Chapter 12

Effects of Excess Dietary Tryptophan on Laying Performance, Antioxidant Capacity and Immune Function of Laying Hens

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Additional information is available at the end of the chapter

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Abstract

Present study was conducted to establish Tryptophan (Trp) needs of Xinyang green-shell laying hens by evaluating its effect on laying performance, egg quality, antioxidant capacity, and the immune functions. A total of 525 laying hens, 28 weeks of age, were randomly allocated to five treatment groups, each of which included 5 replicates of 21 hens. Hens were fed the basal diet based on corn and soybean meal for 12 weeks. L-Trp was added to the control diet at 0.0 0.02, 0.04, 0.06, and 0.08%, respectively, to achieve 0.15, 0.17, 0.19, 0.21 or 0.23% Trp. Laying rate, average egg weight, and feed conversion ratio (FCR) were significantly increased by Trp levels from 0.19 to 0.23%. Dietary Trp from 0.17 to 0.19% increased egg internal quality (albumen height and haugh unit) rather than external quality. Supplementing with Trp increased glutathione peroxidase and total superoxide dismutase activity, total antioxidative capacity concentration and decreased malondialdehyde concentration. Serum IgA concentration increased at 0.21–0.23% dietary Trp, while serum IgM increased linearly in response to dietary Trp levels. We suggest that the optimum level of dietary Trp was ranged from 0.19 to 0.21% for Xinyang green-shell laying hens under the current study conditions.

Keywords: laying hens, Tryptophan, laying performance, egg quality, antioxidant capacity, immunoglobulins

1. Introduction

Tryptophan (Trp) is a nutritionally essential amino acid in animals with a wide range of physiological roles. It is considered to be a substrate for protein synthesis [1], a feed intake enhancer in livestock and poultry [2], a contributor to improved growth performance [3], and a factor in

the generation of hormone-like substances [4]. In addition, it has been reported that a deficiency of Trp decreased antibody production in rats [5], indicating that Trp may have a role in immune function. Apart from being a structural component of all proteins, Trp is a precursor of serotonin [5-hydroxytryptamine (5-HT)]. Serotonin (a neurotransmitter) has many functions in the central nervous system to inhibit aggression and modulates stress response, including social and environmental adaptability [6]. Recent findings suggested that dietary Trp may have beneficial effects on the enzymatic and non-enzymatic antioxidant capacity in laying hens [7], rats [8], and fish [9].

Tryptophan concentration in animals is the lowest of all the amino acids, and thus, it can easily become rate-limiting for protein anabolism [10]. Practical diets composed of vegetable protein sources typically result in the essential amino acids such as Trp being limiting to a similar extent after that of total sulphur amino acids, lysine, threonine and isoleucine in poultry [11]. Although many researchers conducted studies to evaluate the requirements of Trp in poultry, results of dose-response studies addressing the Trp need are variable [7, 11]. This might due to the effect of a variety of factors, such as genotype, age, and diet. Present study was conducted to establish Trp needs of chicks using Xinyang green-shell laying hens, a local strain hybridized by varieties of White Leghorns (female) and domestic green-shell (male), by evaluating the effects of different levels of Trp supplementation on their laying performance, egg quality, antioxidant capacity, and the immune functions.

2. Material and methods

The experiment was conducted in accordance with the Chinese guidelines for animal welfare and approved by the Animal Welfare Committee of Animal Science College, Zhejiang University.

2.1. Birds and housing

Xinyang green-shell laying hens (n = 525), 28 weeks of age, were randomly allocated to five treatment groups, each of which included 5 replicates of 21 hens. Hens were fed the basal diet based on corn and soybean meal. L-Trp (Ajinomoto, Japan) was added to the control diet at 0.0 0.02, 0.04, 0.06, and 0.08%, respectively, to achieve 0.15, 0.17, 0.19, 0.21 or 0.23% Trp. Ingredient composition and calculated nutrients are presented in **Table 1**. Hens were kept in three-layer complete ladder cages (3 birds per cage) under the same managerial conditions in a ventilated room. The temperature inside the barn was 21–27°C, and relative humidity was 60–70%. The photoperiod was 16L: 8D throughout the experiment. Each cage was equipped with two nipple drinkers and one feeder. Diets were offered twice daily for ad libitum intake and laying hens had free access to water. The experiment lasted 12 week, including a one-week acclimation period and an 11-week experimental period.

