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J Vet Res 65, 183-192, 2021
DOI:10.2478/jvetres-2021-0023

Acute and sub-chronic toxicity study of recombinant bovine interferon alpha in rodents

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Received: September 17, 2020 *Accepted: April 9, 2020*

Abstract

Introduction: Recombinant bovine interferon alpha (rBoIFN-α) has been demonstrated to have antiviral activity. However, no conduct of acute or chronic toxicity tests has been reported. **Material and Methods:** Specific pathogen-free Sprague Dawley rats were administered doses at different concentrations through intraperitoneal or intravenous injection. After the administration (single for an acute toxicity test over 14 days or daily for a sub-chronic toxicity test over 30 days), the rats' behaviour and other indicators and the degree of toxic reaction were continuously monitored. Blood was collected for haematological and serum biochemical examinations. At the end of the experiments, the rats were sacrificed for necropsy and histopathological tissue analysis. **Results:** The external performance, behaviour characteristics, and changes in body temperature and body weight of the rats in each subgroup were comparable to the normal control subgroup. Except for a few cases, there were no lesions in the viscera's pathological structures, and the blood parameters and biochemical indicators were not noticeably different from those of the control subgroup. **Conclusion:** This study suggests that rBoIFN-α seems to be safe for rats, and its use may foster the development of the cattle industry in China by protecting livestock health.

Keywords: recombinant bovine interferon-alpha (rBoIFN-α), acute toxicity, sub-chronic toxicity, pathology, rat.

Introduction

Interferon (IFN) is a protein which induces the body to produce a broad spectrum of antiviral, antitumour, and immunomodulatory effects after viral infection. It mainly inhibits the growth and reproduction of viruses and exerts antitumour activity by inhibiting viral gene transcription or degrading viral RNA. Interferon-producing cells are divided into three categories I, II, and III according to their biochemical characteristics and the role they play in the body's immunity. Alpha-type interferon works with the aforementioned broad-spectrum of antiviral effects and exhibits them with high-efficiency *in vivo* and *in vitro*. It inhibits the synthesis of viral proteins and can act selectively on the infected cells, exerting no or a weak effect on normal host cells, and is considered nowadays to be one of the most promising biological agents (3, 11). Since Isaacs and Lindenmann (7) discovered IFN, many studies have shown that besides antiviral activity it acts as a macrophage activating factor and reduces tumour cell division. However, despite its known activity against viruses, currently only a few drugs have been approved by the US Food and Drug Administration (FDA) for the treatment of viral hepatitis B and C (20). The therapeutic efficacy of alpha-interferon has been recognised by scholars from all over the world (6, 9, 19). At present, there are more than 60 countries and regions in the world where human interferon preparations are used to treat approximately 30 viral diseases. Relatively speaking, the study of animal interferon has lagged far behind, and it is still at the stage of basic research and clinical trials (23).

Bovine IFN-α (BoIFN-α) has been proven effective against bovine viral diarrhoea virus (BVDV) (18), foot-and-mouth disease virus (FMDV) (2), and bovine

herpesvirus type 1 (12) infections. In addition, there are studies suggesting that recombinant bovine IFN-α has a preventive role in controlling bovine respiratory diseases (17), and that its administration to growing calves could reduce mortality and the incidence of respiratory diseases.

For the reason that IFN is species-specific, research on its toxicology, pharmacokinetics, and general pharmacology is beset by species-specific problems (5). Bovine viral diseases can only be treated with bovine IFN-α and not with human IFN-α. The recombinant human IFN-α drug has been on the market for many years. However, the research on BoIFN-α is still in the laboratory stage, and its development and application in antiviral therapy has yet to gain momentum.

Our group has recently produced recombinant bovine IFN-α (rBoIFN-α) in the *E. coli* expression system through molecular biology technology (22), which has laid the foundation for rBoIFN-α research and development. So far, no toxicological evaluation of rBoIFN-α has been published; its acute toxicity and safety characteristics of rBoIFN-α should be fully tested before its clinical application. Therefore, this study investigates the acute and sub-acute toxicity effects of rBoIFN-α in rats and is original research. It also provides a detailed assessment of the toxicological parameters of rBoIFN-α.

Material and Methods

Reagents and instruments. Recombinant BoIFN-α (purified product), developed by the Department of Microbiology of Anhui Medical University (22), has an interferon activity unit and potency of 1.0×10^8 IU/mL. The main test equipment included a Mettler general analytical balancer (Greifensee, Switzerland), a DK-98-Ⅱ electric thermostatic water bath box (Huanghua Faithful Instrument, Hebei, China), an Eppendorf desktop low-speed centrifuge (Hamburg, Germany), a Nikon YS100 biological microscope (Tokyo, Japan), a SYSMEX XT-2000i blood cell analyser (Kobe, Japan), a poweam A8 automatic biochemical analyser (Nanjing Poweam Medical Co., Ltd, Nanjing, China), a Leica microtome tissue slicer (Wetzlar, Germany) and Ming-Mei MS60 pathology system (Taipei,Taiwan).

Animals. Healthy specific pathogen-free (SPF) Sprague Dawley rats of body weight 90.00–120.00 g/rat were purchased from the Experimental Animal Center of Anhui Medical University, according to the test-required random grouping demands. The rats were divided into subgroups, half of each group male and half female, and they were treated with intraperitoneal or intravenous injections (these in the tail) using a 0.5 gauge needle. The rats were housed in a well-ventilated animal room under barrier maintenance conditions. The animal room was kept at a temperature of 23 ± 3.0℃, relative humidity of 50 ± 10%, and in a light cycle of 12 h of light and 12 h of darkness (07:00–19:00). Before administering the test substances, the rats were acclimated for 10 days. The male and female rats were separated in a cage with stainless steel wire mesh hung inside the cage (220 × 410 × 200 mm). Before starting the experiment, granular solid rat feed was sterilised by radiation, and tap water was sterilised with ultraviolet light.

