Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.010

RESEARCH ARTICLE

Changes of Antioxidant Function and the mRNA Expression Levels of Xanthine Oxidase in Primary Chick Kidney Cell Culture Caused by Nephropathogenic Infectious Bronchitis Virus Infection

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ARTICLE HISTORY (16-165)

Received: June 30, 2016
Revised: September 07, 2017
Accepted: September 08, 2017
Published online: November 21, 2017

Key words:
Antioxidant activity
Avian gout
Chick kidney cells
Nephropathogenic infectious bronchitis virus
Xanthine oxidase

ABSTRACT

To investigate the variations of morphological, antioxidant function and xanthine oxidase (XOD) mRNA transcription of chick kidney (CK) cells underlying the nephropathogenic infectious bronchitis virus (NIBV) infection. Following NIBV infection there was a time-dependent increase in lactate dehydrogenase (LDH) both in cells and medium. Meanwhile, NIBV infection of CK cells resulted in dysregulation of antioxidant function of CK cells, as superoxide dismutase (SOD) activity was decreased and malonaldehyde (MDA) concentration was increased in cells while there was elevation of SOD activity in medium. Furthermore, the xanthine oxidase (XOD) activity and the uric acid (UA) concentration of infected group were significantly increased both in cell and medium, when cell XOD mRNA transcription showed height in infected group than in the control group. Taken together, our results indicated the metabolic disorder of XOD was an important pathological mechanism of NIBV infection; the results partially elucidate the potential mechanisms of hyperuricemia induced by NIBV.

To Cite This Article: Liu W, Liu P, Wang T, Lin H, Huang Q, Deng G, Gao X, Liu G, Guo X, Zhang C, Cao H and Hu G, 2018. Changes of antioxidant function and the mRNA expression levels of xanthine oxidase in primary chick kidney cell culture caused by nephropathogenic infectious bronchitis virus infection. Pak Vet J, 38(1): 51-55. http://dx.doi.org/10.29261/pakvetj/2018.010

INTRODUCTION

Infectious bronchitis virus (IBV), the pathogenic agent of infectious bronchitis (IB), has been effectively controlled by the wide use of vaccines but still caused a major economic lose in poultry production since first reported in 1931 (Lin *et al*., 2012; Itoo *et al*., 2014; Awad *et al*., 2016). The strains of nephropathogenic infectious bronchitis virus (NIBV) was one of the most prevalent virus that exhibited a severe renal tropism and might cause very high mortality in the poultry industry (Ziegler *et al*., 2002; Gaba *et al*., 2010). For instance, chicken infected NIBV could cause the kidney lesions and turning grey due to urate crystals deposition, then result in visceral gout (Lee *et al*., 2004; Liu *et al*., 2015). Lots of studies have found the significant role of Xanthine Oxidase (XOD) in human and avian gout disease (Ernst and Fravel, 2009; Grassi *et al*., 2013).

XOD is an enzyme which exists in a great variety of organisms and it could oxidize hypoxanthine to xanthine and oxidize xanthine to urate. In our previous study, we found that NIBV infection can increase the expression and activity of XOD in serum, resulting in a decrease in antioxidant activity in poultry's body (Hou *et al*., 2012). Earlier study proved that XOD play important role in urate homeostasis and become a therapy target of drugs in gout or hyperuricemia of human diseases (Nuki and Simkin, 2006). In our previous study, we found that NIBV infection can increase the expression and activity of XOD in serum, resulting in a decrease in antioxidant activity in poultry's body (Lin *et al*., 2015). In addition, superoxide dismutase (SOD) is an important ingredient of body' anti-oxidative enzyme system, it is considered as the first line of defense of superoxide radial and against toxicity from ROS (Sotootero *et al*., 2000; Samuel *et al*., 2011). The over production of ROS can result in oxidative injury by lipid pre-oxidation as well as mitochondrial dysfunction (Surendran and Rajasankar, 2010). Some previous studies documented

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that total XOD and UA, and ROS were founded significantly up-regulated in gout orhyperuricemia (Nuki and Simkin, 2006; Agarwal and Banerjee, 2011).

