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Research Article

Pharmacokinetic Studies of the Recombinant Bovine Interferon-alpha in Cattle

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Abstract

In order to evaluate the pharmacokinetics of recombinant bovine interferon-alpha (rBoIFN-α) in cattle, which has potential for its antiviral and immunomodulatory activities, 12 animals of 6-month age were classified into 4 groups (n=3) to receive rBoIFN-α through IV, IM or SC routes at a dose of 5.0×10^3 IU/kg. Serum rBoIFN-α titer was evaluated using cytopathic effect (CPE) inhibition bioassay. Then, the standard pharmacokinetic parameters were calculated using the DAS (Drug and statistics) software. The concentration-time profiles of serum rBoIFN-α following IM administration, SC administration and IV administration were characteristics of the 1-, 1-, and 2-compartment open models, respectively. After a single dose of IV administration, the drug rapidly dispersed and was rapidly eliminated from the body ($T_{1/2\alpha}$=0.15±0.02 h, $T_{1/2}$=6.48±0.49 h). After IM and SC administrations, the drug is rapidly absorbed and slowly eliminated from the body (For IM administration, T_{max}=6.12±0.32 h, $T_{1/2}$=8.19±0.74 h) (For SC administration, T_{max}=4.06±0.56 h, $T_{1/2}$=7.29±0.55 h). The bioavailability of rBoIFN-α after IM administration is 53.74%, which is higher than the bioavailability of SC administration (27.96%). Therefore, the results showed that the drug administration effect can be preferably obtained following a single dose IM injection using the rBoIFN-α aqueous preparation. We hope that this study will provide valuable information for the clinical application of rBoIFN-α as an potential antiviral agent.

Keywords: *Recombinant bovine interferon-α, Cytopathic effect inhibition assay, Bioavailability, Pharmacokinetic study*

Sığırlarda Rekombinant Bovine İnterferon-alfa Üzerine Farmakokinetik Çalışmalar

Öz

Bu çalışma potansiyel antiviral ve bağışıklık düzenleyici fonksiyonlara sahip olan rekombinant bovine interferon-alfa (rBoIFN-α)'nın sığırlarda farmakokinetik özelliklerini değerlendirmek amacıyla yapılmıştır. Çalışmada 6 aylık 12 hayvan 4 gruba ayrılmış (n=3), hayvanlara IV, IM ve SC yollarla 5.0×10^3 IU/kg dozda rBoIFN-α verilmiştir. Serum rBoIFN-α titresi, sitopatik etki inhibisyon biyotesti kullanılarak değerlendirilmiştir. Sonrasında, standart farmakokinetik parametreler DAS (Drug and statistics) yazılımı kullanılarak hesap edilmiştir. İntramusküler, SC ve IV yollarla rBoIFN-α verilmesi sonrası konsantrasyon-zaman profili sırasıyla 1-, 1- ve 2-kompartman açık model özelliklerini göstermekteydi. Tek doz IV uygulama sonrası ilaç hızlı bir şekilde dağıldı ve hızlıca vücuttan elimine edildi ($T_{1/2\alpha}$=0.15±0.02 s, $T_{1/2}$=6.48±0.49 s). İlaç IM ve SC uygulama sonrasında hızlıca absorbe edildi ve yavaşça vücuttan elimine edildi (IM uygulama için T_{max}=6.12±0.32 s, $T_{1/2}$=8.19±0.74 s) (SC uygulama için T_{max}=4.06±0.56 s, $T_{1/2}$=7.29±0.55 s). rBoIFN-α'nın IM uygulama sonrası biyoyararlanımı %53.74 olup bu değer SC uygulamadaki değerden (%27.96) daha yüksek olarak tespit edildi. Elde edilen sonuçlar, ilaç uygulama etkisinin tercihen tek doz IM rBoIFN-α sıvı preparasyon enjeksiyonu ardından elde edilebileceğini göstermiştir. Bu çalışmanın, potansiyel bir antiviral ajan olarak rBoIFN-α'nın klinik uygulaması için değerli bilgiler sağlayacağı düşüncesindeyiz.

