCTTTCTCCCGCTCTTTCTG
CAT	NM_001031215.2	GGTTCGGTGGGGTTGTCTTT	CACCAGTGGTCAAGGCATCT
SOD1	NM_205064.1	GGTGCTCACTTTAATCCTG	CTACTTCTGCCACTCCTCC
GSH-Px1	NM_001277853.2	GACCAACCCGCAGTACATCA	GAGGTGCGGGCTTTCCTTTA
GSH-Px3	NM 001163232.2	AAGTGCCAGGTGAACGGGAAGG	AGGGCTGTAGCGGCGGAAAG

IL: interleukin; pIgR: Polymeric immunoglobulin receptor; iNOS: inducible nitric oxide synthase; TNF-α: tumour necrosis factor α; Nrf2: nuclear factor (erythroid 2)-like 2; CAT: catalase; SOD1: superoxide dismutase 1; GSH-Px: glutathione peroxidase.

in triplicate, and no template control was included. The mRNA levels were standardized as the ratio to β-actin in arbitrary units by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analysis

The results were analyzed by 2-factorial ANOVA using Univariate General Linear Model using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Differences among all treatments were separated by Duncan's multiple test. Results are presented as the mean ± pooled SEM. $p<0.05$ was considered statistically significant.

RESULTS

Spleen index and immunoglobulin levels

As shown in Table 3, hens fed adequate VA (7000 IU/kg) had the highest spleen index ($p<0.01$) and sIgA content in jejunum ($p<0.05$). Similar case for VK_3 was observed that adequate VK_3 (2.0 mg/kg) elevated plasma IgG content in contrast to deficient (0 mg/kg) and excess VK_3 (4.0 mg/kg) at week 97 ($p<0.01$). VA and VK_3 exhibited interactive effects on the plasma IgG content ($p<0.05$). Deficient or adequate VA and adequate VK_3 significantly increased the plasma IgG content. Furthermore, VA and VK_3 did not show significant effects on the IgA and IgM content in plasma ($p>0.05$).

Immune function-related gene expression in the jejunum and spleen

Compared with deficient VA (0 IU/kg), adequate and excess (14000 IU/kg) VA significantly down-regulated the mRNA expression of IL-1β in jejunum (Table 4), iNOS, TNF-α in spleen (Table 5) ($p<0.01$) and up-regulated expression of pIgR mRNA in jejunum ($p<0.05$). Adequate VA had the highest expression of IL-10 mRNA in spleen ($p<0.05$). Compared with deficient and adequate VK_3, excess VK_3 increased the

Table 3 – Effects of VA and VK_3 on the spleen index and immunoglobulin of aged Roman Pink hens at 97 weeks of age.[1]

Item	VA (IU/kg)									SEM	*p-value*		
	0			7000			14000						
	VK_3 (mg/kg)			VK_3 (mg/kg)			VK_3 (mg/kg)						
	0	2.0	4.0	0	2.0	4.0	0	2.0	4.0		VA	VK_3	Interaction
Spleen index, (g/kg)	0.56	0.53	0.54	0.72	0.68	0.56	0.63	0.66	0.59	0.043	0.008	0.107	0.384
Jejunum													
sIgA, (μg/mL)	169.15	150.00	151.59	188.75	208.40	165.52	171.96	161.79	160.11	14.175	0.035	0.288	0.490
Plasma													
IgA, (μg/mL)	367.64	380.94	440.55	476.32	503.71	458.36	374.24	532.37	380.38	49.557	0.137	0.262	0.280
IgG, (mg/mL)	18.88^{b}	27.76^{a}	18.17^{b}	19.80^{b}	28.16^{a}	16.10^{b}	16.01^{b}	20.88^{b}	21.54^{b}	2.091	0.404	< 0.001	0.042
IgM, (mg/mL)	0.87	0.81	0.83	0.77	0.63	0.82	0.68	0.81	0.52	0.101	0.141	0.834	0.244

[1]Results are presented as the mean ± pooled SEM (n = 8).

