Proteomic alteration of albumen by dietary vanadium in commercial egg-type layers

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ABSTRACT Vanadium (**V**) is an ultratrace metal with the insulin-tropic properties and is often researched as the diabetes drug. However, in animals, V has been reported to have toxic effects on the development, immunity, oxidation–reduction equilibrium, gastrointestinal function, and so forth. Especially in poultry, supplementation of more than 10 mg of V/kg in the layer diets has been shown to adversely affect the egg production and egg quality. In this study, we supplemented 0 mg of V/kg, 5 mg of V/kg, and 10 mg of V/kg in the layer diets for 35 D and examined the quantitative proteomics of albumen for finding the possible target signaling pathway and mechanism of V action and made the preliminary verification. In contrast to the control group, V resulted in a significant drop in the albumen height, and in oviduct ampulla, the activity of total antioxidant capacity and glutathione peroxidase significantly decreased ($P = 0.01$, $P = 0.02$), the content of malonic dialdehyde significantly increased ($P = 0.01$), and the apoptosis rate significantly increased in the 5-mg V/kg and 10-mg V/kg treatment groups ($P < 0.01$). V affected 36 differentially accumulated proteins in albumen, with 23 proteins upregulated and 13 proteins downregulated. The expressions of innate protein albumen lysozyme (Q6LEL2), vitellogenin-2 (P02845), and the F1NWD0 protein in albumen belonged to the P53 family were significantly reduced, in contrast to the control ($P < 0.05$), and the expression of riboflavin-binding protein (P02752) was significantly improved ($P < 0.05$). The Hippo signaling pathway-fly, which is suitable for the key protein P53 as the most significantly affected network, might be important for discriminating V.

Key words: albumen, vanadium, quantitative proteomic

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INTRODUCTION

Vanadium (**V**) is an ultratrace metal and is present in humans and animals as an inorganic enzyme cofactor. Many studies have focused on the insulin-tropic properties of V salts in diabetes and regulation of lipid metabolism (Barceloux and Barceloux, 1999; Srivastava and Mehdi, 2010). But in the recent years, there has been an increased interest in toxicological effects of V. V has a distinct effect on mobilization of intracellular calcium through alteration in Na, K-ATPase and Ca-ATPase activity (Aureliano et al., 2005) and induces nuclear factor of activated T cells activation through hydrogen peroxide (Abdelhamid et al., 2010). The loss of body weight; hematological and biochemical alterations; immunotoxicity; nephrotoxicity; and gastrointestinal, reproductive, and developmental toxicity have been reported to occur after exposure to V compounds (Bressman et al., 2002; Davis et al., 2002; Miles and Henry, 2004; Markopoulou et al., 2009; Liu et al., 2011, 2012; Zhao and Yang, 2013). In particular, poultry are more vulnerable to V than other species (Domingo et al., 2000). Supplementation of more than 10 mg of V/kg in the layer diets has been shown to adversely affect the layer health, egg production, and egg quality (Bressman et al., 2002; Davis et al., 2002; Miles and Henry, 2004; Liu et al., 2012).

Chicken eggs are known for their nutritional edible value and contain a variety of biologically active molecules for the important raw materials in medicine, food, and cosmetics areas. A fresh and high-quality egg should have a firm and gelatinous albumen for anchoring

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Table 1. Composition, energy, and nutrient level of the basal diet.

Composition (%)		Energy and nutrient level (%)	
Corn	64.80	Metabolizable energy (Mcal/kg)	2.68
Soybean meal	24.14	Crude protein	15.73
Soybean oil	0.20	Calcium	3.65
Calcium carbonate	8.66	Available phosphorus	0.32
Calcium hydrophosphate	1.16	Methionine	0.36
NaCl	0.35	Lysine	0.79
Choline chloride	0.16	Cysteine	0.25
Vitamin premix[1]	0.03		
Mineral premix[2]	0.5		
Total	100		

[1]Provided the following per kilogram of diet: 10,000 IU vitamin A; 2,500 IU vitamin D_3; 10 IU vitamin E; 2 mg vitamin K_3; 1 mg thiamine; 5 mg riboflavin; 1 mg pyridoxine; 15 mg cobalamin; 0.25 mg folic acid; 20 mg nicotinic acid; 2.2 mg pantothenic acid; 0.1 mg biotin.

[2]Provided the following per kilogram of diet: 60 mg Fe ($FeSO_4 \bullet 7H_2O$); 8 mg Cu ($CuSO_4 \bullet 5H_2O$); 60 mg Zn ($ZnSO_4$); 600 mg Mn (MnO_2); 0.15 mg Se ($Na_2SeO_3 \bullet 5H_2O$); 0.35 mg (KI) and 0.3 mg Co ($CoSO_4 \bullet 5H_2O$).

the yolk, have antimicrobial effect, and be good looking. The albumen contains approximately 75% water, 12% protein, 12% fat, and other essential nutrients, such as vitamin, represents a shock absorber, constitutes an antimicrobial barrier, and provides substantial nutrients to the developing embryo (Mann, 2007). Albumen quality is commonly evaluated by albumen height and measured in Haugh units (**HU**). V reduced the HU of albumen, but the protein composition changes of albumen were not clear, and the mechanism of V affecting albumen quality is yet to be studied.

Proteomics is an indispensable technology in the research of expression and function patterns of all proteins in organisms. It can be used to identify the components of small protein complexes and large organelles and to determine protein–protein interactions, organelle composition, and post-translational modifications and used in sophisticated functional screening (Tyers and Mann, 2003). But very limited proteomics research has been presented regarding the quality and nutritional characteristics of chicken albumen. The literature is mainly focused on the egg as a reproductive stage. Qiu et al., (2012) reported the degradation of albumen in relation to higher temperature during storage, with the formation of a lysozyme–ovalbumin complex, through a two-dimensional gel electrophoresis (**2-DE**)-proteomic approach. One hundred forty-eight albumen proteins were identified by the combinatorial hexa-peptide libraries in conjunction with liquid chromatography-electrospray ionization ion-trap tandem mass/mass spectrometry (**LC-ESI-IT-MS/MS**) (Ambrosio et al., 2008). Mann, (2007) identified 158 proteins in chicken albumen using an LTQ Orbitrap Velos (Thermo Electron, Bremen, Germany). Guyot et al., (2016) found 20 proteins in albumen with natural antibacterial effect, including 3 newly identified proteins vitelline membrane outer layer protein 1, beta-microseminoprotein (LOC101750704), and pleiotrophin. The distribution of proteins in albumen is extremely uneven, and the highly abundant proteins make the isolation and identification of the albumen proteins difficult (Mann and Mann, 2011). The immunoaffinity method can remove some high-abundance proteins, but this method will inevitably remove some low-abundance proteins at the same time. These low-abundance proteins in albumen often have important physiological significance, such as acting as allergic antigens (Ambrosio et al., 2008). Isobaric tags for relative and absolute quantitation (**iTRAQ**) is a liquid-based proteomics technique which labels the lysine residues and N-terminus of all peptides with isotopes of identical masses, thus allowing accurate quantitative comparisons between 2 (2-plex) and 8 (8-plex) samples (Wu et al., 2006). With the wide application of iTRAQ technology, many new proteins were discovered and reported, but the application of iTRAQ to egg albumin proteomics was less.

