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RESEARCH ARTICLE

Clinicopathology of Gout in Growing Layers Induced by Avian Nephrotrophic Strains of Infectious Bronchitis Virus

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ABSTRACT

To evaluate the effect of avian nephrotrophic strains of infectious bronchitis virus (IBV) on serum electrolytes, liver and renal function indexes in growing chickens of a layer strain. 120 healthy chickens (35 days old) were randomly divided into 2 groups (virus and control), each group with 60 chickens. The chickens in the virus group and the control group were challenged by 0.2mL artificial nasal drip with virus and sterile saline solution per chicken, respectively. Blood parameters of chicken were evaluated on the 8th, 15th and 22nd day after challenge, respectively. The results showed that Ca, P, K values in the virus group were statistically ($P<0.001$) lower than in the control group on the 22nd day; Mg values in the control group were statistically ($P<0.05$) higher than in the viruses group on the 15th day. UA and CR values in the control group were significantly lower than in the virus group (UA, $P<0.001$; CR, $P<0.05$) on the 15th day. BUN values in the control group were significantly higher than in the virus group on the 22nd day. TP, ALB, GLB and GGT activity in the virus group were significantly higher than in the control group on the 15th day. Our results demonstrated that chickens infected with nephrotrophic strains of IBV can cause liver and kidney damage and the results also provided some evidence for avian visceral gout diagnosis.

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INTRODUCTION

Avian infectious bronchitis (IB) is one of the most significant diseases of the intensive poultry feeding, caused by the IB virus (IBV), one of the most highly infectious and contagious pathogens of chicken worldwide (Lin *et al*., 2012). IBV is a single-stranded positive sense RNA virus, belonging to the genus coronavirus family (Cavanagh, 2003). The primary tissue of avian IBV infection is respiratory tract, and the bird shows symptoms such as gasping, coughing, rales, and nasal discharge (Itoo *et al*., 2014), and the infection causes pathologic change in kidney, oviduct and gonads et al, resulting in nephritis and reduced egg production (Cavanagh, 2005; Worthington *et al*., 2008). The most prevalent strain of IBV was Massachusetts strain; its outbreaks caused visceral gout and nephrosis in commercial young broiler chicken and widely spread throughout the country in a short period. Therefore, it caused huge economic losses (Gaba *et al*., 2010).

Visceral gout was closely related to IBV (Ziegler *et al*., 2002; Poorbaghi *et al*., 2012) which was one of the most common factors resulted in mortality in poultry, hyperuricemia is the most typical clinicopathology of visceral gout. Early studies on visceral gout were focused on conditions of layer house (Ejaz *et al*., 2005; Guo *et al*., 2005; González-Senac *et al*., 2014). Besides, high protein level in diet is also a major etiological agent in production of visceral gout. Other factors like cryptosporidiosis, vitamin A deficiency, water deprivation can also cause gout. Recently, IBV has been recognized as a very important factor of visceral gout (Singh *et al*., 2013), avian IBV was involved in disease outbreak of nephropathogenicity and visceral gout in broiler chickens (Fateh *et al*., 2009). Survivors from visceral gout have damaged kidneys (Wideman, 2006). It was reported that gout IBV virus infected birds can cause kidney damage. For instance, kidney tissue samples collected from the

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field outbreak of visceral gout in commercial broilers in Anand District of Gujarat, India were processed for virus isolation, identification and molecular characterization, and consequently nucleotide sequence analysis found 99% nucleotide sequence identify with avian IBV strain 4/91 (Gaba *et al*., 2010). Chicken infected IBV isolates (IRFIBV32) have wide tissue distribution including respiratory and digestive systems (Boroomand *et al*., 2012). And the serotypes 4/91 of Infectious bronchitis virus in mucosa of tracheas was found and it accounts for 47.9 % by the RT-PCR test in poultry farms in Fars province of Iran (Poorbaghi *et al.,* 2012).

As rare research were based on measuring clinicopathological change evaluation in liver and kidney function of the visceral gout disease, this study tried to infect chicken with IBV and determine the serum electrolytes changes, indicators of liver and kidney function, meanwhile analyze the relationship between infectious IBV and visceral gout. This study aimed to understand the prevalence of visceral gout and evaluate the pathogenesis of chicken.

