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RESEARCH ARTICLE

Lipopolysaccharide Upregulating P-Glycoprotein in Small Intestine Alters Pharmacokinetics of Orally Administered Enrofloxacin in Broilers

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Lipopolysaccharide (LPS) modulates P-glycoprotein (P-gp) expression alters pharmacokinetics of its substrates in rodents. However, its influence on P-gp expression in chickens still poorly characterized. This study evaluated LPS effects on P-gp expression in liver, small intestine and kidney by immunohistochemistry and pharmacokinetics of enrofloxacin in LPS-treated and non-treated broilers. The highest immunoreactivity of P-gp was found in the apical membrane of enterocytes, proximal tubules of kidney and biliary canalicular membranes of hepatocytes at 2 to 6 h after LPS administration. The oral enrofloxacin significantly decreased $AUC_{0-\infty}$ (LPS: 19.27±0.65 *vs* control: 23.64±1.27 μg•ml^{-1}•h^{-1}, P<0.05), C_{max} (LPS: 0.83±0.07 *vs* control: 1.57±0.11 μg•ml^{-1}, P<0.01) and K_a (LPS: 0.24±0.10 *vs* control: 1.67±0.11 h^{-1}, P<0.05), and increased T_{max} (LPS: 7.79±1.29 *vs* control: 1.94±0.10 h, P<0.05) and $t_{1/2}$a (LPS: 4.03±1.02 *vs* control: 0.41±0.03 h, P<0.05) in LPS-treated broilers as compared to control. Verapamil significantly attenuated LPS effects on the pharmacokinetics of oral enrofloxacin. However, IV enrofloxacin didn't show significant changes in key parameters among three groups. The results implied that LPS reduced $AUC_{0-\infty}$ of oral enrofloxacin through up-regulating P-gp in small intestine. This study suggests that dosages should be adjusted initially to achieve therapeutic concentration at the site of action during LPS infection in broilers.

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INTRODUCTION

Enrofloxacin is a frequently used fluoroquinolone antimicrobial agent in poultry for the treatment of infections caused by *Escherichia coli* (*E. coli*) in China. It is also effective against some rickettsial organisms, mycoplasmas and gram-positive bacteria. (Andersson and MacGowan, 2003; Ivanov and Budanov, 2006). The descriptive pharmacokinetic behavior of enrofloxacin has been determined in various healthy animals like sheep, (Otero *et al.*, 2009) goats, (Rao *et al.*, 2002) pigs, (Wang *et al.*, 2016) rabbits, (Araneda *et al.*, 2013) rheas (de Lucas *et al.*, 2008) and chicken (Guo *et al.*, 2010). P-glycoprotien (P-gp), an important member of the ABC transporters, has an essential function in the absorption, distribution and elimination of drugs from small intestine, liver and kidney (Funakoshi *et al.*, 2003). It has been demonstrated that enrofloxacin is a substrate of P-gp (Kato *et al.*, 2010; Tomita *et al.*, 2010; Guo *et al.*, 2013). However, the pharmacokinetics of enrofloxacin has been rarely noticed in broilers after modulating P-gp by LPS.

LPS, an endotoxin found in the outer membrane of Gram-negative bacteria, brings out significant influence on the immune system of animals (Dixon and Darveau, 2005). LPS administration is a common model to investigate the effects of inflammatory conditions in various animal species because it can stimulate a wide spectrum of biological activities. (Dixon and Darveau, 2005; Yang and Lee, 2009). It has been well established that the disease conditions cause variation in P-gp expression in animals (Barnes, 2001; Moriguchi *et al.*, 2007; Kato *et al.*, 2010; Masereeuw and Russel, 2012). Thus, the bioavailability and clearance of various P-gp substrate drugs are prone to modification by inflammatory stimuli causing therapeutic failure or adverse drug reactions (Langmann *et al.*, 2003). It has been reported that *E. coli* LPS altered the pharmacokinetics of fluoroquinolone (FQs) in goats and pigs (Post *et al.*, 2003;

Ismail, 2006). However, little is known regarding the linkage of P-gp expression and the disposition of oral enrofloxacin in broilers, particularly after LPS-induced inflammation. Considering the frequent use of enrofloxacin in poultry, we investigated whether LPS affects enrofloxacin absorption by regulating P-gp expression in broilers or not. To our knowledge, this is the first report showing the influence of LPS on P-gp expression in the small intestine, kidney and liver. Our data implied that LPS up-regulated P-gp expression in the small intestine of broilers in a different way than that of rodents and humans. This ultimately altered the pharmacokinetic parameters of oral enrofloxacin in broilers.