Usually, the ovomucin contents and composition in albumen is responsible for the HU (Omana and Wu, 2009; Liu et al., 2017). But lysozyme and ovalbumin may have some important effects on the albumen thinning too (Hawthorne, 1950). In this study, we compared the albumen quality and the oviduct in laying hens treated with V with the control by iTRAQ, the robust technique which presents more sensitivity and better accuracy of quantification aiming to research the albumen protein composition, protein–protein interactions, and the effect of V on the laying hen oviduct health.

MATERIALS AND METHODS

Birds, Diets, and Management

At 67 wk of age, a total of 90 Lohmann laying hens were randomly divided into 3 treatment groups involving 3 V levels (0, 5, and 10 mg V/kg diet). V was added in the form of ammonium metavanadate (Sigma-Aldrich). The composition and nutrient level of the basal diet are shown in Table 1. There were 30 replicates with 1 bird each. The birds were housed individually in stainless-steel cages (38.1 cm width × 50 cm length × 40 cm height), and the room environment was maintained at 22°C, with a daily lighting schedule of 16-h light and 8-h dark; hens were provided food ad

libitum for 35 D. The experimental protocol used in the study was approved by the Animal Care and Use Committee of Sichuan Agricultural University.

Sample Collection

All eggs were collected to measure egg quality at 7, 21, and 35 D. On the 35th D, 24 eggs were collected from every treatment group, and the albumen was carefully separated from yolk and gently homogenized using a magnetic stirrer for 15 min to reduce the viscosity and stored in the liquid nitrogen. All the procedures were carried out at 4°C. Eight egg whites from each treatment group were mixed randomly. Then, 3 independent biological replicates were performed for protein extraction and iTRAQ analysis.

On the 35th D, 18 hens (6 hens from each group) were sacrificed by cervical dislocation, and the mucous membrane of the oviduct ampulla was quickly removed and kept in liquid nitrogen to measure antioxidant enzyme activities and MDA content.

Preparation of Peptide

Two hundred ìL of pooled albumen sample was centrifuged at 14,000 × g for 15 min after mixing with 1 mL of TCA (1:5) at −20°C for 12 h. And this washing step was repeated twice for obtaining the albumen protein. The protein was freeze-dried. Forty milligrams of dry albumen protein powder was splitted by ultrasound with 800 μL of splitting solution (20 mmol HEPES, 9 mol Urea, pH 8.0) on ice and centrifuged at 14,000 × g for 30 min The supernatant was collected, and protein concentration was determined using the modified Bradford assay protocol (Bio-Rad, Berkeley, CA).

Thirty ìL of each sample was boiled in a water bath for 5 min with the dithiothreitol (**DTT**) buffer (100 mmol). Two wash steps with 100 ìL of dissolution buffer (25 mmol ammonium bicarbonate) were performed with centrifugation at 14,000 × g for 10 min after each wash step. Finally, 40 ìL of trypsin (Promega, Madison, WI) buffer (4 ìg of trypsin in dissolution buffer) was added and digested at 37°C for 16 − 18 h. The filter unit was transferred to a new tube and centrifuged at 14,000 × g for 10 min. Resulting peptides were collected as a filtrate, and the peptide concentration was analyzed at 280 nm.

iTRAQ Analysis

iTRAQ labeling was performed in accordance with the company manual. The tryptic peptides were incubated with iTRAQ reagent - 8 Plex Multiplex Kit (AB Sciex Inc., Applied Biosystems, Foster City, CA) (113 for the control group; 114 for 5-mg V/kg group; 115 for 10-mg V/kg group). The labeled samples were fractionated using the AKTA Purifier 100 (GE Healthcare, Chicago, IL) with strong cation-exchange chromatography (SCX) followed by polysulfoethyl column (4.6 × 100 mm, 5 ìm, 200 A; PolyLC Inc., Columbia, SC). SCX buffer A was 10 mmol KH_2 PO_4, pH 3.0, in 25% (v/v) acetonitrile, and SCX buffer B was 10 mmol KH_2 PO_4, pH 3.0, 500 mmol KCl, in 25% (v/v) acetonitrile.

The fractions were then analyzed by nanoLC-MS/MS (Q Exactive, Thermo Finnigan, San Jose, CA). Dissolved buffer C was 0.1% (v/v) formic acid in Milli-Q water, and buffer D was 0.1% formic acid in 84% acetonitrile. Samples were separated using the EASY column (Thermo Fisher Scientific, Waltham, MA; 75 ìm × 100 mm, 3 ìm, C18; Thermo Scientific) at a flow rate of 300 nL/min with a segmented gradient from 0 to 50% (v/v) buffer D from 0 to 55 min, from 50 to 100% (v/v) from 55 to 57 min, and then at 100% (v/v) from 57 to 60 min.

Q Exactive MS survey scan was acquired (m/z 300–1,800), with up to ten of the most intense multiply charged ions from the survey scan being sequentially subjected to product ion analysis. Product ion spectra were allowed to accumulate in the activation type of high energy collision dissociation (**HCD**) with isolation window of 2 m/z, resolution of 17,500 m/z at 200, microscans of 1, maximum IT of 60 ms, normalized collision energy of 30 eV, and underfill ratio of 0.1%.

Proteomic Data Analysis

Protein identification and quantification were performed using the Protein Discoverer 1.0 software (Thermo Fisher, Waltham, MA) with Mascot, version 2.2 (Matrix Science, England, United Kingdom), database using the search parameters as shown in Table 2. The peptide false discovery rate was set as 1%.

The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. A P value <0.05 and fold change >1.2 were considered significant difference.

Bioinformatics Analysis

To better understand the annotation and distribution of differently expressed protein functions, the Blast2GO program was used to obtain gene ontology (GO) annotations. GO immune system process annotations for gene

Table 2. Mascot search parameters.

