Modulation of growth and immunity by dietary supplementation with resveratrol in young chickens receiving conventional vaccinations

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Objective—To determine the effects of resveratrol (RES) on growth and immune status in chickens receiving conventional vaccinations.

Animals—Two hundred forty 1-day-old layer chickens.

Procedures—Chickens received conventional vaccinations throughout the study and were randomly assigned to 1 of 4 treatments in 6 replicate pens/treatment. Treatments included 1 control group (basal diet) and 3 experimental groups fed the basal diet plus 200, 400, and 800 mg of RES/kg of diet. At 40 days of age, 1 bird/pen was randomly selected to have blood and tissues collected to determine serum immunity indices; mRNA relative expression of proinflammatory cytokines in splenocytes; mRNA relative expression of nuclear transcription factor-κB, growth hormone receptor, and insulin-like growth factor-1 in hepatocytes; cell proliferation; and apoptosis.

Results—Average daily gain, antibody titers against Newcastle disease virus and avian influenza viruses H5 and H9, and insulin-like growth factor-1 expression were quadratically increased with increasing RES concentration. In hepatocytes, growth hormone receptor gene mRNA relative expression was quadratically increased and nuclear transcription factor-κB gene mRNA relative expression was linearly decreased with increasing RES concentration. In splenocytes, interleukin-1β and tumor necrosis factor-α mRNA relative expression was linearly decreased with increasing RES concentration. Resveratrol supplementation delayed cell proliferation and reduced apoptosis in immunocytes. With increasing RES concentration, proliferation index and relative weight of the thymus, ratio of CD4+ to CD8+ cells, and CD4+ cell count were quadratically increased, and IgM concentration was linearly increased.

Conclusions and Clinical Relevance—Dietary resveratrol supplementation improved growth, protected immunocytes against antigen-induced apoptosis, and upregulated immune response in chickens that received conventional vaccinations. (*Am J Vet Res* 2014;75:752–759)

Regular vaccinations are indispensable for combating infectious disease in commercial intensive poultry production by stimulating the innate immune response. However, growth inhibition can be induced because of greater proinflammatory cytokines resulting from the vaccinations.[1–4] In addition, immunosuppressive effects of conventional vaccinations may be caused by apoptosis of blood leukocytes, decreased lymphocyte numbers in the secondary lymphoid organs, and immune cell apoptosis, which may result in unsuccessful vaccination.[5] Apoptosis is a process of fundamental importance for regulation of the immune response as well as a defense mechanism, such as in immune reactions or when cells are damaged by pathogens or noxious agents.[6–8] Persisting vaccine challenge can induce specific T-cell sequestration, dysfunction, and deletion at vaccination sites.[9] Accordingly, conventional vaccinations in commercial poultry production may decrease performance and immune response and increase disease suscepti-

Abbreviations

ADG	Average daily gain
FasL	Fas ligand
GAPDH	Glyceraldehyde phosphate dehydrogenase
GHR	Growth hormone receptor
H5	Avian influenza virus H5
H9	Avian influenza virus H9
IGF-1	Insulin-like growth factor
IL	Interleukin
NDV	Newcastle disease virus
NF-κB	Nuclear transcription factor-κB
RES	Resveratrol
RT-qPCR	Real-time quantitative PCR
TNF-α	Tumor necrosis factor-α

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bility and mortality rate.[10–12] This can cause economic loss. As a result, investigating new and safe feed additives to modulate growth performance and immunity, especially in young chickens, which have an immature immune system, may provide new ways for improving health in poultry production.