MATERIALS AND METHODS

Animals and reagents: The male and female, a day old, Ross 308 broilers were selected randomly and bought from a nearby poultry farm (Lishui, Jiangsu Province, China) and were accepted by Nanjing Agricultural University Animal Care and Use Committee. The birds were given feed according to commercial standards (without antibiotics and additives), kept at 25^0C and water was provided *ad libitum*. There was no colibacillosis among the broilers, aged 5-weeks, at the outset of the experiment. The antibody, mouse monoclonal anti-P-gp (C219) prescribed for immunohistochemistry (IHC), was bought from Covance (Princeton, New Jersey, USA). Rabbit anti-mouse IgG-horseradish peroxidase (HRP) was purchased from Boster (Wuhan, Hubei, China). *E. coli* strain (O55:B5) LPS was procured from Sigma-Aldrich (St. Louis, MO, USA). The Enrofloxacin and Verapamil were bought from China Institute of Veterinary Drug Control (Beijing China) and Sigma (St. Louis, MO, USA) respectively. The rest of the reagent grade compounds were obtained locally.

Immunohistochemisty: Immunohistochemistry was employed for the localisation of P-gp expression. For this, twenty aged 5-week, healthy broilers, were chosen randomly into four groups (5-broilers/group). Group-1 did not receive LPS treatment and served as a control, whereas Groups-2, 3 and 4 were treated with LPS (5mg/kg) intramuscularly. Following LPS treatment, broilers were euthanized by carbon dioxide asphyxiation machine and tissue samples of kidney, liver, jejunum and ileum were collected at 2, 6 and 12 h from different groups. Immunohistochemical staining was done as reported (Guo *et al.*, 2013). The three semi quantitative measurements, for P-gp staining were done under a light microscope (BX45-DP72; OLYMPUS, Tokyo, Japan) supported with Plan Apo objectives linked with a CCD camera (U-TV0.63XC; OLYMPUS, Tokyo, Japan) and the positive staining was assessed by estimating the area sum and integrated optical density by Image-Pro Plus 4.1 software, as explained earlier (Guo *et al.*, 2014).

Pharmacokinetic analysis of enrofloxacin in broilers: an experimental design: A total of thirty healthy broilers, 5-weeks old, were randomly divided in six groups (5-broilers/group). Group-1 and Group-2 were administered only enrofloxacin at a single dose of 10mg/kg BW orally and intravenously, respectively, and served as controls. Group-3 and Group-4 received LPS (5mg/kg BW) intramuscularly. After two hrs of LPS administration, single dose of enrofloxacin 10mg/kg BW (via crop tube gavage) was given orally and intravenously respectively. Groups-5 and 6, 30 minutes before oral and intravenous enrofloxacin (10mg/kg BW) administration, verapamil (15mg/kg BW) was administered orally following 2 h of LPS challenge. The blood samples (3 ml) from each bird of all groups were gathered from right brachial vein before and after the administration of enrofloxacin at 5, 10, 15, 20, 30, 45 minutes and 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h in plastic tubes, and were rapidly centrifuged at 4000 g for 10 min. Then, plasma samples were stored at -20°C until further analysis.

