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Original Article

Suppresses of Astragalus Polysaccharide on E. coli-Induced Injured Intestinal Microvascular through TLR4-NF-κB Signal Pathways in Chickens

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■Keywords

Astragalus polysaccharide, *Escherichia coli*, Intercellular adhesion molecule -1, Nuclear factor - κB, Vascular cell adhesion molecule -1

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ABSTRACT

To investigate the hypothesis that APS can attenuate *E. coli*-induced microvascular dysfunction in chicken intestine, 60 0-day old male chickens were divided into three groups with 5 replications of 4 chicks. Chicken in the APS group were treated with 15 mg APS daily while the others were given 0 mg APS for 6 days. Then all 7-day old chicken were injected intraperitoneally by *E. coli,* except for the chicken in the control group. After 4 h, all chicken's intestinal samples were collected to detect gene expressions involved in inflammatory factors and adhesion molecules. Results showed that APS attenuated the signs of edema and hemorrhage in 7-day old chicken intestinal mucosa which were induced by *E. coli* injection. Consistently, APS significantly reduced the increasing mRNA levels of inflammatory factors such as Tumor necrosis factor-a (TNF-a), interleukin (IL) -1β and inducible nitric oxide synthase (iNOS) ($p<0.05$), the same results were observed in vascular adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Moreover, we observed that APS supplementation in water suppressed significantly ($p<0.05$) the decline of reparative factors such as epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF) in *E. coli* injected group. Furthermore, supplementation with APS substantially blocked ($p<0.05$) the increase in Toll-like receptor-4 (TLR4) and Nuclear factor (NF)-κB mRNA abundance ($p=0.087$) induced by *E. coli* infection. This study indicated that microvascular injured chicken intestine induced by *E. coli* would be attenuated with feeding APS, and the mechanism of repairing were probably mediated through TLR4-NF-κB signal pathways.

INTRODUCTION

Astragalus polysaccharide (APS) is extracted from *Astragalus membranaceus* and plays an extensive role in various biological activities, such as being anti-inflammatory, anti-oxidant and anti-bacterial (Guo *et al.*, 2004; Yan *et al.,* 2009; Wang *et al.,* 2013). *Escherichia coli* (*E. coli*) is a kind of opportunistic pathogen which widely resides in the intestine tract of healthy animals and provokes inflammatory response when stressful practices stimulate *E. coli* activity (Mehaisen *et al.*, 2016). Moreover, Lipopolysaccharide (LPS), released by *E. coli*, is the main component leading to the host's inflammatory response. Tumor necrosis factor-a (TNF-a), interleukin (IL) -1β, IL-6 and inducible nitric oxide synthase (iNOS) are important inflammatory cytokines and mediate inflammation response in diseases or injuries (Kalaiyarasu *et al.,* 2016; Zhang *et al.,* 2015). Previous reports showed that APS supplementation in diet inhibited LPS-induced, increase in TNF-α, IL-1β and IL-6 mRNA expression in 35-day old broiler's jejunal mucosa (Liu *et al.,* 2015) and the mRNA levels of iNOS, TNF-α and IL-1β in

microglial cells (Luo *et al.,* 2015). However, it remains unknown whether APS suppresses the expression of inflammation factors provoked by *E. coli* in 7-day old broiler's intestinal tissue.

Vascular endothelial cells, the main component of blood vessel, release low level of adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) to protect endothelial cells. The increase in E-selectin expression on the membrane of endothelial cells causes mononuclear cells adhesion to the vascular endothelium (Jubeli *et al.,* 2012), then over-expression of ICAM-1 and VCAM-1 stimulate immune cells and monocytes recruitment and migration into the intimal area of the vascular wall, firm adhesion and transmigration of the cells into the tissue, and causes local thrombosis and vascular cells dysfunction (Conatans & Conri, 2006; Videm & Albrigtsen, 2008). It was shown that *E. coli* injection significantly increased ICAM-1 and VCAM-1 mRNA abundance in the kidneys (Thorgersen *et al.,* 2013) and in 28-day old chicken ileum intestinal tissue (Zhong *et al.,* 2014). Astragaloside IV, the main component of APS, significantly suppressed LPS-induced expression levels of E-selectin and VCAM-1 in human vascular endothelial cells (Zhang *et al.,* 2003; Hao *et al.,* 2004). Reparative factors, such as epithelial growth factor (EGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) play important roles in the formation of new blood vessels and on the reconstruction of damaged blood vessels due to inflammation. Therefore, it would be interesting to evaluate whether APS regulates the expressions of adhesion factors and reparative factors in broilers intestinal mucosa with *E. coli* injected.