Item	Value
Type of search	MS/MS ion search
Enzyme	Trypsin
Mass values	Monoisotopic
Max missed cleavages	2
Fixed modifications	Carbamidomethyl (C), iTRAQ8plex (N-term), iTRAQ8plex (K)
Variable modifications	Oxidation (M)
Peptide mass tolerance	±20 mg/kg
Fragment mass tolerance	0.1 Da
Protein mass	Unrestricted
Database	uniprot_Gallus_gallus_24,072_0605.fasta
Database pattern	decoy

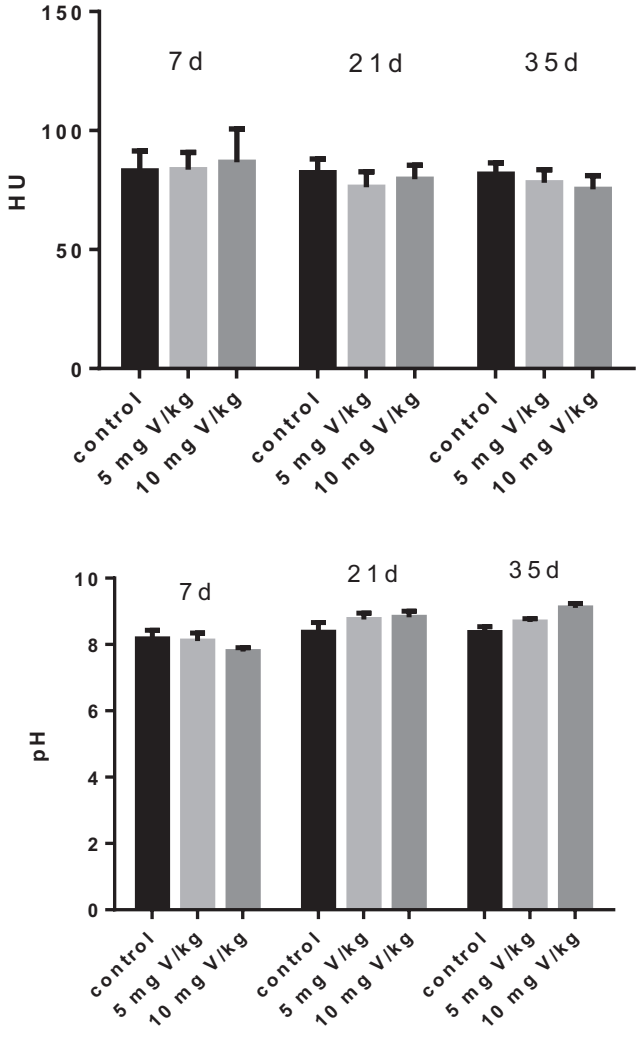


Figure 1. The HU and pH value of albumen affected by V. On the 21st day, V significantly reduced the albumen HU ($P = 0.01$). On the 35th d, the pH value of albumen significantly raised ($P < 0.01$); there also appeared a significant difference between 5 mg V/kg and 10 mg V/kg ($P < 0.01$). HU, Haugh unit.

enrichment (right-sided hypergeometric test) with Benjamini–Hochberg multiple test correction were implemented. Differently expressed proteins were selected for pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for metabolism, environmental information processing, other cellular processes, diseases, and drug development.

Egg Quality

The HU value of eggs was evaluated using an egg multitester (EMT-7300, Robotmation Co., Ltd., Tokyo, Japan). The pH value of albumen was determined using a pH meter (Sartorius PB-10, Ltd., Germany).

Antioxidant Enzyme Activities and MDA Content

Oviduct ampulla tissues were homogenized in 50 mmol sodium phosphate buffer (pH 7.0) and then centrifuged at 12,000 g for 20 min at 4°C. The supernatant was used to measure the antioxidant enzyme activities and MDA. MDA content and catalase (**CAT**), glutathione peroxidase (**GSH-Px**), total antioxidant capacity (**T-AOC**), and total superoxide dismutase (**T-SOD**) activity were determined using the assay kits (Nanjing Jiancheng Bioengineering Co., Ltd., China).

Apoptosis of Oviduct Ampulla

Oviduct ampulla tissues were observed with the terminal deoxynucleotidy transferase–mediated dUTP nick-end labeling (**TUNEL**) method. TUNEL (Roche Group, Switzerland) assays were performed in accordance with the manufacturer's protocol. Apoptotic cells were stained brown, and normal cells were stained purplish blue. The percentage of TUNEL-positive cells was determined by counting at least 200 cells in 5 randomly selected fields.

Statistical Analysis

Statistical analysis was conducted using single-degree-of-freedom contrast in general linear models analysis in SAS, version 9.0, to compare all adding V diets with the control, and the Tukey test was used to evaluate differences among each group sample. *P*-values less than 0.05 were considered significant.

RESULTS

HU and pH of Albumen

From the 21 days, V significantly reduced the albumen HU ($P = 0.01$), and the decline aggravated

Table 3. Effect of V on oviduct ampulla antioxidative status of laying hens.

Item	GSH-Px (U/mg prot)	CAT (U/mg prot)	T-AOC (U/mg prot)	T-SOD (U/mg prot)	MDA (mmol/mg prot)
V, mg V/kg					
0	6.98	10.32[a]	0.08[a]	31.89	0.47[b]
5	6.61	6.68[b]	0.03[b]	26.65	0.70[a]
10	7.02	5.30[b]	0.04[b]	24.36	0.79[a]
SEM	0.35	0.85	0.09	1.16	0.19
P-value	0.48	0.02	0.01	0.14	0.01

[a,b]means $P < 0.05$.

The activity of total antioxidant capacity (T-AOC) and glutathione peroxidase (GSH-Px) decreased significantly ($P = 0.01$; $P = 0.02$) in V-containing treatment, and the content of malonic dialdehyde (MDA) was increased significantly ($P = 0.01$), but V had no significant effect on the activity of total superoxide dismutase (T-SOD) ($P = 0.14$) and catalase (CAT) ($P = 0.48$).