MATERIALS AND METHODS

Experimental animals: One hundred and twenty 35-d-old healthy hens were chosen and divided into virus and control groups randomly, each group with 60 chickens (3 replicates and 20 chickens per replicate). Chicken in the virus group were inoculated with 0.2mL artificial nasal drip according to the results of LD_{50} embryos (10^{-5}/0.2mL) per chicken, and the control group was treated with 0.2mL sterile saline solution per chicken. Over the entire experimental period, the growers were raised with *ad libitum* consumption of feed prepared according to National Research Council (Table 1).

Samples collection and conservation: Blood samples from 120 chickens were collected separately in the morning of the 8th, 15th and 22nd days (12 blood samples were collected from each group every time) by jugular venipuncture under sterile conditions into non-anticoagulant tubes before feeding. And sera were prepared by hatching blood samples in 37ºC for one hour, and then centrifuged at room temperature (1,800×g, 10 min). The sera were stored at -20°C for biochemical measurements.

Production of pathological sections: Segmental nephridial and hepatic tissue on the 8th, 15th and 22nd days were fixed in formalin, dehydrated by alcohol, transparented in dimethylbenzene, immersed with wax, paraffin-embedded, cut into slices, and then H&E stained, sealed it. Lastly, we observed the results by using optical microscope and took pictures at the same time.

Laboratory analyses: Samples were analyzed at the Clinical Veterinary Laboratory, College of Animal Science and Technology, Jiangxi Agricultural University. Serum electrolyte potassium (K), sodium (Na), chlorine (Cl), magnesium (Mg), calcium (Ca), phosphorus (P) concentration and blood urea nitrogen (BUN), creatinine (CR), uric acid (UA) content were measured by an automatic biochemical analyzer. Liver indicators like total protein (TP), albumin (ALB), globulin (GLB), alanine aminotransferase (ALT), aspartate amino-transferase (AST), alkaline phosphate (ALP) and glutamyl transpeptidase (GGT) were measured by using the method of the International Federation of Clinical Chemistry (IFCC) (Liu *et al*., 2012).

Samples analysis: All data were analyzed with the SPSS 19.0, using variance analysis, Student's t-test and multiple comparisons to proceed significance analysis. Significant differences in frequencies were considered at $P<0.05$.

RESULTS

Serum electrolytes: The measured concentrations showed constant values of electrolytes in the serum of experiment chicken (Table 2); on the 22nd day, Ca, P, K values in the viruses group were extremely statistically ($P<0.001$) far lower than in the control group, P value in the virus group was significantly higher ($P<0.05$) than the control group in the 8th day and the values in 8th day Ca, P, K values in the viruses group were all higher than the control group with P in virus group significantly higher than the control group, in the 15th day the values became similar and in the 22nd day the values in virus group became to very significantly lower ($P<0.05$) than the in the control group. Mg values were statistically higher in the virus group on the 15th day when compared to the control groups. The values of Na and Cl in the control groups showed a steadily increasing trend but in the virus group they changed little.

Kidney function: The serum UA, CR and BUN activity values in chicken were compared between the control group and the virus group, they also showed significant differences (Table 2). UA values in the control group on the 15th day were extremely significantly lower than virus group ($P<0.001$). CR values in the control group on the 15th day were much lower than virus group ($P<0.05$). BUN on the 22nd day, control group were extremely significantly higher than virus group.

Liver functions: The metabolic status of liver function enzymes in serum were statistically different during different time points (Table 3), TP, ALB, GLB values in the virus group were statistically higher than in the control group ($P<0.05$) and GGT values were extremely significantly higher ($P<0.001$) on the 15th day; GLB and GGT values of control group were significantly higher than they in the virus group on the 22th day. ALT, AST and ALP values in control group and virus group showed no significant differences ($P>0.05$), but the values in control group and virus group differed on different time points.