The dynamic characteristic of XOD and antioxidant ability of CK cells infected by NIBV in previous studies has not been reported previously. Therefore, our study used CK cell culture model to investigate the changes of mRNA expression of XOD, XOD and UA and antioxidant indexes in CK cells. The results will help us to clarify XOD dynamic characteristic and antioxidant ability of CK cells post infected by NIBV. The mechanism of this study can help us put forward a comprehensive prevention and control measures of avian gout.

MATERIALS AND METHODS

Experimental animals: The use of all animals in this experiment were carried out according to the rule of Committee of Animal Welfare, Agricultural University of Jiangxi, China.

CK cell cultures: Kidneys were isolated and washed with sterile phosphate buffer saline (PBS) from 14-day-old chicks, minced and disaggregated in Type I collagen enzyme solution. After enzyme digestion, cells were washed with PBS, centrifuged and resuspended in Dulbecco's modification of Eagle's medium (DMEM) which containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were placed in 6-well and/or 96-well tissue culture plates and maintained at 37°C with 5% CO_2.

IBV passage in CK cells: Cells were incubated for 72hr, and then divided into two groups (infected and control group). Cells in the infected group were treated with 0.2 ml of 50% tissue culture infectious dose of NIBV ($10^{-3.583}$/0.2 ml, date not shown) for 1.5hr, while control group was treated with 0.2 ml of PBS.DMEM containing 3% fetal bovine serum (FBS) was applied after washing three times with PBS in infected and control group, respectively.

Measurement of infected cell indices: The samples were harvested at twelve-hourly intervals post infection. The ability of CK cells was evaluated by MTT assay. The CK cells monolayers were infected with 0.2mL of 50% Tissue Culture Infectious Dose ($TCID_{50}$) of NIBV at 37°C for 1.5 h. At 12, 24, 36, 48, 60, 72 hrs post-infection (hpi), cells and culture medium were collected. Cells were washed three times with PBS, isolated by trypsin and then stored at -80°C. The activities of XOD, LDH, SOD and the concentrations of MDA and UA were measured using quantitative determination kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Six replicates were used for each time.

Total cellular RNA isolation and cDNA synthesis: Under low temperature position, total cellular RNA in the two groups (infected group and control group) were extracted using RNAiso Plus reagent (Takara, Japan) strict accordance with manufacturer's protocol, respectively. The RNA was diluted in nuclease-free water, and quantitated by UV spectrophotometry at 280nm 260nm. The integrity of isolating total RNA was confirmed by 0.8% agarose gel electrophoresis, and cDNA synthesis (PrimeScript™ II 1st Strand cDNA Synthesis Kit, 6210A, Takara, Japan) was prepared as Lin's described (Lin *et al*., 2015).

Real-time quantitative PCR (RT-qPCR): Real-time PCR was carried out using ABI Prism®7500 Sequence Detection System according to the instruction of Premix EX TapTM II SYBR q-PCR Kit. Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as normalized to quantify XOD gene transcription. According to the published sequences of XOD (GenBank, BG713540.1) and avian GAPDH (GenBank, NM_204305.1), the primers were designed as described in table 1. The PCR system included 10 uL SYBR Premix Ex Tap II (2×), 0.8 uL 10 pmol/ml forward and reverse primers respectively, 0.2 ul ROX referencing dye (50×), 2 ul cDNA templates, and 6 uL water. The PCR was performed at 95°C for 30 s, and 40 cycles of 95°C for 30 s, 60°C for 30s. Amplification curves and melt curves were analyzed with the ABIPrism®7500 Sequence Detection Software and for each PCR system Ct values were obtained.

Statistical analysis: The data were pre-processed by excel and analyzed by SPSS 17.0 statistical software. The results were presented as mean±SD and $P<0.05$ ($P<0.01$) was considered as data statistically (highly) significant.

RESULTS

Cell-toxicity of NIBV: We observed CPE at 24 hpi and more than half of the cells died, fell off and floated in the medium at 72 hpi in the infected group whereas no obvious changes in the control group. The variability of CK cells in the infected group was significantly reduced at 24, 36 and 72 hpi in the infected group compared with the control group ($P<0.05$). The biggest difference between infected group and control group was observed at 72 hpi (Fig. 1).