As a natural product, RES (3,4′,5-trihydroxystilbene) is a phytoalexin produced by plant species in response to environmental stress or pathogenic attack. In the immune system of various animals, RES inhibits cytokine production,[13] and the NF-κB inhibitory activity and anti-inflammatory activity of RES have been confirmed.[14,15] In addition, the immunomodulatory effect of RES includes suppression of lymphocyte stimulation as well as its effect on apoptosis of stimulated lymphocytes.[16] Activation-induced lymphocyte apoptosis was also reduced in the presence of RES. Resveratrol appears to protect activated human B lymphocytes from apoptosis by upregulating the antiapoptotic protein Bcl-2.[17] Low-dose RES enhances the cell-mediated immune response of mice.[18] Despite the apparent importance of RES effects on inflammatory response and immunomodulation, data concerning the relationships between RES and growth performance and immunologic function have not been reported in poultry. The objective of the study reported here was to examine the effects of RES supplementation on growth performance and immunity in chickens receiving conventional commercial vaccinations and to explore the potential mechanisms through which RES functions. Our hypothesis was that RES can improve growth by inhibiting cytokine production and upregulated immune response by protecting immunocytes against antigen-induced apoptosis in chickens that received conventional vaccinations.

Materials and Methods

Animal care and diets—Two hundred forty 1-day-old commercial crossbred (LuoDaoHong × White LaiHang) layer chickens with an initial body weight of 35.6 ± 0.6 g (mean ± SD) were used in this study. The experimental procedures and the animal use and care protocols were approved by the Committee on Ethical Use of Animals of the Department of Science and Technology of Henan Province. Chickens were wing banded, weighed, and randomly assigned to 1 of 4 dietary treatments. Chickens were placed in 6 replicate pens for each treatment with 10 birds/pen in wire-floored battery brooders in an environmentally controlled room with electrical heating. The room temperature was maintained at 30° to 35°C from days 0 to 3, then gradually reduced 2° to 3°C/wk until it reached 22°C. Chickens were exposed to a 23-hour light and 1-hour dark photoperiod. All experimental treatments used the same corn-soybean meal basal diet (**Appendix 1**) fed in mash form.

Experimental design—The 4 treatments included a control group fed the basal diet and 3 experimental groups fed the basal diet plus 200, 400, or 800 mg of RES/kg of diet (in powder form), respectively (high-performance liquid chromatography testing confirmed that 50% of the supplement was RES and 50% was inert carrier). The RES supplemental concentrations were chosen according to results of a preliminary experiment and previous studies.[19,20] The RES was naturally derived from giant knotweed and was provided by a biotechnology company.[a] The RES was gradually mixed into the basal diet to guarantee uniform mixture. Diets were quantitatively fed to each chicken. Water and feed were provided ad libitum during the 40-day experimental period. At the end of 20 and 40 days, birds and residual feed were weighed. Weight gain and feed conversion for each phase were calculated.

Vaccination procedure—A conventional vaccination procedure was applied in this study, as follows: day 0, IM injection of Marek's disease virus vaccine[b] (in hatchery); day 7, intranasal and ocular administration of NDV (LaSota 4)[c–e] and infectious bronchitis virus (H120)[c–e] vaccines and SC administration of NDV oil emulsion vaccine[c–e]; day 14, oral administration of infectious bursal disease virus vaccine[c–e]; day 21, oral administration of infectious bursal disease virus vaccine; day 28, SC administration of avian influenza oil emulsion vaccine[c–e]; day 35, oral administration of NDV (LaSota 4) and infectious bronchitis virus (H120) vaccines.

Sample collection and preparation—At 40 days of age, 1 bird/pen was randomly selected, and 5 mL of blood was drawn from the heart (2 mL of blood without anticoagulant used for serum analysis; 3 mL blood with anticoagulant used for enumeration of CD4+ and CD8+ cells). Serum was prepared by centrifugation of clotted blood samples at 4,520 × *g* for 20 minutes. After bleeding, chickens were euthanized by administration of ether, and liver, spleen, bursa of Fabricius, and thymus were aseptically obtained. A portion of the thymus was preserved in neutral-buffered 10% formalin for immunohistochemical evaluation, and the remainder of the organs were immediately frozen in liquid nitrogen and stored at –80°C for assays of mRNA expression of genes and apoptosis.

