TOXIC AND IMMUNOMODULATORY PROPERTIES OF AN IRON(II) COORDINATION COMPLEX OF ISOLEUCYLTRYPTOPHAN DIPEPTIDE

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Results from toxicological and pharmacological studies of the iron(II) coordination complex of the immunoactive dipeptide isoleucyltryptophan (timofer) were presented. It was established that timofer was practically nontoxic and exhibited high immunostimulatory activity when combined with viral and bacterial vaccines.

Keywords: immunoactive peptides, coordination complexes, toxicological activity, pharmacological activity, drugs.

Several studies have demonstrated that metal-ion (Fe, Zn, Ag) coordination complexes of low-molecular-mass tryptophan-containing peptides possess immunoactive and other pharmacological properties [1 – 6]. Previously, the synthesis and dosage form preparation of the iron(II) complex of isoleucyltryptophan dipeptide (timofer) were reported [7, 8].

The present work reports toxicity data and the immunomodulatory activity of timofer.

EXPERIMENTAL PART

The toxicological properties of timofer were studied according to current rules for preclinical trials [7].

Acute toxicity and tolerance of timofer were studied in mice and dogs. Timofer was injected i.m. once into white laboratory mice of both sexes (20 – 22 g) in a total volume of <0.5 mL, which is the maximum volume for this animal species, in tests to determine its acute toxicity. The injected doses were 1,000, 1,500, 2,000, and 2,500 µg/kg. Test ani-

mals in control experiments received i.m. normal saline (0.9% NaCl).

Tolerance to timofer was assessed in experiments on four mongrel dogs of both sexes (8 – 12 kg). The drug was injected i.m. in 15 mL, which is allowed for this animal species. The injected dose in this instance was 1,500 µg/kg, which was greater than 650 times the therapeutic dose for humans considering the interspecies dose tolerance. The animals were observed for two weeks after the start of the experiment. The effects of timofer on the biochemical blood parameters were studied after i.m. injection in rats of both sexes for 120 d at doses of 8 and 80 µg/kg.

Studies of embryotoxicity in white laboratory rats estimated the pre- and post-implantation embryonic death, morphological (anatomical) development abnormalities, and an overall delay of embryo development after injection of timofer at doses of 8 and 80 µg/kg, which were equivalent to the therapeutic and 10 times the therapeutic dose for humans considering interspecies dose tolerance. Cyclophosphamide, which possesses embryotoxic activity, served as the reference drug and was injected once on the 12th day of pregnancy at a dose of 20 mg/kg (positive control).

The specific pharmacological activity of timofer at a dose of 148 µg/mL per 100 kg of live mass was studied in combination with the associated vaccines against enteric rota- and coronaviruses and calf colibacteriosis. The experi-

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ment used 35 calves that were divided into two groups (test, 20 calves; control, 15 calves). The associated formulated vaccine against enteric rota- and coronaviruses and calf colibacteriosis was prepared at the Ya. R. Kovalenko All-Russian Research Institute of Experimental Veterinary Medicine and was injected i.m. twice at an interval of 10–15 d at doses of 5 mL.

RESULTS AND DISCUSSION

The completed studies showed that mice were unaffected by timofer injected at these doses. Signs of intoxication were not observed after injection of timofer over the whole range of studied doses.

Deaths of test animals were not noted during dynamic observation over 14 d.

Body-mass dynamics and the demand for food and water of the test animals differed significantly from those of the control mice.

Pathological changes as compared to the controls were not found from dissections and macro- and microscopic inspection of the internal organs of surviving test animals that received the test drug.

Dogs that received timofer had normal demands for food and water and body-mass changes. Lethal outcomes did not occur. Pathological changes were not found from dissections and macro- and microscopic inspections of the internal organs of test animals.

The chronic toxicity of timofer was studied after the dose for such studies was determined initially. In this instance, the previously developed timogen (ZAO Medico-biological Sci-

entific-Production Complex Cytomed, St. Petersburg, trade name TIMOGEN[®]) was selected as the closest pharmacological and chemical-technical analog.

The one-time therapeutic dose of previously studied timogen for humans is 50–100 µg of dipeptide (0.5–1 mL) for administration once per day.