Detection of enrofloxacin in plasma by HPLC: Agilent 1200 high-performance liquid chromatography (HPLC) system was used to examine the plasma concentrations of enrofloxacin, as explained previously (Guo *et al.*, 2014). The peak area measurement quantified Enrofloxacin. Before evaluation of experimental samples, the quantification and extraction of enrofloxacin in plasma was carried out by complete validation of analytical procedures. The determination coefficients for enrofloxacin exhibited by linear regression were >0.99. From plasma, the recoveries were >82%. In chicken plasma, the limit of quantification (LOQ) and limit of detection (LOD) of enrofloxacin were 0.05 and 0.02 µg/mL respectively. The coefficients of variation of inter and intraday were under 11%.

Pharmacokinetic analysis: The 3p97 practical pharmacokinetic software, a compartmental model method (Version 97, Chinese Pharmacologic Association, Beijing, China) was employed to get pharmacokinetic calculations of each individual set of data. Akaike′s Information Criterion was used to find the best fit.

RESULTS

Clinical assessment in LPS-treated broilers: All broilers showed clinical signs like depression, lethargic, loss of appetite, fever, watery whitish diarrhea till 12 h after LPS administration. Afterwards these signs were diminished and broilers started taking food and water slowly and gradually. However, of note, none of the broiler was fully recovered till 24 h following LPS treatment. Five broilers died after collecting blood samples at different times, possibly because of the LPS-induced toxicity. Thus, 5-broilers from each group were randomly selected for analyzing pharmacokinetics.

Localisation and P-gp expression level in liver, kidney and small intestine of broilers: Immunohistochemistry was employed to examine P-gp expression in liver, kidney, jejunum and ileum of broilers (5weeks-old) at 2, 6 and 12 h after treatment with LPS. No immunostaining of P-gp was noticed in the negative controls (incubation without P-gp antibody) (data not mentioned). Positive staining was noticed in jejunum, ileum, liver and kidney in non-treated as well as LPS-treated broilers. Obvious P-gp immunoreactivity was visualized in the biliary canalicular membrane of hepatocytes in non-treated

broilers. Nevertheless, in LPS-treated broilers, P-gp was scattered internally inside the cytoplasm far from the biliary canalicular membrane in liver. The staining was most intense at 2 h in the liver, and then declined gradually in the liver of LPS-treated broilers (Fig. 1A). In kidney, the immunostaining was viewed in the apical membranes of proximal tubule cells of non-treated broilers, but P-gp was disseminated extensively throughout the cytoplasm in LPS-treated broilers. However, the strongest staining was observed at 2 h in the kidney in LPS-treated broilers (Fig. 2A). In non-treated broilers, P-gp immunoreactivity was detected in the apical membrane of the intestinal epithelium of jejunum and ileum, whereas in LPS-treated broilers, positive staining of P-gp remained confined in the membrane of intestinal epithelium but the intensity was markedly increased. The highest immunoreactivity was perceived at 2 h in the ileum while at 6 h in jejunum in LPS-treated broilers (Fig. 3A and 4A).

We further semi-quantified the P-gp expression level of liver, kidney, jejunum and ileum from non-treated and LPS-treated broilers using Image-ProPlus 4.1 software. In liver and kidney, the total P-gp levels were enhanced (Fig. 1B and 2B) but the level in the biliary canalicular membrane and apical membrane of proximal tubule cells were not altered ($P>0.05$) via IOD and positive area estimates. LPS treatment ($P<0.05$) increased P-gp staining in the ileum and jejunum as compared to non-treated broilers (Fig. 3B and 4B).

Pharmacokinetic evaluation of enrofloxacin after LPS administration: The pharmacokinetic parameters of enrofloxacin by oral and intravenous administration were presented in Tables 1 and 2, respectively. The plasma concentration-time curves of enrofloxacin were showed in Fig. 5 and 6. Pharmacokinetic profile of enrofloxacin showed in Table 1 revealed that LPS treatment significantly decreased $AUC_{0-\infty}$ (LPS: 19.27±0.65 *vs* control: 23.64±1.27 $\mu g \cdot ml^{-1} \cdot h^{-1}$, $P<0.05$), C_{max} (LPS: 0.83±0.07 *vs* control: 1.57±0.11 $\mu g \cdot ml^{-1}$, $P<0.01$) and K_a (LPS: 0.24±0.10 *vs* control: 1.67±0.11 h^{-1}, $P<0.05$), in addition to that increased T_{max} (LPS: 7.79±1.29 *vs* control: 1.94±0.10 h, $P<0.05$) and $t_{1/2}a$ (LPS: 4.03±1.02 *vs* control: 0.41±0.03 h, $P<0.05$) of enrofloxacin orally administrated in broilers. However, the key pharmacokinetic parameters of IV administered enrofloxacin were not significantly altered by LPS treatment (Table 2).