Toll-like receptor-4 (TLR4) is an important recognition receptor of LPS (Robert & Brend, 2011) and regulates the expressions of inflammatory factors by activating downstream Nuclear factor (NF)-κB (Li *et al.,* 2016; Lidia *et al.,* 2016). Nevertheless, whether TLR4-NF-κB signal pathway is involved in the effect of APS on the expression of inflammatory factors and adhesion factors in chicken intestinal tissue with *E. coli*-treated is still unclear.

E. coli is commonly found in the chicken's intestinal microbiota. Various stressful practices including climatic and hygienic stress activate *E. coli*, which in turn induces inflammatory bowel disease and releases inflammatory cytokines (Binion & Rafiee, 2009; Star *et al.,* 2008). Inflammatory cytokines could provoke distinct reactions and dysfunction of endothelial cells (Wang *et al.,* 2014). Injury of chicken intestinal microvascular is harmful to animal health. Therefore, the aim of this study was to investigate whether APS ameliorates inflammatory processes and vascular dysfunction in 7-day old chicken intestinal tissue with *E. coli* injected, and whether TLR4-NF-κB signal pathway is involved in the protective effect of APS.

MATERIAL AND METHODS

Escherichia coli

Escherichia coli 01 C84010A were obtained from the Poultry Diseases Research institute of Henan Agricultural University (Zhengzhou, China). Bacteria strain was incubated in nutrient broth at 37°C for 18 h with agitation at 200 rpm/min for recovery. Then bacteria strain was harvested by centrifugation at 5000×g for 10 min and then re-suspended in phosphate-buffered saline (PBS, pH 7.0). The number of bacterium was determined by plate counting. The *E. coli* titer was more than 1×10^9 colony forming unit (cfu)/mL.

Birds and Experimental Procedures

Fertilized Gushi broiler eggs (52.66 ± 0.41 g) were obtained from the breeding chicken farm of Henan Agricultural University (Zhengzhou, China) and incubated in an electric incubator with an automatic rotator at 37.5 ± 0.5 °C and 60 % relative humidity (Wansheng Company, Nanjing, China). On hatching day, 60 male chickens were selected and randomly divided into three groups (n = 20 per group) with 5 replications of 4 chicks in a box. The birds were reared in separated boxes with 22: 2-hour light: dark cycle and allowed to access feed corn-soybean-based basal diets and water ad libitium. The basal diets were formulated to meet the nutrient requirements as recommended by the National Research Council (1994). Chicken in the control group (Con.) and the *E. coli* group (*E. coli*) were given 1.5 mL sterile saline daily before receiving standard water and feed ration. The chickens in the APS group (APS) received 1.5 mL APS daily (10 mg/mL, obtained from CENTRE Biology Co. Ltd) before receiving standard water and feed ration. On day 7, chickens were injected with 1 mL of PBS in the abdomen in the control group, and in the *E. coli* group and the APS group, chickens were injected with 1 mL of PBS which contained *E. coli* about 1×109 cfu/mL, respectively. Two birds were randomly selected from each box and a total of 10 birds from every group were euthanized with cervical dislocation under deep Nembutal anesthesia (45 μg/g of body weight, intraperitoneal injection; Shanghai chemical factory, Shanghai China) after 4 h of the *E. coli* injection.

Approximately 2 cm of ileum intestinal tissue were collected and snap-frozen in liquid nitrogen, and then stored at - 80 °C until RNA extraction.

All experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Ethics Committee of Henan University of Technology.