Anahtar sözcükler: *Rekombinant Bovine interferon-α, Sitopatik etki inhibisyon testi, Biyoyararlanım, Farmakokinetik çalışma*

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INTRODUCTION

Interferon (IFN) belongs to the large-scale protein family with anti-viral, cell proliferation regulatory and immuno-regulatory activities [1,2]. It was originally reported by Isaacs and Lindenmann in 1957, that the influenza virus infected chicken cells could produce a soluble factor, which was endowed with the resistance to homologous and heterologous viruses [3]. Currently, IFN is generally classified into three categories, including type I, type II and type III IFNs [4]. Among them, type I IFN includes IFN-α, IFN-β, IFN-ω, IFN-τ and IFN-κ that play important roles in suppressing virus replication and cell growth [5,6], exerting their biological effects through the growth of co-receptor IFNAR [7]. Type II IFN only consists of one member IFN-γ [8], which plays a key role in adaptive immune responses, and is crucial for activating macrophages and natural killer (NK) cells [9,10]. Type III IFN are constituted by IFN-λ and interleukin (IL)-28/29 [4,11,12]. Typically, type I IFN and Type III IFN have anti-viral activities [13]. Some effects of various IFNs may overlap, however, in comparison with other types of IFN, type I IFN has displayed the strongest anti-viral activity [14].

IFN-α, which belongs to type I IFN, has been used as one of the most effective therapeutic drug to prevent or treat specific viral diseases. For example, human IFN-α shows favorable prospect in treating hepatitis B, hepatitis C, viral hemorrhoids, multiple sclerosis, and other diseases [15-17]. Porcine IFN-α has been gradually adopted to treat some viral infections, such as PRV [18], PRRSV [19] and CSFV [20]. Bovine IFN-α (BoIFN-α) has also been proved to have anti-viral effect on the infection of bovine viral diarrhea virus (BVDV) [21], Foot and Mouth Disease Virus (FMDV) [22]. Moreover, the recombinant bovine IFN-α (rBoIFN-α) has been generated in the yeast expression system through Molecular Biology technology [23]. This technique allows rBoIFN-α to move further toward practical application in preventing and controlling the viral diseases in bovine industry.

In order to elucidate the pharmacokinetic profiles of recombinant interferon-α, many studies have been performed in human or animals, such as the pharmacokinetic profile of *Escherichia coli*-derived human interferon type alpha in mice [24], Recombinant Human Interferon Alpha2b Formulations in healthy human volunteers [25], recombinant leukocyte A interferon in patients with disseminated cancer [26], recombinant interferon alpha-C in patients with metastatic renal cell carcinoma [27], recombinant human interferon-alpha I in African green monkeys [28], recombinant alpha A interferon in African green monkeys [29], human recombinant interferon (Re-IFN-alpha A) in cynomolgus monkeys [30], recombinant leukocyte A interferon in beagle dogs [31], recombinant human interferon-alpha 2C in rat and marmoset [32], recombinant feline interferon in cats [33], recombinant chicken interferon-α in broiler chickens [34]. However, till now, to the best of our knowledge, no information on the pharmacokinetic characteristics of rBoIFN-α has been reported in scientific literatures. Moreover, due to the natural aspect of species-specificity in IFN [35], the bovine viral diseases can only be treated with bovine IFN and cannot be treated with human IFN. Therefore, our study aims to investigate the pharmacokinetic characteristics of rBoIFN-α in cattles by calculating the serum rBoIFN-α bio-activities at different time points using cytopathic effect (CPE) inhibition bioassay. Our study is original and provides the detailed evaluation of the parameters of rBoIFN-α pharmacokinetics. We hope that this study will provide scientific contributions to the research on rBoIFN-α.

MATERIAL and METHODS

Animals and Materials: In this study, twenty four 6-month-old cattles were used, including 12 males and 12 females. All cattles were derived from the commercial cattle farm at the age of 5 months. All animals were fed *ad libitum* with commercial diet for a month in the Experimental Animal Research Center of Anhui Province (Hefei, Anhui, China). The animals weight from 186.5 kg to 226.2 kg (200±26.3 kg) and were randomly classified into 4 groups, with 6 animals per group.

The rBoIFN-α freeze-dried powder for animal injection was offered by Anhui Jiuchuan Biotech Co., Ltd (batch number: 20151024, Wuhu, Anhui, China), which was produced through yeast *(Pichia pastoris)* foreign gene expression method as previously described [23]. The rBoIFN-α was obtained following the procedure of protein purification, sterilization and freeze-drying. The product titer equaled 1.0×10^6 IU/vial. Within the four cattle group, group 1 was given IV injection of rBoIFN-α at a dose of 5.0×10^3 IU/kg. Group 2 and group 3 were given IM injection and SC injection of rBoIFN-α at the same dose, respectively. Group 4 (the normal control group) was also injected with normal saline through the same way.

