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**Συγκριτική μελέτη του φαρμακοκινητικού προφίλ της IgY κοτόπουλου σε σχέση με την IgG ιπποειδούς σε κουνέλια**

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## Comparative study on Pharmacokinetic Profiles of Chicken IgY to Horse IgG in Rabbits

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**ABSTRACT.** Chicken IgY antibody has been extensively reported for various applications as an alternative to mammalian IgG. To evaluate the pharmacokinetics profile of chicken IgY in comparison with horse IgG. Chicken IgY antibody was prepared by immunizing the white leghorn chicken with tetanus toxoid (TT) and then extracted anti-TT-IgY from immune egg yolks by PEG-6000 extraction. The titer of anti-TT-IgY was determined by ELISA in order to select the hyper immune eggs for further extraction. Rabbits were injected with 300 µg/kg of Chicken IgY and horse IgG by intravenous (i.v.) and intramuscular (i.m.) administrations. Then, the concentrations of IgY and IgG in rabbit serum were determined using indirect ELISA. Pharmacokinetic parameters were estimated using non-compartmental analysis. IgY can reach higher concentration ( $C_{max} = 4.54$  mg/L after i.m. injection) within shorter time ( $T_{max} = 0.12$  h after i.m. injection) than IgG ( $C_{max} = 3.99$  ng/mL after i.m. injection;  $T_{max} = 0.11$  h after i.m. injection), while the IgY lifespan ( $T_{1/2} = 26.98$  h and 31.98 h after i.v. and i.m. administration, respectively) in the body was comparable with IgG ( $T_{1/2} = 27.94$  h and 29.58 h after i.v. and i.m. administration, respectively). IgY might be a promising choice as an alternative to mammalian sourced antibodies.

**Keywords:** tetanus, IgY, IgG, pharmacokinetics; intravenous administrations; intramuscular administrations

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## INTRODUCTION

Tetanus, known as lockjaw, is a fearful health problem in both human and animals caused by neurotoxin produced by the anaerobic bacterium *Clostridium tetanus*, usually through contaminated wounds and will affect body's muscles and nerves (Hertz and Sørensen, 2014). Prevention by appropriate wound care (cleaning and antibiotics) and immunization are vital. At present, tetanus can be prevented by the administration of tetanus toxoid, which induces specific antitoxins. In addition, antibody used for the prevention and treatment of tetanus dates from 1890, tetanus antitoxin is an important drug prevention and treatment of tetanus, such as tetanus antiserum (TAT) were obtained from tetanus toxoid immunized horse (Keller and Stiehm, 2000). TAT in an effort to toxin neutralization is a crucial part of the treatment, while its obviously adverse reactions (the high incidence of anaphylaxis) limited the clinical application (Shen and Zhang, 2012). And its preparation went against the animal welfare and required high production costs, which can in turn hamper its use (Luo, 2002).

It has been reported very long ago, that egg yolk extracts laid by hens hyper immunized against tetanus toxin were capable of protecting mice from the toxin effect (Klemperer, 1893). However, antitoxin or antivenoms are mostly produced in hyper immunized mammals (Theakston and Warrell, 1991). Recently, numerous studies are supporting the immunotherapeutic use of avian IgY antibodies (Diaz et al., 2014).

IgY is the functional equivalent of IgG in birds, reptiles and amphibian. It has gained significant attention because of several attractive advantages than mammalian IgG including low-cost, easiness and high yield (Schade et al., 2005; Sun et al., 2013). Besides this, IgY has high affinity and does not react with Fc receptors and thereby possess low risk of adverse reactions in immunotherapeutic applications (Vázquez et al., 2010). However, the information on clinical success for IgY usage has not explored well to draw strong conclusions. Notably, the investigations on the applications of IgY to control non-enteric diseases; especially for intravenous administration (i.v.) are scanty. Only limited numbers of studies (with preliminary findings) have been reported on IgY against snake venoms and some other toxins (Polson et al., 1980; Diaz et al., 2014). Thus, it is indispensable to understand the consequences after i.v. or i.m. admin-

istration of IgY. Because, the effect of IgY therapy is mainly associated with knowledge about IgY pharmacokinetics (PK). The previous study have investigated the pharmacokinetics of antivenoms IgY in rabbits after i.v. administration, IgY PK lacks the fast initial decay, last less time in the body, reaches a smaller volume of distribution in steady state (Diaz et al., 2014). However, it would be still interesting to compare the pharmacokinetic characteristics of IgY and IgG, and to evaluate the feasibility of IgY in parenteral administration with regard to its pharmacokinetic and safety concerns.

