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

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-β s, Tβ Rs 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 10⁹ CFU/mL (colony-forming units). The final concentration of the microorganism for the assay was about 6×10⁹ 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 6×10⁹ 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 froze 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 ddH₂O 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 −ΔΔC(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.
RESULTS

Clinical Signs 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.

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-β2, 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).

Table 2 – Sequences of PCR primers.

| gene | GenBank number | Primer sequence (5'-3') | Orientation | Product size(bp) |
|------|----------------|-------------------------|-------------|-----------------|
| TGF-β2 | NM_001031045.3 | TATCATCACCAGCAGCCTG | Forward | 177 |
| TGF-β3 | NM_205454.1 | ACCCTGTTGCTTACGTCCTG | Reverse | 211 |
| TGF-β4 | JQ423909.1 | CCGGACGAGTAGAAGAAAC | Forward | 258 |
| TβR I | NM-204246.1 | GCTGTGTTGTTGATGATT | Forward | 156 |
| TβR II | NM-205428.1 | GACCAACCGCAAAGTACAT | Forward | 129 |
| Smad2 | NM-204561.1 | GTATGTTCCATTCTCCGCA | Forward | 100 |
| Smad3 | NM-204475.1 | GAGCCGCAGAAGTAAGCAT | Forward | 135 |
| Smad7 | XM-004949015.1 | GAGCATCAAGAGCATGGTG | Forward | 106 |
| GAPDH | K01458 | GGTGGTGTTAGGTGTCTC | Forward | 264 |

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 factor-α; IL-6 = Interleukin 6; IL-1β = Interleukin 1β; ZO1/2 = Zonula occludens-1/2; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

2 GenBank accession number.

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.
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, TβRI; E, TβRII; 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-β3, 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 TβRI and TβRII receptors. TβRI and TβRII receptor are two transmembrane serine/threonine kinases (Derynck & Feng 1997; Franzen et al., 1993; Wrana et al., 1994). In the present study, TβRI, TβRII 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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