Serum assays—Serum concentrations of IL-1β, IL-6, TNF-α, growth hormone, and IGF-1 were analyzed with commercially available chicken ELISA kits,[c–g] according to the manufacturer's instructions. Serum concentrations of antibodies against NDV, H5, and H9 were measured with a hemagglutination-inhibition assay[21] at the Institute of Poultry Disease, Henan Agricultural University, China.

mRNA relative expression of proinflammatory cytokines NF-κB, GHR, and IGF-1—The mRNA relative expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in splenocytes and NF-κB, GHR, and IGF-1 in hepatocytes was determined by means of RT-qPCR assay. Total RNA was isolated from spleen and liver tissue with a reagent according to the manufacturer's instructions.[h] Integrity of the RNA was verified electrophoretically by ethidium bromide staining and quantified. Purity of the RNA was determined with UV-clear microplates at an optical density of 260 nm. The optical density 260 to optical density 280 ratios of all samples were from 1.8 to 2.0.

Total RNA was reverse transcribed. Briefly, 2 μL of RNA isolated from each cell sample was added to a 40-μL reaction mix containing 1.0 μL of oligo-dT18,[i]

1.0 µL of dNTPs,[j] 1.0 µL of RNasin inhibitor,[i] 2.0 µL of M-MLV transcriptase,[i] 8.0 µL of M-MLV reverse transcriptase reaction buffer,[i] and 25 µL of RNase-free water. Primers were designed (span exon-exon boundaries) on the basis of sequences of IL-1β (GenBank accession No. DQ393267), IL-6 (GenBank accession No. NM-204628), TNF-α (GenBank accession No. AY765397), NF-κB (GenBank accession No. D13721), GHR (GenBank accession No. NC-006127), IGF-1 (GenBank accession No. NM-001004384), and GAPDH (GenBank accession No. NM-204305) of chickens. Primers were used for PCR amplification of cDNA and quantification by use of RT-qPCR assay. The primer sequences used were tabulated (**Appendix 2**).

The mRNA expression levels of the target genes (IL-1 β, IL-6, TNF-α, GHR, IGF-1, and NF-κB) and the housekeeping gene GAPDH were determined with RT-qPCR assay. The assay was performed by means of an RT-qPCR kit.[k] The reaction system consisted of a 25-µL final volume including 2 µL of cDNA, 12.5 µL of DNA polymerase,[k] 0.5 µL of a reference dye,[k] 1 µL of 20pM primer mix of each gene, and 9 µL of double-distilled water. The reaction conditions for the RT-qPCR machine,[l] were 1 cycle of enzyme inactivation for 3 minutes at 95°C followed by 40 cycles of amplification, including initial denaturation at 95°C for 15 seconds, annealing at 60°C for 40 seconds, and elongation at 95°C for 15 seconds.

A melting curve with 1 peak was obtained to determine the possibility of nonspecific amplification or primer-dimer formation. On the basis of the cycle threshold (CT), the relative content of mRNA was calculated and normalized as the mRNA relative expression of IL-1β, IL-6, TNF-α, GHR, IGF-1, and NF-κB. The relative expression of the target gene was calculated by the 2–ΔΔCT method[22]:

$$\Delta\Delta CT = (CT_{target} - CT_{housekeeping})\text{ at time x} - (CT_{target} - CT_{housekeeping})\text{ at time 0}$$

Relative expression of target gene = 2–ΔΔCT. Where the housekeeping gene was GAPDH.

Cell proliferation and apoptosis—The spleen, bursa of Fabricius, and thymus were thawed and placed in ice-cold Hank balanced salt solution containing 140mM NaCl, 5mM KCl, 2.5mM $CaCl_2$, 1.1mM $MgCl_2$, 5.6mM glucose, and 10mM HEPES, at a pH of 7.4. The tissues were cut into small pieces and homogenized with a tissue grinder. The cell suspensions were washed in Hank balanced salt solution.

The cell cycle, cell proliferation index, and apoptosis ratio were detected with a propidium iodide staining assay by use of flow cytometry.[m] The specific method has been discussed by Ormerod.[23] Samples were analyzed by collection of 10,000 events; forward light scatter (forward scatter), right angle light scatter (side scatter), and 2-color fluorescence were analyzed. The light scatter and the fluorescence signals were set in a logarithmic gain and were stored in list-mode data files. The obtained data were further analyzed with a software program.[n] Cell cycle was detected. Flow cytometry was used to detect the P-glycoprotein on the surface of the cells, the intracellular concentration of daunomycin, and the immune type of the cells. Proliferation index, which indicates cell proliferation activity, was determined:

$$\text{Proliferation index} = (S + G2/M)/(G0/G1 + S + G2/M) \times 100\%$$

where G0 is the rest phase in cell division. G1 is the gap that precedes DNA synthesis. S is the DNA synthesis phase, G2 is the gap between DNA synthesis and mitosis, and Mis the mitotic phase.