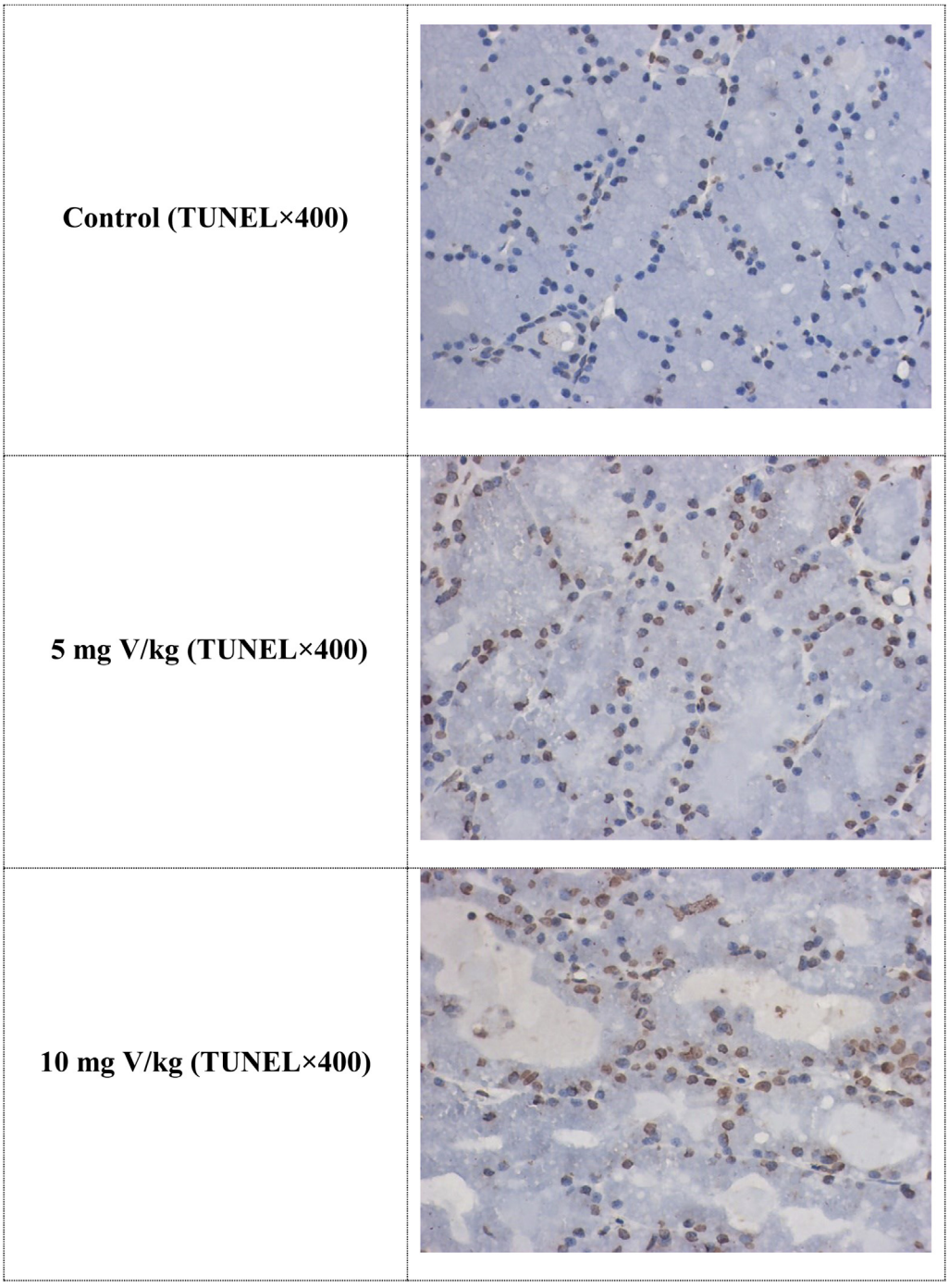


Figure 2. The apoptosis in the tissue of the laying hen oviduct ampulla (HE staining × 400). In contrast to the control, the apoptosis rate of the laying hen oviduct ampulla in the 5 mg V/kg and 10 mg V/kg treatment groups were significantly increased ($P < 0.01$), but it had no significant difference between 5 mg V/kg and 10 mg V/kg ($P > 0.05$). HE, hematoxylin and eosin; TUNEL, terminal deoxynucleotidy transferase–mediated dUTP nick-end labeling.

with time ($P < 0.01$). On the 35th D, the pH value of albumen significantly raised ($P < 0.01$), and there was a significant difference between 5 mg V/kg and 10 mg V/kg treatment groups ($P < 0.01$) (Figure 1).

Antioxidant Enzyme Activities and Malondialdehyde

Dietary V significantly decreased the GSH-PX activity and T-AOC ($P = 0.01$, $P = 0.02$) and increased MDA content ($P = 0.01$), and there was no significant difference between CAT and T-SOD enzyme activities ($P > 0.05$) (Table 3).

Apoptosis of Oviduct Ampulla

In contrast to the control, the apoptosis rate of laying hens' oviduct ampulla in the 5 mg V/kg and 10 mg V/kg V treatment groups significantly increased ($P < 0.01$), but no significant difference was observed between

5 mg V/kg and 10 mg V/kg ($P > 0.05$). treatment groups (Figure 2).

Quantitative Proteomics and Identified Proteins

A total of 379 proteins of albumen samples were identified using the iTRAQ quantitative proteomic method. Student t test analysis of the 3 V treatment groups revealed the number of differentially expressed proteins as shown in Figure 3. As can be seen from Figure 3, there were 20 different proteins between the control group and 5-mg/kg V treatment group, among which the expression of 11 increased and 9 decreased. When the dosage of V was increased to 10 mg/kg, the number of different proteins increased to 38, among which expressions of 20 proteins increased and 18 proteins decreased. Ten of the different proteins between the control group and 5-mg/kg V treatment group overlapped with the different proteins between the control group and 10-mg/kg of V treatment group (Figure 4).

Thirty-six different proteins were found by the single-degree contrast analysis of albumen samples comparison of V and control groups, with 23 proteins upregulated and 13 proteins downregulated (Table 4). The expression of intrinsic proteins in albumen, albumen lysozyme (Q6LEL2), was significantly decreased ($P < 0.05$), and the expression of F1NU63 (ovostatin precursor) and ovalbumin (P01012) was significantly increased ($P < 0.05$). The expression levels of proteins derived from oviduct cells, 6 proteins including F1NWD0 (tumor protein 63 isoform x2) and E1C7S9 (wd repeat and socs box-containing protein 2), decreased significantly; the expression of cytoplasmic 1 (P60706), 5' exonuclease apollo (Q5QJC3), beta-2 microglobulin (P21611), clusterin (Q9YGP0), and Golgi apparatus protein 1 (Q02391) significantly increased ($P < 0.05$). The

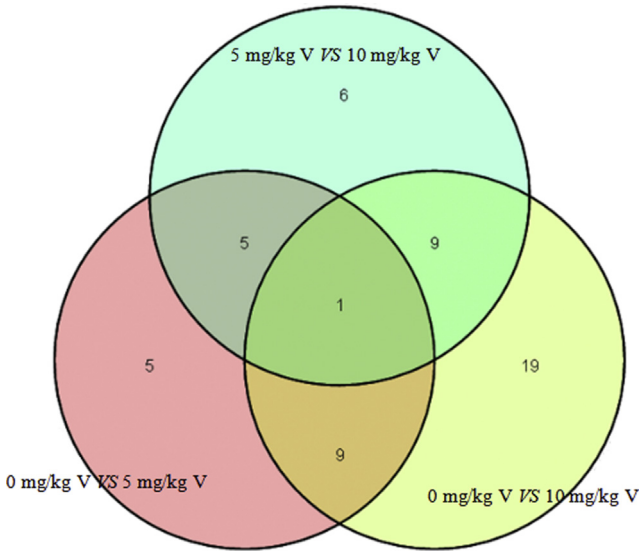


Figure 3. Different protein Venn diagram by the student t test analysis in treatments. There were 20 different proteins between the control group and 5 mg V/kg treatment, 38 different proteins between the control group and 10 mg V/kg treatment, and 10 of them were overlapped.

expression of albumen proteins from blood, 6 proteins including serum albumin (P19121) and Vitellogenin-2 (P02845), significantly decreased ($P < 0.05$), and the expression of 10 proteins including riboflavin-binding protein (P02752) and serum lysozyme (B8YJT7) increased significantly ($P < 0.05$).