Observation of pathological sections: The liver and kidney tissues of majority infected hens showed significantly pathological changes according to the observation of pathological section pictures on the 8th, 15th and 22nd days (Fig. 1). Compared with the normal groups, the renal section pictures of the virus groups showed renal tubular epithelial circular cell nuclei in the kidney tissue

Table 1: Feed composition and nutrients (%)

Ingredient	Starter	Ingredient	Starter
Corn	66	Premix[1]	3
Wheat bran	5	ME (Mcal/Kg)	144.31
Soybean meal	22	Calcium	0.875
Fish protein concentrate	2	Available phosphorus	0.57
Vegetable dregs	2	Crude protein %	17.37
Limestone	0		

[1]Premix provided per kg of diet:copper, 6.0mg; iron, 40mg; manganese, 70mg; zinc, 50mg; ioidne, 0.30; Selenium, 0.10mg;vitamin A, 10000IU; vitamin D, 2000IU; vitamin B_1, 0.5mg; vitamin B_2 4.0mg; vitamin E, 10mg; choline chloride, 400mg; vitamin $B_{12,}$ 0.01mg; pantothenic acid, 8mg; niacin, 30mg; folic acid, 0.5mg; vitamin K_3, 2mg; vitamin B_6, 2mg.

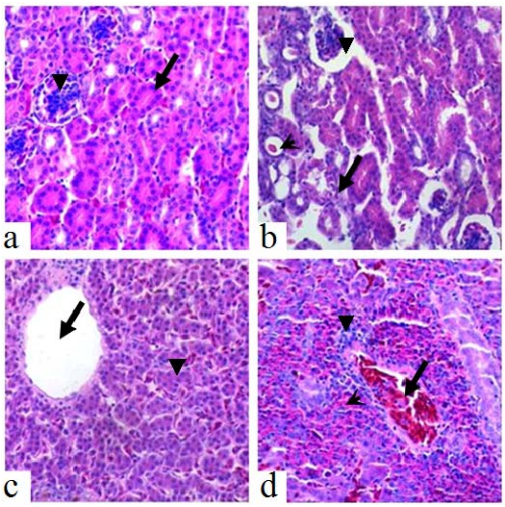

Fig. 1: (HE, 10×40), a: Control group kidney, normal renal tubular (⬇), normal glomerulus (▼); b: Virus group kidney, distorted renal tubular (⬇) enlarged capsular space (▼), urate deposition (▼). c: Control group liver, normal vena centralis (⬇), normal hepatocyte (▼); d: Virus group liver, vascular wall is thickened (⬇), neutrophils aggregation(▼), hemosiderin (▼).

became irregular shape, and separated from basement membrane. Urate deposition can be seen in the renal capsule, tissue were bleeding and glomerulus were vicarious hypertrophy; the hepatic section photos showed that focal or cord-like aggregations of a great number of neutrophils are present in liver; Hepatocytes showed degeneration and necrosis; Hemosiderin showing yellow brown granules was present in cytoplasm of macrophages in liver.

DISCUSSION

Serum electrolytes: The balance of cations, especially Ca, P, K, Na, Cl and Mg in feed stuff and tissues of birds is a prerequisite for body homeostasis maintenance. On the contrary, disorders of immune mechanisms may occur if lack of electrolyte balance in feed and in body (Tykalowski *et al*., 2011). In our study, Ca, P and K levels in control group were significantly higher than virus group on 22nd day, but compared with virus group Mg was significantly lower on 15th day ($P<0.001$) and P was lower on 8th day ($P<0.05$). The reason for this result was probably because IBV infected chicken and could cause kidney damage (Wideman, 2006; Cong *et al*., 2013), leading to its reabsorption of Ca and P decreased. On the contrary, K and Mg cannot be discharged in the kidneys. For this reason, blood Ca and P levels are directly related to the synthesis of parathyroid hormone (PTH) in renal and intestinal. Previous studies have reported that the relationship between Ca, P and vitamin D and concluded that the declining of Ca, P led to a decrease in the level of vitamin D, and then the PTH synthesis reduced, and eventually the feedback of kidneys and intestines to PTH reduced, so the blood Ca and P levels were alerted directly or indirectly (Eraslan *et al*., 2005). On the other hand, Ca, P and Mg dynamic distribution in blood electrolytes showed significant increase in the control group. It is likely because the maintenance of Ca, P and Mg is essential for performing various functions in the process of chicken growth. About 99% of body Ca, 80% of P, and 60-70% of Mg are present in the skeleton (Zelenka, 2012). Ca is a major element in poultry nutrition and higher Ca level is required for bone development, blood-clot formation, and muscle contraction and provides good eggshell quality and dietary, particularly in the finish periods calcium phosphate level increased at the third week of age (Talpur *et al*., 2012). Similar results were also discovered by Zelenka (2012), his study suggested that the remarkably high allometric coefficients of Ca, P and Mg in both slow-growing laying-type cockerels and fast-growing male broiler hybrids were possibly connected with the rapid growth of skeletal tissues which requires an adequate mineral nutrition during this period of growth from hatch until the age of 22 days (Zelenka, 2012). The changes in Na levels were statistically significant high in the control group from the 8th to 15th periods. In avian species, dietary Na levels are an important consideration of bone mineralize-tion in view of skeletal defects and deformities encountered in fast-growing birds (Zduńczyk and Jankowski, 2014). In conclusion, chicken infected with IBV may result in the observed disturbances in blood electrolytes.