Apoptosis in the thymus—Expressions of Bcl-2, Fas, FasL, and caspase-3 apoptosis were determined with a streptavidin-peroxidase immunostaining kit according to the manufacturer's instructions. Analysis of all samples was conducted by calculating the ratio of labeled cells to total cells in 10 fields of view by light microscopy at 400× magnification. To qualitatively assess the number of labeled cells, slides were subjectively scored as ++++ when a high number of cells were labeled and ++ when a moderate number of cells were labeled.

CD4+ and CD8+ cells—Absolute counts of CD4+ cells, CD8+ cells, and their ratios in peripheral blood mononuclear cell samples were determined by flow

Table 1—Growth performance variables (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Variable	**0**	**200**	**400**	**800**	**SEM**	**Linear**	**Quadratic**
1–20 days*							
ADFI (g)	15.55	15.06	15.51	14.97	0.44	0.32	0.87
ADG (g)	6.59	7.21	7.40	7.23	0.25	0.04	0.02
F:G	2.30	2.05	2.07	2.05	0.13	0.12	0.16
21–40 days*							
ADFI (g)	29.42	29.52	30.62	31.12	1.47	0.20	0.91
ADG (g)	12.38	12.24	12.45	13.26	0.36	0.01	0.17
F:G	2.80	2.81	2.87	2.67	0.07	0.07	0.06
1–40 days*							
ADFI (g)	22.48	22.29	23.05	23.07	0.79	0.34	0.90
ADG (g)	9.49	9.73	10.25	9.92	0.25	0.10	0.05
F:G	2.36	2.29	2.26	2.32	0.09	0.69	0.23

*Feeding period (age of chickens).
ADFI = Average daily feed intake. F:G = Feed-to-gain ratio.

cytometry.[m] Isolation of the mononuclear cells was conducted according to the method of Dalgaard et al.[24] Monoclonal antibodies used were specific for chicken cell surface markers.[o]

IgA, IgM, and IgG in serum—Serum concentrations of immunoglobulins (IgA, IgM, and IgG) were analyzed by use of commercially available chicken ELISA quantitation kits according to the manufacturer's instructions.[p]

Statistical analysis—Data were analyzed with a statistical analysis package[q] and expressed as mean ± SEM. Analysis evaluated the effect of RES on each group independently by use of ANOVA with linear and quadratic contrasts. For all comparisons, a value of $P < 0.05$ was considered significant.

Results

Growth performance—The effects of the different treatments were determined (**Table 1**). The ADG increased quadratically ($P < 0.05$) with increasing RES during days 1 to 20 and 1 to 40 of the trial. The group fed 400 mg of RES/kg had the highest ADG.

Serum assays—Anti-NDV, anti-H5, and anti-H9 antibody titers of chickens in the 4 groups were determined (**Table 2**). At 40 days of age, these antibody titers were increased quadratically with increasing RES ($P < 0.05$). The group fed 400 mg of RES/kg had the highest antibody titers.

The concentration of IGF-1 in serum was increased quadratically ($P < 0.05$) with increasing RES (**Table 3**). The highest IGF-1 concentration was observed in the group fed 400 mg of RES/kg.

mRNA relative expression of proinflammatory cytokines NF-κB, GHR, and IGF-1—Gene mRNA relative expression levels associated with the different treatments were determined (**Table 4**). In hepatocytes, GHR mRNA relative expression was increased quadratically and NF-κB mRNA relative expression was decreased linearly with increasing RES ($P < 0.05$), and the group fed 400 mg of RES/kg had the highest GHR mRNA expression. In splenocytes, IL-1β and TNF-α mRNA relative expression was decreased linearly with increasing RES ($P < 0.05$) .

