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Preclinical Safety Evaluation: Acute and Repeated-Dose Toxicity of a New Intranasal Recombinant Vector Vaccine TB/FLU-04L Against Tuberculosis

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ABSTRACT

Background Vaccination against tuberculosis is one of the most successful medical measures to reduce morbidity and mortality. The BCG vaccine has been in use for more than 100 years, but its efficacy is still controversial. New vaccine candidates may offer better protection than available BCG vaccine. In this work, we studied the acute and the repeated-dose toxicity study of a new vector vaccine TB/Flu-04L against tuberculosis.

Materials and Methods The study was conducted on 60 BALB/c mice and 150 Wistar rats. The vaccine was administered intranasally and intravenously for the acute toxicity study. For the repeated-dose toxicity study, rats were intranasally immunized by 6.5 $\log_{10}$ $TCID_{50}$ or 7.5 $\log_{10}$ $TCID_{50}$ three times with 21-day intervals. Mortality, temperature, body weight, food and water consumption, hematological and biochemical parameters, urine analysis, as well as cardiovascular, respiratory, and central nervous system parameters were evaluated. A macroscopic examination of internal organs was performed.

Results The TB/FLU-04L vaccine did not cause death among the mice and rats in the acute toxicity study. There were no pathological abnormalities in animal condition, behavior, food and water consumption, temperature, and body weight during the observation period. The results suggest that intranasal repeated-dose administration of the TB/FLU-04L vaccine does not exhibit significant toxicity in rats.
Hematological and biochemistry analysis and the histological examination identified no toxicity-associated changes.

Conclusions The toxicity study in mice and rats showed that the intranasal vector vaccine TB/FLU-04L had no toxic effect. The tests confirm no adverse effects for laboratory animals in the studied parameters.

Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (Mtb), is one of the deadliest diseases according to the WHO data. Approximately 1.4 million people died from TB-related illnesses in 2019 [1]. Despite the progress made in the diagnosis and treatment of TB, a tense epidemiological situation persists in some regions of the world. Therefore, many countries pay great attention to the prevention of this disease.

The only tuberculosis vaccine currently available is BCG which is an attenuated strain of *Mycobacterium bovis*, although its efficacy against TB in adults remains controversial. Furthermore, drug-resistant TB is becoming a critical worldwide issue [2, 3]. BCG is the oldest vaccine currently in use, as it has been available for almost 100 years. Nevertheless, the population needs better quality TB vaccines to boost the immune system after the initial BCG vaccination. The past decade has seen an explosive increase in the development of new potential TB vaccine candidates. Various types of

novel vaccine candidates are being discussed, including antigen/adjuvant subunit vaccines, viral vector vaccines, multi-component vaccines, whole-cell mycobacterial vaccines which come as either live recombinant or dead whole-cell ones, the viable BCG-based vaccine, attenuated strain Mtb, and DNA vaccines [4].

The new TB/Flu-04L vaccine candidate has been developed by the Research Institute for Biological Safety Problems (Kazakhstan) in collaboration with the Smorodintsev Research Institute of Influenza (Russia). The vaccine is constructed from influenza virus and suggests an intranasal administration route, as it is known that mucosal vaccination provides significantly better immune protection against pulmonary tuberculosis than systemic vaccination [5]. Recombinant viral vector vaccines have several advantages over protein-based or inactivated vaccines. Even in the case of a replication deficiency, infectious viral vectors are able to induce a full range of humoral and cellular immune responses initiated on the surfaces of the mucous membrane. It is important to note that viral vectors may have auto-adjuvant activity by stimulating innate immune systems [6]. In a preclinical mouse model of TB, an intranasal boost of TB/FLU-04L can significantly improve the protective efficacy of BCG [7].

This study reports the results of preclinical testing of TB/FLU-04L which is a prerequisite for the subsequent clinical trials. Preclinical trials aim to assess the potential toxicity of a new therapeutic drug in animals before the medicine can be tested in human participants.