Table 1: Primes used in this study

Genes	Accession no.	Sequence(5'-3')	Productsize/bp
XOD	BG713540.1	F:AAGTGGCACGACAAGCAGTACA R:TACTCCAACTTTCTCGAGGACGAT	461bp
GAPDH	NM_204305.1	F:GGTGCTAAGCGTGTTATCATCTCA R:CATGGTTGACACCCATCACAA	63bp

F=forward; R=reverse; GAPDH=glyceraldehyde 3-phosphate dehydrogenase.

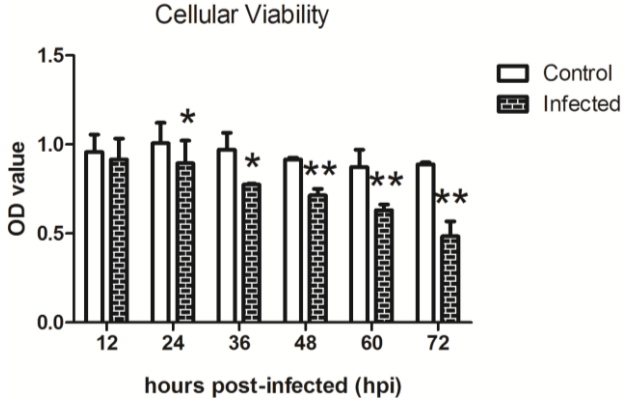

Fig. 1: The cellular viability in cell and medium of CK cells at different groups with difference hours post infected, N=6 per group at each time, ∗P<0.05 and ∗∗P<0.01.

Activities of LDH, XOD and SOD: Fig. 2-3 showed the activities of LDH, XOD and SOD both in cell and medium in infected and control group at 12, 24, 36, 48, 60 and 72 hpi, individually. In the infected group, the activity of LDH in cells significantly increased (P<0.05) at 36 hpi and extremely significantly increased (P<0.01) at 60 and 72 hpi than that in control group. Similarly, the activity of LDH in infected group in medium extremely significantly increased (P<0.01) since 36 hpi compared to the control group (Fig. 2AB). XOD activity in cell and medium were significantly increased (P<0.01) at 36 and 48hpi than in control group and it was also extremely significant increased (P<0.01) at 60 and 72 hpi in medium (Fig. 3AB). In the infected group, SOD activity in cell was extremely significant decreased at 72 hpi (P<0.01) compared to control group. While, it was extremely significantly increased (P<0.01) since 24hpi than it in control group in medium (Fig. 2CD).

UA and MDA Contents: The contents of UA and MDA in cell and medium were showed in Fig. 4, respectively. UA in cell (Fig. 4A) and medium (Fig. 4B) were extremely significant higher than control group since 48 hpi (P<0.01). MDA concentration in infected group was observed statistically higher than that in the control group since 48hpi (P<0.01; Fig. 4C) in cell, no statistical difference in medium (P>0.05; Fig. 4D).

Transcription profile of xanthine oxidase: The transcription levels of cell XOD mRNA at 24, 48 and 72 hpi were showed in Fig. 5, results indicated that levels of XOD transcription in infected group were extremely significant increase than in the control group (P<0.01).

DISCUSSION

Previous study documented that epithelial cell was the primary site of replication of avian NIBV, the increased level of virion in CK cells lead to destructions of the cellular structure and composition, which caused the cell membranes damage (Sun *et al*., 2014). Interestingly, the characteristic syncytia and plaque was observed in CEK cells after transfected by IBV Sczy3 virus (Wei *et al*., 2015) and intracytoplasmic brownish colouration was observed by immunoperoxidase in primary chick embryo chorioallontoic membrane cells after infection by IBV (Ghetas *et al*., 2015). The results are similar to our study: post infected by NIBV, the cytopathic effect was found and the cell viability of CK cells was significant reduced. And discovered the cell LDH activity in the virus group was significantly increased in comparison with control group in our study. As an intracytoplasmic enzyme, LDH is released if the plasma membrane is damaged and it is used to assess cytotoxicity reaction (Bopp and Lettieri, 2008). Hence, this phenomenon suggested that NIBV inhibits cell proliferation and damages the membrane and biological structures of CK cells.