To further investigate whether LPS modulates the pharmacokinetics of oral enrofloxacin by influencing intestinal P-gp or not, the pharmacokinetics of enrofloxacin was also studied following verapamil (a potent and selective P-gp inhibitor) in LPS-treated broilers. The results (Table 1) showed that verapamil treatment significantly increased C_{max} (1.83-fold), Ka (6.92-fold) and $AUC_{0-\infty}$ (1.27-fold) and decreased T_{max} ($P<0.01$) and $t_{1/2}a$ ($P<0.01$) of oral enrofloxacin when P-gp was inhibited by verapamil. However, the key pharmacokinetic parameters of IV administered enrofloxacin were not significantly altered by verapamil in each group (Table 2). The data indicated that LPS up-regulated P-gp, altering the absorption of orally administered enrofloxacin in broilers.

Table 1: Pharmacokinetic parameters of enrofloxacin orally administered alone and co-administrated with verapamil in non-treated and LPS-treated broilers (mean±SEM, n=5)

Parameters	Unit	Enro	LPS+Enro	LPS+Ver+Enro
ke	h^{-1}	0.07±0.003	0.09±0.006	0.06±0.007
ka	h^{-1}	1.67±0.11	0.24±0.1**	1.66±0.37#
t1/2a	h	0.41±0.03	4.03±1.02*	0.48±0.08##
t1/2e	h	8.99±0.25	8.45±0.61	9.78±0.92
T_{max}	h	1.94±0.1	7.79±1.29*	2.14±0.24##
C_{max}	$\mu g \cdot ml^{-1}$	1.57±0.11	0.83±0.074**	1.52±0.16##
$AUC_{0-\infty}$	$\mu g \cdot (ml \cdot h)^{-1}$	23.64±1.27	19.27±0.65*	24.43±1.82#
CL/F	$L \cdot (h \cdot kg)^{-1}$	0.43±0.02	0.52±0.02*	0.41±0.03#
V/F	L•kg	5.52±0.41	6.32±0.35	5.93±0.72

*P<0.05, **P<0.01 significant difference vs. non-treated LPS broilers. #P<0.05, ##P<0.01 significant difference vs. enrofloxacin alone in LPS-treated broilers.

Table 2: Pharmacokinetic parameters of enrofloxacin administered intravenously alone and co-administrated with verapamil in non-treated and LPS-treated broilers (mean±SEM, n=5)

Parameters	Unit	Enro	LPS+Enro	LPS+Ver+Enro
Alpha	h^{-1}	0.60±0.15	0.46±0.079	0.51±0.12
Beta	h^{-1}	0.04±0.01	0.05±0.007	0.04±0.01
A	$\mu g \cdot mL^{-1}$	2.51±0.1	2.73±0.34	2.81±0.26
B	$\mu g \cdot mL^{-1}$	1.04±0.07	1.56±0.5	1.02±0.13
T1/2α	h	1.29±0.26	1.66±0.29	1.65±0.40
T1/2β	h	17.87±1.66	13.82±1.59	17.52±2.77
$AUC_{0-\infty}$	$mg \cdot h \cdot L^{-1}$	32.00±3.75	34.59±3.43	31.82±2.97
Cl	$L \cdot min^{-1}$	0.32±0.04	0.30±0.03	0.32±0.04
V_C	$L \cdot kg^{-1}$	2.81±0.04	2.34±0.08	2.63±0.12

