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Original Article

Effects of E. Coli Infection on the Expressions of TGF-β/Smads Signaling Pathway in Broiler Intestine

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■Keywords

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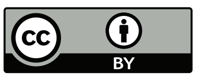

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ABSTRACT

This experiment aimed to investigate whether *Escherichia coli* (*E. coli*) infection could affect the TGF-β/smads signaling pathway in the jejunal tissue of chickens. One-day-old Cobb 500 broilers were randomly divided into 2 groups and treated with intraperitoneal *E. coli* or broth injection. Clinical signs of the birds were assessed every day. Spleen and bursa of Fabricius of the birds, post-infection (pi), were collected to evaluate immune organ index. Jejunal tissues were collected to ascertain the expression of TGF-βs, TβRs, and Smads. The results showed that the infected birds had significantly higher index of the spleen (24hrs and 48hrs pi) compared with birds in the control group ($p<0.05$). The relative gene expression of TGF-β4 increased ($p<0.05$), while the expression of Smad7 down-regulated in the *E. coli* group ($p<0.01$). There was no significant difference in TGF-β2, TGF-β3, TβR I, TβR II, Smad2, Smad3 expression ($p>0.05$). In conclusion, TGF-β/Smad signaling pathway was associated with the immune response of broilers in *E. coli* infection and TGF-β4 was the main subtype interacting with *E. coli* infection.

INTRODUCTION

The intestinal mucosa is an important assurance for health which possesses a complex epithelial barrier to a broad spectrum of inflammation, oxidative stress, and microbes (Howe *et al.,* 2015). *Escherichia coli* is one of the most common pathogens of animal intestinal flora (McDonald *et al.,* 2001) and avian pathogenic *E. coli* (APEC) cause great economic loss every year in poultry (Moniri & Dastehgoli 2005). The intestinal tract is damaged when APEC is colonized in the intestinal. Epithelial cells represent a dynamic continuum of cellular structure and function, and cells at the tip of the villus have a specialized absorptive and digestive function (Barnard *et al.,* 1989). Thus, the disruption of the intestinal barrier not only affects the absorption of the nutrients but also induces diseases, such as multiple organ dysfunctions, systemic inflammatory response syndrome, diarrhea, and others.

Transforming growth factor-β (TGF-β) superfamily, produced by a wide range of cells, composed of multifunctional cytokines implicate in the processes of various diseases (Tohidi et al.2012). TGF-β can suppress inflammatory responses to intestinal bacterial antigens and play an important role in the induction of immune tolerance (Ihara *et al.,* 2017). Studies demonstrated that TGF-β has shown a remarkable ability to protect the epithelial barrier function from the penetration of foreign antigens through countering the effect of T-cell cytokines (Monteleone *et al.,* 2001; Planchon *et al.,* 1994).

TGF-β plays its biologic role primarily through the canonical Smads signaling pathway which has three isoforms that are involved in several developmental processes as TGF-βs, TβRs and Smads (Derynck & Zhang

2003; Heldin *et al.,* 1997; Moustakas *et al.,* 2001). A variety of diseases would happen if these factors were changed or defected in broilers (Hahm *et al.,* 2001). However, little data is available about changes of TGF-βs, TβRs and Smads signaling pathway in early *E. coli* infection in broilers. Therefore, the present study aimed to gain more insight into the changes of the TGF-β/Smads signaling pathway in broilers infected with *E. coli*.

MATERIALS AND METHODS

Animals and diets

One-day-old male broilers (Cobb 500) were bought from a local commercial hatchery (Dayong co. Ltd, Shangqiu, Henan, China). The broiler chicks were maintained in chicken coops and raised in an environmentally controlled room and the temperature was maintained at 34-36 ºC during the experiment. All of the birds had free access to water and commercial corn-soybean basal diets (Table 1). The birds were kept under incandescent lighting on a light schedule of 24hrs light.

Table 1 – Nutrition content of diet used in the experiment.