Total RNA Extraction and RT-PCR

Total RNA extraction was performed according to the manufacture's instruction of TRIzol total RNA kit (Invitrogen Biotechnology Co, Ltd, USA). Briefly, frozen ileum intestinal tissue samples were ground in liquid nitrogen, and a portion of about 100 mg was used for RNA extraction. Then, the total RNA was treated with DNase I (D2215, Takara) to eliminate possible contamination of genomic DNA. The total RNA concentration was determined by nanodrop 2000 (Thermo scientific, USA) and their integrity were verified through 1.4% agarose-form- aldehyde gel electrophoresis.

Total RNA (2 μg) were reverse transcribed into cDNA according to the manufacture's instruction. 2 μL of diluted cDNA (1:20) were used for Quantitative Real-Time PCR(RT-qPCR) analysis. Primers were synthesized by Invitrogen Biotechnology Co Ltd (Shanghai, China). Primer sequences for chicken GAPDH, TLR4, NF-κB, TNF-α, IL-1β, IL-6, E-selectin ICAM-1, VCAM-1, iNOS, FGF2, EGF and VEGF are shown in Table 1, and chicken GAPDH was selected as a reference gene. RT-qPCR was performed in Mx3000P system (Stratagene, La Jolla, CA, USA) in a final volume of 10μL containing 2 μL cDNA, 8 pmol of primers, and 5 μL of SYBR premix (Toyobo Ltd, Osaka, Japan). Each RT-qPCR was performed in duplicate. The RT-qPCR data were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmettgen, 2001). The abundance of mRNA was presented as the fold change relative to the average level of the control group for 4 h after *E. coli* challenge.

Statistical Analysis

Values of mRNA abundance are expressed as the fold change relative to that of the control. Data were calculated as means ± SEM. All statistical analysis were performed with one-way ANOVA using the general linear model procedure of the SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA). Results were considered significance at $p<0.05$.

Table 1 – Primer sequences of the target genes.

Target genes	GenBank accession	Primer sequences
GAPDH	NM 204305.1	F: 5′- GGTGGTGCTAAGCGTGTTAT-3′
		R: 5′- ACCTCTGTCATCTCTCCACA-3′
TLR4	NM 001030693	F: 5′- GTTTGACATTGCTCGGTCCT-3′
		R: 5′- GCTGCCTCCAGAAGATATGC-3′
NF-κB	NM 205134.1	F: 5′-ATCGTACCGGGAACAACACC-3′
		R: 5′-CTCAGAGGGCCTTGTGACAG-3′
TNF-α	BAC 55966	F: 5′-C ACAGAATGTAAGCCCTGTCC-3′
		R: 5′-TGGAGTTCTGCGATCCTGCATT-3′
IL-1β	Y 15006	F: 5′-AACATCGCCACCTACAAG-3′
		R: 5′-TACTCGGTACATACGAGATGGAAA-3′
IL-6	AJ 309541.1	F: 5′- CATGGACTGGAGCACAAGTA-3′
		R: 5′- TGGAGAGCAGCCCATGTAA-3′
iNOS	U 46504	F: 5′-TTGGAAACCAAAGTGTGTAATATCTTG-3′
		R: 5′- CCCTGGCCATGCGTACAT-3′
VCAM-1	XM 422310.3	F: 5′- ACCCAAATGGACTACCCCCT-3′
		R: 5′- AGGATCACTGGGAAAAGAGTAAAGT-3′
ICAM-1	XM 003642866.3	F: 5′-CACGTTCCAAGCAAGACTGA-3′
		R: 5′-CCACAGCAAGCTGATGAAGA-3′
E-selectin	XM 422246.4	F: 5′-GCCACTTCAGCTGCCTAGAA-3′
		R: 5′-CGTAACCGTTTTGCAAGCCA-3′
VEGF	AY 168004	F: 5′-GAAAGGGGAAGGGTCAA-3′
		R: 5′-GAGAAATCAGGCTCCAGAA-3′
bFGF	NM 205433	F: 5′-GCGATCCGCACATCAAAC-3′
		R: 5′- AATCTGCCATCCTCCTTCATAG-3′
EGF	NM 204849.1	F: 5′-TCCGAGAGTTGCCTTCTTGT-3′
		R: 5′-AGGCACGGGTCTCTCTTCTT-3′

RESULTS

Clinical signs

After 4 h of the intraperitoneal injection of *E. coli*, chickens were subjected to somnolence and diarrhea in the *E. coli* group. No similar symptom had been observed in chickens of the control group and APS group. During necropsy, the signs of edema and hemorrhage in the chicken's intestine mucosa in the *E. coli* group had been observed, but similar symptoms were not found in the chicken in the Con. group and the APS group.