Materials and Methods

The tuberculosis vaccine

The recombinant vector vaccine TB/FLU-04L for TB prevention was developed in the Research Institute for Biological Safety Problems (RIBSP CS MES RK) and Smorodintsev Research Institute of Influenza (SRII), and it is based on attenuated influenza strain Flu NS_{106}/ESAT-6_Ag85A expressing mycobacterial antigens Esat-6 and Ag85A. The vaccine candidate was produced in WHO-certified Vero cells cultured under serum-free conditions. The harvest was purified by consequent clarification, concentration, and diafiltration and formulated in a sucrose-phosphate-glutamate stabilizing buffer (SPGN). The stabilizing buffer was used as a control in the animal studies (Placebo). The vaccine candidate TB/FLU-04L was used in 1 immunization dose (1 ID), which equals 6.5 $\log_{10}$ Tissue Culture Infectious Dose ($TCID_{50}$), and in 10 ID (7.5 $\log_{10}$ $TCID_{50}$).

Laboratory animals

All experiments in animals were carried out in agreement with European and national directives for the protection of experimental animals and were approved by the competent local ethical committees. The research was conducted on 60 BALB/c mice (18–20 g body weight) and 150 Wistar rats (160–190 g body weight) of both sexes, obtained from an accredited laboratory animal nursery of the branch "Stolbovaya" of the Federal state budgetary institution of science "Scientific center for biomedical technologies of the Federal medical and biological agency" (Moscow). These animals were randomized and divided into groups according to the study design (▶ **Table 1**).

In the acute toxicity study, mice and rats were immunized intranasally (i.n.) and intravenously (i. v.) with 10 ID/animal of the TB/FLU-04L vaccine. The vaccine was administered 3 times with a 2-hour interval between injections. Clinical signs and body weight were assessed every week. The animals were sacrificed on the 14th day after immunization and their internal organs were histologically examined.

For the repeated-dose toxicity study, rats were immunized intranasally 3 times with 21-day intervals. The research was conducted following national requirements for animal care and non-clinical methodological recommendations [8]. Body weight, temperature, food and water consumption, behavior, respiration rate, and the state of the cardiovascular system were assessed; blood and urine samples were also analyzed. Histological examination of internal organs was performed in control animals (Placebo) and rats treated with the maximum vaccine dose (10 ID).

Statistical analyses

The Prism 8.0 (GraphPad Software, Inc., USA) software was employed for statistical analyses. Data were analyzed for Gaussian distribution using the Shapiro–Wilk test. Differences between groups of normally distributed data were assessed using one-way ANOVA. Kruskal-Wallis test was applied to non-normally distributed data, followed by Dunn's *post hoc* analyses.

▶ **Table 1** Study design.

Type of study	Test system	Number of animals (males + females)	Dose, method of administration
Acute toxicity study	Mice	N = 20	10 ID – 6.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., (0.2 ml) i. v.
	Rats	N = 20	10 ID – 6.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., (0.2 ml) i. v.
		N = 20	Placebo, (0.02 ml) i/n, (0.2 ml) i. v.
		N = 20	10 ID – 7.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., (0.2 ml) i. v.
		N = 20	10 ID – 7.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., (0.2 ml) i. v.
		N = 20	Placebo, (0.02 ml) i/n, (0.2 ml), i. v.
Repeated-dose toxicity study	Rats	N = 30	1 ID – 6.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., triple immunization
		N = 30	10 ID – 7.5 $\log_{10}$ $TCID_{50}$ (0.02 ml), i.n., triple immunization
		N = 30	Placebo, (0.02 ml), i.n., triple immunization

1 ID – 1 immunization dose, 10 ID – 10 immunization dose, $TCID_{50}$ -Tissue culture infectious dose, i.n. – intranasal immunization, i. v. – intravenous immunization.

Results and Discussion

In both the acute toxicity study in mice and rats and the repeated-dose toxicity study in rats, the maximum 10 ID dose of TB/FLU-04L did not cause death among the experimental animals and led to no changes in their appearance and motility, behavioral reactions, and food and water consumption. The temperature and body weight of the animals treated with the vaccine did not significantly differ from those of the Placebo group. Triple immunization with 21-day intervals did not affect the cardiovascular, respiratory, and central nervous system parameters.

The effect of the TB/FLU-04L vaccine on kidney function

The obtained results indicated no significant differences in the kidney function between the experimental and control (Placebo) animals after the triple immunization. The exception was an increase of red blood cell levels in females with 1 ID after the third immunization (▶ **Table 2**).

At the same time, females with 10 ID demonstrated a slight decrease in the urine output coupled with an increase in urine density and protein level. However, there were no statistically significant differences in the urine protein-creatinine ratio (P/C-Ratio) in all the examined groups. Proteinuria in females was not absolute due to the release of more concentrated urine. The revealed differences, although statistically significant, did not exceed the intraspecific variation and the physiological norm, were due to an extremely small intragroup variance, and had no clinical significance.