Ingredient	content (%)
Crude Protein	≥ 18.0
Coarse Fibre	≤ 8.0
Crude Ash	≤ 9.0
Calcium	0.6-1.3
Total Phosphorus	≥ 0.5
NaCl	0.3-0.8
Lysine	≥0.85
Methionine	0.36-0.9
Moisture	≤14.0

E. Coli culture condition

The *E. coli* (O1: K1) strain used was kept in our laboratory. The bacterial strain was cultivated in Luria-Bertani (LB) broth for 24hrs at 37 ºC, after which a single colony was inoculated and cultivated in LB broth at 37 ºC for 18hrs with shaking. After this time the culture was diluted in brain-heart infusion and an inoculum of approximately 10^9 CFU/mL (colony-forming units). The final concentration of the microorganism for the assay was about 6×10^9 CFU/mL.

Experimental Procedure

Forty-eight chickens of one-week of age were randomly assigned to 2 groups and 12 birds were sampled at each time point (6 for each). One group was injected with 0.5 ml 6×10^9 CFU/mL *E. coli* according to the pre-test. Meanwhile, the other group was the control group and received the same amount of LB-Miller broth.

Sample collection

The chicken were weighed before they were killed by exsanguination after receiving the injection. The jejunum samples of 6hrs post injection (pi) were rapidly isolated and freezed immediately with liquid nitrogen and then preserved in a freezer at -70 ºC for subsequent isolation of total RNA. The spleen and bursa of Fabricius were excised and weighed at 6hrs, 12hrs, 24hrs, and 48hrs pi, and the indices (organ weight/body weight ratio) were calculated.

RNA Isolation and cDNA Synthesis

The TRIzol reagent (Invitrogen) was used to isolate total RNA and the method was performed according to the manufacturer's instruction. The RNA integrity was assessed and purity was determined. Ratios of absorption (260/280nm) of all samples were between 1.8 and 2.0. The RNA sample (1μg) was reversely transcribed into cDNA using First Strand cDNA Synthesis Kit (Dingguo Changsheng, Beijing), and synthesized cDNA was kept in a freezer under -20 ºC.

Quantitative Real-time PCR Analysis of Gene Expression

The expression of genes mRNA was performed on the Mastercycler ep realplex Real-Time PCR Detection System (Eppendorf) using GoTaq® qPCR Master Mix (Promega, USA) according to the kit's instructions. Polymerase chain reaction system was performed in 10 μL containing 2 μL of the synthesized cDNA, 5μL GoTaq® qPCR Master Mix (Promega, USA), 0.5μL of each candidate gene or reference gene (GAPDH) specific primer (Table 2) and RNase Free ddH_2O 2 μL. At the final step of the PCR, dissociation curves of the products were identified. Fluorescent data were used to derive the C(*t*) at default threshold values. The resultant value was expressed relative to GAPDH, which showed no variation among treatment groups. The fold changes of relative gene expression were analyzed using the $2^{-\Delta\Delta C(t)}$ method (Livak & Schmittgen 2001).

Data Analyses

The predictive Analytics Software (PASW) version 18.0 software (SPSS Inc. USA) was used to process data. Independent-samples t-tests were used to test for significant differences between the *E. coli* infected and control group. Differences between infected and control group were considered statistically significant at $p<0.05$. Values were expressed as means ± SE.