Effects of APS on mRNA Expression of TNF-α, IL-1β, IL-6 and iNOS

TNF-α, IL-1β, IL-6 and iNOS play important roles in intestinal inflammation, their mRNA expression is shown in Figure 1 and Figure 2. As shown in figure 1, the mRNA abundance of TNF-α and IL-1β in chicken's intestine in *E. coli* group showed an increase by 76.59% and 79.36% respectively, and were higher

than those in the Con. group ($p<0.05$), whereas APS significantly decreased the mRNA levels of TNF-α and IL-1β induced by *E. coli* ($p<0.05$). Figure 2 shows that the mRNA level of iNOS in chicken's intestines in *E. coli* group significantly increased compared with that in the control group ($p<0.05$) , and APS inhibited the increase in iNOS mRNA abundance induced by *E. coli*. But there were no differences in the gene expression of IL-6 in chickens among the control group, *E. coli* group and APS group.

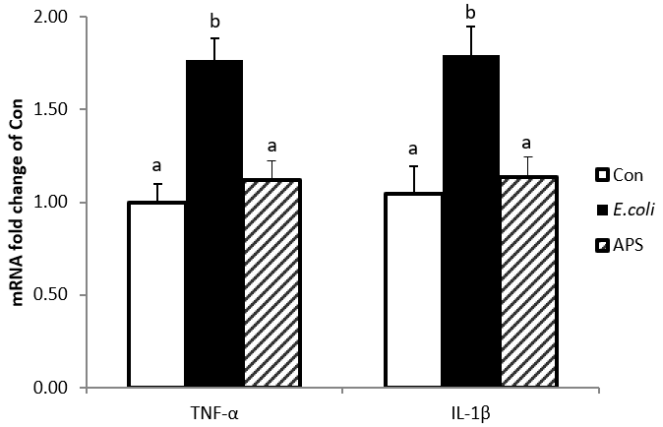

Figure 1 – Expression of TNF-α and IL-1β mRNA. Solid white bar: Con. group; Solid black bar: *E. coli* group; Diagonally striped bar: APS group. Values are means ± SEM (n = 10). Bars with different letters (a, b) are significantly different at $p<0.05$.

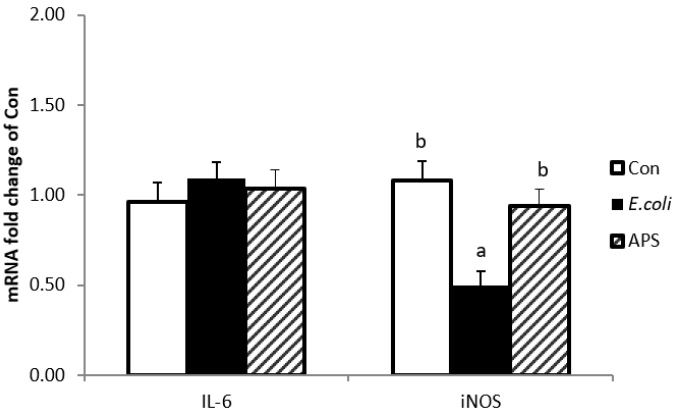

Figure 2 – Expression of IL-6 and iNOS mRNA. Solid white bar: Con. group; Solid black bar: *E. coli* group; Diagonally striped bar: APS group. Values are means ± SEM (n = 10). Bars with different letters (a, b) are significantly different at $p<0.05$.

mRNA Expression of VCAM-1, ICAM-1 and E-selectin

The microvascular of ileum intestinal are abundant and serves as an organ for nutrient exchange. The increase of mRNA expression of adhesion molecules as VCAM-1, ICAM-1 and E-selectin implies endothelial cells dysfunction. VCAM-1, ICAM-1 and E-selectin expressions in ileum intestinal tissue were measured by RT-qPCR (Fig. 3). In this figure, it is shown that the gene level of VCAM-1 in chicken intestine in the *E. coli* group significantly increased by 79.18% compared with that in the Con. group. Furthermore, ICAM-1 and E-selectin mRNA abundance in chickens intestine tissue in the *E. coli* group also increased by 44.24% and 49.01% respectively after 4h with the *E. coli* injection compared with that in the Con. group, what's more, they were significantly different ($p<0.05$). However, APS significantly inhibited the increase in the gene expression levels of VCAM-1, ICAM-1 and E-selectin compared with the *E. coli*-induced.

