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Dietary *RRR*-α-tocopherol succinate attenuates lipopolysaccharide-induced inflammatory cytokines secretion in broiler chicks

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The anti-inflammatory effects of two esters of α-tocopherol (α-TOH), *all-rac*-α-TOH acetate (DL-α-TOA) and *RRR*-α-TOH succinate (D-α-TOS), on broilers repeatedly challenged with lipopolysaccharide (LPS) were investigated. Three hundred and twenty 1-d-old broiler chicks were allotted into four treatment groups and fed on a control diet (30 mg/kg DL-α-TOA) or diets containing 10, 30, 50 mg/kg D-α-TOS. Half of the birds from each treatment group were challenged with 0·9 % NaCl solution or LPS (250 μg/kg body weight) at 16, 18 and 20 d of age. The results indicated that the pretreatment of birds with 50 mg/kg D-α-TOS markedly reduced serum PGE_2 secretion and increased the concentrations of serum or hepatic α-TOH. When LPS-challenged birds were pretreated with 30 or 50 mg/kg D-α-TOS, the increases of plasma and splenic concentrations of interferon-γ, IL-1β, IL-2, IL-6, IL-4 and IL-10 were dramatically attenuated. Also, a significant decrease of hepatic reactive oxygen species (ROS) and hepatic or splenic phosphokinase C (PKC) activities was found in birds pretreated with 30 or 50 mg/kg D-α-TOS. Furthermore, D-α-TOS inhibited the activation of NF-κB by preventing the degradation of inhibitory-κBα. In conclusion, D-α-TOS is able to prevent LPS-induced inflammation response *in vivo*. The beneficial effect may depend on suppressing the secretion of various plasma and splenic inflammatory mediators through inhibiting NF-κB activation and by blocking ROS signalling, in which PKC may play an assistant role.

***RRR*-α-tocopherol succinate: Cytokines: Inflammatory response: NF-κB**

Research on the biology of avian cytokines and their role in regulating immunity has lagged behind the pioneering work in mouse and human systems. Chicken cytokines exhibit relatively low amino acid sequence homology to mammalian cytokines due to the large phylogenetic distance between aves and mammals[1]. Cytokines released from activated macrophages and lymphocytes are primarily responsible for the metabolic effects characterising the immunological challenge[2,3].

Many of the chicken cytokine genes have been sequenced[4]. Although avian cytokines may exhibit unique functions that are not present in their mammalian counterparts, their properties are similar to mammals. In chickens, as in mice, it has become clear that cell-mediated and antibody-mediated immune responses are regulated by distinct subsets of $CD4^+$T lymphocytes, classified as Th1 and Th2[5]. In predominant Th1 responses, enhancement of several cytotoxic mechanisms and inflammatory reactions is expected because of the secretion of interferon-γ (IFN-γ), IL-1β, IL-2 and IL-6, among others, whereas Th2 type produces cytokines such as IL-4 and IL-10, which provide efficient help to B lymphocytes to trigger strong antibody responses[6,7]. Several sequenced chicken cytokines, including IL-2, stimulate the proliferation of activated NK cells, B lymphocytes, cytotoxic effector T lymphocytes and antibody production[1]. IL-2 can generate manifold cytokines, including IFN-γ, IL-4, IL-6 and so on[8]. Among other cytokines that have been sequenced in the chicken are IL-1β, IL-6 and IFN-γ, which possess biological activities similar to that of mammals[8]. Chicken IL-1β functions like its mammalian counterpart in mediating the inflammatory response and increasing antibody production[9]. IFN-γ also participates in the immune response mediated by T, B and other immune cells, and is generated mainly by $CD4^+$ and $CD8^+$T cells. It exhibits pleiotropic effects on leucocytes and has an antiviral effect and can regulate the immune response[10,11]. Although many cytokines have been identified and are known to have important functions, it is also clear that overproduction of these molecules could have potentially deleterious effects[12].

Lipopolysaccharide (LPS) is a key structural component of the outer membrane of Gram-negative bacteria, and the effects of LPS induced *in vivo* are initiated by their interaction with host cells, in particular macrophages, and their subsequent release of pro-inflammatory cytokines and inflammatory mediators[13,14]. Moreover, the observed LPS effects on broiler chicks are mediated through toll-like receptors-4[15] with activation of transcription NF-κB, which is a common and major transcription factor largely involved in the

Abbreviations: D-α-TOS, *RRR*-α-tocopherol succinate; DL-α-TOA, *all-rac*-α-tocopherol acetate; I-κBα, inhibitory-κBα; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MDA, malonaldehyde; PKC, protein kinase C; ROS, reactive oxygen species; VE, vitamin E; α-TOH, α-tocopherol.

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expression of pro-inflammatory genes[16]. Dampening NF-κB activation, thereby limiting the inflammatory response, has been suggested as a strategy to prevent chronic inflammatory diseases[17].

Interest in using nutritional factors to modulate the immune response through their effects on cytokine release in chickens has increased in the last decade[18]. Among the factors that have been found to modulate immune functions, vitamin E (VE) and its different forms have been considered. α-Tocopherol (α-TOH) is the most bioavailable form of VE. This natural antioxidant is lipid-soluble, and due to this property, it exerts preferentially its antioxidant activity in lipid-rich membranes, which concerns immune cells. In terms of immunomodulatory properties, α-TOH was shown to exert anti-inflammatory actions, including the modulation of T cell differentiation and proliferation and PGE_2 production by macrophages and the reduction of pro-inflammatory cytokine synthesis from activated macrophages and monocytes[19,20].