[a-d]Values within a row with different superscripts differ significantly at $p<0.05$.

VA: Vitamin A; VK_3: vitamin K_3; sIgA: secretory immunoglobulin A; IgA, IgG and IgM: immunoglobulin A, G and M.

Table 4 – Effects of VA and VK_3 on the immune function-related gene expression in jejunum of aged Roman Pink hens at 97 weeks of age.[1]

Item	VA (IU/kg)									SEM	*P-value*		
	0			7000			14000						
	VK_3 (mg/kg)			VK_3 (mg/kg)			VK_3 (mg/kg)						
	0	2.0	4.0	0	2.0	4.0	0	2.0	4.0		VA	VK_3	Interaction
IL-1β	0.99	0.95	1.00	0.68	0.57	0.57	0.73	0.56	0.63	0.097	< 0.001	0.420	0.952
IL-8	1.00	0.79	0.55	0.68	0.66	0.48	0.69	0.65	0.61	0.155	0.361	0.179	0.810
pIgR	1.00	1.18	1.09	1.02	1.23	1.96	1.17	1.14	1.77	0.158	0.045	0.001	0.058
iNOS	1.01	1.18	1.12	0.94	0.94	1.15	1.05	0.92	1.07	0.106	0.492	0.357	0.540
TNF-α	0.99	1.38	1.51	1.32	1.24	1.51	1.20	1.46	1.10	0.142	0.662	0.156	0.082

[1]Results are presented as the mean ± pooled SEM (n = 8).

[a-d]Values within a row with different superscripts differ significantly at $p<0.05$.

VA: Vitamin A; VK_3: vitamin K_3; IL: interleukin; iNOS: inducible nitric oxide synthase; pIgR: polymeric immunoglobulin receptor; TNF-α: tumour necrosis factor α.

Table 5 – Effects of VA and VK_3 on the immune function-related gene expression in spleen of aged Roman Pink hens at 97 weeks of age.[1]

Item	VA (IU/kg)									SEM	*p-value*		
	0			7000			14000						
	VK_3 (mg/kg)			VK_3 (mg/kg)			VK_3 (mg/kg)						
	0	2.0	4.0	0	2.0	4.0	0	2.0	4.0		VA	VK_3	Interaction
IL-1β	1.00	0.72	0.73	0.98	0.90	1.01	0.84	0.89	0.85	0.109	0.255	0.502	0.492
IL-6	1.00	0.84	1.05	0.97	0.84	0.90	1.11	0.89	0.98	0.171	0.800	0.480	0.984
IL-8	0.99	1.20	1.29	1.11	1.26	1.15	1.24	1.13	1.27	0.111	0.816	0.406	0.449
IL-10	1.01	0.90	0.92	1.44	1.17	1.05	1.15	0.96	0.92	0.130	0.036	0.083	0.842
iNOS	0.99^{a}	0.93^{ab}	0.85^{abc}	0.93^{ab}	0.87^{abc}	0.66^{d}	0.76^{cd}	0.77^{cd}	0.81^{bc}	0.046	0.002	0.012	0.028
TNF-α	0.99^{a}	0.60^{b}	0.58^{bc}	0.58^{b}	0.60^{b}	0.46^{d}	0.60^{b}	0.56^{bc}	0.50^{cd}	0.026	< 0.001	< 0.001	< 0.001

[1]Results are presented as the mean ± pooled SEM (n = 8).

[a-d]Values within a row with different superscripts differ significantly at $p<0.05$.

VA: Vitamin A; VK_3: vitamin K_3; IL: interleukin; iNOS: inducible nitric oxide synthase; TNF-α: tumour necrosis factor α.

mRNA expression of pIgR in jejunum ($p<0.01$) and decreased the mRNA expression of iNOS ($p<0.05$) and TNF-α ($p<0.01$) in spleen. Interactive effects between VA and VK_3 were observed that adequate VA and excess VK_3 decreased the splenetic mRNA expression of iNOS ($p<0.05$) and TNF-α ($p<0.01$).