Kidney function: Kidneys are considered to be the second target organ damaged by toxins absorbed through the gut. In avian species, kidney function was evaluated by serum concentrations of UA, BUN, and CR. Previous researches have confirmed that significant increases in UA and CR levels were related with nephrotoxicity in broiler chickens. Among them, UA is a kind of prime importance. Like other chickens, 60-80% of nitrogen of broiler chickens is ureoteliced and eliminated in the form of UA. Among many other factors, acid fluctuate in serum was connected with filtration rate which was effected by water consumption and kidney health (Khosravinia *et al*., 2013). In our study, it is found that UA ($P<0.001$) and CR ($P<0.05$) in the virus group on the 15th day was significantly higher than in the control group, suggesting an increase in the level of UA and CR, resulting in the kidney damage, our pathological sections of kidney also proved it (Fig. 1), it is also reported IBV can cause kidney damage, leading to nephritis in layer chicken (Gaba *et al*., 2010). Besides, kidney damage caused by IBV can also effect UA level; in our study, BUN level in the virus group on the 22th day was significantly lower than in the control group, UA is the main end-product of nitrogen metabolism. Its presence in tissues reflects the extent of dietary protein, nutritional state or direction of protein metabolism and reproductive status (Khan *et al*., 2013).

Table 2: The serum electrolytes and kidney function concentrations in chicken in control group (C) and viruses group (V)

Parameter	8d		15d		22d	
	C	V	C	V	C	V
Ca (mmol/L)	1.69±0.13	1.95±0.11	2.14±0.07	1.96±0.22	2.52±0.05^{A}	2.00±0.10^{B}
P (mmol/L)	1.83±0.13^{b}	2.54±0.34^{a}	2.18±0.05	2.66±0.35	2.34±0.11^{A}	1.64±0.06^{B}
K (mmol/L)	5.13±0.85	7.08±1.19	5.53±0.35	5.12±0.18	7.05±0.50^{A}	4.37±0.32^{B}
Na (mmol/L)	138.50±5.08	135.50±3.28	150.33±0.92	136.66±6.70	146.50±0.67	137.50±6.03
Cl (mmol/L)	105.17±4.21	101.83±3.63	111.50±1.20	102.67±5.85	109.33±1.26	104.50±4.67
Mg (mmol/L)	0.96±0.06	1.02±0.04	1.11±0.04^{b}	1.40±0.08^{a}	1.20±0.08	1.03±0.03
UA (μmol /L)	285.5±31.2	285.3±53.2	287.6±35.7^{B}	601.3±41.4^{A}	265.9±38.1	202.5±26.4
CR (μmol /L)	23.02±2.17	23.48±2.38	21.72±1.95^{b}	46.48±8.25^{a}	20.55±1.35	17.85±1.56
BUN (mmol/L)	0.847±0.10	0.97±0.13	0.64±0.04	0.96±0.22	0.84±0.10^{a}	0.56±0.061^{b}

Mean±SE bearing different superscript letters within the same row between C and V at the same day (a, b: P<0.05; A, B: P<0.001).