In chicks, many parameters of the immune system, including resistance to infection, host antiviral activity, cytokine IFN-γ, specific antibody production, number of antibody-producing cells, *in vitro* mitogenic responses of lymphocytes and the phagocytic ability of macrophages, are altered by supplementing diets[21–25]. In many cases, protective immunity in chickens is increased by VE supplementation as evidenced by its ameliorating effect against LPS, *Escherichia coli*, *Eimeria tenella*[26], toxin[22] and heat stress[26,27]. Also, VE has been recognised to interfere with inflammation either by scavenging reactive oxygen species (ROS), modulating the synthesis of inflammatory lipid mediators, or by influencing signal transduction pathways including NF-κB and protein kinase C (PKC)[28–30], in antioxidant-independent manners in various animal models[18,31].

It is common to include VE in poultry feeds in the form of *all-rac*-α-TOH acetate (DL-α-TOA)[32]. However, the main difficulty with VE treatment is the acute administration since the marked lipophilicity hampers tissue distribution and therefore the cellular bioavailability. Tocopherol esters may have advantages over processing, storage and absorption because of their greater stability and water solubility compared with tocopherol alone[33]. They usually are rapidly converted to the natural forms by intestinal or epidermal esterases, and thus can be considered to be pro-vitamins, ultimately performing the same functions in the body as the natural α-TOH[34].

RRR-α-TOH succinate (D-α-TOS) is a new hydrophilic, natural analogue of VE with special properties. Early work indeed demonstrated that, as a *RRR*-α-tocopherol analogue, D-α-TOS not only possesses the physiologically active functions of D-α-TOH, but also has distinct medical value through immunomodulation and cytoprotection with its hydrophilic property[34,35]. Interestingly, it was also found that D-α-TOS, but not α-TOH and DL-α-TOA, appears to be far more effective in many aspects, for example, in protecting isolated hepatocytes against many different oxidative challenges[36]. It has been found to have several unique biochemical properties *in vitro* and *in vivo,* whereas α-TOH, α-TOA and α-TOH nicotinate were ineffective[37]. Among these esterified forms, α-TOS has specifically been shown to exhibit superior rates of absorption, especially in the liver, compared with the non-esterified forms[38].

Use of D-α-TOS in humans has been recommended because of its unique anti-tumour property in the family of VE[39]. Little is known about dietary α-TOS-mediated inflammatory inhibition *in vivo*. Further understanding of the anti-inflammatory mechanisms of α-TOS *in vivo* may provide novel nutritional therapies for inflammation accompanied by various immunological challenges in poultry production. So, in the present study, we explored the effects of supplementary conventional VE derivatives synthetic (*all-rac-*) α-TOA and natural (*RRR-*) α-TOS on LPS-induced inflammatory response with specific reference to the mechanisms including ROS, NF-κB and PKC in broiler chicks.

Materials and methods

Animals and management

A total of 384 one-day-old commercial Arbor Acres broiler chicks in cages were randomly assigned to eight dietary treatments based on the initial body weight ($P>0·05$). Six replicate cages containing eight chicks each were assigned to each treatment. All the birds were placed in wire cages in a three-level battery on their respective diets and housed in an environmentally controlled room maintained at 34–36°C during the first 14 d and then gradually decreased to 26 ± 1°C by 21 d of age, after which it was maintained at room temperature (15–28°C) and kept constant until the end of the experiment. The light regimen was a 12 h light–dark cycle (6.00–18.00 hours light). Birds were allowed to consume both feed and water *ad libitum*. Fresh diets were prepared once a week and were stored in sealed bags at 4°C. All the procedures were approved by the Institutional Animal Care and Use Committee of the Nanjing Agricultural University (Nanjing, People's Republic of China).

Experimental protocols

A 2 × 4 factorial design was used for injection with LPS (250 μg/kg body weight) or saline (sterile 9 g/l (w/v)) as one factor; the other factor was VE treatment (30 mg/kg DL-α-TOA (the control group), or 10, 30 and 50 mg/kg D-α-TOS (groups TOS1, TOS2 and TOS3, respectively)). The LPS (*E. coli* serotype O55.B5; Sigma Chemical, St Louis, MO, USA) was dissolved in sterile 9 g/l (w/v) NaCl solution at 0·5 mg/ml, so that injection of 0·5 ml/kg body weight of solution would achieve the desired dosage. D-α-TOS used in the experiment was provided by Spring Fruit Biological Products Company Limited (Taixing, Jiangsu, People's Republic of China), with the purity of 99·9 %. DL-α-TOA was provided by Huamu Institute of Animal Science and Technology (Nanjing, People's Republic of China), with the purity of 50·0 %. At 16, 18 and 20 d of age, abdominal injections of LPS or an equivalent amount of sterile saline were given in the lower abdominal region to half of the birds in each dietary treatment. Each replicate (eight birds) was the experimental units for dietary treatment and challenge status (i.e. injected or uninjected). The basal diets were of the maize–soyabean type. The basal diets contained α-TOH (7·85 mg/kg), according to the actual determination of the dietary ingredients. The percentage of all other major ingredients remained the same across the treatments. The diets were

Table 1. Ingredients and nutrient composition of broiler diets on fed basis

Ingredients (g/kg)	1–20 d
Maize	591
Soyabean meal (43 %, crude protein)	306
Maize gluten meal	38
Lard	17
Limestone	13·1
Dicalcium phosphate	17·7
NaCl	4·2
L-Lys (99 %)	1·5
DL-Met (98 %)	1·5
Premix*	10
Calculation of nutrients (g/kg)	
Metabolism energy (MJ/kg)	12·27
Crude protein	212
Ca	10
Available P	4·3
Lys	10·8
Met	5
Met + Cys	8·2

* Premix provided per kg of diet: transretinyl acetate, 24 mg; cholecalciferol, 6 mg; menadione, 1·3 mg; thiamin, 2·2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyidoxine·HCl, 4 mg; biotin, 0·04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamine), 0·013 mg; Fe (from ferrous sulphate), 80 mg; Cu (from copper sulphate), 7·5 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 65 mg; iodine (from calcium iodate), 1·1 mg; Se (from sodium selenite), 0·3 mg; Bacitracin Zn, 30 mg.

formulated based on the National Research Council (1994) to meet the nutrient requirements of the broiler (Table 1).