Sample Collection: Blood samples (5 mL) were collected by jugular venipuncture (using contra-lateral vein from that to which the IV dose was administered) at 0 (just prior to treatment), 0.25, 0.50, 1, 2, 3, 4, 6, 8, 12, 24 and 48 h following treatment administration [36]. Samples were collected using a 1-inch by 20-gauge sterile needle and were then deposited into blood tubes. The blood tubes were placed in an ice bath, and protected from light, allowing to clot. After that, they were centrifuged at approximately 3.000×g for 10 min within 2 h after blood coagulation, and the supernatant was transferred by pipette into duplicate plastic tubes. The obtained sera were stored at approximately -70°C prior to assay. The animal experimental protocol performed in this study was approved by the Institutional Ethics Committee of Anhui Medical University (approval number: LLSC20170364).

rBoIFN-α Analysis in Serum: The IFN titers in Madin-Darby bovine kidney (MDBK) cell line, which was infected by

vesicular stomatitis virus (VSV), were determined through the cytopathic effect (CPE) inhibition bioassay. In brief, the MDBK cells were inoculated into the 96-well microtest plates at the density of 3×10^4 cells per well and incubated in DMEM containing 3% fetal calf serum (FCS) at 37°C and 5% CO_2 humid air for 12 h. The monolayers of MDBK cells were treated with 100 µL of 4-fold serial diluted rBoIFN-α liquid. After 24-h incubation, cells were attacked by VSV at the volume of 100$TCID_{50}$/well (50% tissue culture infection dose) and continued to be cultured until the appearance of 100% CPE in the virus-infected cells (virus control well without rBoIFN-α treatment). Prior to plaque counting, the culture was stained with crystal violet. One IFN unit was defined as the highest dilution of rBoIFN-α that inhibited 50% CPE in the case of 100% CPE was observed in the non-IFN treated wells. The rBoIFN titers (IU) was expressed as the reciprocal of the dilutions resulting in 50% cell lysis through the computation with Reed-Muench method [37]. A recombinant human IFN-α (rhIFN-α1, 3×10^6 IU/mL, Lot number 97/04) was provided by the China Food and Drug Inspection Institute (Beijing, China) and was used as a positive control for CPE inhibition bioassay. The precision of the IFN standard, expressed in % RSD, was 2.1%; and the accuracy of IFN standard, expressed in relative mean error (RME), was ≤9.35%. In the current work, both the precision and accuracy values met the requirements.

Data Processing and Statistical Analysis: The mean ± standard deviation (X±SD) was adopted to explore the results about the rBoIFN-α titers. The data of serum rBoIFN-α concentrations at all time points following IV, IM and SC administrative injections were computed through the curve fitting formula using the DAS (Drug and statistics) software (Version 2.0, Wenzhou Medical University, Wenzhou, Zhejiang, China) [38], along with the adoption of noncompartmental analysis. The standard pharmacokinetic parameters included plasma concentration-time related area under curve (AUC [IU/L×h]), clearance rate [CL (L/h]), maximal plasma concentration (C_{max} [$\times10^4$ IU/L]), elimination half-life ($t_{1/2}$ [h]), time to reach peak concentration (t_{max} [h]), and mean retention time (MRT [h]). These serum concentration data were uniformed with the animal's body weight by a comparable analysis. The AUC values after subcutaneous administration was computed through the linear-up/log-down trapezoidal method. To calculate $AUC_{0-\infty}$ and CL, a terminal rate was determined with the slope to 48 h. Moreover, non-paired tailed t test were adopted to compare the data of the anti-viral activity in sera collected from the group of rBoIFN-α-treated animals with the group of normal-saline-treated control animals in each day. The statistical significance level was set to $P<0.05$.

The formula for bioavailability calculation was according to the following equation:

Bioavailability = F=(AUCs.c. or i.m. × D i.v.)/(AUCi.v. × Ds.c. or i.m.) × 100%

RESULTS

The experimental cattles were given IV, IM or SC injections of rBoIFN-α at the dose of 5.0×10^3 IU/kg, and the blood rBoIFN-α efficacy was determined through the antiviral activity in the VSV-infected MDBK cell lines.