2.2. Laying performance parameters and egg quality

During the experimental period, feed residues were collected and weighted weekly to enable estimation of average daily feed intake (ADFI). Eggs from each replicate were counted and

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Ingredients	%	Nutrient levels[2]	%
Corn	62.00	ME/(MJ/Kg)	10.86
Soybean meal	6.60	CP	16.36
Peanut meal	14.50	Ca	3.30
Limestone	9.00	TP	0.32
Wheat bran	5.60	Lys	0.72
Met	0.10	Met	0.30
Lys·HCl	0.20	Thr	0.52
Premix[1]	2.00	Trp	0.15
Total	100.00		

[1]Premix provided the following per kilogram: Vitamin A, 9900 IU; vitamin D3, 2625 IU; vitamin E, 49.5 mg; vitamin K3, 6 mg; vitamin B1, 3 mg; vitamin B2, 10.5 mg; vitamin B6, 6 mg; vitamin B12, 0.03 mg; niacin, 60 mg; folic acid, 3 mg; pantothenic acid, 18 mg; biotin, 0.3 mg; Cu, 9 mg; Fe, 120 mg; Mn, 140 mg; Zn, 120 mg; I, 1.1 mg; Se, 0.4 mg.
[2]Values were calculated from data provided by Feed Database in China (2013).

Table 1. Ingredients and nutrient composition of basal diet.

weighted daily to calculate laying rate and average egg weight. Egg mass was calculated by multiplying egg weight by egg production. Feed conversion ratio (FCR) was calculated as grams of feed intake per gram of egg mass produced. Health status and mortalities were visually observed and recorded daily during the entire experimental period. The magnitude of performance parameters such as laying rate was adjusted for hen mortalities.

At the end of the experiment, 30 eggs from each treatment were randomly collected to assess egg quality parameters. The eggs were weighed and cracked, and albumen height, haugh units, yolk colour, eggshell thickness, and eggshell strength were measured with a digital egg tester (DET-6000, NABEL, Kyoto, Japan). Eggshell thickness (without the shell membrane) was measured at the middle part of the egg.

2.3. Blood sampling and laboratory analyses

At the end of the experiment, 12 h after feed withdrawal, two birds were randomly selected from each replicate, and blood samples were collected from the axillary vein. Blood samples were drawn into Eppendorf tubes (10 ml) and centrifuged at 3000 × g for 10 min to separate out serum. The obtained serum was stored in 1.5-mL Eppendorf tubes at −70°C until analyses and thawed at 4°C before analysis. Serum concentrations or activities of total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), total antioxidative capacity (T-AOC), and malondialdehyde (MDA) were measured spectrophotometrically (UV-2000, Unico Instruments Co. Ltd., Shanghai, China) using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Immunoglobulins in the serum were analysed by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using a sandwich enzyme linked immunosorbent assay (ELISA) using chicken specific IgA, IgG, IgM ELISA quantitation kits (R&D company, System, Inc., McKinley Place NE Minneapolis, MN), respectively, according to the instructions of the manufacturer, and absorbance was measured at 450 nm.

2.4. Statistical analysis

The data were expressed as means ± SE and analysed statistically by one-way ANOVA, using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL). When significant differences were found ($P < 0.05$), Tukey post hoc tests were performed.

3. Results

The laying performance parameters (**Table 2**) were significantly ($P < 0.05$) affected by dietary Trp levels. Laying rate, average egg weight, and FCR in hens fed 0.21–0.23, 0.19–0.21, and 0.21% dietary Trp were significantly improved ($P < 0.05$), respectively, compared with those hens fed on the control diet. No significant difference of ADFI was observed among all the groups ($P > 0.05$).

Results of egg quality characteristics are provided in **Table 3**. The highest value of albumen height ($P < 0.05$) came from 0.19% Trp group. Haugh unit in 0.17 to 0.19% Trp group was significantly higher ($P < 0.05$) than that of other groups. No significant difference of yolk colour, eggshell strength, and eggshell thickness was observed among all the groups ($P > 0.05$).

