

Full Length Research Paper

Effects of modified pulsatilla decoction on avian colibacillosis and concentrations of nitrogen oxide (NO) and tumor necrosis factor- α (TNF- α) in serum

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To investigate the mechanism of the Chinese herbal formula modified pulsatilla decoction (MPD) on chicken colibacillosis, the oral herbal liquid was used to treat chickens experimentally infected with *Escherichia coli*. The serum nitrogen oxide (NO) and tumor necrosis factor- α (TNF- α) concentrations were determined. The results showed that the MPD oral liquid administrated at a dose of 1.5, 1.0 and 0.5 ml/kg-BW achieved effective rates of 70, 82 and 84% in the chickens infected with *E. coli*, respectively. The effective rates of the MPD oral liquid treated groups were significantly higher than that of the challenged group ($p < 0.01$). The MPD oral liquid at a dose of 1.0 ml/kg-BW reduced serum NO and TNF- α concentrations. Therefore, the MPD preparation has good curative effects on chicken colibacillosis, and the efficacy may be achieved by inhibiting the increase of serum NO and TNF- α concentrations caused by *E. coli* infection and resisting endotoxin-induced factors, which protects the body from inflammatory injury and controls the development of colibacillosis.

Key words: Colibacillosis, Chinese herbal medicine, nitric oxide, tumor necrosis factor- α .

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis in chickens, turkeys, and other avian species (Gibbs et al., 2004). Colibacillosis continues to contribute to increased mortality and economic losses in the poultry industry throughout the world, which presents as a localized or systemic infection and includes colisepticemia, coligranuloma, air sac disease, pericarditis or swollen-head syndrome (Dho-Moulin and Fairbrother, 1999). Antibiotics and chemical drugs are used for its treatment, but no good healing effect can be achieved due to widespread drug-resistant strains. Moreover, the quantity of antibiotics used in animal husbandry often exceeds their medical use (Witte,

1998). Antimicrobial resistance has emerged as a global public health problem in recent years (Harrison and Lederberg, 1998; Swartz, 1997). Drug residues reduce quality of animal products and even threaten human health and environment. Therefore, it is of great value to develop or find more effective, more natural and less environment harmful products to control avian colibacillosis.

Chinese herbal medicine (CHM) has been used in controlling avian colibacillosis in China (Zhu et al., 2010) and leaves less residues and does not often lead to drug resistance. As a kind of somewhat natural product it has been used to prevent and treat animal bacterial diseases and shows very promising market potential. CHM can inhibit or kill bacteria. More importantly, it can regulate the expression of many cytokines, improve overall health and immunity state of body, and thus protect the body against disease. *E. coli* releases endotoxin in the pathogenesis of avian colibacillosis, which helps *E. coli* to cause pathological damage and induces target cells to secrete various kinds of inflammatory mediators and cytokines (Zhu et al., 1995). Among the cytokines, nitrogen oxide (NO) and tumor necrosis factor- α (TNF- α) as very

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Abbreviations: MPD, Modified pulsatilla decoction; NO, nitrogen oxide; TNF- α , tumor necrosis factor- α ; APEC, avian pathogenic *Escherichia coli*; CHM, Chinese herbal medicine; CFU, colony-forming units; SEM, standard error of the mean.

important cytokines play a dual role: they kill foreign pathogens and at the same time do injure the body by mediating inflammatory response (Wang et al., 2002). According to diagnosis and treatment based on an overall analysis of the illness and the patient's condition under the philosophy of traditional Chinese veterinary medicine, we prepared an herbal preparation and treated chickens experimentally infected with *E. coli*. Then we determined serum NO and TNF- α concentration to investigate the possible mechanisms of its action.

MATERIALS AND METHODS

Chickens and *Escherichia coli*

One-day old Isa Brown laying hens were selected and raised until the age of 15 days in accordance with the feeding standard for laying hens (NY/T33-2004; the Ministry of Agriculture of the People's Republic of China). Avian pathogenic *Escherichia coli* (APEC) for challenge (*E. coli* O₁; CVCC249) were purchased from the China Institute of Veterinary Drug Control (Beijing, China).

Preparation of herbal extracts

The modified formula pulsatilla decoction contains several herbs including *Radix pulsatillae*, *Cortex phellodendri* and other materials were prescribed according to herb formula. The herbs were soaked in 10 times weight of distilled water for 30 min and brought to boiling quickly, followed by decoction with mild fire for 20 min. The decoction was strained through four-layer gauze to a bowl, and the dregs were extracted again in five times weight of distilled water by simmering with mild fire for 20 min. The decoction was also strained to the same bowl. The decoction mixed together was then evaporated to a concentration equivalent to 0.5 g/ml crude herb. The herbal extracts were repackaged and stored at 4°C after autoclaving.