Jejunal antioxidant status

As presented in Table 6, compared with deficient and adequate VA, excess VA increased CAT and T-SOD activity in jejunum ($p<0.01$). Laying hens fed adequate and excess VA had higher GSH-Px activity than that of hens fed deficient VA ($p<0.01$). Furthermore, excess VK_3 significantly increased the T-SOD activities ($p<0.01$) and adequate VK_3 increased the CAT activity in jejunum ($p<0.05$) in contrast to deficient VK_3 while adequate VK_3 had the highest T-AOC activities ($p<0.01$) in jejunum. VA and VK_3 did not significantly affect the MDA activity in jejunum at week 97 ($p>0.05$).

Table 6 – Effects of VA and VK_3 on the antioxidant status in jejunum of aged Roman Pink hens at 97 weeks of age.[1]

Item	VA (IU/kg)									SEM	*p-value*		
	0			7000			14000						
	VK_3 (mg/kg)			VK_3 (mg/kg)			VK_3 (mg/kg)						
	0	2.0	4.0	0	2.0	4.0	0	2.0	4.0		VA	VK_3	Interaction
MDA, ng/mgprot	1.28	1.22	1.04	1.14	0.88	0.96	1.21	1.29	1.33	0.178	0.152	0.766	0.780
T-AOC, U/mgprot	0.55	0.64	0.67	0.51	0.68	0.70	0.56	0.58	0.67	0.037	0.696	< 0.001	0.476
CAT, U/mgprot	3.45	3.12	2.58	2.64	3.70	2.89	4.28	4.63	3.95	0.298	< 0.001	0.030	0.213
T-SOD, U/mgprot	717.01	698.15	818.17	640.52	796.61	834.72	990.54	1064.54	1075.60	45.930	< 0.001	0.008	0.362
GSH-Px, U/mgprot	29.77	30.64	30.75	46.81	45.20	44.62	45.39	50.87	41.12	5.662	0.002	0.764	0.901

[1]Results are presented as the mean ± pooled SEM (n = 8).

[a-d]Values within a row with different superscripts differ significantly at $p<0.05$.

VA: Vitamin A; VK_3: vitamin K_3; MDA: malondialdehyde; T-AOC: total antioxidant capacity; CAT: catalase; T-SOD: total dismutase; GSH-Px: glutathione peroxidase.

Antioxidative gene expression in the jejunum

VA and VK_3 did not significantly affect the gene expression of GSH-Px1 and GSH-Px2 in jejunum at week 97 (p>0.05) (Table 7). Compared with deficient and adequate VA, excess VA increased the mRNA expression of Nrf2 in jejunum (p<0.01). Adequate and excess VK_3 significantly increased the mRNA expression of Nrf2, SOD1 and CAT in contrast to deficient VK_3 in jejunum (p<0.01). Moreover, excess VA and adequate VK_3 had the highest expression of Nrf2 mRNA in jejunum (p<0.01).

DISCUSSION

The old laying hens usually have decreased immune capacity, intestinal dysfunction, poorer egg quality, and production performance (Claudio *et al.*, 2000; Liu *et al.*, 2016; Zhu *et al.*, 2019). Although many studies have examined the effects of the interactions among lipid-soluble vitamins on immunity, growth, and development in broilers and layers, few studies have explored the effects of interaction between VA and VK in the diet of aged laying hens. In our present study, it was found that although VA and VK_3 did not significantly affect the laying performance of aged Roman Pink laying hens, they improved the eggshell quality and yolk color as well as the antioxidative status in eggshell gland, which has been reported by Guo *et al.* (2021). Therefore, the immune function and intestine antioxidant capacity were determined in present study to see whether VA and VK have beneficial effects on intestinal health of aged laying hens.