Acute toxicity test

Preliminary test. One hundred healthy rats were divided into two groups, 50 of them for intraperitoneal administration and the other 50 for intravenous administration. Within each group, the rats were randomly divided into five subgroups of 10, half of them male and half female. Subgroup A received a dose of 1.5×10^8 IU/kg, subgroup B one of 3.11×10^8 IU/kg, subgroup C 6.25×10^8 IU/kg, subgroup D 12.5×10^8 IU /kg, and subgroup E 25×10^8 IU/kg. The animals were fasted for 12 h before administration, but they were allowed to drink water *ad libitum*. Intraperitoneal administration was performed 2 to 3 times on the day of the experiment (within 24 h), and the interval was 6 h. Symptoms of toxic reactions and the rats' weight changes were recorded. During the investigations, the time of occurrence and disappearance of various toxic reactions in each animal was also recorded. The changes in body weight, body temperature, diet, drinking water and abnormal behaviour were monitored twice a day. At the end of the experiments, the blood of the animals was collected for haematological analysis and investigation of serum biochemical indicators. Then all the rats were euthanised by cervical dislocation. The viscera of the cadavers were pathologically dissected, including the heart, liver, spleen, lungs, kidneys, gastrointestinal tract, a testis (♂), uterus (♀), and an ovary (♀) to find if there were any abnormal changes in organs. We recorded the number of deaths in the preliminary test, determined the lethal dose, and then conducted a formal test.

Formal test. Since there were no deaths of tested animals in the preliminary test, no mortality occurred at the maximum dose level in the preliminary study. Therefore, the dose levels were set according to the preliminary test results and an additional trial was added. The dose group design, test method, and observation indexes were the same as the preliminary test. Rats for intravenous administration received a single-dose injection of 0.75, 1.5, 3.11, 6.25, 12.5, and 25×10^8 IU/kg body weight (b.w.), and the volume of phosphate buffered saline (PBS) was equivalent to 10 mL/kg overnight fasting after weighing. The control group (0 IU/kg) received only the vehicle, namely PBS. Rats for intraperitoneal administration received 0.75, 1.5, 3.11, 6.25, 12.5, 25, or 50×10^8 IU/kg b.w. in a single-dose with PBS, equivalent to 5 mL/kg b.w.

Maximum tolerance test. Twenty rats were randomly selected (both male and female initial body weight considered to be a baseline 105.00 g), and administered rBoIFN-α by intravenous injection at a dose of 50×10^8 IU/kg b.w. The physiological state of the rats was observed every hour during the first 24 h

after the administration, and whether there was a poisoning reaction or abnormal behaviour was recorded. The rats were observed continuously for 14 days after administration, the feeding conditions were kept unchanged, and the diet, mental state, voluntary activities, and faeces of the rats were monitored. When the observation period ended, all the test rats were euthanised by cervical dislocation, and a post-mortem examination was performed to observe whether there were abnormal changes in the liver, spleen, and other solid organs.

Subchronic toxicity test. Forty rats were selected and randomly divided into four subgroups of 10, again of them half male and half female: a high-dose subgroup dosed with 50×10^8 IU/kg b.w., a middle-dose subgroup allocated a 5×10^8 IU/kg b.w. dose, a low-dose subgroup receiving 1×10^8 IU/kg b.w. and a control group administered only a PBS as placebo. Each group was housed in its own cage. The drug dosage design refers to the results of the acute toxicity test and maximum tolerance test. In accordance with the "Guidelines for the 30-day and 90-day feeding trials of veterinary drugs" announced by The Ministry of Agriculture of the People's Republic of China Announcement No. 1247 (August 2009, in Chinese), each dose subgroup was administered continuously for 30 days.

Observations of general symptoms. The rBoIFN-α was administered by intravenous injection. During the test, aspects of general health status of the rats comprising exercise, feeding, water drunk, faeces, body surface characteristics, morbidity, and mortality were monitored twice a day.

Feed intake and body weight recording. The rats were fed *ad libitum*, and the remaining feed was weighed every day to calculate the average daily feed intake during the test. During the test period, the amount of water drunk and residual water was weighed and recorded every day. Body weights were also measured and recorded seven times a week, and the average weight gain, weight gain rate, and feed utilisation rate were calculated using the formulae below:

Weight gain (g) = weight after test (g) − weight before test (g);

Weight gain rate (%) = weight gain (g) / weight before test (g) × 100;

Feed utilisation rate (%) = weight gain (g) / feed consumption (g) × 100.

On the 1st day after drug withdrawal (the 31st day of the test) and 1 week later (the 38th day of the test), ten rats were selected from each group, fasted for 12 h, and not given drinking water, and blood was collected retro-orbitally without the bodily sensation for haematology and blood biochemistry examination; after blood collection, the rats were euthanised by cervical dislocation, systematic necropsy and pathological examination were performed, and the observations were recorded.

Organ index assessment. After blood collection, the rats were sacrificed. Necropsies were performed to observe whether there were pathological changes in the main organs. The heart, spleen, liver, thymus, kidneys, lungs and other solid organs were taken, the wet weight was measured and the organ indexes were calculated using the formula: organ index (%) = organ weight (mg)/animal weight (g) × 100%.