## MATERIALS & METHODS

### 2.1. Experimental animals and sampling procedures

White Leghorn chickens (25-week old ~ 2 kg of body weight) were purchased from local farm and adult New Zealand rabbits (18-week old ~ 3.0 ± 0.25 kg of body weight) were procured from the Fourth Military Medical University (Xi'an, China). Hens and rabbits were kept in individual cages with food and water ad libitum. All experimental procedures in this study were carried out in accordance with university guidelines for care and use of laboratory animals. Purified anti-tetanus horse IgG was kindly provided by the Chifeng Boen pharmaceutical Ltd. Co. (Chifeng, Inner Mongolia, China).

Rabbits were randomly divided into four groups (N=4) with six animals in each (n=6): IgY i.v. group; IgY i.m. group; IgG i.v.; and IgG i.m. group. The experimental was conducted as the method previously described by Díaz et al (Sun et al., 2013). Briefly, experimental animals were immobilized with a harness, and catheterized in the right ear marginal vein with a retention needle. After injection of IgY or IgG (300 µg/kg), the opposite side vastus muscle or the marginal vein from the opposite side ear was used to draw blood samples. A blank sample was drawn from the catheterized ear at t=0. Blood samples (400 µL) were drawn at 2 min intervals from 0 to 20 min, then at 10 min intervals up to 60 min, then at 3 h intervals for up to 12 h, then at 24 h and at days 3, 5, 7, 9, 11 and 15. Blood samples were incubated for 1 h at 37 °C and centrifuged for 10 min at 5000 g at 4 °C. Serum was separated and kept frozen at -20 °C until used.

### 2.2. Preparation and characterization of specific IgY

Inactivated tetanus toxoid 500 µL (1300 UI/mL) mixed with Freund's complete adjuvant (FCA; Sig-

ma) was intramuscularly injected to 25-week-old white Leghorn chickens at different sites of breast muscles. The booster injection with same dose of vaccine and Freund's incomplete adjuvant (FIA; Sigma) was given at 21, 35, 49 and 63 days after the first immunization. Eggs were collected daily and stored at 4 °C. IgY was extracted as per the method previously described by Polson et al (Zhang and Zheng, 2012). In brief, 3.5% (w/v) polyethylene glycol (PEG-6000) in PBS, 10 mM, pH 7.2) was added to yolk. After gentle shaking at room temperature for 10 min, mixture was subjected to centrifugation at 16,000 g for 20 min at 4 °C. PEG-6000 was added to supernatant at a final concentration of 12% (w/v). After gentle shaking at room temperature for 10 min, the mixture was centrifuged as above. The pellet was dissolved in 10 mL of PBS and 12% (w/v) PEG-6000 was added and again centrifuged as above. Finally, the pellet was dissolved in 1.2 mL of PBS and dialyzed against PBS for overnight at 4 °C. The purity of IgY was evaluated by SDS-PAGE and stored at -20 °C. The titer of specific IgY was determined as described in our previous report (He et al., 2014).

### 2.3. Determination of serum IgY and IgG concentrations

The standard IgY and IgG (Zen bioscience, Chengdu, China) were serially diluted in sterile tubes using CBS. Microtiter plates were coated with different dilutions of standard IgY (100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL) and IgG (100 µg/mL, 50 µg/mL, 10 µg/mL, 2 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL) (Zen bioscience, Chengdu, China) separately and incubated overnight at 4 °C. After incubation, the plates were washed 3 times with PBS containing 0.5% Tween-20 (PBST) and unbound sites were blocked with PBS containing 5% skim milk (PBSM) for 1 h at 37 °C. Subsequent washing with 0.5% PBST three times, HRP conjugated goat anti-chicken IgY (Abcam, Canada) or goat anti-horse IgG (Abcam, Canada) was added. Plates were incubated for 1h at 37 °C. Finally, 100µL TMB was added to each well as substrate and incubated for 15 min at room temperature, the reaction was stopped by adding 50 µL of H<sub>2</sub>SO<sub>4</sub> (2 mol/L). The OD450 was measured on a micro plate reader. Standard curves were plotted to determine IgY and IgG concentration. Rabbit serum

samples were diluted to 5-fold with CBS and coated to microplates at 4°C overnight (triplicates). The remaining steps were performed as described above.