Therefore, the base therapeutic dose for timofer was also 50–100 µg (0.5–1 mL) once per day.

The drug was injected i.m. to laboratory white rats over 120 d at doses of 8 and 80 µg/kg, which were equivalent to the therapeutic and 10 times the therapeutic dose for humans considering interspecies dose tolerance.

The parameters of rat peripheral blood (hemoglobin, erythrocytes, leukocytes, color parameter, platelets) of test animals did not differ statistically significantly from the control for timofer injection over 120 d at both doses.

The tests showed that timofer did not affect the general condition of the rats and did not change the biochemical blood parameters in the test animals (Table 1).

Rabbit experiments confirmed the rat data that a single (over 120 d) i.m. injection per day of timofer at doses of 4 and 40 µg/kg, which were equivalent to the therapeutic and 10 times the therapeutic dose in humans considering interspecies dose tolerance, did not change the general conditions of the animals and did not have toxic effects on them. Timofer at both doses did not cause deviations in the biochemical blood parameters. Pathological changes were not observed according to dissection and macro- and microscopic inspection of internal organs of test animals that received the test drug.

TABLE 1. Effect of Timofer on Biochemical Parameters of Rat Blood with Injection for 120 d ($n = 10$)

Dose, mg/kg	Test conditions	Studied parameter							
		glucose, mmol/L	total protein, g/L	urea, mmol/L	aspartate aminotransferase, mmol/h·L	alanine aminotransferase, mmol/h·L	alkaline phosphatase, IU/L	cholesterol, mmol/L	total bilirubin, mmol/L
8.0	Initial	7.8 ± 0.6	74.3 ± 1.8	6.21 ± 0.17	71.8 ± 2.4	34.5 ± 2.0	116 ± 7.7	1.94 ± 0.13	1.23 ± 0.05
	30 d	8.5 ± 0.9	67.2 ± 2.0	6.30 ± 0.19	85.9 ± 2.0	38.7 ± 2.2	126 ± 6.0	1.82 ± 0.10	0.95 ± 0.07
	60 d	9.0 ± 0.7	65.3 ± 2.5	6.18 ± 0.14	88.6 ± 2.2	39.9 ± 2.5	118 ± 5.3	1.75 ± 0.14	1.30 ± 0.08
	120 d	12.2 ± 0.5	64.3 ± 2.3	6.32 ± 0.18	92.3 ± 2.7	46.6 ± 2.4	122 ± 4.7	1.92 ± 0.07	1.22 ± 0.07
80.0	Initial	7.5 ± 0.4	68.9 ± 2.5	6.20 ± 0.12	72.4 ± 2.8	33.7 ± 2.5	101 ± 5.5	1.80 ± 0.12	0.93 ± 0.09
	30 d	9.1 ± 0.7	63.7 ± 1.9	6.42 ± 0.18	74.0 ± 2.3	45.8 ± 1.9	128 ± 6.3	2.13 ± 0.11	1.15 ± 0.04
	60 d	10.8 ± 0.9	71.3 ± 2.3	6.34 ± 0.10	89.6 ± 2.5	39.6 ± 2.7	136 ± 4.8	2.31 ± 0.09	1.08 ± 0.11
	120 d	9.6 ± 0.7	69.2 ± 2.1	6.41 ± 0.13	90.5 ± 2.8	47.0 ± 2.3	140 ± 5.1	2.46 ± 0.14	1.27 ± 0.08
Control	Initial	8.0 ± 0.5	67.6 ± 1.9	6.34 ± 0.18	78.3 ± 2.3	36.3 ± 2.0	95 ± 7.6	1.89 ± 0.10	0.93 ± 0.06
	30 d	8.9 ± 0.9	69.1 ± 2.0	6.27 ± 0.14	80.9 ± 2.7	40.7 ± 2.2	101 ± 6.0	1.96 ± 0.07	1.12 ± 0.09
	60 d	10.2 ± 0.4	67.8 ± 1.7	6.31 ± 0.11	79.4 ± 2.4	38.3 ± 1.9	113 ± 8.0	2.18 ± 0.15	0.82 ± 0.07
	120 d	9.6 ± 0.8	64.6 ± 1.9	6.29 ± 0.17	82.7 ± 2.2	40.4 ± 2.1	109 ± 5.5	2.16 ± 0.09	11.4 ± 0.10

Thus, timofer did not have toxic effects on the test animals if injected multiply over a long time.