Blood and biochemical assessment. After the last administration, the rats were fasted for 12 h and were weighed. The femoral artery was punctured for collection of 0.5 mL of blood into sterile Eppendorf tubes with anticoagulant EDTA. The haematological parameters were: white blood cell count, red blood cell count, haemoglobin, neutrophil, basophil, eosinophil, lymphocyte, monocyte, mean corpuscular haemoglobin concentration and platelet count, the last item was analysed using an automated cell counter (Hospitex, Florence, Italy). Another 5 mL of blood was collected and centrifuged at 2,500 rpm for 15 min to separate the serum. Serum biochemical analysis was performed to quantify aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, blood urea nitrogen, and creatinine.

Histopathological examination. After necropsy, samples were taken from liver, heart, spleen, lungs, testis and kidneys, and were fixed in formalin buffer (10%) for at least 24 h. Then, the samples were dehydrated in an ethanol gradient series, clarified in xylene, embedded in paraffin, cut into 4–5 μm sections, and stained with haematoxylin and eosin (H&E) for inspection under a light microscope. Generally, the control group and the high-dose group were checked at the first step. If the high-dose group was abnormal, then the middle-dose group and the low-dose group were checked in turn.

Maximum dose calculation. During the test, the maximum dose was calculated according to kilogram body weight, which was equivalent to 1000 times the normal dose to cattle. It is implied that a rat was dosed with an amount which would be suitable for an animal with weight in kilograms like the rat's weight in grams, i.e. a 100 g rat received the drug in the dose for a 100 kg animal.

Statistical analysis. All data were expressed as mean ± SD (standard deviation), and SPSS 18.0 software (SPSS Inc, Chicago, IL, USA) was used for analysis and processing. One-way analysis of variance (ANOVA) was applied to compare the differences among groups, and the unpaired two-tailed Student's *t*-test was performed to compare differences between two groups. In all analyses, $P < 0.05$ was set to be statistically significant, and $P < 0.01$ was set to be highly statistically significant. For the reason that no animals died during the experiment, the LD_{50} could not be calculated.

Results

Acute toxicity test results. The mortality rate of pre-test rats was 0, and no fatal intoxication occurred within 24 h. In the formal test, rats in each dose group

were drowsy and mentally depressed within 30 min of administration. Their physiological state returned to normal after 45 min, no symptoms of poisoning nor abnormal feeding were evident in the period up to 24 h from administration, and the rats exhibited normal behaviour, produced normal faeces, and gained weight normally and all consistently with the pre-test results. Necropsies of the rats revealed that there were no abnormal changes in the main organs such as the heart, liver, spleen, lungs, kidneys, thymus, and stomach. After intravenous administration of different doses of rBoIFN-α, the body weight of rats still showed an increasing trend. The average weight gain of rats in each dose subgroup was 5.18–7.40 g, and the weight gain rate was 19.09%–31.05%. The difference between the groups was not significant ($P > 0.05$). According to the classification criteria for a dose to be classified as acutely toxic in clinical medicine in China, it could be determined that rBoIFN-α was not toxic to the tested rats. Since no rat died during the test, the LD_{50} of rBoIFN-α could not be calculated, and a maximum tolerance test was required to verify its safety.

The maximum tolerance test results. No rats died during the maximum tolerance test in any dose group. Clinical observations found that the rats were depressed and their respiration rate was also slow for 30 min after intravenous administration, but their mental and physiological state gradually recovered thereafter, with normal drinking and feeding resuming. The rats did not show symptoms of poisoning in the period up to 24 h from administration. After 14 days of continuous observation, there were no symptoms of poisoning or any deaths. All the rats' signs of breathing, visible mucosa, and fur were normal, and their physiological behaviours such as drinking, feeding and defecation were normal. The heart, liver, spleen, and other solid organs did not have any characteristic pathological changes visible to the naked eye. According to drug toxicity evaluation criteria, when a preparation's $LD_{50} > 5$ g/kg, it is considered non-toxic. In this study, when the dose of the rBoIFN-α in the maximum tolerance test reached 5 g/kg, the rats were still alive and healthy, indicating that the rBoIFN-α is safe.

Subchronic toxicity test results

Clinical manifestations. During the experiment, the physiological activities of the rats were normal, and their faeces were normal as well. The animals' drinking and feeding were consistent with those of the blank control group. Fig. 1 depicts the feed intake of rats in each group trending steadily upwards from the first week to the fourth week of the test, and the maximum feed intake being reached by the fourth week. The body weight results in Fig. 2 are for males and low-dose groups. It indicates the body weight of rats in each treatment group gradually increasing with age, and does not portray any significant difference between each drug dosage subgroup and the blank control subgroup.

Organ indexes. After the several-week-long subchronic toxicology experiment, necropsy revealed that the heart, liver, spleen, lungs, kidneys, and other organs of the rats in each group had a shiny surface, uniform colour and tight texture. However, there were a small number of abnormal cases in the experimental results: after intravenous injection, one male rat in the middle-dose subgroup and one female rat in the high-dose subgroup had dark red spots on the lungs.