### 2.4. Determination of rabbit IgG titer against chicken IgY and horse IgG

The titer of rabbit anti-IgY and anti-IgG antibodies were measured using indirect ELISA. Briefly, the IgY and IgG were coated to plates overnight at 4 °C. After three times with PBST, unbound sites were blocked with 5% PBSM and incubated for 2 h at 37 °C. After washing three times with PBST, rabbit serum was added to each well and incubated for 2 h at 37 °C. After washing with PBST, HRP-conjugated goat anti-rabbit antibody (Abcam, UK) (100 µL/well) was added to plates for 1 h at 37 °C. After washing, the remaining steps were performed as described above.

### 2.5. Pharmacokinetics analysis

The measured plasma concentration data were processed using software DAS 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) as previous description (Huang et al., 2014), calculating pharmacokinetic parameters for each group. Pharmacokinetic parameters were estimated using non-compartmental analysis. All data were presented as the Mean ± Standard deviation.

Pharmacokinetic abbreviations:  $AUC_{(0-\infty)}$ , area under the plasma concentration-time curve at time = ∞;  $AUMC_{(0-\infty)}$ , area under the first moment of the concentration-time curve at time = ∞;  $CL_z$ , total body clearance;  $T_{1/2z}$ , biological half time;  $MRT_{(0-\infty)}$ , mean residence time at time = ∞;  $C_{max}$ , maximum concentration;  $T_{max}$ , time to reach the maximum concentration;  $V_z$ , volume of blood.

### 2.6. Pharmacokinetic appendix

#### 2.6.1. Non multi-exponential pharmacokinetics analysis

Since the concentration vs time curves deviated markedly from a tri-exponential model, pharmacokinetics parameters were calculated from  $AUC$  and  $AUMC$  values obtained by trapezoidal numerical integration of the curves (Vázquez et al., 2010).

$$AUC \approx \sum_{i=2}^n \left[ \frac{C_i + C_{(i-1)}}{2} \times (t_i - t_{(i-1)}) \right] + \frac{C_n}{\lambda_z}$$

Where  $n$  is the number sampled points, and  $t_i$  is the time at which was sampled. Likewise,

$$AUCM \approx \sum_{i=1}^n \left[ \frac{t_i + t(i-1)}{2} \times (c_i * t_i + c(i-1) \times c(i-1) \times t(i-1)) \right]$$

If  $t_n$  is long enough to be taken as a good approximation to infinity, Eqs. (1) and (2) reduce to just the sum terms between brackets. Since in our case the concentration vs time curves for IgY were towards zero after 216 h at the end of the observation period,  $C_n$  was taken as 0 or, equivalently,  $\lambda_z = t_z - 1$  was taken as infinity.

### 2.6.2. Determining pharmacokinetics parameters

The expressions to calculate pharmacokinetics parameters were described in Table 1 and summarized here.  $CL_z$ ,  $V_z$  and  $T_{1/2z}$  are statistical parameters. They calculated with the following formula (Zhang and Zheng, 2012).

The half-lives of distribution and elimination were given by the expression:

$$T_{1/2z} = \frac{0.693}{Ke}$$

Where,  $Ke$  is the elimination rate constant.

The total body clearance ( $CL_z$ ) was given by the equation:

$$CL_z = \frac{C}{AUC}$$

Where,  $C$  means the dose of the drug.