The expression of protein FIP0Z6 in the 5-mg V/kg group was 5.83 times higher than that in the control group. We did not find the annotation of FIP0Z6 protein in the chicken database, but through the analysis of BLAST alignment, we found it had a similar sequence with the protein unc-45 homolog B of rats and humans. The protein unc-45 homolog B acted as a cochaperone for HSP90 and was required for proper folding of the myosin motor domain, and it played an important role in sarcomere formation during muscle cell development (Hansen et al., 2014).

With increasing doses of V, the number of different proteins compared with the control was increased. Ten of those different proteins were overlapping (Figure 4, Table 4). It was worth noting that the F1NWD0 protein (downregulated) belonged the P53 family and was similar to the tumor protein 63 isoform x2.

GO Enrichment Analysis of Differentially Accumulated Proteins

To further investigate the possible mechanism of V effect on laying hens, an overview of the accumulated different proteins affected by V was analyzed by the GO annotations including biological processes, cellular components, and molecular functions. Proteins assigned to each category are presented in Figure 5.

The different proteins in albumen were mostly located in the cell, organelle, and extracellular region. Single-organism process, metabolic process, cellular process, biological regulation, multicellular organismal process, and developmental process were mainly involved in physiological processes of these 20 proteins, and binding was the main biological function.

When the dose of V in laying hen diet increased to 10 mg V/kg, the GO analysis results of different proteins were similar to those of 5-mg V/kg group. Maximum numbers of proteins were found in the organelle, followed by in the cell and extracellular region. Furthermore, these proteins took part in the single-organism process, cellular process, metabolic process, biological regulation, and response to stimulus, and the binding and catalytic activity were the main biological functions.

KEGG Network Analysis

Network analysis of the 2 groups of differentially expressed proteins (0 mg V/kg vs. 5 mg V/kg, control vs. 10 mg V/kg) both indicated that the Hippo signaling pathway-fly as the most significantly affected network might be important for discriminating V, and the different proteins in the control vs. 10-mg V/kg groups were mapped to the KEGG database categories with $P \leq 0.05$, as shown in Figure 6.

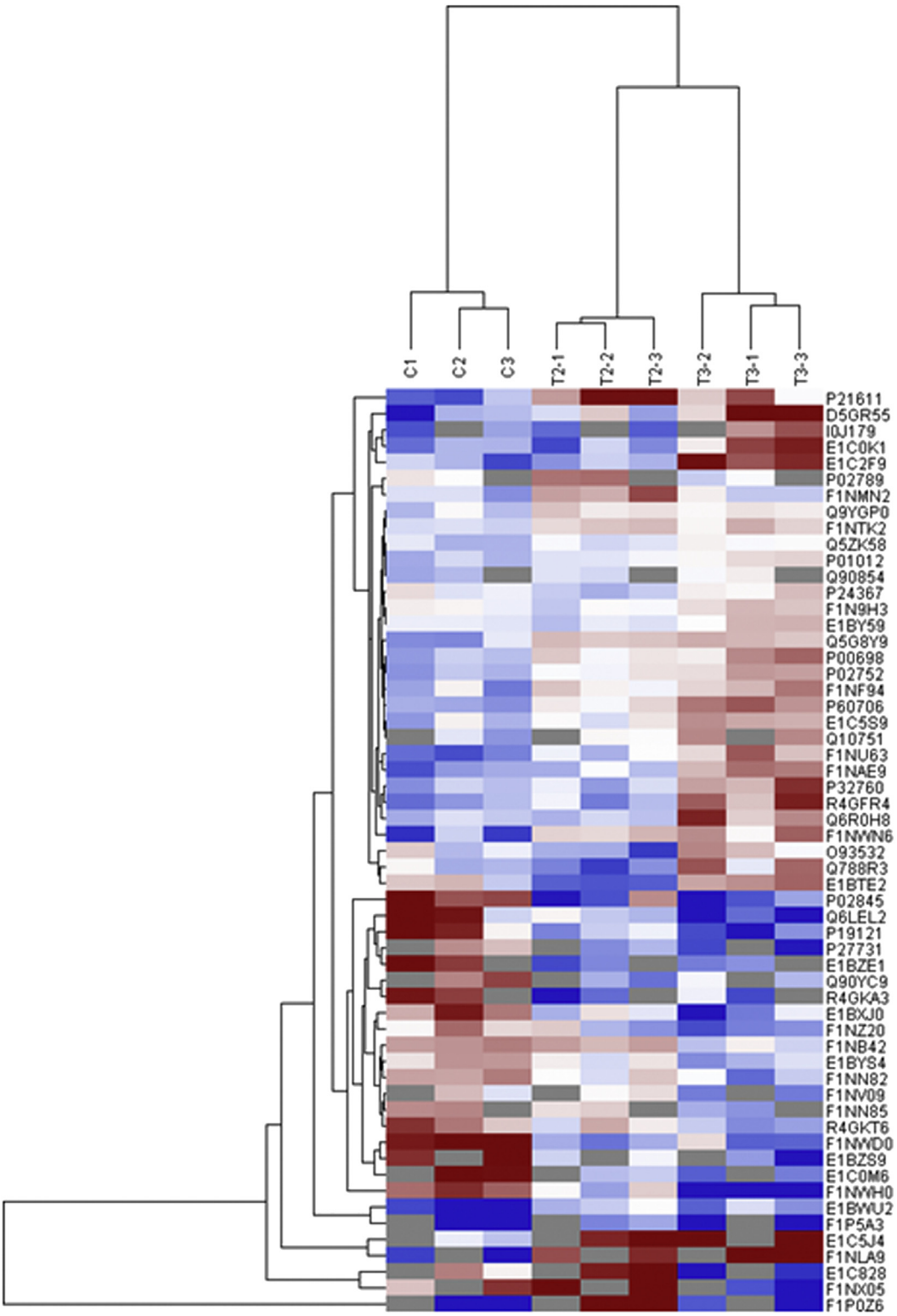


Figure 4. Clustering was based on protein expression levels in albumen. The C1, C2, and C3 belong to the control group, T2-1, T2-2, and T2-3 belong to the 5-mg V/kg group and T3-1, T3-2, and T3-3 belong to the 10-mg V/kg group, the same as below. Bar color represents a logarithmic scale from −0.5 to 0.5. Red bars indicate upregulated proteins, and blue bars indicate downregulated proteins.

DISCUSSION

Moisture content can be as high as 85–90% in albumens and directly affect the protein concentration. In this trial, the V had no significant effect on the water and crude protein content of albumen. It suggested that the less thick albumen induced by V was not caused by the changes in the total protein content but the protein composition.