Table 1 showed the pharmacokinetic results of rBoIFN-α in the tested animals. The pharmacokinetic features of intravenous injection of rBoIFN-α conformed to the two-compartment open model, which was associated with

Table 1. *The main pharmacokinetic parameters of rBoIFN-α for injection (n=3, X±SD)*

Parameters	Intravenous Injection Group	Intramuscular Injection Group	Subcutaneous Injection Group
T_{max} (h)	-	6.12±0.32	4.06±0.56
C_{max} (IU/L)	2400.32±128.48	1205.42±104.32	975.36±84.49
AUC (0-t) (IU/L×h)	17717.22±1421.38	12377.34±983.32	8023.41±628.29
AUC (0-∞) (IU/L×h)	29443.22±1562.47	15815.12±1014.26	8232.19±643.36
CL (L/h)	33.98±1.76	-	-
C0 (IU/h)	3049.35±486.32	-	-
MRT (h) Mean Residence Time	9.44±0.45 h	12.76±0.69	11.73±0.58
$T_{1/2\alpha}$ (h)	0.15±0.02	-	-
$T_{1/2\beta}$ (h)	6.65±0.44	8.96±0.85	7.69±0.66
$T_{1/2ka}$	-	1.12±0.27	1.81±0.34
$T_{1/2}$ (h)	6.48±0.49	8.19±0.74	7.29±0.55
Bioavailability (F)	-	53.74%	27.96%
Vdss (L)	128.64±6.86	-	-
k10 (1/h)	5.33±0.76	-	-
k12 (1/h)	0.19±0.04	-	-
k21 (1/h)	0.14±0.02	-	-

first-order elimination. Besides, the elimination half-life ($T_{1/2}$) of intravenous injection was (6.48±0.49) h *(Fig. 1)*.

The pharmacokinetic features of intramuscular injection and subcutaneous injection of rBoIFN-α conformed to the one compartment open model, Their T_{max} were (6.12±0.32) and (4.06±0.56) h, respectively, and their elimination half-life ($T_{1/2}$) were (8.19±0.74) and (7.29±0.55) h, respectively *(Fig. 2* and *Fig. 3)*. The bioavailability of rBoIFN-α from the group of intramuscular administration was 53.74%.

Gender differences among all animal pharmacokinetic parameters were not statistically significant at any dosing level (data not shown). Moreover, no possible safety issues were observed at any dosing level during or after rBoIFN-α treatment in this study.

DISCUSSION

So far, the clinical application of interferon (IFN) is hindered due to the incomplete knowledge about its mechanisms of action. Nevertheless, some supportive evidence indicates that, the route of administration, namely, the pharmacokinetic behavior of drugs, is a significant factor affecting the efficacy of treatment. The pharmacokinetic characteristics of human IFN have been fully described. Its blood concentration will be rapidly decreased soon after IV administration, and the distribution volume is close to 20-60% of body weight. Animal study suggests that, the catabolism type of IFN belong to the category of the natural processing of proteins. The Clearance value of the entire IFN family varies from one to another (range: 4.8-206 L/h), which may reflect the natural digestion and the regeneration of proteins. The terminal elimination half life of IFN-α is 4-16 h. In comparison, IM and SC administration would render prolonged but really good absorption of IFN-α, which was more than 70% [39].