The apparent volume of distribution ( $V_z$ ) was calculated using the equations:

$$V_z = \frac{C \times AUCM}{AUC \times AUC}$$

MRT is the mean residence time or average time a molecule staying in the body and calculated using the following equation:

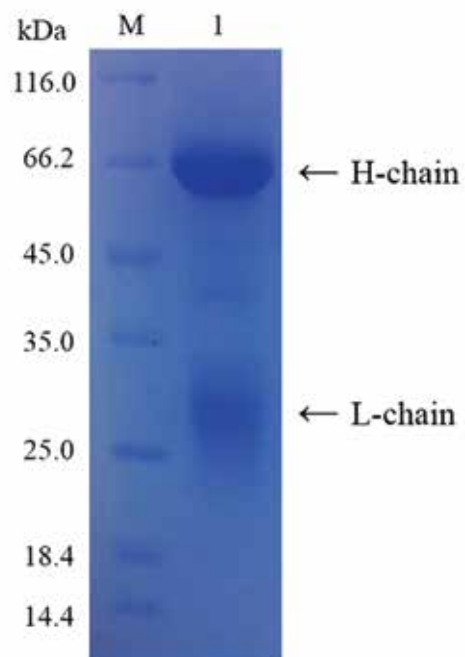
$$MRT = \frac{AUMC}{AUC}$$

## RESULTS

### 3.1. Preparation of anti-tetanus IgY antibody and evaluation of IgY titer

The purity assessment of IgY extract by SDS-PAGE showed two protein bands i.e., a heavy chain (66 kDa) and a light chain (27 kDa) (Fig. 1). Indirect ELISA was used to monitor the titer of specific IgY against tetanus toxoid. After third immunization, the titer of anti-tetanus IgY was increased up to 1:68,000 and remained stable for one month.

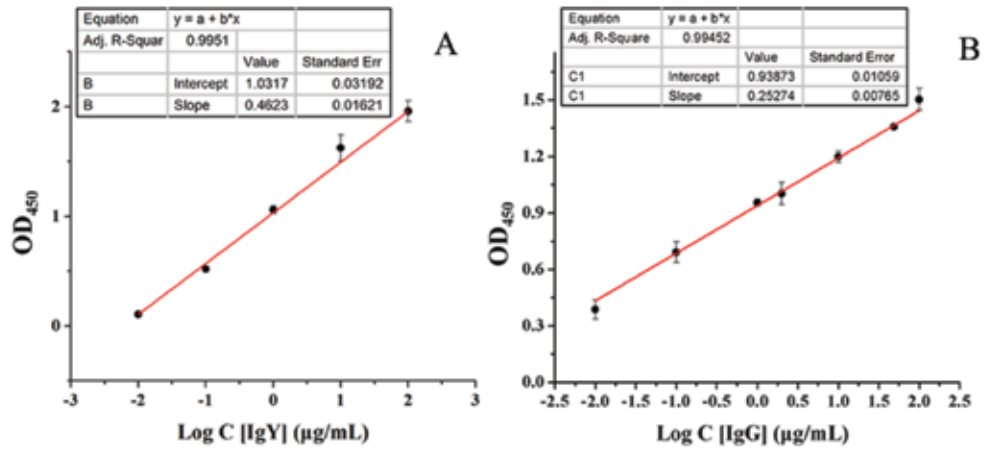
**Figure 1.** SDS-PAGE analysis of IgY extract.  
Note: Lane M: molecular weight marker; Lane 1: IgY isolated by PEG-6000.



**3.2. Development of standard curve for IgY and IgG**

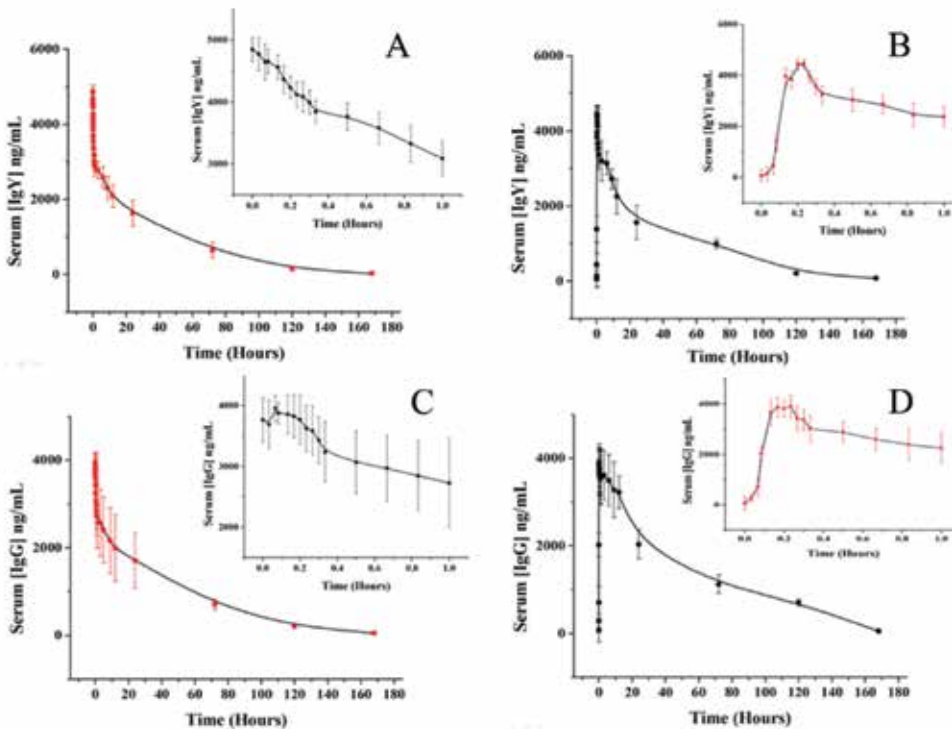
A series of OD450 values were obtained from ELISA. OriginPro.8 was used to plot the standard curve (Fig. 2). The values showed an excellent linear relation  $y = 0.4623x + 1.0317$  ( $R^2 = 0.9951$ ) for IgY and  $y = 0.2527x + 0.9387$  ( $R^2 = 0.9945$ ) for IgG.