Most albumen proteins were synthesized by oviduct epithelial cells and secreted in the oviduct ampulla, and the rest of the albumen protein came from oviduct

Table 4. Differential proteins from single-degree contrast analysis of albumen sample comparison of V and control groups.

Source	Accession	Sequence desc.	MW [kDa]	Calc. pI	Unique peptides	Control	5 mg V/kg	10 mg V/kg	Level
Secretion of oviduct epithelial cell	P02752	Riboflavin-binding protein	27.19	5.24	13	0.9	1.01	1.09	up
	Q6LEL2	Egg white lysozyme	4.95	9.61	1	1.4	0.95	0.72	down
	F1NU63	Ovostatin precursor	166.15	6.16	1	0.8	0.96	1.11	up
	P01012	Ovalbumin	42.85	5.29	26	0.92	0.97	1.03	up
Cell	R4GKT6	Microtubule–actin cross-linking factor 1 isoform x9	347.32	5.14	1	1.24	1.02	0.89	down
	P60706	Cytoplasmic 1	41.71	5.48	2	0.89	1.02	1.18	up
	Q5QJC3	5 exonuclease apollo	50.8	7.4	1	0.94	1.12	1.26	up
	P21611	Beta-2 microglobulin	13.03	6.28	5	0.79	1.3	1.1	up
	E1C7S9	WD repeat and SOCS box-containing protein 2	46.69	8.43	1	1.14	0.98	0.93	down
	F1NWD0	Tumor protein 63 isoform x2	65.22	7.36	1	1.4	0.87	0.88	down
	Q90YC9	Neuroblastoma suppressor of tumorigenicity 1	19.48	5.16	1	1.15	0.86	0.95	down
	R4GKA3	Glioma tumor suppressor candidate region gene 1 protein	60.85	8.37	1	1.33	0.76	0.88	down
	E1C0K1	Extracellular fatty acid-binding protein	20.17	5.72	2	0.86	0.85	1.21	up
	F1P5A3	Histone acetyltransferase type b catalytic subunit	47.93	5.49	1	0.63	0.87	0.65	up
	P00698	Lysozyme	16.23	9.07	3	0.91	1.03	1.12	up
	R4GFR4	Metalloproteinase inhibitor partial	19.96	9.25	2	0.84	0.91	1.21	up
	Q9YGP0	Clusterin precursor	51.32	5.67	19	0.96	1.04	1.02	up
	E1C6M9	Cadherin-1 isoform x1	97.69	5.17	2	0.98	1.05	1.02	up
	Q90854	G-protein–activated inward rectifier potassium channel 1	55.38	8.06	1	0.9	0.96	1.01	up
Cell membrane	F1NWH0	ATP-binding cassette subfamily a member 1	244.1	7.78	1	1.25	0.98	0.58	down
Cell organelle	Q02391	Golgi apparatus protein 1	129.63	6.86	22	0.98	1.05	1.06	up
Yolk resident	P02845	Low-quality protein: vitellogenin-2-like	204.68	9.1	7	1.54	0.88	0.77	down
	E1BXJ0	Protein-l-isoaspartate (d-aspartate) o-methyltransferase–like isoform x1	27.14	6.89	3	1.23	0.97	0.83	down
	F1NN82	Oxysterol-binding protein 3 isoform x3	97.33	6.86	1	1.11	1.01	0.91	down
	P27731	Transthyretin	16.3	5.24	1	1.14	0.88	0.74	down
	E1BS40	Angiopoietin-related protein 3	56.33	5.67	2	0.94	0.98	1.1	up
	F1NTK2	Alpha-2-macroglobulin–like protein 1	93.75	8.12	26	0.96	1.06	1.05	up
Blood	Q10751	Angiotensin-converting enzyme	137.73	5.64	1	0.97	1	1.15	up
	Q5G8Y9	Apolipoprotein d	21.69	5.8	5	0.85	1.07	1.08	up
	P19121	Serum albumin	69.87	5.74	3	1.41	0.92	0.76	down
	F1NGB1	Nucleobindin-2	53.8	5.15	6	1.13	1	0.99	down
	Q5ZK58	Acid ceramidase	44.62	8.12	8	0.94	0.97	1	up
	B8YJT7	Lysozyme	16.24	9.1	2	0.95	1.07	1.07	up
	F1NTQ2	beta-hexosaminidase subunit beta	63.37	6.49	8	0.96	1.05	1.06	up
	F1NWN6	PREDICTED: uncharacterized protein C11orf63 homolog isoform X1	84.23	7.39	1	0.84	1.06	1.12	up
	F1P0Z6	Protein unc-45 homolog a	100.55	6.65	1	0.55	3.09	0.74	up