Sample collection and procedures

At 20 d of age, blood samples were collected within 2 h post-injection and separated by centrifugation at 350 ***g*** for 15 min at 4°C. Serum samples were frozen at −20°C for further analysis. After collection of blood samples, all the birds were killed by exsanguination. After decapitation, liver and spleen samples were excised, frozen in liquid N_2 and stored at −80°C. Simultaneously, fresh liver tissue was prepared for the isolation of hepatocytes for the further assay of ROS.

Preparation of splenic and hepatic tissue

According to the method of Zhu *et al.*[40], splenic and hepatic tissues were weighed and homogenised using an Ultra-Turrax homogeniser (Tekmar Company, Cincinnati, OH, USA) with the whole protein extraction kit (KenGen, Nanjing, People's Republic of China) on ice and then centrifuged at 2000 ***g*** for 20 min at 4°C. The supernatant was collected and stored at −80°C until analysed. Its protein concentration was determined using the Bradford method.

α-Tocopherol and malonaldehyde concentrations assay

α-TOH was analysed as described[41], with modifications. Briefly, samples were saponified by mixing the whole liver homogenate (500 μl) or serum (100 μl) with a 2 % pyrogallol solution (5 ml) and heated for 2 min in a 70°C shaking water-bath. The tubes were removed and 0·25 ml of 11 M-KOH was added. The tubes were heated again in a shaking 70°C water-bath for 30 min, and then placed in an ice bath. Then 2 ml of hexane (used to extract the VE) and 0·5 ml of water were added to the saponified samples and shaken vigorously for 2 min. A quantity of 1 ml of the hexane layer was transferred to a 4 ml glass test-tube for analysis. Standards of 1, 2, 4, 6, 8 and 10 μg/ml of α-TOH were prepared simultaneously. A 0·2 % bathophenanthroline solution (200 μl) was added to all the samples and standards and thoroughly mixed. Then 200 μl of 1 mM-$FeCl_3$ were added and samples were vortexed. After 1 min, 200 μl of an H_3PO_4 solution were added and vortexed again. The tubes were read on a spectrophotometer at 534 nm. The standard curve was used to calculate the concentration of α-TOH in each sample. The concentrations of α-TOH were expressed as μg/ml serum or per mg of protein.

The concentrations of plasma and hepatic malonaldehyde were determined by measuring the thiobarbituric acid-reacting substances, using a corresponding diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, People's Republic of China) according to the instructions of the manufacturer. MDA values were expressed as μg/ml serum and μg/mg protein.

Intracellular reactive oxygen species assay

The isolation of fresh hepatocytes was carried out in accordance with the method developed by Kim & Takemura[42] and modified by Zhou *et al.*[43]. Briefly, the fresh liver was carefully excised and transferred onto a glass petri dish, and rinsed twice with PBS (136·9 mM-NaCl; 5·4 mM-KCl; 0·81 mM-$MgSO_4$; 0·44 mM-KH_2PO_4; 0·33 mM-Na_2HPO_4; 5·0 mM-$NaHCO_3$, pH 7·6) without Ca^{2+}. The liver was dissected into small pieces and was digested with PBS containing 0·1 % collagenase (Sigma) at room temperature for 20 min on a shaker. The softened liver tissue was agitated and filtered through 70 μm nylon mesh. The resulting cell suspension was transferred to a sterilised centrifuge tube and then centrifuged three times at 90 ***g*** for 3 min in the buffer containing 1·5 mM-$CaCl_2$ at 10°C. After the last wash, the cell pellet was re-suspended in Dulbecco's modified Eagle's medium/F12 medium (Sigma). Cells were counted using a haemocytometer (Reichert, Buffalo, NY, USA), and those with more than 90 % viability by the trypan blue exclusion method were used for the following experiment.

Accumulation of intracellular ROS was assayed using 2′,7′-dichlorofluorescein diacetate, which is de-esterified to 2′,7′-dichlorodihydrofluorescein by cellular esterases, and then 2′,7′-dichlorodihydrofluorescein is oxidised by ROS to form fluorescent 2′,7′-dichlorofluorescein[44]. An increase in fluorescence intensity is used to quantify the generation of net intracellular ROS. Briefly, 500 μl hepatocytes suspension (1×10^7 cells/ml) was incubated in PBS buffer with 10 μmol/l 2′,7′-dichlorofluorescein diacetate in the dark for 1 h at 25°C and the fluorescence intensity was determined using a fluorescence microplate reader (Infinite M200; Tecan, Mannedorf, Switzerland) with excitation at 488 nm and emission at 525 nm[45]. All the samples were assayed in triplicate.

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Cytokines and PGE_2 assay

The plasma and splenic concentrations of IFN-γ[46], IL-2[47], IL-4[46], IL-1β and IL-10 were assayed by quantitative sandwich ELISA techniques using commercially available cytokine ELISA kits. Briefly, the desired number of specific antibody-coated wells (monoclonal antibody against chicken IL-2, IFN-γ, IL-4: Adlitteram Diagnostic Laboratories, San Diego, CA, USA; monoclonal antibody against chicken IL-1β and IL-10: BlueGene, Shanghai, People's Republic of China) was secured in the holder. Standards and specimens were dispensed into appropriate wells. The biotin conjugate reagent was added to the specimens' wells, and the enzyme conjugate reagent was added to each well, gently mixed for 15 s, and then incubated at 36 ± 2°C for 60 min. The incubation mixture was removed, and the microtitre wells were rinsed five times with distilled water. Residual water droplets were removed by touching the wells sharply onto an absorbent paper. Then, 3,3′,5-tetramethylbenzidine substrate solution was added to the wells, gently mixed for 5 s and incubated at 36 ± 2°C for 15 min. The reaction was stopped by adding stop solution and gently mixed for 30 s. The optical density at 450 nm was read within 30 min using an automated microtitre plate reader (Bio-Rad, Hercules, CA, USA). The average absorbance values (A450) for each set of reference standards, control and samples were measured and a standard curve was constructed. The results were expressed according to the calibration curve obtained from serial dilutions of each standard. The procedures for the specific antibody production, purification and verification were used according to the established methods[48–50]. The detection limits were 1 pg/ml, 19·5 pg/ml, 0·08 ng/ml, 1 pg/ml and 3·9 ng/m for IFN-γ, IL-1β, IL-2, IL-4 and IL-10, respectively.