DISCUSSION

Despite the common use of enrofloxacin, seldom studies have investigated the correlation of its pharmacokinetics with the change of P-gp expression after LPS treatment in broilers and other farm animals. This prompted us to investigate whether LPS alters the disposition of enrofloxacin by modulating the P-gp expression in, liver, small intestine and kidney or not. Here, for the first time, we showed that high levels of P-gp expression were induced in kidney, small intestine and liver upto 12 and 2 h respectively after LPS administration, which altered the pharmacokinetics of oral enrofloxacin in broilers. In current study, we noticed that upregulation of P-gp caused by LPS had a significant role to modify the pharmacokinetics of oral enrofloxacin, which was supported by the data of diminished C_{max}, $AUC_{0-\infty}$ and K_a in LPS-treated broilers. However, the pharmacokinetic parameters of enrofloxacin administered by IV were not altered by LPS and verapamil. Therefore, the plasma concentration of enrofloxacin was reduced after LPS administration implied that intestinal uptake of enrofloxacin was restricted in LPS-treated broilers. The result endorses the concept that the over-expression of intestinal P-gp performs a main role in detoxification of P-gp substrates from blood to the intestinal lumen and may protect broilers against toxic compounds from bacterial infections (Tsuji and Tamai, 1996; Funakoshi *et al.*, 2003). Contrary to this, P-gp may also restrict the absorption of some oral antimicrobial agents, thus causing an ineffectiveness when treating certain infectious diseases (Haritova *et al.*, 2008). P-gp is efflux transporter, it pumps out its substrates from the interior of the cells towards the luminal site of the intestine, influencing the absorption of the drugs (Schrickx and Fink-Gremmels, 2008). In this study, it has been shown that LPS upregulated P-gp expression level in the small intestine.

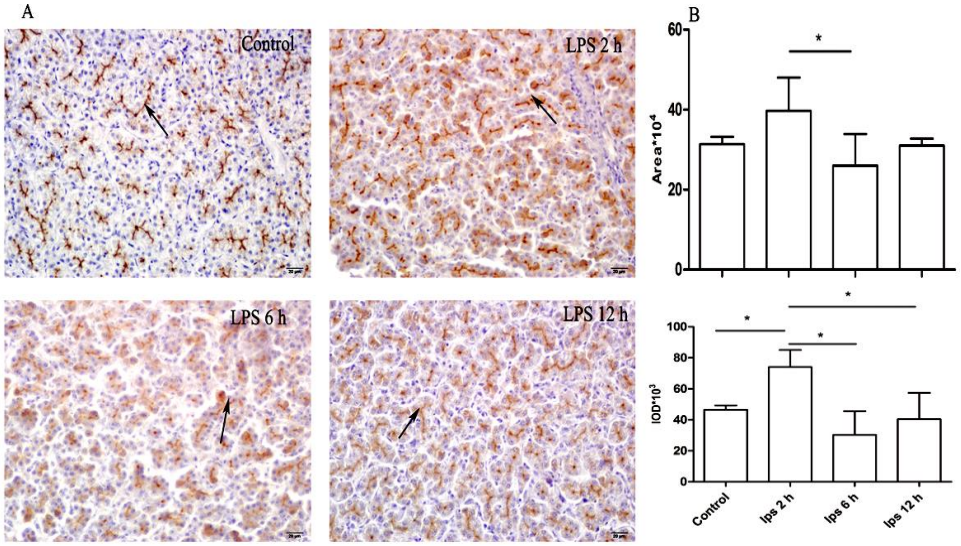

Fig. 1: The immunoreactivity of P-gp (A) and semi-quantification of P-gp (B) in liver of non-treated and LPS-treated broilers at different times. P-gp was probed using mouse monoclonal anti-P-gp antibody (C-219). The IOD was measured to determine the immunoreactivity of P-gp staining and area of positive staining of its expression. Digital images of the surface epithelium area of five sections from each broiler were determined. Every section was divided into five subsections. The software, Image Pro Plus 4.1, was used to determine the IOD and positive area of staining. These figures are representatives of 5 typical broiler samples. Magnifications are indicated by the bar *P<0.05, **P<0.01.