Hematological parameters in the repeated-dose toxicity study

The morphological composition of blood in experimental and control animals (Placebo) did not differ. The animals with 1 ID demonstrated a decrease in mean corpuscular hemoglobin (MCH) level and mean corpuscular hemoglobin concentration (MCHC) after the second immunization. Decreasing MCH levels were observed in animals with 10 ID after the second immunization. The changes in hematological parameters did not go beyond the physiological norm in rats (▶ **Table 3**). The relative and absolute count of white blood cells (WBC) and red blood cells (RBC) did not change after immunizations.

▶ **Table 2** Urine analysis after the third immunization in rats in the repeated-dose toxicity test.

Parameters	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID
Sex	**male**			**female**		
Volume (ml/day/kg)	59.81 ± 10.17	53.43 ± 6.06	51.20 ± 5.16	76.49 ± 20.57	64.73 ± 9.23	34.91 ± 4.53 * ^
Glucose (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Protein (mg/dL)	4.00 ± 4.00	2.00 ± 2.00	14.00 ± 5.10	0.00 ± 0.00	2.00 ± 2.00	20.00 ± 4.47 * ^
P/C-Ratio	0.71 ± 0.10	0.68 ± 0.11	0.83 ± 0.13	0.42 ± 0.09	0.52 ± 0.10	0.59 ± 0.10
pH	6.30 ± 0.12	6.60 ± 0.10	6.50 ± 0.00	6.40 ± 0.10	6.40 ± 0.19	6.50 ± 0.00
Specific gravity (g/ml)	1.017 ± 0.002	1.018 ± 0.001	1.027 ± 0.003 *	1.020 ± 0.003	1.019 ± 0.002	1.041 ± 0.003 * ^
RBC (cell/µl)	0.44 ± 0.12	7.08 ± 6.63	0.88 ± 0.19	0.44 ± 0.13	2.24 ± 0.92 *	0.60 ± 0.14
WBC (cell/µl)	5.80 ± 3.57	19.32 ± 9.18	18.88 ± 9.37	5.32 ± 1.99	25.16 ± 12.27	20.52 ± 11.35

* – compared to Placebo, $p < 0.05$. ^ - compared to 1 ID, $p < 0.05$. RBC – red blood cells. P/C-Ratio – protein to creatinine ratio in urine; RBC – red blood cells. P/C-Ratio – protein to creatinine ratio in urine.

▶ **Table 3** Hematological profile of rats in the repeated-dose toxicity test.

Parameters	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID
Sex	**male**			**female**		
	2nd immunization					
RBC (10^{12}/l)	9.10 ± 0.35	9.10 ± 0.14	9.19 ± 0.25	7.92 ± 0.21	8.82 ± 0.38	8.43 ± 0.17
Hemoglobin (g/l)	149.60 ± 3.61	144.80 ± 2.85	148.40 ± 4.20	143.80 ± 3.32	144.80 ± 4.21	143.8 ± 3.44
MCH (pg)	16.46 ± 0.30	15.90 ± 0.19	16.22 ± 0.48	18.22 ± 0.40	16.50 ± 0.38 *	17.04 ± 0.29 *
MCHC (g/l)	284.60 ± 3.72	282.00 ± 4.22	286.40 ± 3.71	294.00 ± 3.56	278.00 ± 3.70 *	284.80 ± 3.60
Platelets (10^9/L)	726.20 ± 69.24	720.20 ± 30.84	845.60 ± 52.60	845.80 ± 26.83	799.20 ± 63.92	756.60 ± 90.39
WBC (10^9/L)	17.06 ± 1.44	14.52 ± 1.42	14.96 ± 2.72	12.64 ± 1.26	14.34 ± 2.18	11.80 ± 1.78
Basophil (10^9/L)	0.00 ± 0.00	0.02 ± 0.02	0.02 ± 0.02	0.03 ± 0.03	0.02 ± 0.02	0.01 ± 0.01
Neutrophil (10^9/L)	3.60 ± 0.41	2.42 ± 0.21	2.53 ± 0.40	2.44 ± 0.32	2.91 ± 0.61	2.18 ± 0.22
Monocyte (10^9/L)	0.56 ± 0.18	0.50 ± 0.06	0.46 ± 0.07	0.39 ± 0.04	0.50 ± 0.05	0.38 ± 0.07
Lymphocyte (10^9/L)	12.82 ± 0.96	11.53 ± 1.46	11.91 ± 2.37	9.76 ± 1.16	10.84 ± 1.53	9.22 ± 1.53

* – compared to Placebo, $p < 0.05$. ^ - compared to 1 ID, $p < 0.05$. RBC – red blood cells, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, WBC – white blood cells.