Results of antioxidant parameters in serum (**Table 4**) showed that supplementing with Trp increased ($P < 0.05$) GSH-Px and T-SOD activity, T-AOC concentration, and decreased ($P < 0.05$) MDA concentration ($P > 0.05$) but had no effect on CAT activity ($P > 0.05$). Briefly, serum GSH-Px activity in 0.17 and 0.19% Trp group was significantly increased by 27.62% ($P < 0.05$) and 27.42% ($P < 0.05$), respectively, compared with the control group. With the increase of supplemental Trp levels, serum T-SOD activity and T-AOC contents were gradually increased, whereas serum MDA content was gradually decreased.

Regarding serum antibodies (**Table 5**), serum IgA concentration increased ($P < 0.05$) at 0.21 to 0.23% dietary Trp, compared with those receiving 0.15% Trp. Serum IgM concentration increased linearly ($P < 0.05$) in response to dietary Trp levels. No significant effect was observed for serum IgG concentration due to dietary Trp levels ($P > 0.05$).

Items[1]	Dietary L-tryptophan levels, %				
	0.15	**0.17**	**0.19**	**0.21**	**0.23**
Laying rate, %	63.76 ± 0.65^{b}	64.15 ± 0.67^{b}	65.17 ± 1.86^{b}	66.70 ± 0.38^{a}	66.91 ± 1.34^{a}
ADFI, g/hen	85.78 ± 1.14	87.68 ± 2.62	86.81.43 ± 1.18	84.49 ± 2.55	85.52 ± 0.44
Egg weight, g	45.57 ± 0.14^{b}	46.02 ± 0.06^{ab}	46.30 ± 0.15^{a}	46.60 ± 0.04^{a}	46.09 ± 0.36^{ab}
FCR	2.88 ± 0.03^{a}	2.88 ± 0.02^{a}	2.85 ± 0.01^{a}	2.79 ± 0.01^{b}	2.81 ± 0.02^{a}

[1]ADFI = average daily feed intake; FCR = feed conversion ratio.

Means sharing different letters (a, b) in the same row are significantly different ($P < 0.05$).

Table 2. Effect of dietary L-tryptophan on laying performance.

Items	Dietary L-tryptophan levels, %				
	0.15	0.17	0.19	0.21	0.23
Albumen height, mm	4.47 ± 0.17[b]	4.50 ± 0.05[b]	4.96 ± 0.22[a]	4.66 ± 0.13[b]	4.41 ± 0.17[b]
Haugh units	68.94 ± 1.26[b]	70.96 ± 0.60[a]	73.41 ± 1.88[a]	69.10 ± 0.48[b]	68.22 ± 1.54[b]
Yolk colour, points	7.56 ± 0.17	7.22 ± 0.22	7.34 ± 0.19	7.26 ± 0.14	7.46 ± 0.16
Eggshell strength, Kgf	3.67 ± 0.20	3.35 ± 0.35	3.60 ± 0.35	3.58 ± 0.29	3.69 ± 0.32
Eggshell thickness, mm	0.31 ± 0.00	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.00

Means sharing different letters (a, b) in the same row are significantly different ($P < 0.05$).

Table 3. Effect of dietary L-tryptophan on egg quality.

Items[1]	Dietary L-tryptophan levels, %				
	0.15	0.17	0.19	0.21	0.23
GSH-Px, U/ml	3236.74 ± 164.11[b]	4130.87 ± 198.83[a]	4124.35 ± 322.76[a]	3503.48 ± 56.12[b]	3388.70 ± 369.18[b]
T-SOD, U/L	394.85 ± 14.48[b]	396.65 ± 7.25[b]	440.11 ± 8.38[a]	415.92 ± 11.62[a]	418.37 ± 8.8[a]
CAT, U/ml	2.81 ± 0.21	2.67 ± 0.16	2.69 ± 0.13	2.87 ± 0.19	2.83 ± 0.24
MDA, nmol/ml	3.45 ± 0.17[a]	3.12 ± 0.37[a]	2.60 ± 0.34[b]	2.47 ± 0.23[b]	2.44 ± 0.20[b]
T-AOC, U/L	3.85 ± 0.84[b]	4.67 ± 0.42[a]	4.71 ± 0.40[a]	4.49 ± 0.53[a]	5.15 ± 0.46[a]

[1] T-SOD = total superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; T-AOC = total antioxidative capacity; MDA = malondialdehyde.