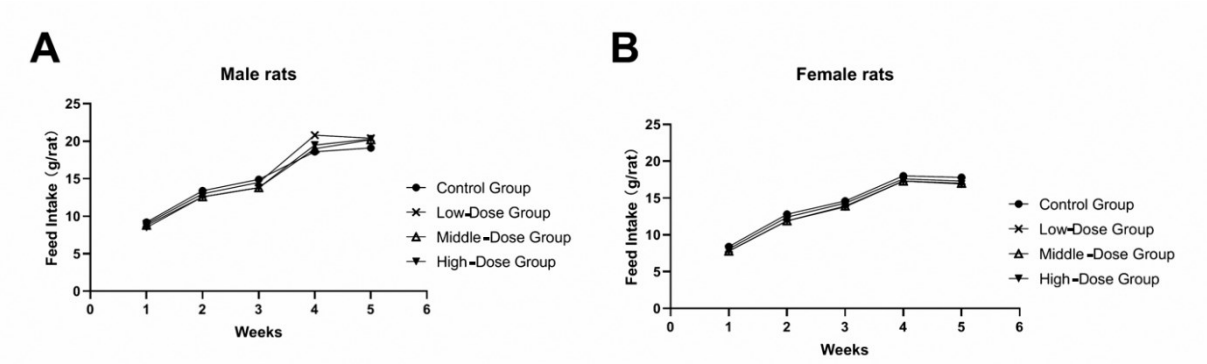

Fig. 1. Effects of rBoIFN-α on feed intake of male (A) and female (B) rats in sub-chronic toxicity test

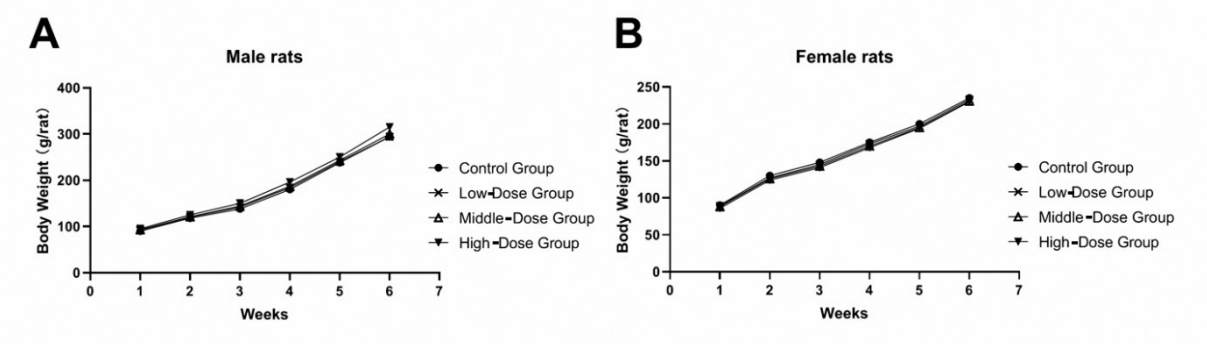

Fig. 2. Effects of rBoIFN-α on weight gain of male (A) and female (B) rats in sub-chronic toxicity test

In the high-dose subgroup, congestion was observed in the lymph nodes of one female rat. Besides, in the high-dose subgroup, there were two other female rats with hyperemia in the ovaries, accompanied by thymic nodules and dark red spots of the thymus being observed in the spleen and thymus. There was also a rough appearance on the surface of the spleen of a male rat in the high-dose subgroup.

Except for the few cases outlined above, compared with the control subgroup, the experimental subgroups showed no significant toxic lesions in the heart, liver, spleen, lungs, adrenal gland, pancreas, intestine, stomach, or ovary. On the first day after drug withdrawal (the 31st day of the test), there was no significant difference between the organ index of rats in each drug dosage subgroup and the blank control subgroup, and they were within the normal physiological range; 1 week after drug withdrawal (the 38th day of the test), the testis index of male rats in the high-dose subgroup was slightly smaller than that of the blank control subgroup ($P < 0.05$), but there was no significant difference between other organ indexes and the blank control subgroup (Table 1).

Table 1. Effects of rBoIFN-α on parenchymal organ indexes in Sprague Dawley rats in the sub-chronic toxicity test

Time	Organ	Gender	High-dose group	Middle-dose group	Low-dose group	Control group
Day 31	Heart	M	3.71 ± 0.28	3.61 ± 0.28	4.02 ± 0.42	3.60 ± 0.22
		F	3.71 ± 0.19	3.88 ± 0.57	3.66 ± 0.34	4.01 ± 0.43
	Liver	M	30.01 ± 3.19	31.53 ± 3.59	31.3 ± 2.28	28.14 ± 2.11
		F	33.78 ± 3.10	27.46 ± 4.40	28.19 ± 1.98	32.35 ± 5.27
	Spleen	M	1.80 ± 0.45	2.01 ± 0.40	2.01 ± 0.21	1.76 ± 0.24
		F	2.01 ± 0.19	2.09 ± 0.49	2.27 ± 0.99	1.89 ± 0.23
	Lung	M	4.72 ± 0.37	5.00 ± 0.54	4.61 ± 0.41	4.30 ± 1.08
		F	5.54 ± 0.79	5.01 ± 0.83	5.00 ± 0.24	5.82 ± 0.79
	Kidney	M	7.67 ± 1.24	7.53 ± 0.64	7.64 ± 0.30	7.53 ± 0.70
		F	7.52 ± 0.35	7.50 ± 1.54	6.35 ± 1.96	7.34 ± 0.83
	Stomach	M	8.74 ± 1.47	8.85 ± 1.64	9.30 ± 1.39	9.30 ± 1.39
		F	9.58 ± 3.20	8.44 ± 1.14	7.10 ± 1.32	7.83 ± 1.73
	Intestines	M	64.34 ± 11.54	64.52 ± 5.79	65.48 ± 16.00	63.52 ± 3.26
		F	55.22 ± 12.14	59.91 ± 11.92	58.32 ± 6.33	65.08 ± 9.62
	Testis	M	11.91 ± 0.70	12.02 ± 0.62	12.26 ± 1.44	12.62 ± 1.00
	Uterus and ovaries	F	3.72 ± 1.13	4.24 ± 0.73	3.88 ± 0.80	3.14 ± 0.63
Day 38	Heart	M	3.45 ± 0.98	3.42 ± 0.21	3.35 ± 0.34	3.55 ± 0.47
		F	3.59 ± 0.53	3.60 ± 0.29	3.80 ± 0.58	3.71 ± 0.45
	Liver	M	33.01 ± 1.98	31.85 ± 2.72	32.61 ± 2.83	32.06 ± 1.23
		F	27.84 ± 3.20	29.50 ± 2.41	31.15 ± 2.75	29.31 ± 2.26
	Spleen	M	1.76 ± 0.17	1.65 ± 0.17	2.01 ± 0.21	2.01 ± 0.43
		F	1.83 ± 0.37	2.01 ± 0.10	2.17 ± 0.91	1.80 ± 0.25
	Lung	M	4.16 ± 0.47	4.42 ± 0.57	4.25 ± 0.36	5.01 ± 1.10
		F	4.27 ± 0.29	4.65 ± 0.38	4.41 ± 0.24	4.64 ± 0.44
	Kidney	M	6.74 ± 0.30	7.04 ± 0.57	6.77 ± 0.17	7.25 ± 0.36
		F	6.59 ± 0.88	6.59 ± 0.58	7.03 ± 0.43	6.72 ± 0.64
	Stomach	M	8.66 ± 1.72	7.78 ± 0.82	8.58 ± 1.45	8.53 ± 0.71
		F	8.33 ± 0.86	8.07 ± 0.89	7.49 ± 1.18	7.68 ± 1.74
	Intestines	M	64.31 ± 8.66	66.15 ± 8.74	66.88 ± 8.01	66.81 ± 7.93
		F	61.07 ± 4.78	64.68 ± 11.63	58.82 ± 4.52	66.23 ± 6.21
	Testis	M	9.59 ± 0.35*	10.33 ± 0.70	9.75 ± 0.43	10.39 ± 0.56
	Uterus and ovaries	F	3.00 ± 0.74	2.85 ± 0.19	3.32 ± 0.49	3.04 ± 0.85