Table 3: The liver functions concentrations in chicken in control group (C) and viruses group (V)

Parameter	8d		15d		22d	
	C	V	C	V	C	V
TP (g/L)	28.12±1.22	29.82±1.63	30.92±1.51^{b}	43.38±4.26^{a}	38.83±1.63	33.87±2.13
ALB (g/L)	11.95±0.54	11.58±0.68	11.82±0.51^{b}	14.93±1.10^{a}	12.67±0.69	12.43±0.89
GLB (g/L)	16.17±0.73	18.23±1.06	19.10±1.22^{b}	28.45±3.23^{a}	26.17±1.39^{a}	21.43±1.49^{b}
ALT (U/L)	3.40±0.51	4.33±1.02	2.17±0.60	2.17±0.60	3.83±0.31	3.17±0.17
AST (U/L)	189.6±5.5	209.8±20.5	197.0±17.8	234.5±10.1	203.2±6.0	175.0±11.7
ALP (U/L)	900.5±88.2	654.3±72.6	912.8±178.3	755.1±166.9	654.0±134.1	906.8±194.4
GGT (U/L)	25.00±1.88	25.50±2.86	25.33±2.09^{b}	37.00±2.08^{a}	32.50±3.13^{a}	22.50±1.57^{b}

Mean±SE bearing different superscript letters within the same row between C and V at the same day (a, b: P<0.05; A, B: P<0.001).

Liver functions: Liver is the primary organ collecting chemicals absorbed in the gut and also as the major detoxification organ; its function always harmed by the toxic properties of absorbed from agents, the leaking of cellular enzymes into the plasma is a noticeable indication of hepatocytes damage (Khosravinia *et al*., 2013). IBV infection was considered closely related with liver damage. Abdel-Moneim *et al*. (2006) reported finding severe congestion of liver, spleen and lungs in birds died after IBV experimental infection. In our study, the liver TP, ALB and GLB activity in the virus group were significantly higher than in the control group on the 15th day (P<0.05), we considered that TP, ALB and GLB increasing is due to water loss in blood and may combined with symptoms as height of dehydration, diarrhea, vomiting and high fever et al, our experiment result is consistent with that reported by Chacon *et al*. (2014), most chicken showed symptom of diarrhea, especially on 15th day in virus group. The results were in accordance with the report of Singh *et al*. (2013). In the natural cases of gout, GLB was found increased because of dehydration and emaciation of the birds (Singh *et al*., 2013). On the other hand, Na loss results in the elevated protein concentration in serum. Our results confirmed that Na values in the virus group were lower than the control group on the 8th and 15th day (Table 2). The last reason was that IBV could hurt kidney severely, adrenocortical hypofunction leading to elevated protein concentration (Worthington *et al*., 2008). Moreover, in our study, AST values in the control group compared to virus group on the 8th, 15th high and 22nd day low, while ALP values were going in the opposite direction, but compared with the values in the control group and virus group in different time, respectively, they have difference, constant exposure of the chickens. GGT values in the virus group were higher than in the control group on the 15th day (P<0.05), and on the 22nd day, significantly lower than in the control group (P<0.05). The reasons of our result probably because of IBV infection can cause liver damage, and the fig 1 had showed the same results. When the plasma membrane of a hepatocyte injured, a variety of cytosolic enzymes will be released into the circulation, thus causing increased enzyme levels in the serum, AST and ALP value are useful quantitative markers to estimate the extent and type of hepatocellular damage in chicken (Khosravinia *et al*., 2013). Elevated plasma ALP might be due to acute hepatocellular damage and renal damage (Singh *et al*., 2013).On the other hand, IBV could hurt kidney, detecting the values of ALP and GGT can reflect the functional integrity of these portions of the kidney (Alasia, 2010). Local ALP concentrations reflect the capability of host defense against endotoxin in the kidney (Pickkers *et al*., 2012). In our study, experiment animals showed severe kidney damage and the serum indicators change reflected the liver damage after nephrotrophic strains of IBV infection.

Author's contribution: XQG conceived and designed the experiment. GFD and JK executed the experiment. CZ and GH analyzed the sera samples while HC and PL analyzed the tissue samples. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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