With regard to bovine interferon, Gillespie et al.[40] and

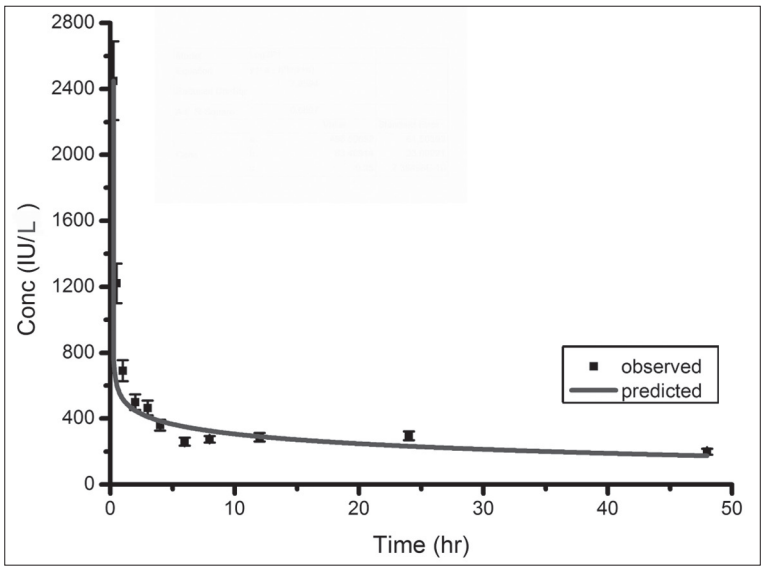
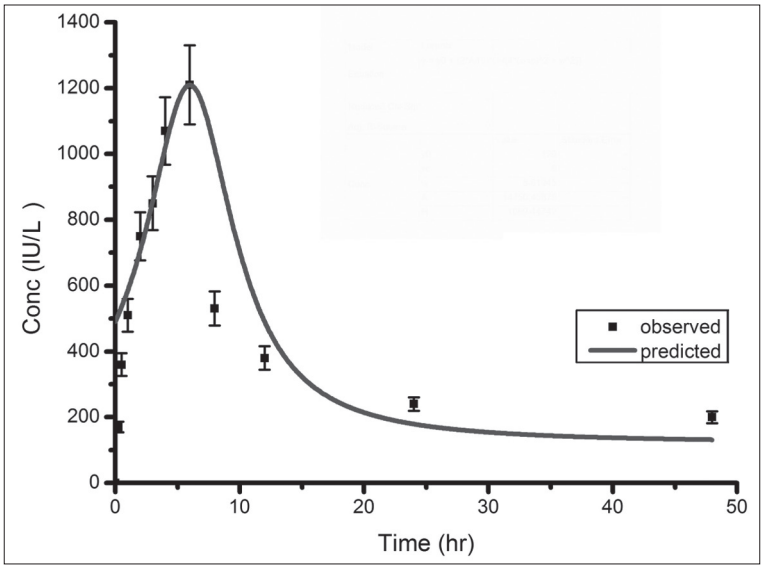


Fig 1. Serum IFN titer-time curve in the rBoIFN-α intravenous administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The intravenous injection group was tested for 3 times

Fig 2. Serum IFN titer-time curve in the rBoIFN-α intramuscular administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The intramuscular injection group was tested for 3 times



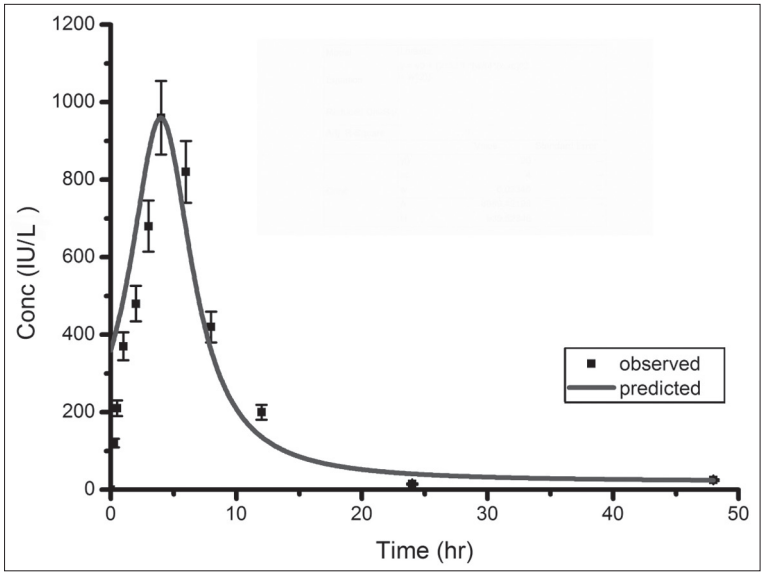


Fig 3. Serum IFN titer-time curve in the rBoIFN-α subcutaneous administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The subcutaneous injection group was tested for 3 times

Gillespie et al.[41] first reported the antiviral effects of *E. coli*-derived bovine recombinant interferon-α against bovine diarrhea virus and its application levels in the blood serum of dairy calves in 1986. rBoIFN-α has also been suggested to have a prophylactic effectiveness in controlling bovine respiratory disease. The administration of rBoIFN-α into growing calves resulted in reduced mortality and incidence of respiratory diseases. Specifically, rBoIFN-α-treated calves affected by respiratory disease showed less severe clinical symptoms, shortening of sick days and less recurrence of respiratory disease [42]. Because the research report on the pharmacokinetic assessment of rBoIFN-α is unavailable, therefore, we performed this study to investigate the pharmacokinetic characteristics of rBoIFN-α following a single injection of IV or SC or IM administrations.