Assessment on efficacy

Three hundred chicks were randomly assigned into six groups, 50 in each group. The chicks except those in the negative control group were administrated with 0.1 ml *E. coli* O₁ culture (1.0×10^9 CFU/ml) via pectorals. After challenge, the chicks were immediately treated with medicines. The chicks in Groups I, II and III were orally administrated with the herbal extracts at a high dose of 1.5 ml/kg-BW, at a medium dose of 1.0 ml/kg-BW, and at a low dose of 0.5 ml/kg-BW, respectively. The chicks in Group IV were orally administrated with florfenicol soluble powder at a standard dose of 20 mg/kg-BW. The treatment was given twice a day for 5 days consecutively. The challenged chicks in Group V and the unchallenged chicks in Group VI were not given any medication. The chickens were monitored for clinical symptoms and death rate every day during 2-week trial period. Dead chickens were dissected to observe pathological changes. Bacteria were isolated and identified according to biochemical characteristics. Effective rate, cure rate and mortality were determined. Each chick was weighed before and after the experiment to calculate relative growth rate.

Treatment and determination of serum NO and TNF- α concentration

Two hundred chicks were randomly assigned into four groups, 50 in

each group. The chicks in Group I, Group II and positive control group were challenged with *E. coli* as stated earlier and then treated as those in the Groups II, IV and V for efficacy assessment, respectively. The unchallenged chicks in negative control group were also not given any medication. Blood samples were collected from hearts of five chickens randomly selected from each group at 12, 24, 48, 72, 96 and 120 h post challenge, respectively. Sera were separated and preserved at -20°C. Serum NO and TNF- α were determined according to the manufacturer's instructions of ELISA kit for gallinaceous total NO and TNF- α produced by R&D Systems (Minneapolis City, MN), respectively. Staffax-2100 microplate reader produced by Awareness (Palm City, FL) was used for detection.

Statistical analysis

Data were expressed as means with standard error of the mean (SEM). All data were analyzed using SPSS 11.5 software. The statistical differences of the effective rate, cure rate and mortality were checked by *t* test, and that of the relative growth rate, serum NO and TNF- α concentrations were compared by χ^2 test. A P value <0.05 was considered statistically significant difference.

RESULTS

Efficacy of the CHM oral liquid against colibacillosis

After exposure to *E. coli* for 8 h, the challenged chicks displayed clinical symptoms of colibacillosis such as wing paralysis, poor appetite and yellow-white diarrhea. Death was seen in each challenged group. Necropsy revealed typical lesions of colibacillosis including much mucus in the trachea, scattered hemorrhagic spots in the liver, fibrinous perihepatitis and pericarditis, mucosal thickening in the intestine with much yellow foamy exudate in enteric cavity, and slightly swollen kidney. The bacteria isolated from liver specimens were identified as *E. coli*. The chicks in the negative control group had good spirit and appetite, and no death and clinical symptoms were observed. Therefore, compared with those chicks in the positive control group, the chicks treated with the herbal medicines had slighter symptoms and less number of deaths.

The effective rate, cure rate, mortality and relative growth rate of each group was shown in Table 1. As can be seen from this table, the chickens treated with herbal medicines had higher effective rate, cure rate and relative growth rate but lower mortality than those in the positive control group ($p < 0.01$), which indicated better efficacy of the herbal medicines. Moreover, these four indicators to assess efficacy were not significantly different between any two groups of the high-dose herbal extract-treated group, the medium-dose herbal extract-treated group and the florfenicol-treated group.

Effects of the CHM oral liquid on serum NO concentration

From the NO concentration of chicks in each group

Table 1. Efficacy of the herbal extracts against colibacillosis (%).

Groups	Effective rate	Cure rate	Mortality	Relative growth rate
High-dose herbal extract-treated group	84 ^{Aa}	76 ^{Aa}	16 ^{Bc}	85.85 ^{Bb}
Medium-dose herbal extract-treated group	82 ^{Aa}	70 ^{ABa}	18 ^{Bc}	84.84 ^{Bb}
Low-dose herbal extract-treated group	70 ^{Ab}	56 ^{Bb}	30 ^{Bb}	74.19 ^{Bc}
Florfenicol-treated group	80 ^{Ab}	72 ^{ABa}	20 ^{Bbc}	82.35 ^{Bb}
Positive control group	48 ^{Bc*}	8 ^{Cc*}	52 ^{Aa}	10.54 ^{Cd}
Negative control group	--	--	--	100.0 ^{Aa}

Note: Within a column, different lowercase letters in superscript represent significant differences at 0.05 (capital letters 0.01) level, and the same lowercase letters in superscript represent no significant difference. * represents self-healing or self-relief of chicks after challenge.