A human IL-6 RIA commercial kits[51] (Institute of Radiation of Science and Technology Development Center of the General Hospital of People's Liberation Army, Beijing, China) was used to determine chicken IL-6 concentrations in serum and spleen tissues according to the manufacturer's instructions. All samples and standards were assayed in triplicate. The inter-assay and intra-assay CV obtained for IL-6 concentrations were 8·2 and 5·3 %, respectively. The detection limits were 50 pg/ml.

PGE_2 was measured using a commercially available ^{125}I RIA kit (College of Medical Science of Suzhou University, Jiangsu, People's Republic of China). Minimum detectability of plasma PGE_2 was 6·25 ng/l with an intra-assay CV $<10\,\%$. There were cross-reactivities of 4·5, 2·4, and $<0{\cdot}1\,\%$ in the kit with PGE_1, PGE_3 and arachidonic acid or its metabolites, respectively.

Splenic and hepatic protein kinase C activity assay

The splenic and hepatic PKC activities were determined using chicken-specific ELISA kit (Adlitteram Diagnostic Laboratories), following the manufacturer's instructions based on the multiple antibody sandwich principle. The minimum detectable concentration of PKC in this assay was estimated to be 1·0 ng/ml. Quantisation of the ELISA results was detected by the absorbance at optical density 405 nm using a Microplate Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). Densitometry values were normalised with respect to total β-actin protein expression to normalise the sample variation.

Preparation of hepatic extracts and Western blotting

Hepatic p65 protein in the nuclear fraction and inhibitory-κBα (I-κBα) protein in the cytoplasm were detected by Western blotting. The total cellular protein and nuclear fractions were extracted according to the instructions of nuclear and cytoplasmic extraction reagents kit (KenGen, Nanjing, People's Republic of China). The protein concentrations were determined by Bradford protein-binding assay. A total of 15 mg cytoplasmic and nuclear extracts was loaded per lane and separated onto 10 % SDS-PAGE, electroblotted on electrochemiluminescence (ECL)-nitrocellulose membrane and analysed by Western blotting. The chicken-specific monoclonal antibody (anti-p65, anti-I-κBα and β-actin 0·1 mg/ml diluted in Tris-phosphate saline, 5 % non-fatty dry milk, 0·05 % Tween) was provided by Beijing Biosynthesis Biotechnology Company Limited (Beijing, People's Republic of China). The antibody was incubated for 4 h at room temperature. After being washed, the secondary antibody (horseradish peroxidase-coupled rat-to-rabbit IgG, 1:5000; KenGen, Nanjing, People's Republic of China) was added. Immunoreactive proteins were detected using ECL-Western blotting detection system according to the manufacturer's instructions. Membranes were exposed to hyperfilm-ECL autoradiography films for 2 min. β-Actin was used as the internal control.

Statistical analysis

Data were analysed using the General Linear Model procedure of statistical package for social sciences 16.0 (SPSS Inc., Chicago, IL, USA) as a 4 × 2 factorial arrangement with dietary treatment and challenge status as main effects. The significant differences among different treatments were evaluated by least significant difference *post hoc* multiple comparisons test. Significance level was set at 0·05.

Results

Effect of dietary RRR-*α-tocopherol succinate on lipopolysaccharide-induced growth inhibition and inflammation*

Before LPS challenge (0–15 d), there was no dietary effect on bird growth performance ($P>0{\cdot}05$) (Table 2). When injected with LPS, the body weight gain of LPS-injected birds was less than that of uninjected ones ($P=0{\cdot}001$). However, the growth depression of birds induced by LPS challenge in group TOS2 or TOS3 was not as great as that of injected birds in the control or TOS1 group. Furthermore, LPS-challenged birds demonstrated hyperthermia (0·95 ± 0·038°C higher than the normal at the time of 2 h post-injection of LPS) and sickness behaviours such as anorexia and lethargy.

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Table 2. Effect of dietary *RRR*-α-tocopherol succinate (α-TOS) on lipopolysaccharide (LPS)-induced growth inhibition in broilers*

Items	LPS (−)				LPS (+)				SEM	P†		
	Cont.	TOS1	TOS2	TOS3	Cont.	TOS1	TOS2	TOS3		Stress	Diet	Interaction
1–15 d												
BWG (kg)	273·7	272·5	282·5	285·2	275·7	274·0	281·3	284·5	1·497		0·064	
FI (g/bird per d)	1·746	1·748	1·711	1·701	1·745	1·727	1·679	1·671	0·010		0·108	
F/G	31·86	31·75	32·24	32·33	32·07	31·55	31·50	31·68	0·239		0·507	
15–21 d												
BWG (kg)	476·9[a]	471·7[a]	482·1[a]	486·5[a]	347·4[c]	345·0[c]	377·0[b]	388·3[b]	4·086	0·001	0·001	0·022
FI (g/bird per d)	1·181[a]	1·182[a]	1·162[a]	1·172[a]	1·262[c]	1·289[c]	1·225[b,c]	1·213[b]	0·900	0·001	0·041	0·314
F/G	93·88	92·90	93·34	94·99	73·04	74·06	76·77	78·40	0·109	0·001	0·279	0·655

Cont., control; BWG, body weight gain; FI, feed intake; F/G, ratio of feed intake and body weight gain.
[a,b,c] Least-square mean values (*n* 6) within a row with unlike superscript letters were significantly different ($P<0·05$).
* On day 20, six birds of each dietary treatment were injected with LPS or sterile saline.
† The *P*-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