Means sharing different letters (a, b) in the same row are significantly different ($P < 0.05$).

Table 4. Effect of dietary L-tryptophan on antioxidant parameters in serum.

Items	Dietary L-tryptophan levels, %				
	0.15	0.17	0.19	0.21	0.23
IgA, ng/ml	1779.98 ± 100.03[c]	1830.65 ± 158.94[bc]	2034.70 ± 121.37[abc]	2226.91 ± 94.59[ab]	2329.05 ± 118.36[a]
IgG, ng/ml	413.24 ± 36.50	448.67 ± 36.83	451.85 ± 34.55	475.49 ± 36.83	499.89 ± 34.09
IgM, μg/mL	1743.24 ± 24.63[b]	1999.39 ± 61.72[a]	2022.86 ± 155.02[a]	2057.07 ± 169.52[a]	2244.93 ± 107.29[a]

Means sharing different letters (a, b) in the same row are significantly different ($P < 0.05$).

Table 5. Effect of dietary L-tryptophan on serum immunoglobulins concentrations.

4. Discussion

Tryptophan plays a significant role in laying hen nutrition because it is considered to be the third-limiting amino acid, after the sulphur-containing amino acids and lysine. Most commercial diets are calculated on an amino acid basis, rather than a protein basis [12]. Therefore, it is important to have an accurate value of amino acids to use as a requirement when formulating

diets. Results of dose-response studies addressing the Trp need are variable, although the [13] suggests a requirement of 160 mg per hen per d for the commercial layer. In a study using Hy-Line hens (53 week), egg production was similar among groups receiving 0.17, 0.21 or 0.23 g/kg Trp, but at 0.19 g/kg Trp, egg production had maximized significantly compared to those given 0.17 g/kg Trp [12]. By using Rhode Island Red × White Leghorn layers (25 week), researchers found that 500 mg Trp/kg diet improved the egg production rate in laying hens [14]. However, in Babcock Brown layers (40 week), the authors found that supplementing Trp had no effect on laying performance [7]. In the current study, we found that the laying performance parameters (i.e. laying rate, egg weight, and FCR) were significantly improved by dietary Trp levels (from 0.19 to 0.23%) in Xinyang green-shell laying hens (28 week). Combining to all the aforementioned, it is clearly that the variable need for Trp in laying hens is partially due to the genotype and age.

Egg quality is important for consumer appeal and encompasses several aspects related to the shell (external quality) and to the albumen and yolk (internal quality). Egg quality has a genetic basis, and the parameters of egg quality vary between strains of hens [15, 16]. However, egg quality is also influenced by diet nutrition such as dietary protein and amino acid content [17]. Limited research has been conducted on the effects of supplemental Trp on egg quality of laying hens. In Ref. [7], the authors found that adding 0.2 or 0.4 g/kg Trp to the basal diet (0.17% Trp) improved egg shell strength quadratically in Babcock Brown layers, but had no effect on egg internal quality. Conversely, in the current study, we found that dietary Trp from 0.17 to 0.19% increased egg internal quality (albumen height and haugh unit) rather than external quality. The mechanism of Trp regulating egg quality is not well understood due to limited references. Thus, further studies are needed to conduct to verify the role of Trp on egg quality.