Table 2. Variation of serum NO concentration in chicks treated with CHM preparations ($\mu\text{mol/L}$).

Groups	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h	At 120 h
Herbal extract-treated group	23.41 \pm 3.48 ^{ab}	27.59 \pm 3.22 ^{bA}	31.45 \pm 4.27 ^B	30.12 \pm 3.55 ^{cB}	25.46 \pm 3.13 ^B	25.17 \pm 3.02 ^{bAB}
Florfenicol-treated group	23.28 \pm 4.16 ^{ab}	29.13 \pm 3.68 ^{abA}	35.75 \pm 3.13 ^B	34.71 \pm 3.34 ^{bB}	27.11 \pm 3.63 ^B	26.46 \pm 3.65 ^{abA}
Positive control group	25.09 \pm 3.28 ^a	33.15 \pm 3.04 ^{aA}	43.59 \pm 3.36 ^A	55.35 \pm 4.29 ^{aA}	33.65 \pm 2.90 ^A	30.43 \pm 3.41 ^{aA}
Negative control group	19.64 \pm 3.16 ^b	17.64 \pm 2.95 ^{cB}	20.35 \pm 3.04 ^C	19.82 \pm 2.59 ^{dC}	18.69 \pm 3.36 ^C	20.23 \pm 2.57 ^{cB}

Note: Within a column, different lowercase letters in superscript represent significant differences at 0.05 (or capital letters, 0.01) level, and the same lowercase letters in superscript represent no significant difference.

Table 3. Variation of serum TNF- α concentration in chicks treated with CHM preparation (pg/ml).

Groups	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h	At 120 h
Herbal extract-treated group	66.50 \pm 7.40	82.17 \pm 8.95 ^{bA}	73.50 \pm 7.82 ^{bB}	71.83 \pm 7.68 ^{bB}	72.50 \pm 4.42 ^{bB}	69.82 \pm 10.17 ^{bB}
Florfenicol-treated group	68.83 \pm 7.54	89.67 \pm 10.48 ^{abA}	78.50 \pm 7.42 ^{bB}	73.17 \pm 7.39 ^{bB}	74.67 \pm 8.12 ^{bB}	72.33 \pm 5.82 ^{bB}
Positive control group	69.83 \pm 6.77	95.17 \pm 9.41 ^{aAB}	111.83 \pm 6.49 ^{aA}	113.00 \pm 9.76 ^{aA}	105.33 \pm 9.89 ^{aA}	94.37 \pm 11.20 ^{aA}
Negative control group	67.33 \pm 6.59	66.83 \pm 8.40 ^{cB}	73.67 \pm 5.57 ^{bB}	68.50 \pm 8.60 ^{bB}	70.27 \pm 6.47 ^{bB}	66.65 \pm 9.20 ^{bB}

Note: Within a column, different lowercase letters (capital letters) in superscript represent significant differences at 0.05 (or 0.01) level, and the same lowercase letters in superscript represent no significant difference.

(Table 2), we can see that the NO concentration of the challenged chicks increased rapidly after 12 h post-infection with a peak at 72 h and then decreased gradually; that of the chicks treated with the herbal preparation was always at a higher level than the negative control, and it peaked at 48 h post challenge without large fluctuation; and that of the unchallenged chicks remained at 20 $\mu\text{mol/L}$ with little variation. Statistical analysis showed significantly lower NO concentration in the herbal extract-treated groups than in the positive control group at 48, 72 and 96 h post challenge, respectively ($p < 0.01$).

Effects of the herbal oral liquid on serum TNF- α concentration

As can be seen from the TNF- α concentration of chicks in each group (Table 3), the TNF- α concentration of

the challenged chicks, treated or not treated with the herbal medicines, rose after challenge and still remained at a high level at the end of trial. It peaked at 24 h in the chicks treated with the herbal extracts or florfenicol, and it peaked at 72 h in the chicks challenged but not treated. In addition, the chicks treated with the herbal extracts had significantly lower TNF- α concentration than the positive control ($p < 0.01$) at any sampling time except at 12 and 24 h.

DISCUSSION

Many kinds of Chinese herbal medicines have been used to treat chicken colibacillosis. *Rabdosia* (Maxim.) Hara decoction can reduce mortality of chickens artificially infected with *E. coli*, and the best efficacy is achieved by oral administration at a dose of 0.2 ml per chicken (He et al., 2009). Chinese herbal medicine prepared by Zhang et

al. (2008) can achieve an 83.3% protection rate in chickens artificially infected with *E. coli*. *Flos Lonicerae* extract can achieve a protection rate higher than 90% in chickens artificially infected with *E. coli* (Li et al., 2006). In this study, the Chinese herbal medicine oral liquid showed good curative effects on experimental infection of *E. coli* in chickens, and the efficacy was as good as that of florfenicol ($p > 0.05$). In addition, the herbal medicine oral liquid also promoted recovery and growth of these chickens and increased weight gain. We recommend the medium dose of 1.0 ml/kg-BW be used to treat avian colibacillosis, as its efficacy was not significantly different from that of the high-dose treatment.