Cell proliferation and apoptosis—The effects of the different treatments on cell cycle, cell proliferation index, and apoptosis in splenocytes, bursa of Fabricius, and thymocytes were determined (**Tables 5–7**). A large proportion of cells were in the G0 or G1 phases of the cell cycle (**Figure 1**). The splenocyte proliferation-to-apoptosis ratio was decreased linearly ($P < 0.05$) with

Table 2—Antiviral antibody titers (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Virus	0	200	400	800	SEM	Linear	Quadratic
NDV	5.00	6.83	7.50	5.50	0.56	0.79	0.01
H5	1.50	3.50	3.50	2.83	0.43	0.78	0.02
H9	1.50	2.83	3.00	2.00	0.49	0.73	0.01

Table 3—Hormone and cytokine variables (mean values [nmol/L]) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Variable	0	200	400	800	SEM	Linear	Quadratic
GH	0.93	1.01	0.99	0.97	0.06	0.76	0.28
IGF-1	17.35	17.81	18.67	18.56	0.42	0.16	0.02
IL-1β	29.98	17.93	17.10	18.61	5.69	0.11	0.77
IL-6	25.31	24.27	22.87	23.67	1.16	0.16	0.14
TNF-α	50.28	47.65	43.05	43.42	2.47	0.08	0.13

GH = Growth hormone.

Table 4—Gene mRNA expression levels (mean values) in hepatocytes (GHR, IGF-1, and NF-κB) and splenocytes (IL-1β, IL-6, and TNF-α) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Gene	0	200	400	800	SEM	Linear	Quadratic
Hepatocytes							
GHR	1.00	1.21	1.71	1.34	0.08	0.06	0.00
IGF-1	1.00	1.37	1.59	1.51	0.21	0.08	0.14
NF-κB	1.00	0.95	0.93	0.92	0.15	0.02	0.06
Splenocytes							
IL-1β	1.00	0.88	0.64	0.63	0.15	0.01	0.20
IL-6	1.00	0.92	0.86	0.88	0.10	0.07	0.17
TNF-α	1.00	0.85	0.78	0.75	0.14	0.01	0.07

Table 5—Splenocyte proliferation variables, splenocyte apoptosis, and spleen weight-to-body weight ratios (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Variable	**0**	**200**	**400**	**800**	**SEM**	**Linear**	**Quadratic**
G0:G1 (%)	79.75	76.70	76.12	77.61	2.08	0.38	0.10
G2:M (%)	17.40	20.83	21.24	19.95	1.58	0.55	0.09
S (%)	2.85	2.47	2.64	2.44	0.52	0.08	0.15
Proliferation index	20.32	23.36	23.93	22.35	1.08	0.48	0.06
Apoptosis ratio (%)	0.28	0.26	0.22	0.22	0.02	0.04	0.82
Spleen weight-to-body weight ratio	1.88	1.80	2.14	2.23	0.20	0.06	0.23

G0 = Rest phase in cell division. G1 = Gap that precedes DNA synthesis. S = DNA synthesis phase. G2 = Gap between DNA synthesis and mitosis. M = Mitotic phase.

Table 6—Bursal cell proliferation variables, bursal cell apoptosis, and bursa of Fabricius weight-to-body weight ratios (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg)					*P* value	
Variable	**0**	**200**	**400**	**800**	**SEM**	**Linear**	**Quadratic**
G0:G1 (%)	74.81	73.60	70.43	72.65	3.18	0.23	0.08
G2:M (%)	9.89	5.70	8.07	5.70	1.15	0.12	0.18
S (%)	15.30	20.70	21.50	21.65	1.86	0.03	0.20
Proliferation index	25.17	26.42	29.59	26.42	2.89	0.10	0.08
Apoptosis ratio (%)	0.88	0.86	0.75	0.76	0.06	0.04	0.28
Bursa of Fabricius weight-to-body weight ratio	1.30	1.35	1.32	1.40	0.04	0.16	0.35

See Table 5 for key.