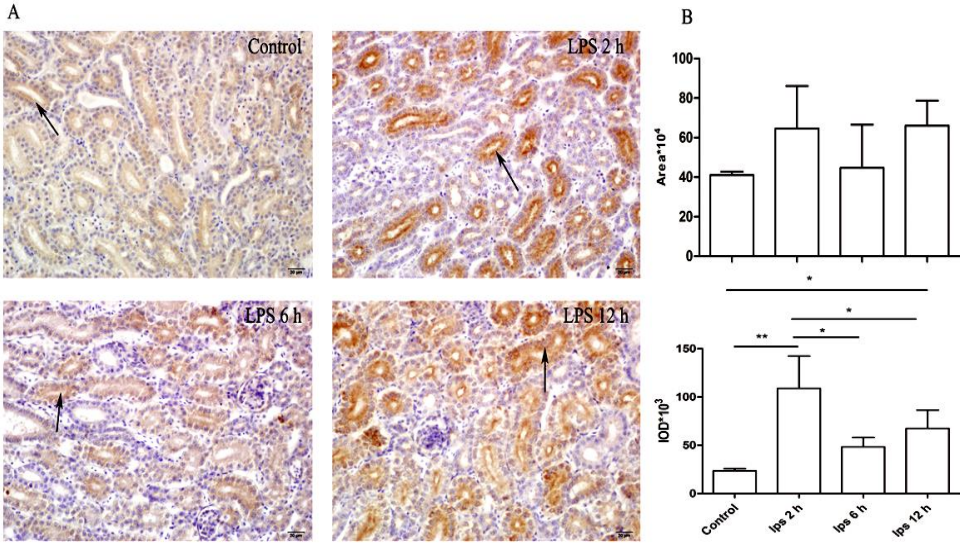

Fig. 2: The immunoreactivity of P-gp (A) and semi-quantification of P-gp (B) in kidney of non-treated and LPS-treated broilers at different times. These figures are representatives of 5 typical broiler samples. Magnifications are indicated by bar. *P<0.05, **P<0.01.

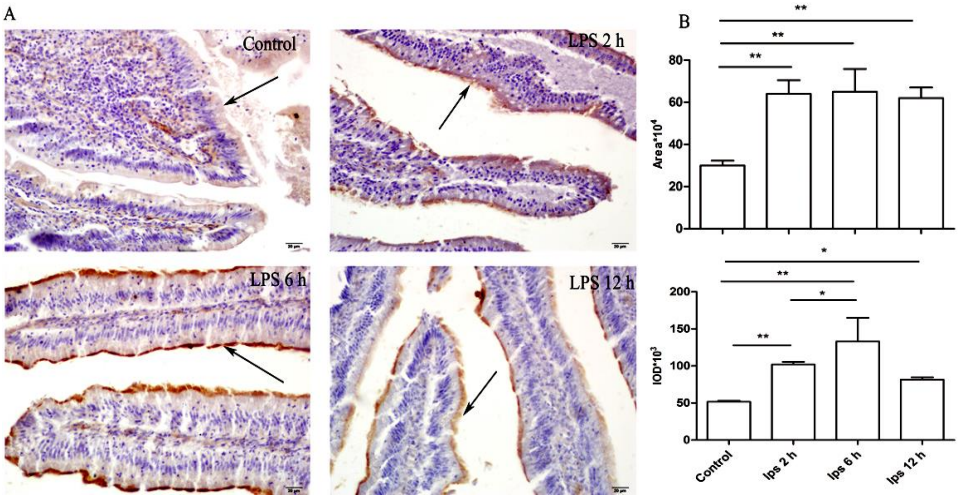

Fig. 3: The immunoreactivity of P-gp (A) and semi-quantification of P-gp (B) in jejunum of non-treated and LPS-treated broilers at different times. These figures are representatives of 5 typical broiler samples. Magnifications are shown by bar. *P<0.05, **P<0.01.