The spleen index can reflect the immune function of the body to some extent. Immunoglobulins is a specific binding antibody produced by the immune response of plasma cells converted from B lymphocytes that can match the corresponding antigen. IgA, IgG and IgM are important immunoglobulins in poultry. In present study, adequate VA increased the spleen index and sIgA content in jejunum while adequate VK_3 increased plasma IgG content. Interactive effects between two vitamins on plasma IgG content were observed that hens fed deficient or adequate VA and adequate VK_3 had higher plasma IgG content. Wang *et al.* (2015) found that fat-soluble vitamin contents in colostrum of cows may be changed in similar patterns and high colostral vitamin A is related with high colostral IgG. Hu *et al.* (2020) reported that gelatin and starch vitamin A supplementation both highly increased serum IgA and IgM level. This discrepancy may be because of the differences in the domestic animal species, age and measuring area. The influence of VK_3 on the immunoglobulin content of poultry was little reported.

Owing to the significant effects of VA and VK_3 on immunoglobulin and spleen index, the expressions of immune function-related genes in jejunum and spleen were detected. The pro-inflammatory cytokines are responsible for different phenomena underlying the inflammatory response including proliferation, differentiation, stimulation and activation of immune cells (Dinarello, 2000), including IL-1β, IL-6, IL-8 and TNF-α (Howren *et al.*, 2009; Felger & Lotrich, 2013; Patil *et al.*, 2018). IL-10 is a prototypical anti-inflammatory cytokine that acts via several mechanisms to ultimately minimize inflammation both within the periphery and in the central nervous system, and is one of the most potent anti-inflammatory cytokines (Lobo-Silva *et al.*, 2016). Moreover, pIgR is one of the most vital components of mucosal immunity that plays an important role in mediating the transcytosis of polymeric immunoglobulins to protect organisms against pathogen invasion (Xu *et al.*, 2021). iNOS is

Table 7 – Effects of VA and VK_3 on the antioxidative gene expressions in jejunum of aged Roman Pink hens at 97 weeks of age.[1]

Item	VA (IU/kg)									SEM	*P-value*		
	0			7000			14000						
	VK_3 (mg/kg)			VK_3 (mg/kg)			VK_3 (mg/kg)						
	0	2.0	4.0	0	2.0	4.0	0	2.0	4.0		VA	VK_3	Interaction
Nrf2	1.00^{d}	0.98^{d}	1.35^{bc}	0.90^{d}	1.12^{cd}	1.55^{ab}	1.20^{cd}	1.72^{a}	1.38^{bc}	0.110	0.003	< 0.001	0.006
CAT	1.00	1.73	1.32	1.24	1.79	2.13	1.16	2.23	1.47	0.222	0.122	0.001	0.182
SOD1	1.00	1.49	1.45	1.21	1.29	1.72	1.10	1.40	1.42	0.129	0.570	0.001	0.322
GSH-Px1	0.93	0.92	0.91	0.96	0.92	1.23	1.18	0.98	0.95	0.088	0.202	0.397	0.072
GSH-Px3	0.93	0.58	0.78	0.66	0.71	0.80	0.73	0.71	0.81	0.084	0.836	0.142	0.160

[1]Results are presented as the mean ± pooled SEM (n = 8).

[a-d]Values within a row with different superscripts differ significantly at p<0.05.

VA: Vitamin A; VK_3: vitamin K_3; Nrf2: nuclear factor (erythroid 2)-like 2; CAT: catalase; SOD1: superoxide dismutase 1; GSH-Px: glutathione peroxidase.