Embryotoxicity studies showed that cyclophosphamide at a dose of 20 mg/kg and timofer at doses of 8 and 80 µg/kg did not affect the number of corpus lutea in the ovaries. All rats became pregnant after both compounds were administered. Injection of cyclophosphamide caused resorption of fetuses in practically all females whereas timofer did not affect this process.

Timofer was not lethal to embryos whereas cyclophosphamide increased significantly by 7.25 times post-implantation death. Both compounds did not cause pre-implantation death of embryos. Intrauterine death of fetuses due to cyclophosphamide was related to their increased lethality in both early and late terms of embryogenesis. This was consistent with the significant increase of the early and late resorption sites. Cyclophosphamide reduced significantly by ~1.5 times the mass and length of embryos. Timofer did not change any of these parameters, in contrast with cyclophosphamide.

The embryotoxic activity of timofer at doses of 8 and 80 µg/kg demonstrated that the fertility of rats in the test and control groups did not differ significantly. The average number of fetuses per female in the control group was 9.7 ± 0.22 ; in the group receiving timofer at doses of 8 and 80 µg/kg, 9.65 ± 0.23 and 9.8 ± 0.23 , respectively. The number of pups did not differ from the control group in rats that received timofer at both doses whereas cyclophosphamide decreased their number by 11.3 – 11.5 times. Pups born to test females did not differ from those of the controls. Morphological studies showed no anomalous development of internal organs and skeletons in fetuses born by test rats.

Cyclophosphamide produced specific embryonic development anomalies such as craniolysis, edema, poly- and oligodactyly, anophthalmia, speech loss, and micrognathia. Various anomalies of the brain, palatal appendages, kidneys, and bladder were noted during studies of the internal organs and skeletons of rat fetuses that received cyclophosphamide. Development anomalies were not noted in fetuses of rats that received timofer. Studies of the ossification of various skeletal sections of rat embryos showed that cyclophosphamide had a pronounced adverse effect that manifested as a significant decrease in the length and number of calcification points of large bones. Timofer at both doses had no effect on these processes.

The condition of offspring of rats that received timofer at both doses during the whole pregnancy was observed for one month. Body mass, physical endurance, pubescence time, eye opening, and peeling of the ear were recorded. The results showed that timofer at the studied doses had no teratogenic effects. Anomalies and deformities were not observed. The mass of the rats and their physical endurance as estimated from the hanging time on a horizontal rod did not differ one month after birth from those of the controls.

Thus, timofer at the therapeutic and 10 times the therapeutic dose had no adverse effects on the pregnancy and development of rat offspring.

The vaccine for studying the specific pharmacological activity of timofer combined with an associated vaccine against enteric rota- and coronaviruses and calf colibacteriosis was selected so that it included viral and bacterial antigens. Bovine blood serum before immunization contained antibodies to rotavirus in the EIA with titer $2.5 \log_2$; to coronavirus in the HAIA, $3 \log_2$; to *Escherichia coli*, 1:50. The antibody titers to rotavirus in the EIA after immunization of the first and second animal groups reached 8.0 and $6.5 \log_2$, respectively; to coronavirus in the HAIA, 9.3 and $7.5 \log_2$; and to *E. coli* in the RA, 1:1600 and 1:800. The lysozymal and bactericidal activities of blood serum from animals before administering the preparation were 65 and 3.1%, respectively.

The antibody titer was greater in animals that receive timofer together with the vaccine than in those that received only vaccine, i.e., to rotavirus, by 4 times; to coronavirus, 3.48 times; *E. coli*, twice. The lysozymal activity increased by 1.13 times; bactericidal, by 1.42 times. Therefore, timofer increased both the level of humoral immune response and nonspecific infection resistance factors and exhibited high immunomodulatory properties.

Thus, the research showed that timofer was a nontoxic drug that exhibited immunomodulatory properties and was promising for the pharmaceutical industry.

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