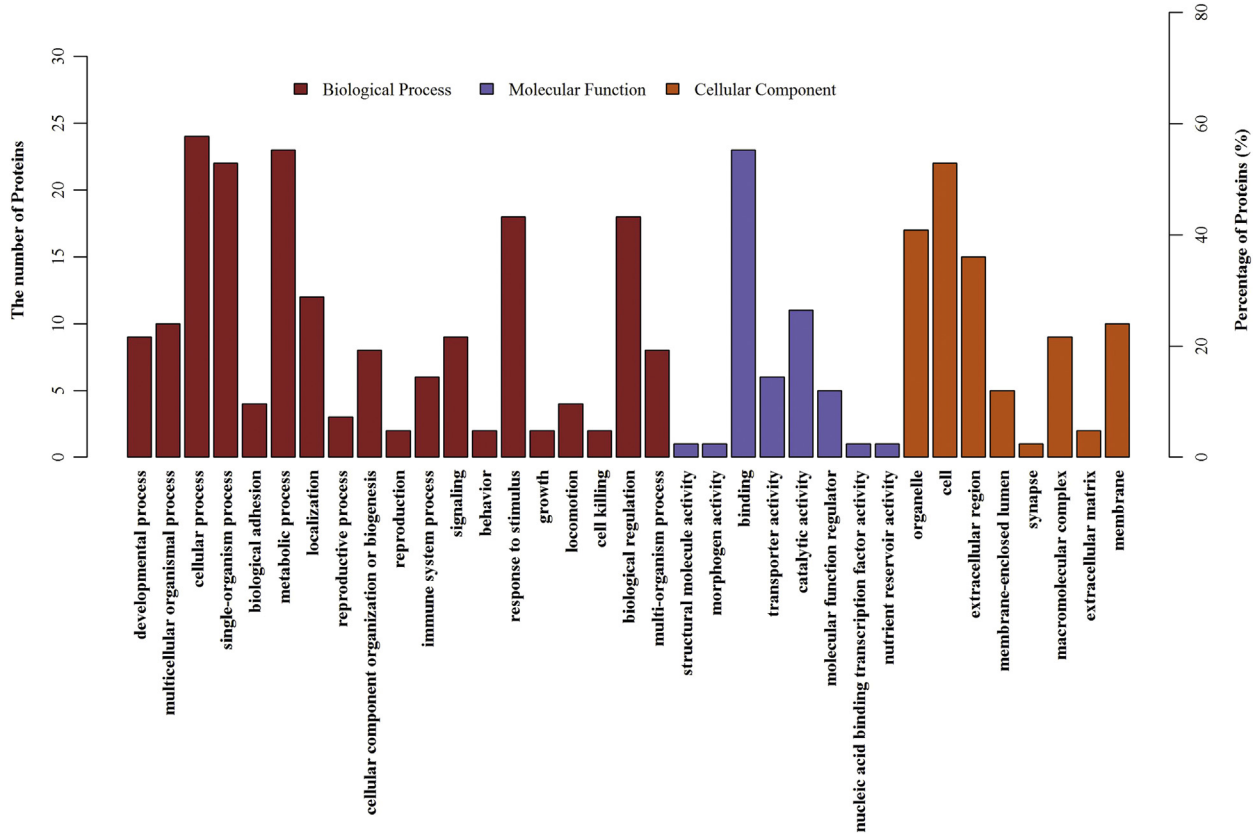
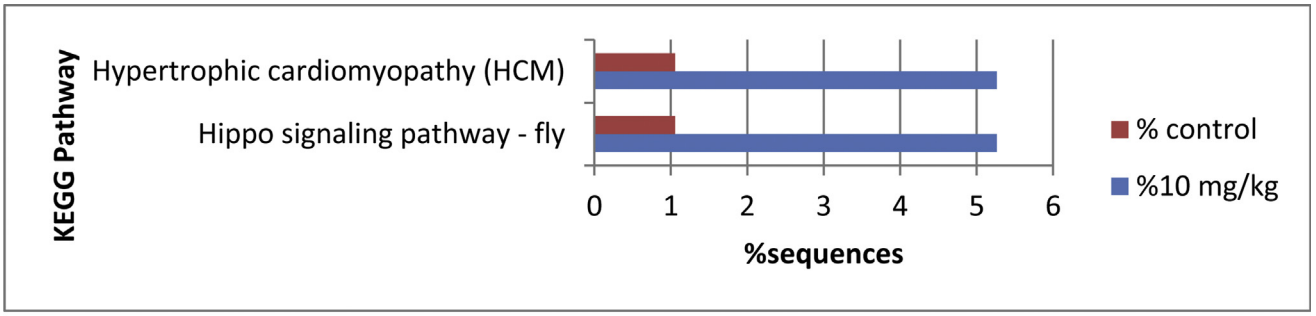


Figure 5. The classification of 36 different proteins in albumen affected by V according to gene ontology annotation. Biological process: the proteins that participated in cellular process (GO:0009987), single-organism process (GO:0044699), and metabolic process (GO:0008152) were differentially expressed. Molecular function: binding (GO:0005488) was ranked at the top of the category for the differential protein. Cellular component: differential proteins were located in the cell (GO:0005623), organelle (GO:0043226), and extracellular region (GO:0005576), respectively.

cell shedding and capillary-tube infiltration. By proteomics analysis, we found that the contents of some inherent proteins in the albumen were changed significantly. The expression of egg white lysozyme (Q6LEL2) which had defense response to bacteria was significantly decreased, but the ovomucin had no significant difference. The ovomucin was often thought to be the key protein for influencing the albumen HU (Omana and Wu, 2009), but the definite mechanism is not yet clear. At present, the cause of thick albumen liquefaction is the depolymerization of á and â subunits in ovomucin. When the â subunits (50-57% carbohydrate) reduced, the proportion of insoluble ovomucin with high carbohydrate will also reduced, which leads to thick albumen liquefaction (Offengenden, et al., 2011). In this trial, the ovomucin contents had no significant change; it is also suggested that the factor affecting thick albumen height is the ovomucin subunit type rather than the ovomucin quantity. He et al., (2017) found that the cottonseed meal in the diet led to the decline of albumen HU, and there was no significant difference in ovomucin content, but the precursor of ovomucin (GI_Accession No: 45382809) was significantly reduced. Egg white lysozyme is the key antimicrobial protein to ensure the quality and incubation of eggs. Lysozyme is always complexed with ovomucin to maintain the gel properties of thick albumen. When the network chain between lysozyme and ovomucin changes, the physical condition of ovomucin also changes, thus destroying the albumen's thick protein gel structure (Kato, et al., 2014). The significant reduction of lysozyme content in albumen may be one of the reasons for reduction of HU induced by V.

The F1NU63 expression was significantly increased in the V group. Through BLAST alignment, it was found to be similar to the ovostatin precursor protein. Ovostatin was a proteinase-binding protein, belonging to the á2-macroglobulin family, which á-macroglobulin could inhibit protease activity (Nagase and Harris, 1983). Too much protease, especially metalloproteinase, could degrade the proteins excessively. The protease inhibitors could increase the strength of gels, and it has been proven successful in Pacific herring surimi with weak gel-forming ability or slow setting properties (Reppond, et al., 2010). The content of protease inhibitors in albumen was increased by V, which inhibited some proteins, thus resulting in albumen degradation.

Damage, death, and falling of oviduct cells can bring cell membranes and intracellular proteins into the albumen. For example, the amino acid sequence of protein Q02391 was highly matched with the Golgi somatic protein 1 through BLAST alignment. Golgi somatic protein 1 belongs to the family of structural protein of organelles and regulates cellular responses to bind fibroblast growth factors (FGFs). V significantly increased the content of protein Q02391 in albumen.