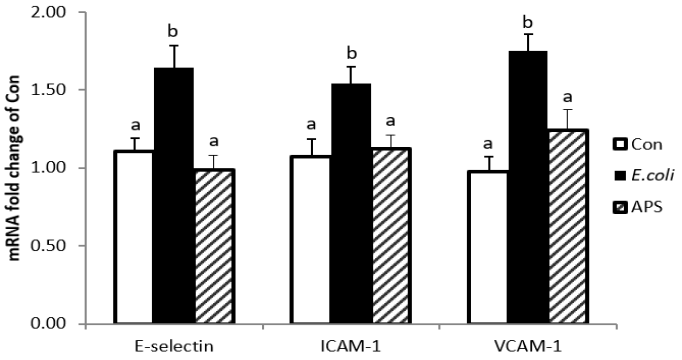

Figure 3 – Expression of E-selectin, ICAM-1 and VCAM-1 mRNA. Solid white bar: Con. group; Solid black bar: *E. coli* group; Diagonally striped bar: APS group. Values are means ± SEM (n = 10). Bars with different letters (a, b) are significantly different at $p<0.05$.

mRNA Expression of EGF, bFGF and VEGF

Figure 4 indicated that mRNA expression of EGF, bFGF and VEGF in intestine tissue fluctuated in three treatments. The mRNA abundance of EGF and bFGF significantly decreased by 44.71% and 47.29% in chicken's intestine tissue after 4 h with the *E. coli* injection compared with that in the Con. group ($p<0.05$), and adding APS in water significantly inhibited the decrease in EGF and bFGF mRNA abundance induced by *E. coli*. But there was no significant difference in the gene expression of VEGF in chicken's intestine in all groups.

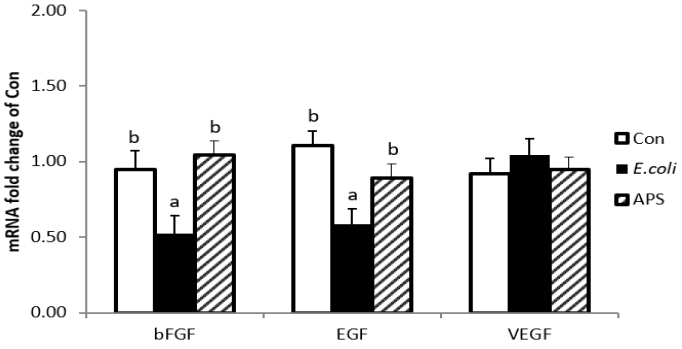

Figure 4 – Expression of bFGF, EGF and VEGF mRNA. Solid white bar: Con. group; Solid black bar: *E. coli* group; Diagonally striped bar: APS group. Values are means ± SEM (n = 10). Bars with different letters (a, b) are significantly different at $p<0.05$.

mRNA Expression of TLR4 and NF-κB

E. coli injection in the abdomen showed an obvious effect on the gene expression levels of TLR-4 and NF-κB (Fig. 5). This figure shows that the level of TLR-4

in chicken's intestine tissue significantly increased by 53.53% (p<0.05) after 4 h of the *E. coli* injection compared with that of the Con. group. The mRNA abundance of NF-κB in chickens with the *E. coli* injection tended to increase (p=0.087). Feeding APS water significantly decreased TLR-4 and NF-κB mRNA abundance induced by *E. coli*.