Effect of dietary RRR-*α-tocopherol succinate on lipopolysaccharide-induced α-tocopherol, thiobarbituric acid-reacting substances contents in plasma and tissues*

In LPS-unchallenged groups, the plasma α-TOH concentrations were significantly enhanced 0·48- and 1·03-fold, respectively, in groups TOS2 ($P<0·05$) and TOS3 ($P<0·01$) compared with group TOS1, and group TOS3 showed an increase ($P<0·05$) of 62·60 and 62·90 % in comparison with the control and TOS2 groups, respectively (Table 3). The hepatic α-TOH concentrations for groups TOS2 and TOS3 were elevated 53·77 % ($P<0·01$) and 73·58 % ($P<0·01$), respectively, compared with group TOS1. Moreover, the hepatic MDA levels for group TOS3 were significantly decreased by 23·65 % ($P = 0·039$) compared with TOS1 group. Injection of LPS decreased ($P=0·004$) plasma and hepatic α-TOH ($P=0·009$) concentrations and increased ($P=0·001$) hepatic MDA levels. The birds fed 30 ($P<0·05$) or 50 mg/kg ($P<0·01$) α-TOS diet had significantly higher hepatic α-TOH in comparison with the birds fed 10 mg/kg α-TOS.

Effect of dietary RRR-*α-tocopherol succinate on lipopolysaccharide-induced cytokines in plasma and tissues*

Before LPS challenge, there was no dietary effect on the secretion of cytokines in plasma and tissues in broilers ($P>0·05$). As shown in Table 4, injection of LPS increased the plasma and splenic levels of cytokines ($P<0·01$). However, the elevation of cytokine secretion induced by LPS challenge in groups TOS2 and TOS3 ($P<0·05$ or $P<0·01$) was not as great as that of injected birds in TOS1 group or the control group (data not shown). It seemed that LPS-induced cytokines release was inhibited by α-TOS in a dose-dependent manner. Birds in group TOS3 after LPS administration had lower ($P<0·05$ or $P<0·01$) levels of plasma and splenic IFN-γ, IL-1β, IL-2, IL-6, IL-4 and IL-10 than that of the birds in TOS1 group or the control group. Furthermore, there was an LPS × α-TOS interaction for plasma IL-1β, IL-2, IL-4, IL-6, IL-10 and splenic IFN-γ, IL-2, IL-6, IL-10 ($P<0·05$ or $P<0·01$). A marked elevation ($P=0·001$) of PGE_2 levels was observed after LPS administration regardless of α-TOS supplementation, while pretreatment of birds with α-TOS dose-dependently inhibited the release of PGE_2 into plasma. Similar to the response in cytokines in plasma and tissues, there was an LPS × α-TOS interaction in plasma PGE_2 ($P<0·01$).

Effect of dietary RRR-*α-tocopherol succinate on lipopolysaccharide-induced protein kinase C activity and reactive oxygen species levels*

A marked elevation ($P=0·001$) of PKC activities in liver and spleen tissues was observed in birds receiving LPS, while the elevation was dose-dependently inhibited by pretreatment of birds with α-TOS (Table 5). Birds receiving a 50 mg/kg dose of α-TOS showed a significant reduction in splenic ($P<0·01$) and hepatic ($P<0·05$) PKC activities compared with the control, TOS1 or TOS2 groups. Treatment of TOS2 resulted in a decrease ($P<0·05$) in splenic PKC activities compared with the other two groups. Similar to the response in PKC activities, LPS challenge increased hepatic ROS

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Table 3. Effect of dietary *RRR*-α-tocopherol succinate (α-TOS) on lipopolysaccharide (LPS)-induced α-tocopherol (α-TOH) and malonaldehyde (MDA) contents in plasma and liver tissues in broilers*

	LPS (−)				LPS (+)					*P*†		
Items	Cont.	TOS1	TOS2	TOS3	Cont.	TOS1	TOS2	TOS3	SEM	Stress	Diet	Interaction
Plasma (μg/ml)												
α-TOH	$1.86^{b,c,d}$	$1.49^{c,d,e}$	2.20^{b}	3.03^{a}	$1.44^{d,e}$	1.18^{e}	$1.92^{b,c}$	2.77^{a}	0·105	0·004	0·001	0·951
MDA	$3.16^{a,b}$	$3.27^{a,b}$	$2.88^{a,b}$	2.74^{b}	$3.44^{a,b}$	3.64^{a}	$3.08^{a,b}$	$2.98^{a,b}$	0·179	0·134	0·086	0·988
Liver (μg/mg protien)												
α-TOH	3.14^{a}	2.12^{c}	3.26^{a}	3.68^{a}	$2.38^{b,c}$	1.70^{c}	$2.98^{a,b}$	3.22^{a}	0·176	0·009	0·001	0·808
MDA	$6.07^{b,c,d}$	$6.47^{b,c}$	$5.29^{c,d}$	4.94^{d}	$7.19^{a,b}$	7.87^{a}	$6.17^{b,c,d}$	$6.06^{b,c,d}$	0·309	0·001	0·001	0·948

Cont., control.
a,b,c,d Least-square mean values (*n* 6) within a row with unlike superscript letters were significantly different ($P<0.05$).
* On day 20, six birds of each dietary treatment were injected with LPS or sterile saline.
† The *P*-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

levels in birds, regardless of dietary treatment, but those fed the 30 or 50 mg/kg dose of α-TOS had lower ($P<0.05$ or $P<0.01$) ROS levels than the other groups.