▶ **Table 4** Clinical biochemistry parameters of rats in the repeated-dose toxicity test.

Parameters	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID	Placebo	TB/FLU-04L, 1 ID	TB/FLU-04L, 10 ID
Sex	**male**			**female**		
	2nd immunization					
Albumin (g/L)	28.7 ± 0.3	29.8 ± 1.2	28.6 ± 1.8	31.0 ± 1.1	27.5 ± 0.9	32.2 ± 1.1
Total Protein (g/L)	69.9 ± 1.0	77.1 ± 1.0	72.9 ± 1.8	79.7 ± 2.6	72.1 ± 1.5	75.0 ± 1.8
Globulins (g/L)	41.2 ± 0.9	47.2 ± 2.0	44.3 ± 1.3	48.7 ± 2.2	44.6 ± 1.7	42.7 ± 0.8
ALT (U/L)	106.8 ± 5.9	86.8 ± 8.2	89.4 ± 8.0	90.1 ± 7.0	95.8 ± 7.4	89.3 ± 3.7
AST (U/L)	248.9 ± 9.2	241.2 ± 20.0	217.1 ± 14.6	258.2 ± 22.4	220.3 ± 11.8	238.9 ± 5.7
Alkaline phosphatase (U/L)	614.1 ± 52.8	399.1 ± 56.4	748.3 ± 82.2	374.7 ± 43.6	574.9 ± 26.9	386.9 ± 48.0
Bilirubin (umol/L)	8.76 ± 0.90	7.12 ± 0.44	9.56 ± 0.47	9.86 ± 0.81	11.22 ± 1.75	8.78 ± 0.96
Glucose (umol/L)	6.74 ± 0.31	6.34 ± 0.28	7.33 ± 0.41	7.08 ± 0.25	7.34 ± 0.28	7.54 ± 0.35 *
Cholesterol (umol/L)	2.23 ± 0.10	2.28 ± 0.17	1.73 ± 0.10 *	2.03 ± 0.17	1.73 ± 0.13 *	2.17 ± 0.15
Triglycerides (mmol/L)	0.79 ± 0.11	0.55 ± 0.05	0.91 ± 0.07	1.05 ± 0.13 *	1.25 ± 0.30	0.84 ± 0.13
Urea (mmol/L)	5.45 ± 0.39	4.55 ± 0.27	5.57 ± 0.45	5.32 ± 0.27	5.31 ± 0.39	4.68 ± 0.29
Creatinine (umol/L)	69.8 ± 5.6	69.0 ± 2.0	70.3 ± 1.8	77.7 ± 3.4	76.9 ± 6.1	67.6 ± 2.2^
Fibrinogen (g/L)	2.34 ± 0.27	2.31 ± 0.15	2.34 ± 0.20	2.29 ± 0.15	2.29 ± 0.26	2.31 ± 0.14
	3rd immunization					
Total Protein (g/L)	68.0 ± 1.1	74.1 ± 1.6	71.5 ± 1.1	81.7 ± 1.4 * ^	72.2 ± 1.2	76.0 ± 1.1^
Globulins (g/L)	40.9 ± 1.0	44.4 ± 0.6	42.1 ± 0.9	49.7 ± 1.6 * ^	44.4 ± 0.8	45.6 ± 1.3^
ALT (U/L)	91.0 ± 3.2	76.2 ± 5.3	100.3 ± 4.7	90.2 ± 3.9	97.3 ± 4.1	68.9 ± 2.0^
AST (U/L)	276.9 ± 7.2	254.2 ± 16.6	246.1 ± 13.6	295.1 ± 20.8	274.5 ± 8.9	239.4 ± 10.6
Bilirubin (umol/L)	6.52 ± 0.48	7.46 ± 1.19	8.62 ± 0.56 *	11.82 ± 1.10 * ^	7.56 ± 0.25	7.58 ± 0.52^
Glucose (umol/L)	8.88 ± 0.32	9.10 ± 0.16	10.18 ± 0.54	8.50 ± 0.23	8.56 ± 0.33	8.66 ± 0.43
Cholesterol (umol/L)	3.17 ± 0.33	2.60 ± 0.09	2.29 ± 0.04 *	2.94 ± 0.54	2.71 ± 0.43	2.51 ± 0.12
Triglycerides (mmol/L	0.43 ± 0.02	0.55 ± 0.13	0.72 ± 0.06 *	0.81 ± 0.09	0.61 ± 0.08	0.48 ± 0.04
Creatinine (umol/L)	63.8 ± 1.6	68.9 ± 3.0	66.3 ± 1.2	90.7 ± 7.5 *	64.3 ± 1.3	71.1 ± 2.9^
Fibrinogen (g/L)	2.29 ± 0.30	2.31 ± 0.21	2.31 ± 0.22	2.33 ± 0.18	2.33 ± 0.34	2.31 ± 0.18