**Figure 2.** Standard curves of IgY and IgG.  
Note: A represents the standard curve of IgY; B represents the standard curve of IgG.



**3.3. Concentration-time profiles of IgY and IgG in rabbit serum**

The concentrations of IgY and IgG in rabbit were determined by ELISA for over a period of time. It is a semi logarithmic plot of serum [IgY] and [IgG] vs time elapsed after i.v. and i.m. administration (Fig. 3). Their levels were undetectable until 216 h.



**Figure 3.** Plasma of IgY and IgG concentrations-time profiles in rabbit following single IV and IM injection at a dosage of 300µg/kg BW (n=6). Note: A, B presents the concentration-time (0 to 1 h and 0 to 168 h) of IgY after IV and IM injections, respectively; C, D presents the concentration-time (0 to 1 h and 0 to 168 h) of IgG after IV and IM injections, respectively.

### 3.4. Pharmacokinetic characteristics of IgY and IgG

The main pharmacokinetic parameters were summarized in Table 1. The rabbits were carefully observed during and after the experimental period, and there was no observable adverse reaction in terms of animal behavior, including appetite, hair, spirits atrophy, allergy, stress and death.

**Table 1.** Pharmacokinetic parameters of IgY and IgG in Rabbit (n=6).

Parameter	IV IgY	IV IgG	IM IgY	IM IgG
Dose ( $\mu\text{g kg}^{-1}$ )	300	300	300	300
$T_{max}$ (h)	--	--	0.12 $\pm$ 0.03	0.11 $\pm$ 0.07
$C_{max}$ (mg L <sup>-1</sup> )	--	--	4.54 $\pm$ 0.33	3.99 $\pm$ 0.31
$T_{1/2z}$ (h)	26.98 $\pm$ 4.35	27.94 $\pm$ 4.31	31.98 $\pm$ 4.22	29.58 $\pm$ 1.48
$AUC_{(0-\infty)}$ (mg h L <sup>-1</sup> )	132.87 $\pm$ 17.66	139.10 $\pm$ 32.00	112.94 $\pm$ 26.98	160.63 $\pm$ 15.25
$AUMC_{(0-\infty)}$ (mg h <sup>2</sup> L <sup>-1</sup> )	4921.33 $\pm$ 956.87	5821.55 $\pm$ 810.92	4435.56 $\pm$ 1113.91	6770.89 $\pm$ 637.99
$MRT_{(0-\infty)}$ (h)	36.85 $\pm$ 4.05	42.7 $\pm$ 4.98	39.53 $\pm$ 3.84	42.33 $\pm$ 4.27
$CL_z$ (mL h <sup>-1</sup> kg <sup>-1</sup> )	2.00 $\pm$ 0.00	2.00 $\pm$ 1.00	3.00 $\pm$ 1.11	2.00 $\pm$ 0.00
$V_z$ (L kg <sup>-1</sup> )	0.09 $\pm$ 0.02	0.09 $\pm$ 0.02	0.13 $\pm$ 0.06	0.08 $\pm$ 0.01