Following IV administration, serum concentrations of rBoIFN-α rapid declined exponentially. rBoIFN-α serum concentration verses time data can be best fitted to a two-compartment open model, which was supported by disposition characteristics of recombinant IFN-α reported in human [25] and animals [28,30-33]. The elimination half-life ($t_{1/2\beta}$) (6.65±0.44 h) determined in the present study is longer than that reported in cats (about 0.51±0.08 h) [33], rats (2.8-6.3 h) [32], beagle dogs (about 4.5 h) [31], African green monkey(about 4.0 h) [28], but shorter than that determined in marmosets (Callitrix jacchus) (10-14 h) [32]. Clearance of rBoIFN-α observed in cattles (33.98±1.76 L/h) in the present study is faster than those reported in cats (2.56±0.61 L/h) [33], rats (1.34L/h) [32], beagle dogs (1.59±0.15L/h) [31], African green monkey (about 5.34 L/h) [28]. Besides, the clearance of the drug is also faster than that has been reported in marmosets (Callitrix jacchus) (12L/h) [32]. The drug is widely distributed in the body as determined by apparent volume of distribution Vdss (128.64±6.86 L) observed in the present study. This is in similar with that reported in marmosets [32], suggesting wider distribution of drug into the tissues of cattles.

Following IM administration, the pharmacokinetics of rBoIFN-α were well described by a classic one-compartment open model. After rBoIFN-α was injected IM, peak serum rBoIFN-α concentration (1205.42±104.32 IU/L) was achieved at 6.12±0.32 h (Tmax), which is lower than the peak recombinant IFN-α concentrations observed in African green monkey [28] and healthy human volunteers [25]. Elimination half-life following IM injection of the drug in the present study is longer than that reported in beagle dogs (about 4.7 h) [31], African green monkey (about 7.0 h) [28], healthy human volunteers (7.8±3.5 h) [25]. The absolute bioavailability (F) of rBoIFN-α following IM injection observed in the present study indicates that there was moderate good absorption of the drug from the IM injection site (F=53.74%). This value is similar to that reported in marmosets (ranged from 40-80%) [32] and slight higher than that reported in beagle dogs (42%) [31].

Following SC administration, the pharmacokinetic parameters of rBoIFN-α were slightly lower than those determined through the route of IM injection. Based on the observed serum drug concentration following IM administration of the drug in the present study, IM injection of rBoIFN-α may be used as a therapeutic route in cattles.

ELISA and cytopathic effect (CPE) inhibition bioassay are commonly used to quantitatively measure IFN concentration. Usually, ELISA is a rapid and simple way to quantitatively measure the protein concentration. Nevertheless, regarding to IFN-α, it can not determine the serum bioactivity of rBoIFN-α in due course. More significantly, Cytopathic effect (CPE) inhibition assay is a widely-used routine titer determination system for biological activity determination of human interferon. Furthermore, at the present time, no stable and reliable BoIFN-α ELISA kit approved by the competent authority is available. Consequently, CPE inhibition bioassay is employed in the current study to

quantitatively detect the bioactivity of rBoIFN-α in animal serum according to the descriptions of experimental protocols reported by several published articles [43-45].

The cytopathic effect (CPE) inhibition method employed in our study is a well-established and widely recognized method for analysis of interferon [46] and was referred to the 2015 edition of the pharmacopoeia of the People's Republic of China. Most of the interferon bioassays rely on the same biological end-point: quantification of a viral cytopathic effect of host cells [47]. Host cells and the virus selected may differ depending on the interferon of interest In general, biological fluid containing interferon is added 10 plates seeded with monolayers of host cells and incubated; the medium from each well is aspirated, followed by a washing of the cells. The cells are then challenged with a cytopathic virus. The interferon titre is read as the reciprocal of the dilution in which 50% of the cell monolayer is protected, determined by visual inspection [47,48] or spectrophotometric detection [43,49]. The method's principle was based on IFN protect cells against virus attacks ability to compute the titer of IFN, the determination results were expressed by the international units (IU).

In conclusion, rBoIFN-α was well tolerated in this study. It disseminated and disposed rapidly following a single dose of IV injection, while it was quickly adsorbed and slowly metabolized after a single-dose IM injection in cattles. Based on these data, rBoIFN-α has a potential for the treatment of viral infections without alteration of the dose and dose intervals in cattles. rBoIFN-α may be beneficial and potentially applicable in cattle industry. However, in the future, more detailed studies concerning effective dosages, timing of administration and characterization of the condition should be done to access the efficacy of rBoIFN-α in clinical practices.

Author Disclosure Statement

The authors declare that they have no competing interests in this study.

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