* represented significant difference compared with the control group ($P < 0.05$). M represented male and F represented female

Table 2. Effects of rBoIFN-α on haematological indexes in Sprague Dawley rats in the sub-chronic toxicity test

Time	Index	Gender	High-dose group	Middle-dose group	Low-dose group	Control group
Day 31	HGB (g/L)	M	151.30 ± 4.16	149.00 ± 6.48	147.90 ± 4.64	146.00 ± 0.11
		F	144.10 ± 7.98	142.10 ± 8.41	142.90 ± 12.65	147.50 ± 5.37
	RBC (× 10^{12}/L)	M	7.72 ± 0.16	7.65 ± 0.23	7.78 ± 0.33	7.63 ± 0.13
		F	7.63 ± 0.39	7.49 ± 0.35	7.59 ± 0.52	7.64 ± 0.37
	WBC (× 10^9/L)	M	14.45 ± 1.29	13.35 ± 2.26*	15.07 ± 2.66	16.23 ± 1.09
		F	8.54 ± 1.38	10.79 ± 1.64	8.90 ± 2.31	10.11 ± 1.38
	NE (× 10^9/L)	M	1.65 ± 0.29	1.84 ± 0.90	1.86 ± 0.59	2.20 ± 0.42
		F	1.18 ± 0.57	1.01 ± 0.25	1.10 ± 0.64	1.41 ± 0.36
	BA (× 10^9/L)	M	0.01 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
		F	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	EO (× 10^9/L)	M	0.10 ± 0.03	0.08 ± 0.03	0.08 ± 0.04	0.10 ± 0.05
		F	0.10 ± 0.03	0.10 ± 0.03	0.08 ± 0.04	0.12 ± 0.05
	LY (× 10^9/L)	M	12.31 ± 1.08	1.10 ± 2.36	12.66 ± 2.40	13.01 ± 1.10
		F	7.02 ± 1.13	9.56 ± 1.44	7.46 ± 1.82	8.30 ± 1.30
	MO (× 10^9/L)	M	0.26 ± 0.07	0.23 ± 0.11	0.34 ± 0.11	0.33 ± 0.11
		F	0.12 ± 0.08	0.10 ± 0.06	0.14 ± 0.02	0.18 ± 0.05
Day 38	HGB (g/L)	M	145.70 ± 8.87	140.50 ± 26.82	149.60 ± 5.02	155.20 ± 8.38
		F	134.50 ± 5.35	151.50 ± 9.53	143.70 ± 3.49	142.70 ± 8.29
	RBC (× 10^{12}/L)	M	7.39 ± 0.50	7.58 ± 0.73	7.68 ± 0.58	8.06 ± 0.34
		F	7.00 ± 1.27	7.66 ± 0.46	7.40 ± 0.24	7.50 ± 0.28
	WBC (× 10^9/L)	M	10.69 ± 3.91	10.86 ± 1.50	9.01 ± 1.24	11.92 ± 4.08
		F	7.42 ± 2.87	10.32 ± 1.24*	8.65 ± 0.92	7.33 ± 2.14
	NE (× 10^9/L)	M	0.71 ± 0.15	1.41 ± 0.54	1.05 ± 0.47	1.10 ± 0.33
		F	1.00 ± 0.50	1.00 ± 0.21	0.90 ± 0.33	1.00 ± 0.27
	BA (× 10^9/L)	M	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.01
		F	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	EO (× 10^9/L)	M	0.05 ± 0.03	0.11 ± 0.09	0.04 ± 0.03	0.10 ± 0.05
		F	0.07 ± 0.03	0.11 ± 0.04	0.10 ± 0.15	0.09 ± 0.02
	LY (× 10^9/L)	M	9.77 ± 3.84	9.03 ± 1.04	7.65 ± 0.85	10.23 ± 3.69
		F	6.09 ± 2.35	8.81 ± 0.83*	7.32 ± 0.90	6.02 ± 2.31
	MO (× 10^9/L)	M	0.10 ± 0.05	0.18 ± 0.07	0.10 ± 0.04	0.11 ± 0.06
		F	0.10 ± 0.03*	0.16 ± 0.05	0.11 ± 0.04*	0.18 ± 0.07