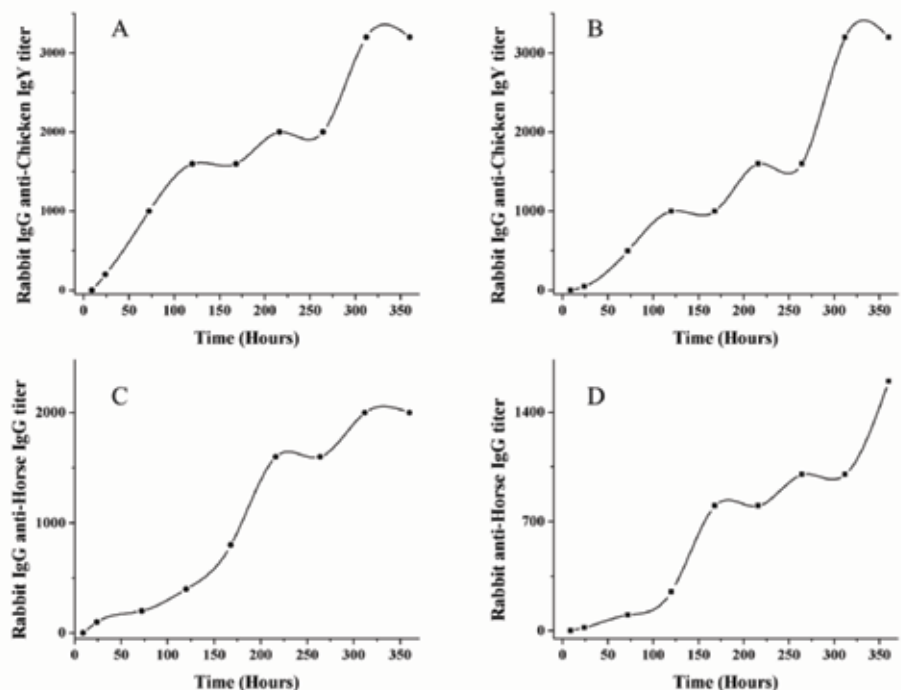
(Notes:  $AUC_{(0-\infty)}$ , area under the plasma concentration-time curve at time =  $\infty$ ;  $AUMC_{(0-\infty)}$ , area under the first moment of the concentration-time curve at time =  $\infty$ ;  $CL_z$ , total body clearance;  $T_{1/2z}$ , biological half-time;  $MRT_{(0-\infty)}$ , mean residence time at time =  $\infty$ ;  $C_{max}$ , maximum observed plasma IgY concentration;  $T_{max}$ , time to reach the maximum concentration;  $V_z$ , volume of blood; BW, rabbit body weight. Data presented as medians and their 95% confidence intervals (between parentheses) calculated for 6 rabbits.)

### 3.5. Titers of rabbit anti-IgY and anti-IgG antibody

After receiving IgY and IgG, anti-IgY and anti-IgG titers were monitored in rabbits (Fig. 4). After i.v. 72 h, titers of anti-chicken and anti-horse IgG reached 1:1000 and 1:200; and attained the peak 1:3200 for IgY and 1:2000 for IgG after 300 h. In i.m. group, titer of anti-IgY was 1:500 and 1:200 for anti-IgG at 72 h. After 300 h, titer was (1:3200) for IgY and (1:1000) for IgG.

**Figure 4.** Titers of anti-IgY and anti-IgG antibody in rabbit serum.

Note: A and B present titers of anti-IgY body in rabbit serum following IV and IM injection, respectively; C and D present titers of anti-IgG body in rabbit serum following IV and IM injection, respectively.