mainly produced by macrophages, and generates large amounts of NO at short intervals (Michel & Feron, 1997), which has a critical role in the immune system by acting as a cytotoxic and tumoricidal agent (Lin *et al.*, 1996). In current study, hens fed adequate or excess VA had the decreased expression of IL-1β mRNA in jejunum and increased expression of IL-10 mRNA in spleen. VA and VK_3 showed main effects on the mRNA expression of iNOS and TNF-α in spleen with interactive effects being observed. Both adequate or excess VA and VK_3 increased the mRNA expression of pIgR in jejunum. Wang *et al.* (2020) reported that there were interactions between maternal and offspring VA on splenic IL-2, IL-1β and IFN-γ expression. Furthermore, Hatanaka *et al.* (2014) reported that VK_3 and VK_5 significantly inhibited the production of TNF-α, IL-4, IL-6, and IL-10 from the activated PBMCs at 10-100 μM. Abbasi *et al.* (2017) implies that appropriate levels of Vitamins D and K in ovo injection has beneficial effects on growth performance, immune system and bone development. The literature about VA and VK_3 supplementation on the diet of aged laying hens is limited and the underlying mechanism in improvement of immune function-related genes expression by VA and VK_3 supplementation in the current study needs further investigation.

VA and carotenoids improve the activity of antioxidant enzymes and maintain balance between oxidation and reduction (Palace *et al.*, 1999). GSH-PX, SOD and CAT are important antioxidant enzymes and T-AOC is a comprehensive indicator of the functional status of antioxidant systems and is a result of the interaction of antioxidant enzymes in the body (Liang *et al.*, 2019). In present study, although there were no interactive effect between VA and VK_3 on antioxidant capacity, excess VA or VK_3 increased the activities of T-SOD and CAT in jejunum. Furthermore, adequate and excess VA and VK_3 increased the activities of GSH-Px and T-AOC, respectively. Consistently, Hong *et al.* (2013) found that adding 3,000 IU/kg VA to the diet increased T-AOC, activities of T-SOD, GSH-Px, and CAT and decreased MDA content in serum of yellow feather broilers aged 43 to 63 D. Conversely, Hu *et al.* (2020) reported that dietary VA supplementation had no significant effects on serum T-AOC and SOD on d 21 and 42, but significantly increased serum GSH-Px activity. Wang *et al.* (2020) reported that maternal or offspring VA did not affect the activities of T-SOD and GSH-Px or the content of MDA. The influence of VK_3 on the antioxidant enzymes of poultry was little reported.

In addition to the activities of antioxidant enzymes mentioned above, we also detected the expressions of antioxidant enzymes and the regulating factor of antioxidant reaction in the jejunum. Transcription factor Nrf2 is a major regulator of cellular antioxidant defence, which controls the basic expression and induced expression of a series of antioxidant response elements-dependent genes to regulate redox homeostasis (Ma, 2013). In present study, excess VA up-regulated the mRNA expression of Nrf2 in jejunum while adequate and excess VK_3 not only increased the mRNA expression of Nrf2 but also CAT and SOD1 in jejunum. Moreover, interactive effect between two vitamins on Nrf2 expression in jejunum was observed. This suggests that VA and VK_3 might modulate the antioxidative capacity of laying hens in a Nrf2-dependent way, but the underlying mechanisms need further investigation.

CONCLUSION

In conclusion, dietary addition of adequate VA (7000 IU/kg) and VK_3 (2.0 mg/kg) can improve the immune function by increasing the immunoglobulin levels, and inhibiting the expressions of pro-inflammatory factors, and can also improve intestine antioxidant capacity by increasing the activity and expressions of antioxidant enzymes in aged laying hens and excess levels did not exhibit superior effects. Further research should be done in future.

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AUTHOR CONTRIBUTIONS

Zhengfan Zhang, Shuangshuang Guo, and Binying Ding conceived and designed the experiments. Zhipeng Liu, Bijun Fang and Jingyun Xu conducted them. Zhipeng Liu, Bijun Fang, Jingyun Xu, Ling Yang, Shuangshuang Guo, and Binying Ding took part in the sample collection. Zhipeng Liu, Bijun Fang and Jingyun Xu conducted the feeding experiment and laboratory experiments. Xinyang Dong analyzed the data and Lanlan Li wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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