Effect of dietary RRR*-α-tocopherol succinate on lipopolysaccharide-induced nuclear translocation of NF-κB and phosphorylations of inhibitory-κBα*

Since VE has been described as an inhibitor of NF-κB nuclear translocation by influencing IκB kinase activity *in vitro*[52], we therefore wondered whether the overall effect of α-TOS on cytokine secretion might be due to its influence on LPS-induced NF-κB nuclear translocation *in vivo*. As shown in Fig. 1(B), 50 mg/kg α-TOS pretreatment significantly inhibited the nuclear translocation of NF-κB in the birds that received LPS injection ($P=0.005$) in comparison to the birds fed 10 mg/kg α-TOS. It was further found that LPS treatment decreased ($P<0.05$) cytosolic I-κBα protein levels in the control group and 10 mg/kg α-TOS group, as shown in Fig. 1(D). However, 50 mg/kg dietary α-TOS resulted in a significant higher I-κBα protein levels in comparison with the control group ($P=0.041$) and 10 mg/kg α-TOS group ($P=0.032$).

Discussion

The commonly accepted conversion factor between D-α-TOS and DL-α-TOA is 1·21[53]. A previous *in vitro* study showed that D-α-TOS can enter into cells as intact ester and delay D-α-TOS hydrolysis until cellular uptake, thus improving the rate of delivery and even mitochondrial localisation, especially in hepatocytes[54,55]. In the present study, plasma and hepatic α-TOH levels were reduced significantly after LPS challenge. The reason may be that increased α-TOH consumption resulted in the depletion of the hepatic α-TOH accumulation. As a pro-VE form, the increase of plasma and liver α-TOH (2·35- and 1·89-fold, respectively) in 50 mg/kg D-α-TOS treatment was impressive in comparison with the 10 mg/kg D-α-TOS treatment. These findings suggest that 30 or 50 mg/kg D-α-TOS treatment provides an efficient delivery of α-TOH, which may be attributed to the hydrophilic property with an anionic charge that increases its water solubility and further its availability[34,35].

Bacterial LPS is often used to mimic bacterial infection and induce inflammation via the neuroendocrine–immune axis communication network[56], which is characterised by the secretion of pro-inflammatory cytokines and inflammatory

Table 4. Effect of dietary *RRR*-α-tocopherol succinate (α-TOS) on lipopolysaccharide (LPS)-induced cytokines in plasma and tissues in broilers*

	LPS (−)				LPS (+)					*P*†		
Items	Cont.	TOS1	TOS2	TOS3	Cont.	TOS1	TOS2	TOS3	SEM	Stress	Diet	Interaction
Plasma (ng/ml)												
IFN-γ (pg)	153.3^{c}	148.7^{c}	140.4^{c}	146.5^{c}	282.8^{a}	288.9^{a}	$268.2^{a,b}$	242.8^{b}	7·100	0·001	0·071	0·198
IL-1β	1.15^{c}	1.07^{c}	1.46^{c}	1.46^{c}	$2.56^{a,b}$	2.75^{a}	$2.39^{a,b}$	2.17^{b}	0·110	0·001	0·888	0·009
IL-2	2.42^{d}	2.45^{d}	2.44^{d}	2.48^{d}	5.03^{a}	5.15^{a}	4.72^{b}	4.28^{c}	0·065	0·001	0·001	0·001
IL-6 (pg)	34.08^{d}	34.28^{d}	33.87^{d}	34.46^{d}	145.81^{b}	169.99^{a}	138.91^{b}	105.61^{c}	2·622	0·001	0·001	0·001
IL-4	3.80^{c}	3.76^{c}	3.78^{c}	3.81^{c}	7.58^{a}	8.12^{a}	6.67^{b}	6.38^{b}	0·154	0·001	0·001	0·001
IL-10	8.36^{c}	8.26^{c}	8.32^{c}	8.38^{c}	17.79^{a}	17.39^{a}	16.97^{a}	14.53^{b}	3·917	0·001	0·026	0·018
PGE_2	255.2^{d}	256.5^{d}	254.4^{d}	254.5^{d}	528.1^{a}	534.1^{a}	499.5^{b}	454.8^{c}	9·296	0·001	0·001	0·001
Spleen (ng/mg protein)												
IFN-γ (pg)	256.0^{d}	283.2^{d}	$333.0^{c,d}$	304.6^{d}	$465.0^{a,b}$	524.0^{a}	$429.4^{a,b}$	$408.2^{b,c}$	7·100	0·001	0·110	0·002
IL-1β	0.49^{c}	0.52^{c}	0.50^{c}	0.49^{c}	1.56^{a}	1.70^{a}	$1.50^{a,b}$	1.26^{b}	0·070	0·001	0·141	0·225
IL-2	4.14^{d}	4.13^{d}	4.15^{d}	4.17^{d}	$9.12^{a,b}$	9.45^{a}	8.53^{b}	6.04^{c}	0·187	0·001	0·001	0·001
IL-6 (pg)	81.36^{c}	81.18^{c}	82.95^{c}	82.06^{c}	200.67^{b}	217.41^{a}	187.65^{b}	133.21^{c}	4·805	0·001	0·001	0·001
IL-4	33.18^{c}	32.26^{c}	32.18^{c}	33.88^{c}	60.30^{a}	62.93^{a}	$55.80^{a,b}$	51.69^{b}	1·716	0·001	0·188	0·085
IL-10	4.12^{d}	4.03^{d}	4.02^{d}	4.19^{d}	$19.59^{a,b}$	20.91^{a}	$18.17^{b,c}$	16.51^{c}	4·893	0·001	0·058	0·038

Cont., control; IFN, interferon.
a,b,c,d Least-square mean values (*n* 6) within a row with unlike superscript letters were significantly different ($P<0.05$).
* On day 20, six birds of each dietary treatment were injected with LPS or sterile saline.
† The *P*-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

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Table 5. Effect of dietary *RRR*-α-tocopherol succinate (α-TOS) on lipopolysaccharide (LPS)-induced protein kinase C (PKC) activity and reactive oxygen species (ROS) levels in broilers*