HGB: haemoglobin; RBC: red blood cell count; WBC : white blood cell count; NE : neutrophils; BA : basophils; EO: eosinophils; LY: lymphocyte; MO: monocyte. * represented significant difference compared with the control group ($P < 0.05$). M represented male and F represented female

Table 3. Effects of rBoIFN-α on blood biochemical indexes in Sprague Dawley rats in the sub-chronic toxicity test

Time	Index	Gender	High-dose group	Middle-dose group	Low-dose group	Control group
Day 31	ALT (U/L)	M	51.10 ± 11.03	54.10 ± 7.19	46.80 ± 5.87	51.40 ± 21.00
		F	40.80 ± 7.07	43.30 ± 5.08	43.60 ± 7.98	36.00 ± 11.90
	AST (U/L)	M	194.70 ± 31.81	190.20 ± 54.94	175.70 ± 11.80	197.30 ± 44.16
		F	203.70 ± 50.69	182.80 ± 27.34	198.20 ± 29.10	188.20 ± 28.74
	BUN (mmol/L)	M	3.48 ± 0.34	4.11 ± 0.84	3.56 ± 0.44	3.55 ± 0.72
		F	5.00 ± 082	5.00 ± 0.65	5.30 ± 0.33	5.91 ± 1.10
	CREAT (μmol/L)	M	29.10 ± 3.11	29.70 ± 5.63	27.50 ± 3.36	30.30 ± 1.67
		F	32.70 ± 3.42	34.10 ± 4.92	33.20 ± 4.83	37.10 ± 3.77
	GLU (mmol/L)	M	3.74 ± 1.34	4.02 ± 0.85	4.41 ± 0.30	4.55 ± 1.58
		F	2.56 ± 0.77	2.47 ± 0.24	2.90 ± 0.49	2.70 ± 0.44
	TP (g/L)	M	56.84 ± 1.79	55.60 ± 3.66	56.15 ± 3.89	57.10 ± 1.80
		F	61.60 ± 2.33	59.72 ± 4.11	60.70 ± 3.26	60.16 ± 3.54
	ALB (g/L)	M	42.00 ± 1.07	41.88 ± 2.99	41.58 ± 2.20	41.30 ± 0.48
		F	47.01 ± 1.06	45.92 ± 2.80	45.58 ± 2.13	44.44 ± 2.25
	CHO (mmol/L)	M	2.03 ± 0.23	2.02 ± 0.14	2.11 ± 0.20	1.81 ± 0.35
		F	1.88 ± 0.36	2.00 ± 0.10	1.70 ± 0.35	1.70 ± 0.30
	TG (mmol/L)	M	0.74 ± 0.26	0.81 ± 0.09	1.01 ± 0.14	0.64 ± 0.31
		F	0.85 ± 0.29	0.79 ± 0.12	0.69 ± 0.05	0.68 ± 0.19
Day 38	ALT (U/L)	M	49.20 ± 2.30	56.20 ± 3.97	65.80 ± 15.98	60.20 ± 15.47
		F	38.50 ± 6.43	40.20 ± 5.64	42.00 ± 9.88	42.70 ± 5.17
	AST (U/L)	M	125.30 ± 13.07	120.20 ± 12.74	153.70 ± 28.60	143.00 ± 18.15
		F	124.10 ± 24.77	121.00 ± 17.03	125.50 ± 13.10	132.40 ± 13.85
	BUN (mmol/L)	M	4.73 ± 0.93	5.01 ± 0.79	4.69 ± 1.06	5.10 ± 0.95
		F	6.00 ± 0.32	5.77 ± 0.97	6.48 ± 0.36	5.96 ± 0.66
	CREAT (μmol/L)	M	29.30 ± 3.91	33.10 ± 6.95	29.00 ± 4.04	31.30 ± 3.91
		F	38.70 ± 4.02	38.50 ± 3.65	39.20 ± 3.65	34.90 ± 2.74
	GLU (mmol/L)	M	6.40 ± 0.71	6.11 ± 0.20	5.32 ± 0.79*	7.10 ± 1.62
		F	4.17 ± 0.74	4.70 ± 0.59	5.11 ± 0.51	4.71 ± 0.70
	TP (g/L)	M	57.24 ± 2.17	58.60 ± 2.46	57.16 ± 0.82	56.86 ± 1.95
		F	62.44 ± 2.64	61.30 ± 4.50	63.64 ± 1.34	63.02 ± 1.77
	ALB (g/L)	M	57.24 ± 2.17	58.50 ± 2.46	57.26 ± 0.82	57.00 ± 1.95
		F	31.70 ± 1.55	31.80 ± 2.21	32.20 ± 2.01	32.20 ± 0.79
	CHO (mmol/L)	M	1.80 ± 0.33	1.91 ± 0.05	1.63 ± 0.14	1.80 ± 0.34
		F	1.80 ± 0.18	1.62 ± 0.25	1.63 ± 0.10	1.77 ± 0.20
	TG (mmol/L)	M	0.90 ± 0.38	1.05 ± 0.52	1.02 ± 0.21	1.18 ± 0.13
		F	0.91 ± 0.02	0.86 ± 0.30	0.92 ± 0.06	1.11 ± 0.26