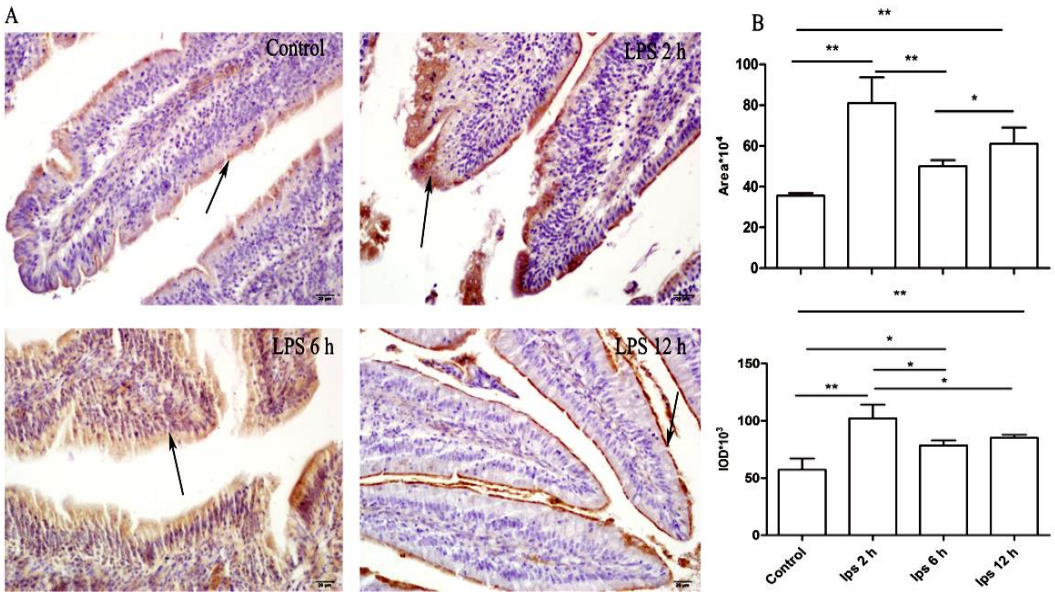

Fig. 4: The immunoreactivity of P-gp (A) and semi-quantification of P-gp (B) in ileum of non-treated and LPS-treated broilers at different times. These figures are representatives of 5 typical broiler samples. Magnifications are shown by bar. *P<0.05, **P<0.01.

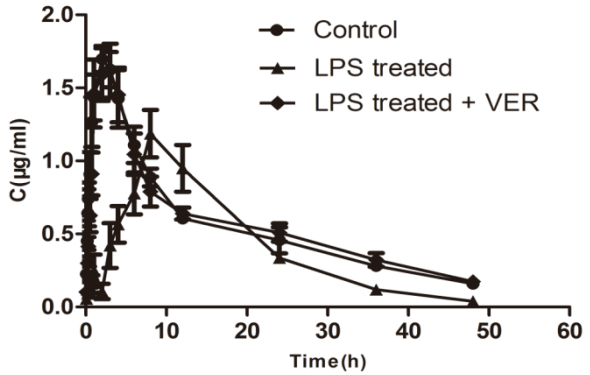

Fig. 5: Mean plasma concentration curves of enrofloxacin (10mg/kg) following a single oral administration in broilers in non-treated (n=5), LPS-treated (n=5) and oral co-administration with verapamil after LPS treatment (n=5). Each point represents the mean±SEM of 5 broilers.

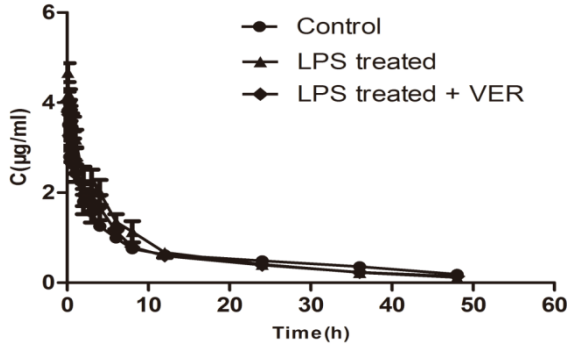

Fig. 6: Mean plasma concentration curves of enrofloxacin (10mg/kg) in broilers following a single IV administration of non-treated (n=5), LPS-treated (n=5) and oral co-administration with verapamil after LPS treatment (n=5). Each point represents the mean ±SEM of 5 broilers.