	LPS (−)				LPS (+)					*P* †		
Items	Cont.	TOS1	TOS2	TOS3	Cont.	TOS1	TOS2	TOS3	SEM	Stress	Diet	Interaction
Liver												
PKC	$0·67^{c}$	$0·68^{c}$	$0·68^{c}$	$0·68^{c}$	$1·64^{a}$	$1·67^{a}$	$1·50^{a}$	$1·06^{b}$	0·069	0·001	0·001	0·001
ROS (μg/ml)	$59·78^{d}$	$60·18^{d}$	$57·08^{d}$	$52·51^{d}$	$165·2^{a,b}$	$172·2^{a}$	$161·0^{b}$	$152·9^{c}$	2·789	0·001	0·001	0·177
Spleen												
PKC	$0·36^{d}$	$0·35^{d}$	$0·34^{d}$	$0·33^{d}$	$1·20^{a}$	$1·23^{a}$	$0·93^{b}$	$0·61^{c}$	0·044	0·001	0·001	0·001

Cont., control.
a,b,c,d Least-square mean values (*n* 6) within a row with unlike superscript letters were significantly different ($P<0·05$).
* On day 20, six birds of each dietary treatment were injected with LPS or sterile saline.
† The *P*-values represent the main effect of the diet, the main effect of LPS challenge and the interaction between the dietary treatments and LPS challenge.

mediators in chicks[57]. The increases in biologically active chicken IL-1β, IL-2, IL-6 and IFN-γ after LPS were reported previously, which are all NF-κB-responsive (LPS-sensitive) genes in chicks[58]. VE and its derivatives decrease the expression of pro-inflammatory cytokines as well as other mediators, such as PG, inducible cyclo-oxygenase and normalise cytokine expression in malnourished, aged and virus-infected chicks or mammals[1,58–60]. An *in vivo* study by Webel *et al.*[12] shows that pigs that received α-TOH before the LPS challenge had substantially lower ($P<0·05$) peak levels of IL-6 and cortisol than pigs that did not receive α-TOH. An *in vivo* study in broiler chicks showed that dietary VE decreased myelomonocytic growth factor (an avian IL-6 analogue) mRNA ($P=0·049$) expression in a dose-dependent manner after LPS challenge, which could explain the immunomodulatory effect of VE in inflammation[1]. In this experiment, the increased secretion of the pro-inflammatory cytokines IFN-γ, IL-1β, IL-2 and IL-6 after LPS administration was observed. The reason may be that VE can modulate the functions of macrophages, and hence normalise the secretion of pro-inflammatory cytokines.

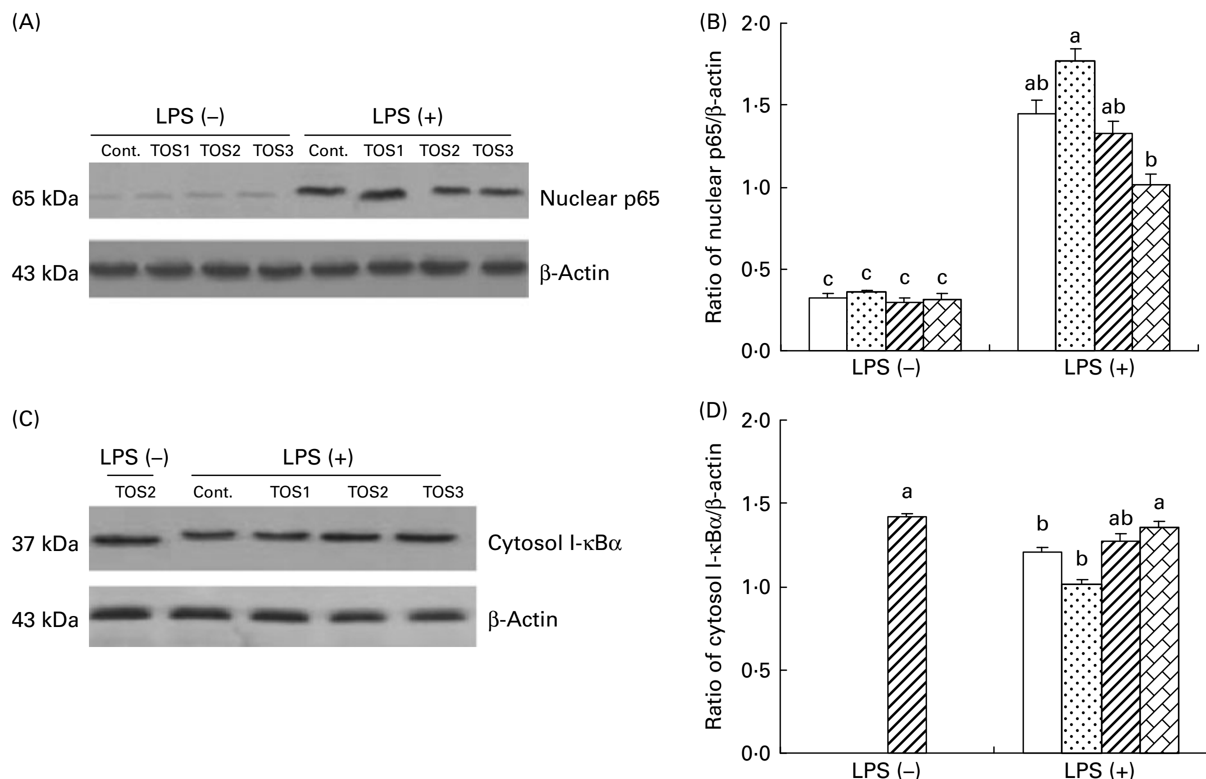

Fig. 1. Influence of dietary *RRR*-α-tocopherol succinate (α-TOS) on lipopolysaccharide (LPS)-induced hepatic nuclear translocation of NF-κB and phosphorylations of inhibitory-κBα (IκB-α). A 4 × 2 factorial arrangement was adopted. Birds received different forms of vitamin E (30 mg/kg *all-rac*-α-tocopherol acetate or 10, 30, 50 mg/kg D-α-TOS) pretreatment and immunological challenge (sterile 9 g/l (w/v) NaCl solution or 250 μg/kg LPS (body weight)) (*n* 6). (A) and (C) Nuclear NF-κB p65 and cytosol I-κBα were immunoblotted with chicken anti-NF-κB p65 and anti-I-κBα antibodies, respectively. (B) and (D) The bands intensities were quantified by the Biorad-Image for Windows Program (Biorad GS-800 Calibrated Densitometer). Experiments were repeated three times and similar results were obtained. Data are presented as means with their standard errors. For at least six independent animals/group, data were analysed by two-way ANOVA. (B and D) □, control (cont.); ⚀, α-TOS1; ▨, α-TOS2; ⊠, α-TOS3. a,b,c Mean values with unlike letters were significantly different ($P<0·01$).