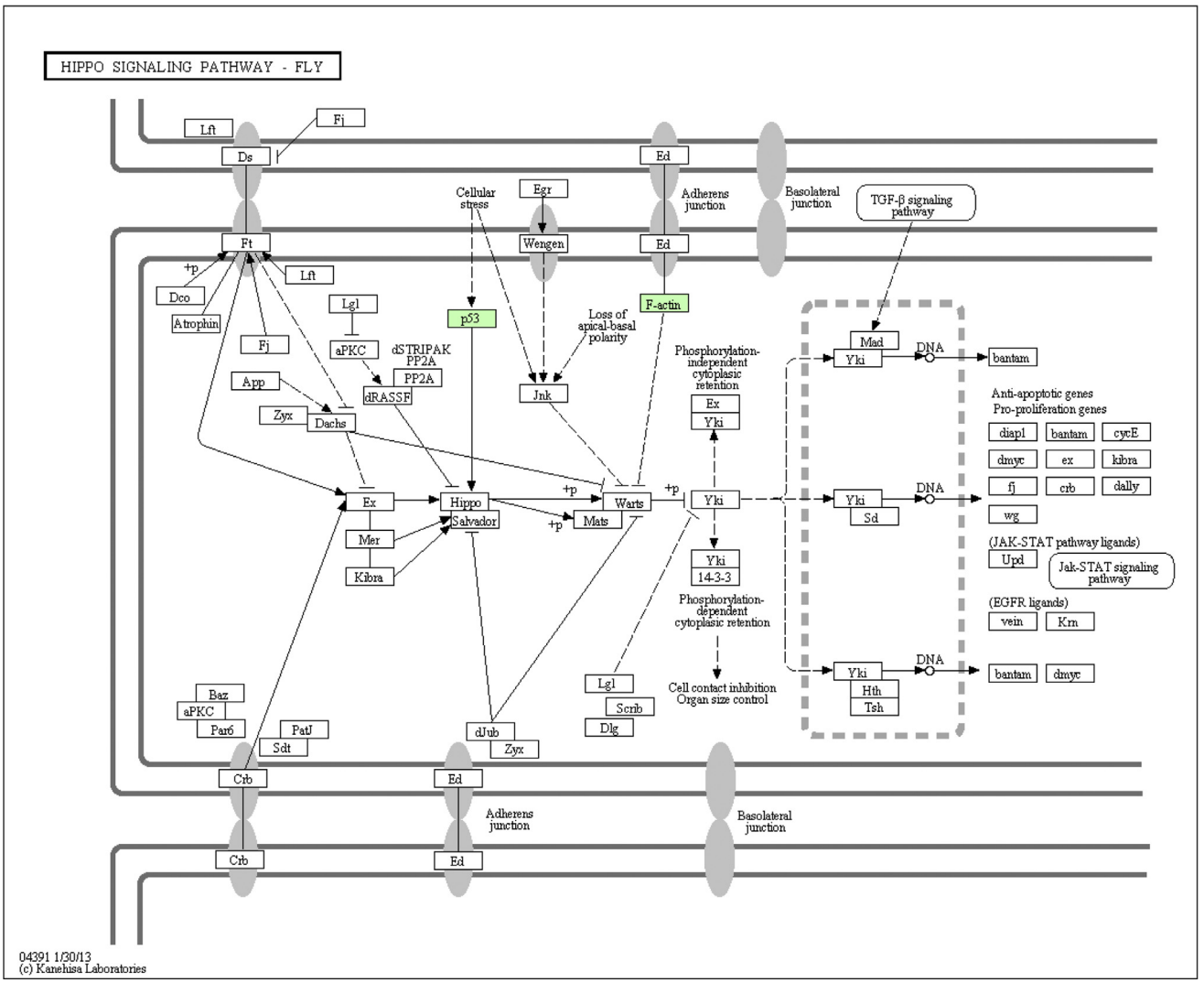


Figure 6. KEGG pathway of "0 mg V/kg vs. 10 mg V/kg V." The Hippo signaling pathway-fly as the most significantly affected network might be important for discriminating V. KEGG, Kyoto Encyclopedia of Genes and Genomes.

And the expression of proteins related to signal transduction on cell membranes, such as G-protein activation–related proteins (Q90854) and calmucin isoform X1 (E1C6M9), also increased significantly in the V treatment groups. The significant increase of these proteins suggested that dietary V may lead to abnormal cell death, apoptosis, denaturation, and falling off of the oviduct of laying hens. Some albumen proteins which were secreted by the other tissues such as the liver and brain were carried by blood to the oviduct, where it enters the albumen through the capillary walls of the oviduct. It is shown that the expression of some of those proteins in albumen of V groups increased and that of others decreased.

The hens' nutrition level, body hormone level, and the health state of the expanded oviduct affected the protein secretion function of oviduct epithelial cells (Gunawardana et al., 2008). Many research studies showed that the V resulted in the impairment of oxidative metabolism, suppression of respiratory chain enzymes, possession of potential chemical asphyxiate properties, and induction of oxidative stress in

mitochondria (Aureliano et al., 2005; Srivastava and Mehdi, 2010; Zhao and Yang, 2013). The studies on rats and humans had shown that V decreased the antioxidant enzyme (SOD and GSH-Px) activity and produced excess reactive oxygen species in the body and caused oxidative stress and induced apoptosis in the lungs, liver, kidney, and small intestinal epithelial cells (Huang et al., 2001; Hosseini et al., 2012; Sanchez-gonzalez et al., 2017). Yuan et al., (2016) also reported that the apoptosis rate of oviduct uterine cells in laying hens was increased by V. Reactive oxygen species generated from V could regulate the growth state of cells through peroxidase proliferator–activated receptor gamma (Zhao and Yang, 2013). However, V-induced cytotoxicity was directly related to not only H_2O_2 reaction but also the production of vanadium peroxide compounds (Capella et al., 2007). Vanadium caused oxidative stress, cell degeneration, and cell apoptosis in the oviduct ampulla of laying hens and affected the normal protein secretion function. Oxidative stress changed the permeability of cell membrane and capillary wall and caused changes in the proteins in albumen from oviduct cells and blood. For example, the proteins of the Golgi apparatus in albumen of dietary V treatment groups were upregulated. On the other hand, V induced hepatotoxicity, such as congestion, functional impairment, and hepatic cell apoptosis (Abdelhamid et al., 2010; Hosseini et al., 2012). Huang et al., (2018) reported that V induced hepatic cell autophagy via the activation of Liver kinase B1(**LKB1**)/adenosine 5′-monophosphate (**AMP**)-activated protein kinase (**AMPK**)-dependent signaling pathway. The proteins that were secreted by the liver and moved along with the blood to the oviduct were changed, for example, serum albumin content was decreased by dietary V.

In accordance with the result of the KEGG analysis on proteomics, we found that V may induce oviduct ampulla apoptosis of hens through the hippo signaling pathways, and P53 may be the key protein. V induces P53 transactivation through hydrogen peroxide and causes mouse cell apoptosis (Huang et al., 2000; Morita et al., 2010). The tumor suppressor gene, P53, can be used to regulate the cell apoptosis by Bax/Bcl-2, Fas/Apol, IGF-BP3, and other proteins (Ray et al., 2016; Kim et al., 2017). Bcl-2 has antiapoptotic effect which prevents the formation of apoptosis factors such as cytochrome C from the mitochondria. And Bax could interact with the voltage-dependent ion channels on the mitochondria and mediate the release of cytochrome C, which has the effect of promoting apoptosis (Sidi et al., 2008). In HaCaT cells, $VOSO_4$ has a dose-dependent effect on the inhibition of Bcl-2 mRNA expression and improvement of Bax mRNA expression (Markopoulou et al., 2009).

Overall, the dietary V reached the oviduct ampulla of laying hens through the circulation of blood and resulted in the oxidative damage of the oviduct ampulla and the increase of cell apoptosis, thus changing the contents of intrinsic proteins of albumen, oviduct ampulla, and blood-derived proteins in albumen.

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