The result of this study showed that antioxidant system of CK cells has changed significantly after IBV infection. The descending of SOD activity and the elevation of MDA concentration in cell were found in infected group which suggest that NIBV infection induce the increase of ROS and the radical formation might have occurred through oxidative damage of lipid since 48hpi. The equilibrium between the ambient levels of the superoxide anion and cellular antioxidant capacity provided by SOD activity was broken at 72hpi. SOD are the free radical scavenger which can remove body's free radical, it have anti-oxidative stress function to maintaining body's in a healthy balance between oxidants and antioxidants (Han *et al*., 2014) MDA is a biomarker of lipid per-oxidation and its content represent the level of body's oxidative stress (Demirbilek *et al*., 2011). Therefore, determination of MDA and SOD can reflect the capability of elimination of free radicals in the body and the extent of CK cells injury during IBV infection (Zhou *et al*., 2006). Earlier vivo studies has proved that the kidney was vulnerable attacked by ROS (Hiromi *et al*., 2014) and NIBV infection could cause serum SOD activity of growing layers increased (Lin *et al*., 2015). The results indicated that abnormal generation of ROS might be a pathogenesis of damages in NIBV injury of CK cells, but the detailed pathogenic mechanism need to have further studies and discussions.

Likewise, we found that cell cultures in infected group sustained the higher levels of XOD mRNA transcript in cell compared with that in control group at 24, 48, 72 hpi (P<0.01) and this result was correlated with the XOD activities both in cell and medium between infected and control groups, and the UA concentration in cell and medium were elevated. Due to UA is the final product of purine nucleotide catabolism in poultry (Marinello *et al*., 2000; Ejaz *et al*., 2008); XOD is closely associated with hyperuricemia (Boban *et al*., 2014; Lemos Lima *et al*., 2015). The study (Hiromi *et al*., 2014) shown

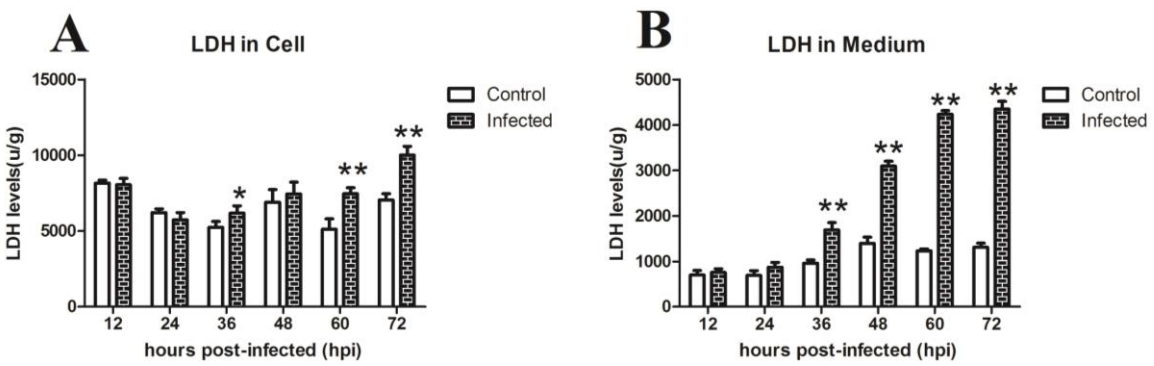

Fig. 2: The levels of LDH in cell and medium of CK cells at different groups with difference hours post infected (U/g), N=6 per group at each time, *P<0.05 and **P<0.01.