* – compared to Placebo, $p < 0.05$. ^ - compared to 1 ID, $p < 0.05$. ALT – Alanine transaminase, AST – Aspartate transaminase; ALT – Alanine transaminase, AST – Aspartate transaminase.

Biochemical parameters in the repeated-dose toxicity study

The first immunization provoked the following changes in biochemical parameters. Male rats with 1 ID showed an increase in total cholesterol level with creatinine concentration; females from this group demonstrated an increase in glucose level and total cholesterol concentration. The rat males with 10 ID showed an increase in creatinine level, while females demonstrated a decrease of alkaline phosphatase activity with an increase in glucose level, urea level, and creatinine concentration. Increased albumin level and total protein concentration were provoked by the redistribution of serum protein fractions.

After the second immunization, males with 1 ID showed a decrease in total cholesterol level (▶ **Table 4**).

Females from this group demonstrated an increase in triglycerides levels. The male rats with 10 ID exhibited a decrease in total cholesterol levels after the second immunization, and a decreased creatinine level and increased glucose and calcium levels were observed in the female rats. These changes seem not to be induced by any toxic effects but can be attributed to the following factors.

The first factor arises from the adequate immune response due to the activity of lipid-binding fractions of blood serum proteins. Another factor is associated with the small intragroup dispersion (▶ **Table 3**).

The third immunization resulted in an increased total bilirubin level and a decreased triglycerides level in the rat males with 1 ID. Females from this group showed an increase in total protein and globulins levels with higher total bilirubin and creatinine concentrations. These differences were characterized by an increase of total protein and globulins levels as a manifestation of the immune response to vaccination, higher total bilirubin and creatinine concentrations, and a decrease in alanine aminotransferase activity. Lower cholesterol levels were observed in males with 10 ID after the third immunization. The presented changes were not provoked by any toxic effects, since the functions of the organs may be temporarily changed while staying within physiological limits of the norm for rats. These differences were not related to sex, dose, or the duration of vaccine administration. The identified statistical differences in the studied parameters were not clinically significant.

Histological examination

According to the results of the full assessment in histological trials, TB/FLU-04L administered to laboratory animals did not initiate inflammation or any alternative pathological manifestations in the form of dystrophic and destructive changes in the lungs, liver, kidneys, spleen, or cardiac muscle. Furthermore, neither dystrophic changes in neurons and glial elements nor impairments of the brain microcirculatory bloodstream circulation were noted. In vaccinated animals, the histological pattern of the organ samples under examination did not differ from the relevant samples of the control group animals.

Conclusion

The results show that the TB/FLU-04L vaccine is well tolerated in acute and repeated-dose toxicity studies. The acute toxicity test identified no lethal effect and no pathological changes in animal condition, behavior, clinical signs, food and water consumption, temperature and body weight, and other vital functions. The vaccine at a dose of 6.5 $\log_{10}$ TCID50 and 7.5 $\log_{10}$ $TCID_{50}$ induced no significant changes in the metabolism and hematopoiesis indicators. There were no statistically significant differences between the sexes, and no morphological changes in the organs were identified. Although some statistically significant changes in blood and urine parameters were found in vaccinated rats, they did not exceed the normal control ranges and were considered random and thus having no toxicological significance. Degenerative processes, changes associated with necrobiosis, inflammation, and reparative changes of experimental animals' inner organs were not observed.

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Conflict of Interest

The authors of this study do not have any conflicts of interest to declare.

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