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PGE_2, one of the most important eicosanoids, is an endogenous inhibitor of the immune response. PGE_2, which is regulated by the availability of its substrate – arachidonic acid – inhibits proliferation of T cells and production of IL-2 and IFN-γ from T cells[61] and is involves in early inflammatory events. A previous *in vitro* study also reported that inhibition of PGE_2 production by α-TOS dose-dependently in intact lung epithelial cells was primarily due to its inhibition of cyclo-oxygenase activity. The present study confirmed that dietary α-TOS could decrease PGE_2 synthesis in LPS-challenged birds. A potential immunoregulatory mechanism of VE is the direct modulation of arachidonic acid metabolism via cyclo-oxygenase and lipoxygenase pathways[62], which lead to the synthesis of PG and leucotrienes, respectively. In addition, the feedback regulation on the cytokines and PGE_2 secretion via the nerve–endocrine–immunological axis may be the indirect action mechanism of VE in chicks[63].

IL-10 is a cytokine with potent immunoregulatory activities and is produced primarily by monocytes and to a lesser extent by lymphocytes. IL-4 has an important role in the proliferation and activity of B and T cells, in stimulating the generation of immunoglobulins and inhibiting the transcriptional activity of NF-κB in response to LPS[64]. As anti-inflammatory cytokines, the enhanced secretion of both IL-4 and IL-10 are simultaneous after LPS challenge. The down-regulation of both may be part of a homeostatic mechanism of TOS in generating the appropriate balance between Th1 and Th2[65].

It is now widely accepted that ROS not only play a critical role in direct tissue injury but also contribute to increased inflammatory responses serving as intracellular signals. α-TOH is able to inhibit intracellular ROS and MDA accumulation, generated during LPS-induced inflammation[66,67]. In the present study, we found that treatment of birds with α-TOS (30 or 50 mg/kg) significantly decreased the LPS-stimulated ROS and MDA levels, which may contribute to the potent immunoregulatory effect of α-TOS by the modulation of the cellular free radical/antioxidant balance[25]. As a lipid-soluble vitamin, α-TOH is present in the outer membrane of cells and cell organelles. ROS and MDA oxidise membrane PUFA and cause cellular dysfunction. Thus, one postulate is that by quenching the lipid peroxyl radical formed by the oxidised PUFA, membranes can be protected from further oxidation. Another postulate is that ROS and MDA themselves serve as intracellular signals that facilitate cytokine expression[25]. Thus, by increasing oxidative defences, α-TOS may have reduced the availability of oxygen free radicals to further stimulate cytokine production pathways. The present finding that α-TOS reduces LPS-induced intracellular ROS and MDA in plasma and liver tissues supports both possibilities.

In chickens, as in mammals, PKC is one of the important intracellular signal transduction systems and a key enzyme implicated in the control of cellular proliferation, differentiation and pro-inflammatory cytokine secretion[68]. While previous *in vivo* research has shown that the specific regulatory interaction of VE with PKC is distinct from VE's antioxidant function[69], this has not been demonstrated in chickens yet. Also, α-TOS has been shown to modulate the protein phosphatase-2A/PKC pathway[70], which can be important for maintaining the inflammatory balance. In the present study, we found that the activity of LPS-induced PKC is inhibited dose-dependently by α-TOS (Table 5). The mechanism may be explained as that activation of protein phosphatase-2A by α-TOS causes dephosphorylation of PKC, at cellular level[71], through which the PKC-dependent ROS signalling pathways are inhibited.

The nuclear factor NF-κB is a central regulator of stress in all cell types[71] and NF-κB pathway is important for the expression of genes that are involved in the control of the host inflammatory response. As shown in the present study, markedly increased expression of nuclear p65 protein in all the groups and cytosol I-κBα degradation in the control and 10 mg/kg dietary α-TOS groups were detected after LPS challenge, indicating increased transcription activity of NF-κB during inflammation. Calfee-Mason *et al.*[72] reported in rats that an increased antioxidant environment (10, 50, 250 mg/kg α-TOA) can inhibit ciprofibrate-mediated NF-κB induction. On the basis of our observation that 50 mg/kg dietary α-TOS potently inhibited the expressions of nuclear NF-κB p65 subunits protein, we propose that 50 mg/kg α-TOS inhibits IκB phoshphorylation, which prevents the subsequent proteolytic degradation of IκB-α, and thereby blocks NF-κB nuclear translocation in macrophages. This would, at least in part, lead to decreased transcription of inflammatory cytokines. These data are consistent with the data observed in the elevated hepatic ROS levels. VE affects free radical-mediated signal transduction events and ultimately modulates the expression of genes that are regulated by free radical signalling.

In conclusion, results from the present study indicate for the first time that α-TOS, a natural antioxidant, exerts potent *in vivo* anti-inflammatory effects in chickens by inhibiting LPS-induced secretion of several major inflammatory mediators. These protective effects are exerted, at least in part, by reduction of NF-κB translocation, presumably through the modulation of the hepatic intracellular ROS level and MDA contents, and PKC may exert an assistant effect. Hence, the present study offers new perspectives for the treatment of inflammatory diseases in young broilers. Further studies are required to determine the upstream molecular mechanisms leading to inhibition of NF-κB activity by α-TOS under inflammatory response.

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