NO is an essential molecular regulator. Too much NO can kill foreign pathogens but do injury to normal tissues, causing pathological changes. TNF- α , a bioactive factor secreted by macrophages (Carswell et al., 1975; Kawakami, 1981), can play the cytotoxic activity by stimulating phagocytes to produce a variety of bioactive molecules (For example, free radicals). If these molecules are not eliminated in time, inflammation will occur. As inflammatory mediators, NO and TNF- α can not only make local inflammation more serious but also cause injury to other tissues through circulatory system and even multiple organ failure. In this study, the chickens infected with *E. coli* had higher serum NO and TNF- α concentration than the healthy chickens. The mechanism may be that *E. coli* propagate rapidly in chicken blood and release large amounts of endotoxin which induces the increase of serum NO and TNF- α concentrations. NO and TNF- α kill *E. coli*, mediate occurrence and development of inflammation, make pathological damage more serious, and cause inflammation in multiple organs.

Chinese herbal medicine and its components can regulate NO and TNF- α *in vivo*. Szechwan lovage alkaloidal can inhibit increase of NO induced by cerebral ischemia-reperfusion injury in rats (Wang et al., 2009). *R. salvia miltiorrhiza* has some regulating effects on peripheral neutrophil apoptosis and NO production in patients with systemic lupus erythematosus (Shi et al., 2008). *Cornus florida* polysaccharide can regulate immune functions by reducing NO and interleukin-6 (Zhang et al., 2008). *Lonicera japonica* Thumb. extract can reduce NO greatly secreted by LPS stimulated rat primary microglial cells and shows anti-inflammatory effect and immunosuppressive effect (Cui et al., 2008). *Radix bupleuri* hemostatic agent can inhibit secretion of NO by reducing the activity of inducible nitric oxide synthase (iNOS) and resist endometrial bleeding (Zhou et al., 2008). *Panax notoginseng* and *alceaefolius* extracts can decrease NO and TNF- α level in rats and relieve carbon tetrachloride- and alcohol-induced liver injury (Liu et al., 2010; Huang et al., 2008). *Polygonum cuspidate* can inhibit pulmonary fibrosis in rats by reducing the level of serum TNF- α (Xia et al., 2010). Cortex moutan can reduce serum lipid, C-reactive protein (CRP), TNF- α and

interleukin-6 of diabetics (Min, 2009). *Oxalis corniculata* L. can treat prostatitis by decreasing interleukin-1 β and TNF- α level (Xiao et al., 2009). Dachengqi decoction can treat severe pancreatitis by decreasing the levels of cytokines and thus preventing intestinal failure for a better prognosis (Sun, 2009). Modified Yupingfeng granule can treat chronic urticaria effectively by inhibiting the expression of TNF- α in peripheral blood (Li et al., 2009). Shashen Maidong decoction can treat rat chronic bronchitis caused by lung Yin deficiency, and one of the mechanisms is that the decoction reduces serum interleukin-6 and TNF- α level (Hong et al., 2009). Qinqiao oral solution can relieve inflammation caused by *E. coli* by reducing serum NO and TNF- α level (Liu et al., 2008). In this study, the serum NO and TNF- α level of the chicken administrated with the herbal extracts was significantly lower than that of the chickens challenged by *E. coli* during the experimental period. At the end of the experiment, the serum NO and TNF- α level of the treated chickens was close to the healthy chickens. These results showed that the TCM compound preparation may inhibit synthesis or secretion of NO and TNF- α , which relieves inflammatory response and injury to internal organs.

Conclusions

The Chinese herbal medicine (CHM) preparation of pulsatilla decoction (MPD) has good curative effects on avian colibacillosis, and the effective rates are higher than 80%. Its mechanism may be that the herbal preparation can inhibit the increase of serum NO and TNF- α concentration caused by *E. coli* infection and resist endotoxin-induced factor, which protects the body from inflammatory injury and controls the development of colibacillosis.

This experiment indicated that CHM (*R. pulsatillae*, *C. phellodendri*, etc.) can affect the expression of cytokines of chicken colibacillosis. It has great significances in defining the mechanism of CHM in preventing and treating animal diseases and promoting the development and utilization of CHM.

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