Table 2 – Sequences of PCR primers.

gene[1]	GenBank number[2]	Primer sequence (5'-3')	Orientation	Product size(bp)
TGF-β2	NM_001031045.3	TATCATCACCAGGACAGCGT	Forward	177
		ACCTTGTGGCTTAGGGTCTG	Reverse	
TGF-β3	NM_205454.1	ACCTTGTGGCTTAGGGTCTG	Forward	211
		ATTCCTTGCCTTCCCAGTTC	Reverse	
TGF-β4	JQ423909.1	CGGGACGGATGAGAAGAAC	Forward	258
		CGGCCCACGTAGTAAATGAT	Reverse	
TβR I	NM-204246.1	GCTGTGGTTGGTGTCAGATT	Forward	156
		GGTTTGCCTTGTGTGCCTAC	Reverse	
TβR II	NM-205428.1	GACCACCGCCAAGTAGCAT	Forward	129
		TGACAGCCTCAGTTCTCCAG	Reverse	
Smad2	NM-204561.1	GTCATCCATTCTGCCATTCA	Forward	100
		ATTCTGCTCACCACCACCA	Reverse	
Smad3	NM-204475.1	GAGCCGCAGAGCAACTACAT	Forward	135
		CGGAGACATAGGATTTGGTGAT	Reverse	
Smad7	XM-004949015.1	GAGCATCAAGAGCAGGTTCG	Forward	106
		GCACAGTCCATTCAGAGCAG	Reverse	
GAPDH	K01458	GGTGGTGCTAAGCGTGTTAT	Forward	264
		ACCTCTGTCATCTCTCCACA	Reverse	

[1] TGF-β2/3/4 = Transforming growth factor-β2,3,4; TβR I / II = Transforming growth factor-β receptor I / II; Smad2,3,7 = drosophila mothers against decapentaplegic protein 2/3/7; TNF-α = Tumor necrosis fact-α; IL-6 = Interleukin 6; IL-1β = Interleukin 1β; ZO1/2 = Zonula occludens-1/2; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

[2] GenBank accession number.

RESULTS

Clinical Sighs of Chicken Infected with *E. coli*

Throughout the experiment, the control group showed no abnormality in clinical signs. By contrast, the chicks infected with *E. coli* demonstrated huddling, shivering and inactivity. At 6hrs pi, the chicks were killed, and it was found that the abdominal cavity and intestinal surface of the experiment group was filled with a yellowish exudate. There were no obvious pathological changes in the tissues in the control group.

Immune Organ Index of Chicken

As shown in Table 3 and 4, compared with the control group, *E. coli* had no significant influence on the index of the spleen at 6hrs and 12hrs after the infection ($p<0.05$), while the index of spleen increased at 24hrs and 48hrs pi ($p<0.05$). A significant difference in the index of bursa of Fabricius was observed on 12hrs pi.

Table 3 – The index of spleen of chicken after challenge.

Item	6hrs (pi)	12hrs (pi)	24hrs (pi)	48hrs (pi)	SEM	*p*
Control	0.422	0.477	0.503	0.479	0.03	<0.05
E.coli	0.463	0.680	0.801*	0.643*	0.05	<0.05

Note: *in same raw indicate significant difference $p<0.05$.

Table 4 – The index of bursa of Fabricius of chicken after challenge.

Item	6hrs (pi)	12hrs (pi)	24hrs (pi)	48hrs (pi)	SEM	*p*
Control	2.489	2.158	2.207	2.26	0.106	<0.05
E.coli	2.43	2.963*	2.521	2.426	0.205	<0.05

Note: *in same raw indicate significant difference $p<0.05$.

Expression of TGF-β/Smads signaling pathway

To determine the mRNA expression of the TGF-β/Smads pathway in the intestine to *E. coli* infection, we studied the production levels of TGF-β 2, 3, 4, and TβR I , II and Smad 2,3,7 in the jejunum of chicken at 6hrs pi (*Fig.1*). Compared with the control group, the gene expression of TGF-β4 was up-regulated significantly after *E. coli* infection ($p<0.05$), but no significant differences of TGF-β2, TGF-β3, and TβR I, TβR II were found ($p>0.05$). Smad2 and Smad3 mRNA also showed no significant differences, while the expression of Smad7 mRNA was significantly down-regulated in the *E. coli* infected intestine at 6hrs pi ($p<0.05$).