Table 7—Thymocyte proliferation variables, thymocyte apoptosis, and thymus weight-to-body weight ratios (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg)					*P* value	
Variable	**0**	**200**	**400**	**800**	**SEM**	**Linear**	**Quadratic**
G0:G1 (%)	76.52	73.70	67.40	67.80	1.58	0.04	0.15
G2:M (%)	9.87	7.90	11.35	7.43	2.05	0.18	0.10
S (%)	13.61	18.40	21.25	24.77	1.88	0.02	0.08
Proliferation index	23.78	27.34	34.60	32.20	1.30	0.12	0.04
Apoptosis ratio (%)	4.59	4.32	3.95	3.72	0.60	0.06	0.20
Thymus weight-to-body weight ratio	3.23	3.58	4.16	3.89	0.34	0.28	0.02

See Table 5 for key.

increasing RES. In the bursa of Fabricius, the percentage of S-phase cells was linearly increased ($P < 0.05$) and the apoptosis ratio was linearly decreased ($P < 0.05$) with increasing RES. The percentage of cells in G0 or G1 was linearly decreased ($P < 0.05$) and the percentage of S-phase cells was linearly increased ($P < 0.05$) with increasing RES. In the thymocytes, the proliferation index and relative weight of thymus were quadratically increased ($P < 0.05$) with increasing RES.

Apoptosis in the thymus—Results of apoptosis-related protein expression in the thymus were determined (**Table 8**). The expression of FasL and caspase-3 was decreased linearly ($P < 0.05$) with increasing RES. Bcl-2 and Fas apoptins did not differ significantly.

Immune cells—The CD4+ to CD8+ ratio and the concentration of CD4+ T cell were increased quadratically ($P < 0.05$). The concentration of IgM was in-

creased linearly ($P < 0.05$) with increasing RES concentration (**Table 9**).

Discussion

Chickens reared in commercial-intensive facilities are usually conventionally vaccinated to protect them against a variety of diseases. Although vaccinations against viruses are typically effective, management measures also may be undertaken to help chickens cope with the subsequent vaccination-induced stress.[25] Persistent antigenic stimuli in the rearing environment have negative effects on the growth of livestock because they cause production of potent proinflammatory cytokines (IL-1β, IL-6, and TNF-α).[1] This restricts anabolic growth factors and thus suppresses growth to ensure that adequate energy and nutrients are available for high-priority immunologic and homeostatic pathways. A quiescent immune system is desirable to obtain maximum growth performance. In the present study, RES supplementation may have improved ADG. This indicated that RES supplementation may be an effective way to improve growth in chickens that receive conventional vaccinations.

The mechanism by which RES may improve growth may be associated with reduced NF-κB activation and inflammatory response. Nuclear transcription factors-play a pivotal role in inflammation, and inhibitor of κB kinase mediates NF-κB activation in response to proinflammatory cytokines and microbial products.[26,27] Resveratrol can inhibit the NF-κB pathway, resulting in suppression of transcription of proinflammatory cytokines (IL-1β and TNF-α). In addition, GHR and IGF-1 mRNA expression levels are improved in a state of low inflammatory response. Thus, dietary RES may reduce the growth-suppressive effects induced by the inflammatory response resulting from conventional vaccinations. Wong et al[2] found that proinflammatory cytokines may affect child growth through systemic mechanisms that reduce concentrations of IGF-1 and cause an abnormal GH response.[2] In the present study, RES supplementation improved GHR and IGF-1 expression.

Conventional vaccinations can induce chronic inflammation, immunocyte apoptosis, and immune dysfunction[2,3] resulting in the suppression of the immune response and unsuccessful vaccination. Various studies have found that induced