This enhanced level of P-gp effluxed more concentration of oral enrofloxacin into the lumen of the intestine prohibiting its absorption resulting oral absorption of enrofloxacin was retarded in broilers. LPS is an extensively used model to develop inflammation influencing pharmacokinetics of FQs as well as P-gp expression which are illustrated in some mammals and cell lines (Mishra *et al.*, 2008; Masereeuw and Russel, 2012; Prasad *et al.*, 2015). It has also been speculated that pro-inflammatory cytokines intervened this declined activity and P-gp expression through nuclear receptors in LPS-treated animals. LPS induces the expression of pro-inflammatory cytokines, activating a variety of cell signaling kinases, such as c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB). Activation of JNK causes the suppression of constitutive androstane receptor (CAR) and pregnane X receptor (PXR), consequently, the expression of their target genes *abcb1* and CYP_{450} were promptly and markedly inhibited (Ghose *et al.*, 2009). However, our data demonstrated that LPS up-regulated P-gp expression in broilers, which was in great contrast to the findings that LPS down-regulates P-gp expression during an acute inflammatory response in some mammals. (Moriguchi *et al.*, 2007; Tomita *et al.*, 2010). To date, a few studies have reported regarding the effects of LPS on P-gp expression in various farm animals. This data in some extent in line with a previous study in which LPS administration increased P-gp expression in the liver, kidney and spleen of chicken (Barnes, 2001). However, Barnes found that LPS did not alter P-gp expression in intestine in chicken, which differed from the observation of this study. Also, another study presented increasing trend of P-gp in cell surface of intestinal epithelial cells treated by LPS, but it decreased P-gp activity (Mishra *et al.*, 2008). Previously, we also found that P-gp was up-regulated by *E. coli* infection in broilers (Guo *et al.*, 2014). This study together with that of Barnes indicated that the influence of LPS on P-gp between mammals and poultry are different. The mechanisms of LPS on P-gp expression in poultry need to be addressed further.

It was interesting to note that a decreasing trend in elimination of oral enrofloxacin was observed in the present study, which was not parallel to up-regulation trend of P-gp in liver and kidney. In immunohisto-chemical study, it was observed that P-gp was located on the biliary canalicular membrane in the hepatocytes and apical membrane of proximal tubules in the kidney of non-

treated broilers, whereas in LPS-treated broilers P-gp was scattered inside the cytoplasm far from the membranes in the liver and kidney (Fig. 1 and 2). This implied that though LPS treatment increased P-gp expression in liver and kidney, it might have badly influenced the P-gp function, as P-gp translocated from cell membrane to cytoplasm. More studies are required to test this hypothesis. This finding is in agreement with the study in which LPS increased the displacement of MRP_2 from the canalicular membrane to the vesicles of cytoplasmic membrane because membrane proteins were not attached properly to the cytoskeleton, following dislodgement in the cell membrane (Apodaca, 2001; Elferink *et al.*, 2004). However, in small intestine P-gp was localized in the intestinal epithelium and did not show its translocation inside the cytoplasm in LPS-treated broilers.

Conclusions: For the first time, it was observed that LPS (5mg/kg) administration up-regulated P-gp expression in the small intestine and kidney at 2 to 12 h and in liver at 2 h of its post administration, which is different from the findings in rodents and humans. LPS-modulated P-gp expression that remarkably altered the pharmacokinetics of orally administered enrofloxacin in broilers. The results suggested that whenever LPS-induced inflammation occurs, this up-regulation must be kept in mind for all P-gp substrate drugs in broilers. Additionally, to remain conscious to treat broilers that initially they must receive higher doses of drugs during LPS-producing infection to achieve therapeutic concentration at the site of action.

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Authors contribution: Participated in research design: Liping Wang, conducted experiments: Shamsuddin Bughio, Tingting Guo, Yong Sun and Fang He, Performed data analysis: Yang Liu and Shamsuddin Bughio.

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