Figure 1 – Relative expression of TGF-β/smads related genes in the jejunum of *E. Coli* infected chicken. A, TGF-β2; B, TGF-β3; C, TGF-β4; D, TβR I; E, TβR II; H, Smad 2; I, Smad 3; J, Smad 7. Data were expressed as means ± SE. * Indicate significant difference $p < 0.05$.

DISCUSSION

The intestinal mucosa is an internal and external surface of the body that possesses biological barrier, mechanical barrier, and immune barrier and therefore forms an important physical barrier against pathogens and toxic macromolecules (Ruemmele & Garnier-Lengliné 2013; Springler *et al.,* 2016). Infections with APEC cause the intestinal mucosal barrier of the chicken to be injured and the permeability of the gut to increase, accompanied by endotoxin translocation. When stimulated, the size of the immune organs will change. The weight of the spleen and the bursa of Fabricius reflects the immune response of chicken to some extent (Rivas & Fabricant, 1988). In this experiment, both the spleen index and the bursa of Fabricius index were observed to increase post-infection. The birds were using the cellular and humoral immunity to defend against *E. coli* infection.

TGF-β is a potent negative regulator of mucosal inflammation and it has been proved that TGF-β rich diet reduced intestinal injury in the acute phase and improved recovery of mucositis in the gut (Boukhettala *et al.,* 2010). But the molecular mechanism of TGF-βs, especially TGF-β4 in avian, is not well known. TGF-β/Smads dependent pathway has not been identified when broilers were infected with *E. coli*. Therefore, we characterized the effect of *E. coli* on the expression of TGF-β/Smads related genes. The results of our study showed that the expression of TGF-β4 significantly increased post *E. coli* infection, while, TGF-β2 and TGF-β3 showed no significant changes. Although the three isoforms of TGF-βs are expressed in inflammatory tissues, the role of TGF-β subjects is not entirely consistent. TGF-β2 was thought to be the least effective in intestinal wound repair (Govinden, 2003). The results of our study showed that TGF-β4 has a closer relationship with the inflammatory reaction induced by *E. coli* in chicken intestinal.

TGF-β signals are transduced by a heteromeric complex formed by TβR I and TβR II receptors. TβRI and TβR II receptor are two transmembrane serine/threonine kinases (Derynck & Feng 1997; Franzen *et al.,* 1993; Wrana *et al.,* 1994). In the present study, TβR I, TβR II had no significant change (Fig1D, E). Smad proteins are critical downstream mediators responsible for propagating biological effects of TGF-β (Heldin *et al.,* 1997). In this study, the expression of Smad7 significantly decreased (p<0.05) (Fig1J). Smad7 is one kind of antagonistic Smads which are key negative regulators of TGF-β/Smads signaling system by a feedback loop (Nakao *et al.,* 1997; Yan *et al.,* 2009).

It has been reported that Smad7 can directly form a stable complex with TβRI receptor, thereby preventing the phosphorylation of R-Smad and hetero-complex formation between R-Smads and Co-Smad by its antagonistic effect (Hayashi *et al.,* 1997; Nakao *et al.,* 1997; Shu,2016). In the present study, the increase of TGF-β4 and decrease of Smad7 expression to suppress *E.coli* induced inflammation in jejunal, suggesting that feed-back control between TGF-β4 and Smad7 may be crucial for *E.coli* infection at first stage of chicken and that anti-inflammatory effects were stronger than pro-inflammatory effects at the first 6hrs after *E. coli* infection. It was related to a decrease in the inflammatory response of the gut.

In conclusion, we demonstrated that TGF-β/Smad signaling pathway was involved in the response of *E. coli* infection of chicken. The expression of TGF-β4, Smad7 indicating that feed-back control between TGF-β4 and Smad7 may be crucial for *E. coli* induced jejunal inflammation at 6hrs after infection of chicken. Targeted enteral therapy with optimized concentrations of TGF-β4 or smad7 might be of interest for the treatment of inflammatory disorders in the intestine of chicken.

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