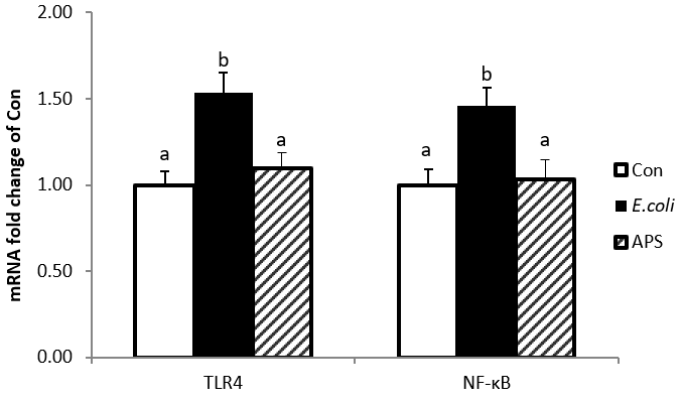

Figure 5 – Expression of TLR4 and NF-κB mRNA. Solid white bar: Con. group; Solid black bar: *E. coli* group; Diagonally striped bar: APS group. Values are means ± SEM (n = 10). Bars with different letters (a, b) are significantly different at p<0.05.

DISCUSSION

In the current study, the signs of edema and hemorrhage in 7-day old chicken intestine mucosa was induced by *E. coli* injection, the expression of inflammation cytokines include TNF-α, IL-1β and iNOS increased, and the adhesion cytokines like ICAM-1, VCAM-1 and E-selectin also increased. However, the unhealthy effects of *E. coli*-induced in chicken's intestinal tissue were alleviated by APS. Meanwhile, APS suppressed the decrease in mRNA abundance of reparative factors of EGF and bFGF when the chickens were injected with *E. coli*. Moreover, further investigations indicated that APS exhibited strong anti-inflammatory activities in the intestinal mucosa and microvascular in 7-day old chicken through the inhibition of TLR-4-NF-κB signaling pathways.

E. coli is a member of the microbiota in digestive tract in healthy poultry and there are physiological mechanisms of clearance of LPS. A variety of stressful practices such as environmental, nutrition or pathogen could activate *E. coli* to release large amounts of LPS and then cause inflammatory response. It has long been known that APS may enhance anti-inflammatory activity by regulating immune function and cytokine expression (Wang *et al.*, 2013). We first investigated the effect of APS on *E. coli*-induced inflammatory response by analyzing the levels of TNF-α, IL-1β and iNOS in 7-day old chicken intestinal tissues. As expected, after 4 hours of the injection of *E. coli*, the significant increases in TNF-α, IL-1β and iNOS mRNA expression in the chicken's intestinal tissue were observed. Interestingly, APS pre-treatment for 7 days significantly inhibited the increase in TNF-α, IL-1β and iNOS mRNA abundance by *E. coli*-induced. Indeed, many publications indicated the anti-inflammation action of APS. For instance, APS supplementation in diet inhibited the increase in TNF-α and IL-1β mRNA abundance in broilers jejunal mucosa with LPS administration (Liu *et al.,* 2015), and APS also effectively restrained the mRNA expression of iNOS, TNF-α and IL-1β in microglial cells (Luo *et al.,* 2015). Moreover, our findings that APS in water for 7 days inhibited the increase in the gene expression of inflammatory cytokines in chicken's intestinal tissue by *E. coli*-induced was consistent with that APS ameliorated the sign of edema of chicken intestine induced by *E. coli*. As for the *E. coli*-injection, it had no effect on mRNA expression of IL-6 in the chicken's intestinal tissue, which contradicts the previous notion that LPS increased the mRNA abundance of IL-6 in chicken macrophage-like cells (Qi *et al.,* 2017; Yang *et al.*, 2008), it may attribute to the differences in species, as well as the dose of *E. coli*, and/or the timing of the sampling.