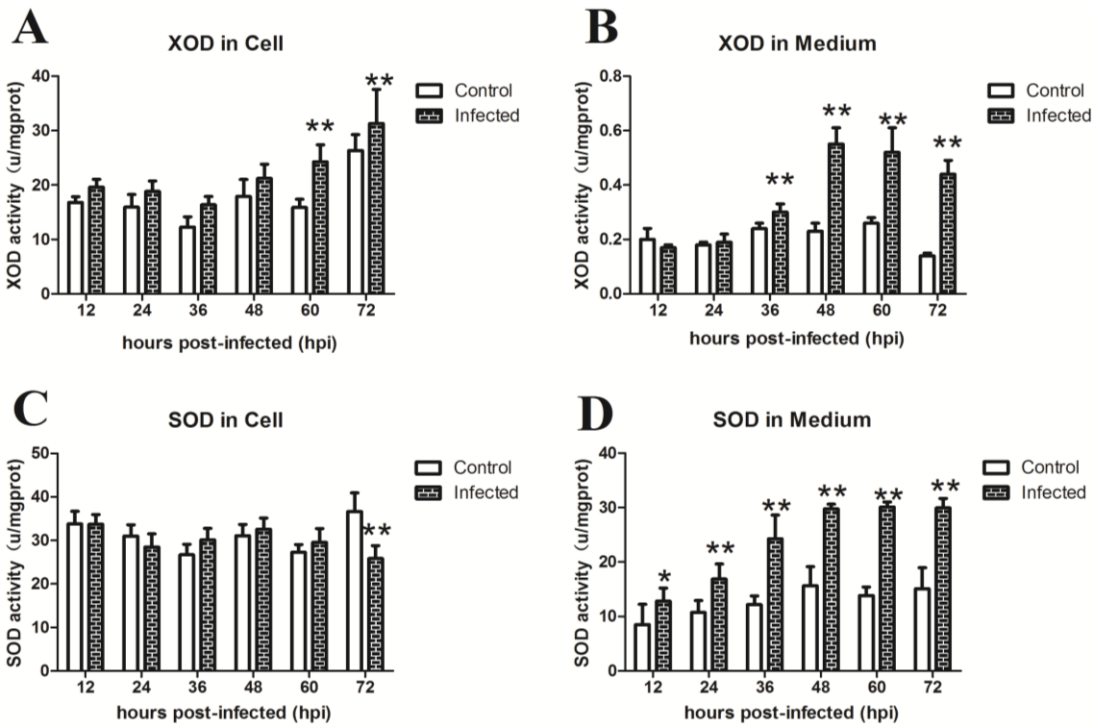

Fig. 3: The activities of XOD and SOD in cell and medium of CK cells at different groups with difference hours post infected (U/mgprot), N=6 per group at each time, ∗P<0.05 and ∗∗P<0.01.