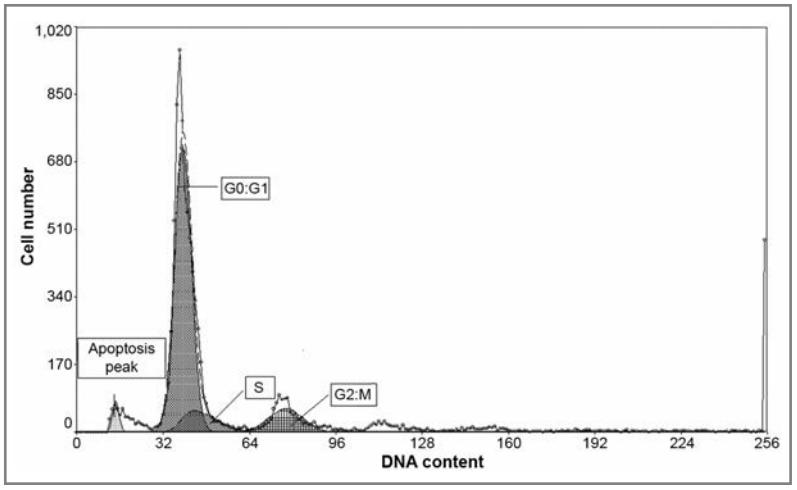

Figure 1—Results of flow cytometric analysis of cell cycle, cell proliferation phase distribution, and apoptosis peak of thymocytes from 40-day-old chickens that were fed a basal diet not supplemented with RES and received conventional vaccinations. G0 = Rest phase in cell division. G1 = Gap that precedes DNA synthesis. S = DNA synthesis phase. G2 = Gap between DNA synthesis and mitosis. M = Mitotic phase.

Table 8—Apoptosis-related protein expression (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Variable	0	200	400	800	SEM	Linear	Quadratic
Bcl-2	6.17	5.67	6.17	6.33	0.27	0.34	0.12
Fas	3.50	2.67	1.83	2.50	0.28	0.20	0.06
FasL	7.17	6.00	5.33	5.17	0.41	0.03	0.10
Caspase-3	3.50	3.33	2.80	2.50	0.39	0.04	0.32

Bcl-2 = B-cell lymphoma-2.

Table 9—Immunity indices (mean values) associated with supplemental feeding of RES in chickens that received conventional vaccinations.

	RES concentration (mg/kg of feed)					*P* value	
Variable	0	200	400	800	SEM	Linear	Quadratic
CD4 (%)	17.68	18.26	20.53	19.18	1.03	0.11	0.04
CD8 (%)	28.29	26.15	23.46	25.37	3.15	0.13	0.06
CD4/CD8	0.62	0.70	0.83	0.72	0.08	0.22	0.03
IgA (g/L)	0.40	0.38	0.42	0.45	0.06	0.10	0.12
IgG (g/L)	0.65	0.68	0.66	0.70	0.12	0.22	0.16
IgM (g/L)	0.24	0.28	0.32	0.35	0.05	0.04	0.08

immunosuppression is at least in part attributable to a reduction in the number of immunocytes.[28–30] Reductions in these cells may reflect changes in the dynamics of immunocyte migration and recirculation or absolute decrease in total cell numbers because of cell apoptosis. Apoptosis is a tightly regulated program of cell death involved in various physiologic and pathological processes, such as the maintenance of immune homeostasis. The induction of apoptosis by various factors may play an important role in pathogenesis because apoptosis of immunocytes might favor bacterial invasion.[31–34] Enhanced immunocyte apoptosis can cause immunodeficiency through cell loss. Resveratrol protects against activation-induced lymphocyte apoptosis in vitro.[16] The present study revealed that RES may delay cell proliferation of splenocytes, bursa of Fabricius cells, and thymocytes and reduce apoptosis in chickens exposed to conventional vaccination conditions. The mechanism of the antiapoptotic activity may be that RES affects protein expression of apoptotic regulators (FasL and caspase-3) that results in initiation of the immunocyte apoptosis program. Resveratrol supplementation may decrease the relative expression of FasL and caspase-3 in chickens that receive conventional commercial vaccinations.