It is known that serotonin, with Trp as its precursor, has many functions in the central nervous system to inhibit aggression and modulates stress response [6], suggesting that dietary Trp may sever as a free radical scavenger, and hence have beneficial effects on the antioxidant capacity of animals. Almost all the phenomena of life and pathological processes are related to the perspectives of free radicals that can induce body damage when they are presented in excessive levels [18]. MDA can generally be used as a biomarker for free radical induced damage and can endogenously reflect lipid peroxidation, which is the consequence of diminished antioxidant protection as concentrations of reactive oxygen species increase [19]. SOD and GSH-Px are the main parameters used to assess oxidative status in the enzymatic system, while T-AOC represents enzymatic and non-enzymatic antioxidant defence systems [10, 18]. In the current study, the higher T-AOC level, T-SOD and GSH-px activities and lower MDA concentrations due to supplemental Trp in laying hens reflect a greater antioxidant defence. In accordance with our results, previous researchers also found that Trp increased the serum SOD activity of laying hens [7] and elevated the GSH content in the hepatic tissues of rats [8]. The present study suggested that appropriate Trp levels in the diet may have a positive effect on both the enzymatic and non-enzymatic antioxidant capacity function of laying hens.

Recent studies have also proved that Trp may play an important role in immune function. Dietary Trp deficiency has been demonstrated to reduce the levels of nutrition and to depress immune function to cause a significant increase in the susceptibility to disease infection, morbidity, and mortality in animals [20]. The addition of Trp to the control diet resulted in increasing the levels of

serum IgA and IgM in the current study. Substantiating the findings on serum immunoglobulins, a previous study showed that a deficiency of Trp decreased antibody production in rats [5]. In addition, a recent study in laying hens also found that the addition of Trp at 0.4 g/kg to the basal diet (0.17% Trp) resulted in quadratically increasing the levels of serum IgM in Babcock Brown layers [7]. From the nutritional viewpoint, we speculated that amino acids affect the synthesis of effector molecules (immunoglobulins, nitric oxide, lysozyme, and complement). It is worth noting that tryptophan is not the only amino acid that affects the immune function. Researchers observed that a deficiency of phenylalanine decreased antibody production in rats [5], while threonine supplementation increased IgG concentrations in the serum of laying hens [21]. In contrast, excess methionine [5] and leucine [22] suppressed humoral immune function in the rat. Our results indicated that Trp plays important roles in the regulation of poultry humoral immune through regulation of the generation of immunoglobulins; however, our understanding of the function of Trp in the regulation of immune response is far from complete, and its involved mechanisms require further study.

5. Conclusion

In conclusion, supplemental Trp to the control diet can improve laying performance, egg quality, antioxidant capacity, and the immune functions in Xinyang green-shell laying hens. We suggest that the optimum level range of dietary Trp is from 0.19 to 0.21% for Xinyang green-shell laying hens under the current study conditions.

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References

[1] Ruan Z, Yang YH, Wen YM, Zhou Y, Fu XF, Ding S, Liu G, Yao K, Wu X, Deng ZY, Wu GY, Yin YL. Metabolomic analysis of amino acid and fat metabolism in rats with tryptophan supplementation. Amino Acids. 2014;**46**:2681-2691. DOI: 10.1007/s00726-014-1823-y

[2] Woodger TL, Sirek A, Anderson GH. Diabetes, dietary tryptophan, and protein-intake regulation in weanling rats. The American Journal of Physiology. 1979;**236**:R307-R311

[3] Cortamira NO, Seve B, Lebreton Y, Ganier P. Effect of dietary tryptophan on muscle, liver and whole-body protein-synthesis in weaned piglets-relationship to plasma-insulin. British Journal of Nutrition. 1991;**66**:423-435. DOI: 10.1079/BJN19910045

[4] Le Floc'h N, Melchior D, Seve B. Dietary tryptophan helps to preserve tryptophan homeostasis in pigs suffering from lung inflammation. Journal of Animal Science. 2008;**86**:3473-3479. DOI: 10.2527/jas.2008-0999

[5] Gershoff SN, Gill TJ, Simonian SJ, Steinberg AI. Some effects of amino acid deficiencies on antibody formation in the rat. Journal of Nutrition. 1968;**95**:184-189

[6] Martin CL, Duclos M, Aguerre S, Morede P, Manier G, Chaouloff F. Corticotropic and serotonergic responses to acute stress with/without prior exercise training in different rat strains. ActaPhysiologicaScandinavica. 2000;**168**:421-430. DOI: 10.1046/j.1365-201x.2000.00683.x