In inflammatory processes of organ tissues, endothelial cells were exposed to various stimuli, including *E. coli*, inflammatory cytokines, and activated (Molema, 2010), then followed by the increase of E-selectin, ICAM-1 and VCAM-1. For example, *E. coli* in ovo administration stimulated the expression of ICAM-1 and VCAM-1 in 35-day old chicken ileum intestinal tissue (Zhong *et al.*, 2014), and kidneys in pig (Thorgersen *et al.*, 2013). But APS had a preventive effect against endothelial dysfunction by inhibiting the expression of ICAM-1 and VCAM-1, improving endothelial survival (Zhu *et al.*, 2013). Astragaloside IV, a novel saponin purified from Astragalus membranaceus, also decreased the LPS-induced of E-selectin and VCAM-1 gene expression in HUVEC (Zhang *et al.*, 2003). To investigate whether APS inhibits *E. coli*-induced acute inflammatory response in chicken's intestinal microvascular, we assessed the gene levels of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 in 7-day old chicken's intestinal tissue. Our results showed that the gene expression levels of E-selectin, ICAM-1 and VCAM-1 in chicken's intestinal tissue were stimulated by *E. coli*, which were associated with the signs of edema and hemorrhage in chicken's intestine in *E. coli* group. However, APS reduced the mRNA expression of E-selectin, ICAM-1 and VCAM-1, and alleviated the signs of intestine edema and hemorrhage with *E. coli*-induced. Inflammatory factors TNF-α and IL-1β also induced the expression

of E-selectin, ICAM-1 and VCAM-1, and caused to vascular endothelial cells dysfunction (Wang *et al.*, 2014; Fong *et al.*, 2016). APS significantly decreased TNF-α-induced expressions of ICAM-1 and VCAM-1 in human vascular endothelial cells (Zhu *et al.*, 2013). In our findings, the increase in E-selectin, ICAM-1 and VCAM-1 mRNA abundance in intestinal tissue of 7-day old chicken which were injected by *E. coli* were consistent with the increase in TNF-α and IL-1β mRNA expression. While APS inhibited the increase in the expression levels of E-selectin, ICAM-1 and VCAM-1 which were associated with the decline in TNF-α and IL-1β mRNA expression in chicken intestinal tissue in APS group. Moreover, the symptoms of edema and hemorrhage had not been seen in chicken's intestine in APS group.

Angiogenesis, the formation of new blood vessels, is regulated by reparative factors. In our study, APS inhibited the decline in the expression levels of EGF and bFGF in chicken intestinal tissue with *E. coli*-induced, consistent with the increased level of TNF-α, IL-1β, iNOS, E-selectin, ICAM-1 and VCAM-1 mRNA expression. But for VEGF, the gene expression was not changed in chicken intestine tissue in all three treatments.

LPS induces the inflammatory response mediated by its receptor TLR4. After binding LPS, TLR4 and its downstream NF-κB are activated, subsequently activated NF-κB translocates into the nucleus to regulate target gene expression (Faure *et al.*, 2000). For example, avian pathogenic *E. coli* challenge activated TLR4 and NF-κB and stimulated the expression of ICAM-1, VCAM-1, TNF-α and IL-1β in 21-day old chicken ileum intestinal tissue (Zhong *et al.*, 2014). APS inhibited the increase in the expression levels of TLR4 and NF-κB in 35-day old broilers jejunal mucosa (Liu *et al.*, 2015) and the translocation of NF-κB in murine (Lu *et al.*, 2016) with LPS-induced. Our findings showed that APS inhibited the increase induced by *E. coli* in both TLR4 and NF-κB mRNA expression in 7-day old chicken's intestine tissue, which coincided with the notion that APS inhibited *E. coli* induced increase of ICAM-1, VCAM-1, TNF-α, IL-1β and iNOS mRNA expression, and alleviated the decline in EGF and bFGF mRNA expression. Meanwhile, we also observed that APS alleviated symptoms of edema and hemorrhage in chicken's intestine mucosa by *E. coli*.

CONCLUSIONS

In this study, we found that *E. coli* injection can induce the 7-day old chicken's intestinal mucosa injured and microvascular dysfunction though increasing the mRNA expression of TNF-α, IL-1β, iNOS, E-selectin, ICAM-1 and VCAM-1, and decreasing mRNA abundance of reparative factors EGF and bFGF. Feeding APS can attenuate *E. coli*-induced chicken intestinal mucosa injured and microvascular dysfunction by modulating the TLR4-NF-κB signaling pathways. We hope that the molecular basis of APS efficacy will be intriguing and worthy for further investigation.

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