The Fas-FasL system plays an integral role in maintaining cellular homeostasis of the immune system. The stress of chronic restraint induces lymphocyte reduction through endogenous opioid-mediated Fas expression, which in turn induces apoptosis.[35,36] Results of the present study revealed that RES may have improved NDV, H5, and H9 antibody titers; the ratio of CD4+ to CD8+; and IgM concentration. This indicated that RES supplementation may be an effective way to increase immune function in chickens that receive conventional commercial vaccinations. Further studies are needed to evaluate the effects of RES and determine suitable concentrations of RES for feed supplementation.

a. Zhengzhou LiNuo Biotech Co Ltd, Zhengzhou, China.
b. Qilu Animal Health Products Co Ltd, Jinan, China.
c. IL-1β kit, R&D Systems Inc, Minneapolis, Minn.
d. IL-6 kit, R&D Systems Inc, Minneapolis, Minn.
e. TNF-α kit, R&D Systems Inc, Minneapolis, Minn.
f. GH kit, JiangLai Biological Science and Technology Ltd Co, Shanghai, China.
g. IGF-1 kit, JiangLai Biological Science and Technology Ltd Co, Shanghai, China.
h. TRIzol, Promega Biotech Co Ltd, Beijing, China.
i. Promega Biotech Co Ltd, Beijing, China.
j. Sigma-Aldrich Biotech Co Ltd, Beijing, China.
k. Takara Biotech Co Ltd, Dalian, China.
l. 7300 Real-Time PCR System, Applied Biosystems Inc, Foster City, Calif.
m. EPICS XL-MCL Flow Cytometer, Beckman Coulter, Brea, Calif.
n. WinMDI, version 2.8, Joe Trotter, Scripps Research Institute, La Jolla, Calif.
o. BD Co, Franklin Lakes, NJ.
p. ZhongShanJinQiao Co Ltd, Beijing, China.
q. SPSS Statistics, version 17.0, SPSS Inc, Beijing, China.

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Appendix 1

Ingredient composition and nutritional content of a basal diet fed to chickens.

Variable	Value
Ingredients	
Corn (%)	65.50
Soybean meal (%)	30.00
Soybean oil (%)	1.20
Dicalcium phosphate (%)	1.60
Limestone (%)	1.20
NaCl (%)	0.30
L-lysine (%)	0.20
Vitamin and mineral premix (%)	1.00
Nutrient content	
Metabolizable energy (Kcal/kg)	3,150
Crude protein (%)	20.30
Calcium (%)	0.90
Total phosphorus (%)	0.60
Lysine (%)	1.15
Methionine + cystine (%)	0.85

Vitamin-mineral premix provided the following per kilogram of complete diet: vitamin A, 8,000 U; vitamin D3, 1,750 U; vitamin E, 15 U; vitamin B12, 10 μg; riboflavin, 5 mg; D-pantothenic acid, 10 mg; niacin, 20 mg; choline chloride, 400 mg; manganese, 70 mg; zinc, 70 mg; iron, 85 mg; copper, 8 mg; iodine, 0.5 mg; and selenium, 0.3 mg.

Appendix 2

Primers used for quantification of GAPDH, IL-1β, IL-6, TNF-α, NF-κB, GH, and IGF-1 mRNA gene expression in a study of dietary reservatrol supplementation in chickens.

Gene	Primer sequence	PCR product length
GAPDH	F: 5′ATGGCATCCAAGGAGTGA3′ R: 5′GGGAGACAGAAGGGAACAG3′	141
IL-1β	F: 5′ACAGTCCTTCGACATCTTCGAC3′ R: 5′GAGCTTGTAGCCCTTGATGC3′	202
IL-6	F: 5′TGAAGTGGTCATCCCAGACTC3′ R: 5′CCTCACGGTCTTCTCCATAAAC3′	213
TNF-α	F: 5′TGAGGCATTTGGAAGCAG3′ R: 5′TTGTGGGACAGGGTAGGG 3′	198
NF-κB	F: 5′AGTGTGTGAAGAAACGGGAACT3′ R: 5′ATAGATGGGCTGGGAGAGGAC3′	194
GHR	F: 5′GCGTGTTCAGGAGCAAAGCT 3′ R: 5′TGGGACAGGCATTTCCATACTT3′	132
IGF-1	F: 5′TGTACTGTGCTCCAATAAAGC3′ R: 5′CTGTTTCCTGTGTTCCCTCTACTTG3′	113

F = Forward. R = Reverse.