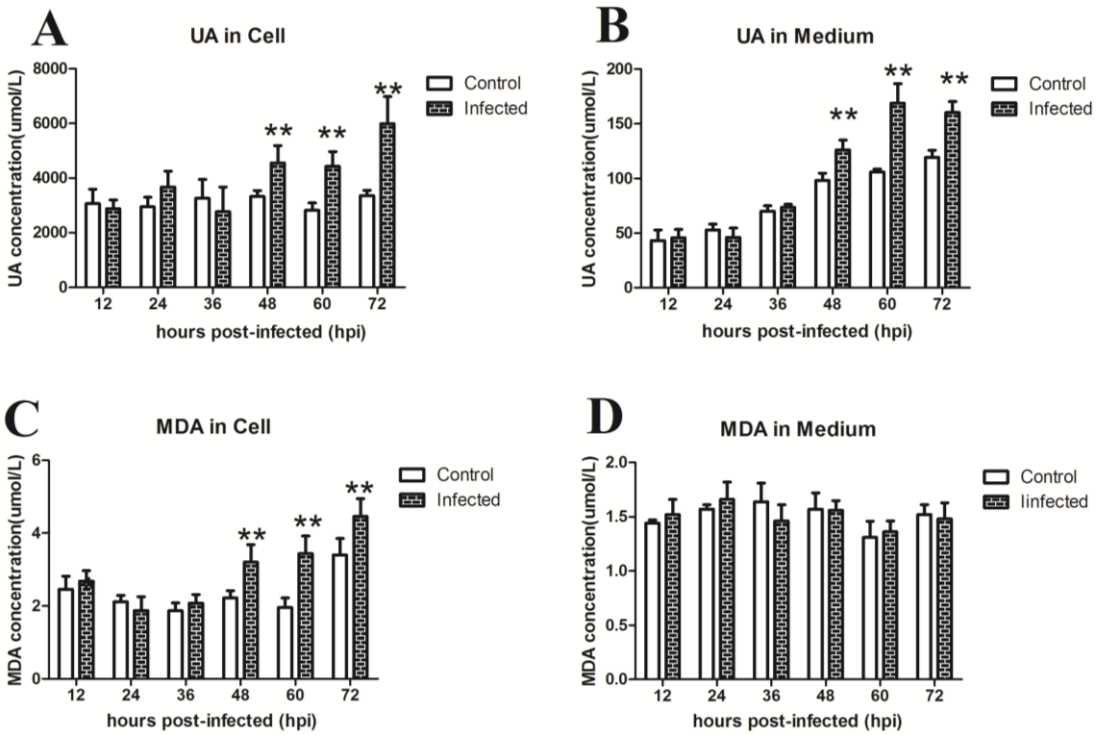

Fig. 4: The concentrations of UA and MDA in cell and medium of CK cells at different groups with difference hours post infected (umol/L), N=6 per group at each time, ∗P<0.05 and ∗∗P<0.01.

that the highest relative expression of cytokines correlated with the infection degree of IBV and IBV infection could provoke renal inflammatory responses (Okino *et al.*, 2014). Our previous study found that NIBV infection induced both renal XOD transcription and its serum activity increase in growing layers, the serum concentration of UA was statistically increased (Lin *et al.*, 2015). Earlier study showed that NIBV triggers UA salt precipitation by invading the tubule cell to initiate kidney tissue damage (Kim *et al.*, 2006; Okino *et al.*, 2014). Results of this present study confirm the increase of XOD transcription and its activity may be due to the NIBV infection induced the increase of pro-inflammatory cytokines, the over production of UA induce the damage of the CK cells. However, further research is required to execute to illustrate the accurate mechanism of the increase of XOD transcription and activities induced by NIBV infection.

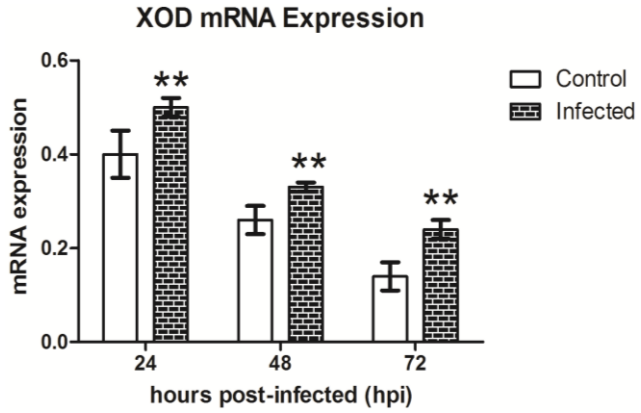

Fig. 5: The levels of XOD mRNA transcript in the chick kidney cells of control group and infected group, N=6 per group at each time, ∗P<0.05 and ∗∗P<0.01.

Conclusions: Based on our results, exposure to NIBV caused increasing level of lipid per-oxidation in CK cells by altering antioxidant enzymes' activities, increasing XOD activity and its gene transcription which increased the cellular contents of UA, and induced CK cell damage.

Acknowledgements: This project was supported by National Natural Science Foundation of China awarded to Xiaoquan Guo (No. 31260627, 30860212), Jiangxi Young scientists target training program to Xiaoquan Guo (No. 20122BCB23022) and The Technology R&D Program of Jiangxi Province awarded to Xiaoquan Guo (No. 2010BNB00501).

Authors contribution: XG and GH conceived and designed the experiments. WL and PL collected the samples and executed the experiments. WL, PL, TW and HL analyzed the sera data. QH, GD, XG, GL, CZ and HC processed the tissue samples and analyzed the tissue samples. All authors interpreted the data, critically revised the manuscript for important intellectual content and approved the final version.

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