[7] Dong X, Azzam M, Rao W, Yu D, Zou X. Evaluating the impact of excess dietary tryptophan on laying performance and immune function of laying hens reared under hot and humid summer conditions. British Poultry Science. 2012;**53**:491-496. DOI: 10.1080/00071668.2012.719149

[8] Raju T, Kanth VR, Reddy PUM, Srinivas J, Sobhanaditya J. Influence of kynurenines in pathogenesis of cataract formation in tryptophan-deficient regimen in Wistar rats. Indian Journal of Experimental Biology. 2007;**45**:543-548

[9] Wen H, Feng L, Jiang W, Liu Y, Jiang J, Li S, Tang L, Zhang Y, Kuang S, Zou X. Dietary tryptophan modulates intestinal immune response, barrier function, antioxidant status and gene expression of TOR and Nrf2 in young grass carp (Ctenopharyngodonidella). Fish & Shellfish Immunology, 2014;**40**:275-287. DOI: 10.1016/j.fsi.2014.07.004

[10] Sainio EL, Pulkki K, Young SN. L-Tryptophan: Biochemical, nutritional and pharmacological aspects. Amino Acids. 1996;**10**:21-47. DOI: 10.1007/BF00806091

[11] Corzo A, Kidd MT, Thaxton JP, Kerr BJ. Dietary tryptophan effects on growth and stress responses of male broiler chicks. British Poultry Science. 2005;**46**:478-484. DOI: 10.1080/00071660500157974

[12] Russell GB, Harms RH. Tryptophan requirement of the commercial hen. Poultry Science. 1999;78:1283-1285. DOI: 10.1093/ps/78.9.1283

[13] NRC. Nutrient Requirements of Poultry. 9th rev. ed. Washington, DC: National Acad. Press; 1994

[14] Ohtani H, Saitoh S, Ohkawara H, Akiba Y, Takahashi K, Horiguchi M, Goto K. Research note: Production performance of laying hens fed l-tryptophan. Poultry Science. 1989;**68**:323-326. DOI: 10.3382/ps.0680323

[15] Pandey NK, Mahapatra CM, Verma SS, Johari DC. Effect of strain on physical egg quality characteristics in White Leghorn chickens. Indian Journal of Poultry Science. 1986;**21**:304-307

[16] Silversides FG, Korver DR, Budgell KL. Effect of strain of layer and age at photostimulation on egg production, egg quality, and bone strength. Poultry Science. 2006;**85**:1136-1144. DOI: 10.1093/ps/85.7.1136

[17] Roberts JR. Factors affecting egg internal quality and egg shell quality of laying hens. Journal of Poultry Science. 2004;**41**:161-177

[18] Zhao X, Yang ZB, Yang WR, Wang Y, Jiang SZ, Zhang GG. Effects of ginger root (Zingiberofficinale) on laying performance and antioxidant status of laying hens and on dietary oxidation stability. Poultry Science. 2011;**90**:1720-1727. DOI: 10.3382/ps.2010-01280

[19] Wang YZ, Xu CL, An ZH, Liu JX, Feng J. Effect of dietary bovine lactoferrin on performance and antioxidant status of piglets. Animal Feed Science and Technology. 2008;**140**:326-336. DOI: 10.1016/j.anifeedsci.2007.02.006

[20] Harden JL, Lewis SM, Lish SR, Suárez-Fariñas M, Gareau D, Lentini T, Johnson-Huang LM, Krueger JG, Lowes MA. The tryptophan metabolism enzyme l-kynureninase is a novel inflammatory factor in psoriasis and other inflammatory diseases. Journal of Allergy & Clinical Immunology. 2015;**137**:1830-1840. DOI: 10.1016/j. jaci.2015.09.055

[21] Azzam MMM, Dong XY, Xie P, Wang C, Zou XT. The effect of supplemental l-threonine on laying performance, serum free amino acids, and immune function of laying hens under high-temperature and high-humidity environmental climates. Journal of Applied Poultry Research. 2011;**20**:361-370. DOI: 10.3382/japr.2010-00308

[22] Aschkenasy A. Prevention of the immunodepressive effects of excess dietary leucine by isoleucine and valine in the rat. Journal of Nutrition. 1979;**109**:1214-1222

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