ALT: alanine aminotransferase; AST : aspartate aminotransferase; BUN : blood urea nitrogen; CREAT: creatinine; GLU: glucose; TP: total protein; ALB: albumin; CHO : cholesterol; TG: triglyceride. * represented significant difference compared with control group ($P < 0.05$). M represented male and F represented female

Haematological parameters. On the first day after drug withdrawal, only the white blood cell count of male rats in the middle-dose subgroup was significantly lower than that of their control group counterparts, and there were no significant differences between other haematological indicators in the experimental subgroups and the blank control group (Table 2). One week after stopping the drug administration, the number of white blood cells and lymphocytes of the female rats in the middle-dose subgroup was significantly higher than that in the rats in the blank control group. The number of monocytes in the high- and low-dose subgroup animals were markedly lower than those in the blank control subgroup rodents, but were all within the normal physiological range (15); the routine blood haematological indexes of male rats in each drug dosage subgroup were not significantly different from those of the blank control subgroup males.

Serum biochemical parameters. On the first day after drug withdrawal, the blood of laboratory animals was taken for biochemical index examination. There were no significant differences between the serum biochemical indexes of the rats in each dose subgroup and those of the rats in the blank control subgroup (Table 3); one week after the withdrawal, the blood glucose of male rats in the low-dose subgroup was significantly lower than that in the control subgroup males. Except for the control subgroup, there were no significant differences on the other serum biochemical indicators between the experimental testing subgroups. Table 3 shows that the alanine aminotransferase of female rats in the low-dose subgroup was slightly higher than that in the control group subjects ($P < 0.05$), and that there were no significant differences between the biochemical indexes of the other subgroups and the control subgroup.

Organ pathology and structure. The main organs (the liver, spleen and kidney) of rats in each treatment subgroup were taken, fixed with 10% formalin to make paraffin sections, and observed under an optical microscope. The results showed that there were no abnormal pathological changes in kidney, liver, or spleen paraffin sections (Fig. 3). In paraffin tissue sections of the rat liver, hepatocytes were closely arranged, and the structure of the liver lobule was intact, without abnormal changes; the red pulp and white pulp areas in the spleen tissue section were clearly defined, and the trabecular structure was not abnormal; the structure of the rat kidney was complete. There were no abnormal changes in the volume and shape of the glomeruli or the regularity of the tubular epithelial cells.

Gross observations. Indicators included death, food consumption, urine, faeces, spirit, activity, hair, body temperature, body weight and changes detected in pathological examination. The findings of gross observation were as follows:

(1) Changes in behaviour characteristics: In the acute toxicity and sub-chronic toxicity experiments, the behavioural characteristics of the experimental rats were basically unchanged after injection. Claws and tails were the normal flesh red colour, with no erosion, inflammation, redness, or swelling. Physical activity, scratching ears, flinching feet, and frequent head shaking were not observed. Fur colour was normal and no shedding was observed. Frequent bites of feed were taken and good appetite was noted. Faeces were basically uniform in size, slightly moist, and black. No urinary incontinence was recorded. The animals were responsive to touch.

(2) Deaths: In the acute toxicity experiment, none of the rats died. In the subchronic toxicity experiment, the rats also all lived until the end of experiment.

(3) Changes in body weight: The results showed that in the acute toxicity test, the weight change was relatively smooth, with a slight upward trend, but the difference was not significant. In the subchronic toxicity test, its change also showed an upward trend, and there were no large fluctuations.

(4) Changes in body temperature: After administration, the body temperature changes of the animals in each group were similar in the acute toxicity and subchronic toxicity experiments. Specifically, the temperature of the rats in each group fluctuated over a range of 36~38℃, and there was no abnormal appearance in body temperature. Body temperature changes did not exceed 2℃, which varied in the normal fluctuation range.

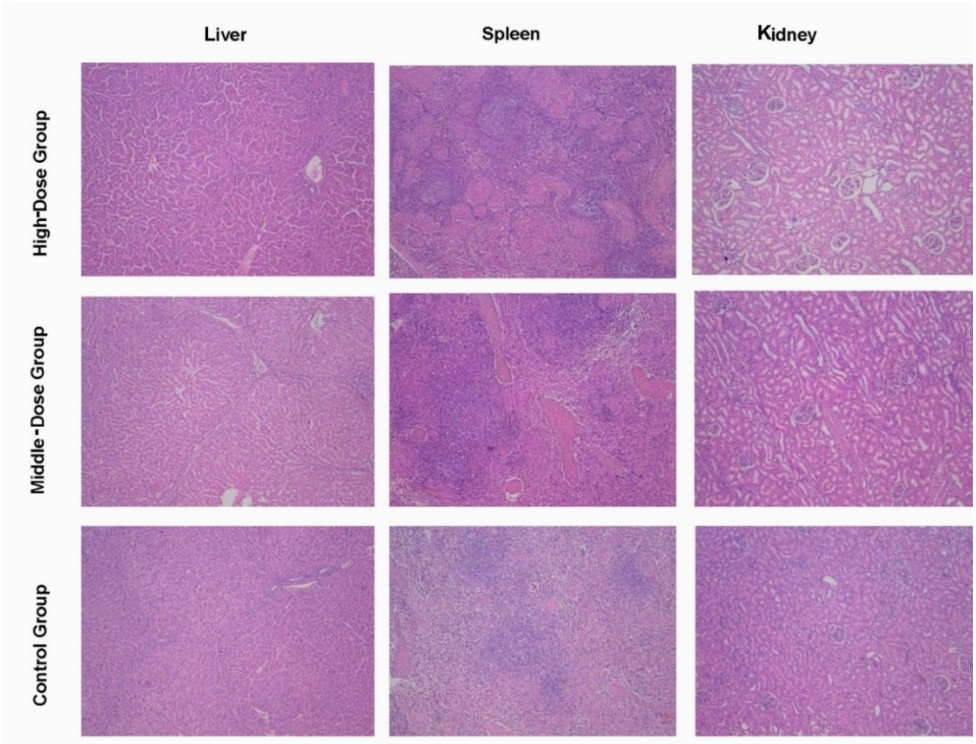

Fig. 3. Effects of rBoIFN-α on the morphology of liver, kidney and spleen in Sprague Dawley rats (HE staining, ×200)