## DISCUSSION

IgY has been clinically used as an effective tool for the treatment and control of various infections especially in veterinary medicine (Spillner et al., 2012; Zolfagharian and Dounighi, 2015). Even though, the IgY has been considered as an promising alternative to conventional IgG to some extent, the pharmacokinetics behavior and safety are not well explored in mammals. Hence, we had designed this study to evaluate the IgY pharmacokinetics profile after i.v. and i.m. administrations in parallel to IgG targeted against the same antigen. In this study, the tetanus toxoid has been used just as a tool, the exact focus of the study was to explore the general bodily effect on IgY (regardless of antigen specificity) as well as IgG administration (for comparison) in order to appraise the IgY for non-enteric passive immunization. However, it is interesting to mention that, more than 100 years ago the first investigated IgG as well as IgY both were targeting tetanus toxin (Behring and Kitasato, 1890; Klemperer, 1893); even, the anti-tetanus serum is still under use. The previous reports by Klemperer in 1893 and Guimarães et al., in 2009 have stated that, IgY treatment could reduce the risk of tetanus toxin (Klemperer, 1893; Guimarães et al., 2009), we also experienced similar neutralization effects of IgY (data not shown). As far as antivenoms produced based on chicken IgY have been tried in humans (Kiem, 2000; Diaz et al., 2014).

Different research groups have reported on IgY pharmacodynamics (PD) profile. Notably, anti-venom IgY generated in chicken was studied to evaluate venom neutralizing potential. Neutralizing potency of IgY anti-B. arietans was particularly efficient, as shown by the capacity of 1440 mg IgY to neutralize 62.2 LD<sub>50</sub> of the venom (de Almeida et al., 2008). Another report stated that, the LD50 of venom for 18 g of mice was found to be 10 mg for Cobra and 3 mg for Krait venoms. The median effective dose (ED<sub>50</sub>) of anti-Cobra venom was 4.48 mg/5 LD<sub>50</sub> and 1.0 ml neutralized 0.127 mg of Cobra venom and the median ED<sub>50</sub> of anti-Krait venom was 3.18 mg/5 LD<sub>50</sub> and 1.0 mL neutralized 0.051 mg of Krait venom (Meenatchisundaram et al., 2008). In neutralization study, a preincubated mixture of both affinity-purified (50mg/kg body weight) as well as partially purified (210 mg/kg body weight) anti-Echis carinatus IgY with 2 LD<sub>50</sub> dose of E. carinatus venom (2×6.65 mg/kg body weight) gave 100% protection in mice when administered subcutaneously (Paul et al., 2007). Even IgY was capable of neutralizing lethal toxic activity of the pool of Bothrops sp venoms from five species, with an ED<sub>50</sub> of 365 mL/2 LD<sub>50</sub> and, 1.0 mL of IgY antivenom neutralized

0.154 mg of venom (Araujo et al., 2010). Indeed, anti-venom IgY can provide a higher bioactivity than antivenoms raised in horses (Thalley and Carroll, 1990). In addition, IgY could protect donkeys from 1 minimum lethal dose (MLD) of Clostridium tetanic through passive immunization before and after infection (Meshad et al., 2013). Furthermore, IgY has equally efficacy to IgG in prophylaxis and treatment of tetanus in mice. In a previous study showed that 1000 units was the best therapeutic dose (Smith and MacIver, 1969).

In the present study, during both i.v. and i.m. administrations, the AUC for IgY was comparable (in i.v.) or lower (in i.m.) than that of IgG. This slight variation was probably impacted by the faster elimination of IgY in rabbit and/or related with the minor difference in binding affinity between rabbit FcRn and horse IgG, which would have controlled the rapid degradation of exogenous IgG. In contrast, the mammalian FcRn could not bind with avian IgY (Murai et al., 2013), there is a clear phylogenetic distance between chicken and rabbit, compared with rabbit and horse; thus the rabbit's immune response to the exogenous IgY was slightly higher than exogenous IgG, nevertheless they are still in a comparable level. However, IgY exhibited higher  $C_{max}$  compared to IgG (i.m.) that is IgY can quickly reach a higher initial concentration than IgG; Thus, it can effectively act against the toxins injected into the body. It is necessary to admit that in our result, IgG i.v. administration in rabbit exhibited lower AUC than that of i.m. administration (Table 1), this could be experimental error caused by individual difference of rabbits, and the possible cross reactivity of anti-horse secondary IgG to rabbit IgG could play a role.

In summary, these findings suggest that, PK profiles between IgY and IgG are still comparable despite of some observable variations. Furthermore, the PK profile could diverse from species to species, as well as to pathological/physiological statuses; even in this study, the rabbits were not challenged with toxins. IgY has been considered as a promising choice for the enteric diseases notably in veterinary medicine; our observations suggest that, further investigations are indispensable to understand the IgY PK profile for non-enteric usage.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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