Discussion

Kim *et al.*'s study (8) confirmed that the recombinant human interferon alpha (rHuIFN-α) preparation has no long-term toxic effect on Sprague Dawley rats, since various blood indicators of rats and the organ indexes did not show drug toxicity. Since the production of genetically engineered recombinant interferon has been approved, many preclinical or clinical trials have been conducted. The dose-limiting toxicity of rHuIFN-α induces nausea, vomiting, fever, fatigue and anorexia (4, 16, 21). However, no acute toxicity test or chronic toxicity test involving rBoIFN-α had been reported prior to this research.

The acute toxicity test results of this study on rBoIFN-α showed that even if the dose of bovine recombinant interferon alpha was as high as 50×10^{10} IU/kg b.w., it did not cause poisoning symptoms or death in the experimental rats, and after continuing to observe for another 14 days, it was found that the feeding and weight gain of the rats were normal, and the post-mortem observation showed that there were no abnormal changes in the heart, liver, spleen or other solid organs. In the maximum tolerance test, the rBoIFN-α was administered in doses of up to 50.000 g/kg and the rats were still alive and healthy at the end of the test. Their signs of breathing, visible mucosa, and fur were not abnormal, and post-medicine necropsy observation revealed that no substantial organs had visible characteristic pathological changes. The results suggested that rBoIFN-α had no toxic effect in rats, and could be expected to protect the health and safety of cattle.

The purpose of the sub-chronic toxicity test is to observe the toxic response of the animal caused by continuous repeated administration of the test drug, including the symptoms and severity of the occurrence and the primary organ evincing toxic effects and its recovery and development, and then to determine the non-toxic dose, according to the references for the safe clinical medication (1). The 30-day administration test in rats can be used as the main reference basis for evaluating the sub-chronic hazards of drugs or poisons. This experiment investigated the toxicity of rBoIFN-α by observing the clinical manifestations and histopathological changes, and determining haematological parameters, serum biochemical indexes, and organ indexes of rats after intravenous injection of drugs (10, 14). These latter included a relatively high gastrointestinal index for the middle- and high-dose groups. Considering the influence of the test animal indicators on the recipient weight, nutritional status, and other conditions, individual indicators might also have significant differences among groups. However, overall, after several-week-long administration of rBoIFN-α, there were no significant differences in rat body weight, blood haematology indicators, blood biochemical indicators, or organ indexes between experimental and control groups. Furthermore, no pathological changes related to drug effects were observed. These null findings implied that rBoIFN-α had no or extremely low toxicity.

It can be seen that use of rBoIFN-α over a sustained period had no significant effect on the haematological indexes of rats. Likewise it can be noted that administration of this recombinant interferon over the experimental duration did not influence serum biochemical indexes in rats. Several-week-long administration of rBoIFN-α induced only mild toxic effects or side effects on the main organs of rats and these effects did not usually injure the morphological structures of those organs.

Regarding the adverse drug reactions, some new findings have been made in experimental animals. For example, dark red discoloration and swelling of the liver were observed in two male rats. Congestion was observed in the ovary of a female rat in the control group and the 5×10^{8} IU/kg group. However, these are individual phenomena and they ought not to be considered treatment-related changes. The incidence is very low and is independent of the dose level.

This study's results showed that rBoIFN-α had no obvious toxic effect on test animals and is safe to use. During the test, the maximum dose was calculated according to kilogram body weight, which was equivalent to 1000 times the normal dose to cattle. Therefore, even as rBoIFN-α was administered in a 1000-fold larger dose than the clinical dose amount, it still had no noticeable acute toxic effect on rats. This result establishes a further experimental basis for the clinical use of rBoIFN-α.

The above results suggest that several-week-long administration of rBoIFN-α has rare adverse effects on the intake and body weight of rats. Besides, the several-week-long administration of rBoIFN-α seems not to cause substantial damage to the parenchymal organs of rats or induce significant abnormal changes. Therefore, this study suggests that rBoIFN-α seems to be safe for rats, and its use may foster the development of the cattle industry in China by protecting livestock health.

*Hai-Yang Yu and Dong-Mei Gao contributed equally to this study and should be considered co-first authors.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was supported by the National Key R&D Program of China under grant no. 2017YFD0500906, the Natural Science Foundation of Anhui Province under grant no. 1808085MC75, the Wuhu Science and Technology Plan Project under grant no. 2019yf44, and funding for scientific research activities of postdoctoral researchers in Anhui under grant no. 2017B194. The funders did not participate in the study design, data collection, data analysis, publishing decision, or manuscript writing.

Animal Rights Statement: Animal experiments and experimental protocols were approved by the Research Ethics Committee of Anhui Medical University (reference number LLSC20170364). Acute and sub-chronic toxicity studies were also conducted according to the Organisation for Economic Cooperation and Development (OECD) Toxicity Research Test Guideline 407 (13). The authors confirm that they have adhered to international standards for the protection of animals used for scientific purposes.

Acknowledgements: The authors are grateful to the whole research staff in Wuhu Interferon Bio-products Industry Research Institute Co., Ltd, (Wuhu, Anhui, P.R. China) for technical assistance.

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