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Research Article

**PRECLINICAL STUDY OF THE ALLERGENIC PROPERTIES
OF CARBAMYLATED DARBEPOETIN****Pavel D. Kolesnichenko^{1*}, Alexander A. Dolzhikov¹, Nina I. Zhernakova¹,
Andrey A. Shaposhnikov¹, Alexander A. Stepchenko², Galina A. Batischeva³, Konstantin
M. Reznikov³**¹Belgorod State University, 85, Pobedy St., Belgorod, 308015, Russia²Kursk State Medical University, 3, K. Marx St., Kursk, 305041, Russia³Voronezh State Medical University, 10, Studencheskaya St., Voronezh, 394036, Russia**Abstract:**

The article presents the results of a study of allergenicity of a new drug from the group of erythropoietin, carbamylated darbepoetin. The study was performed according to the Russian manual of pre-clinical studies and carried out through the following tests: the reaction of active cutaneous anaphylaxis, systemic anaphylactic reaction and delayed type reaction. The results of the reaction of active cutaneous anaphylaxis showed that the drug had caused a stain at the injection site 2.87 ± 0.17 of size, not significantly differenced from the size of the stain caused by placebo solution — 2.74 ± 0.10 even tenfold dose. The systemic anaphylactic reaction has not produced any symptoms, classified according to the Weigle index. In the delayed type reaction in mice the relative size of the edema paw after administration of the shocking dose of the drug was 0.18 ± 0.04 , and did not exceed the control results — 0.16 ± 0.03 . Thus, the standard tests prove the absence of allergenic actions of carbamylated darbepoetin.

Key words: carbamylated darbepoetin, allergenicity.**Corresponding author:****Pavel D. Kolesnichenko,**
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INTRODUCTION:

A search for innovative molecules [1, 2] and its safety inspection are an important tasks of pharmacology. Thus, its safety study is performed in pharmacological targets [3, 4], models in vivo [5, 6], the study of pharmacokinetic parameters [7, 8] and clinical studies [9, 10]. Each new drug must undergo a toxicological evaluation, then there is an obligatory regard to its allergenic effects, i.e. the ability to cause a change in the organism reactivity, manifested in hypersensitivity, which is based on different immunopathological mechanisms. Substances containing protein fractions and high molecular weight compounds should be tested particularly careful [11]. Great potential for the study and application of erythropoietin drugs, the positive advantages and disadvantages of recombinant erythropoietin and analogues were currently revealed. However, undesirable side effects galvanize into the search for new safer poietic drugs [12]. Recently there has been patented a new drug in this group, the carbamylated darbepoetin made by carbamylation of all amino acid residues of lysine, included in the darbepoetin molecule and carbamylated amino acid residue of alanine in the N-terminal of this protein - 9C-DEPO, which is hyper glycosylated derivant of the human recombinant erythropoietin [13]. Theoretically this drug has a great pharmacological potential, because the molecule was modified to not have a negative impact on the hematocrit and to retain therapeutic properties. [14].

The aim of this study is to study the allergenic properties of the carbamylated darbepoetin, solution for injection.

MATERIALS AND METHODS

Experiments to study the allergenic properties of the carbamylated darbepoetin were performed in mature both sexes Guinea pigs-albinos, Wistar rats and BALB/C mice. All manipulations with the animals were performed in strict accordance with Russian legislation and International recommendations of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETSN 124, Strasbourg, 22.06.1998).

For the study of allergenic properties in the reaction of active cutaneous anaphylaxis and the systemic anaphylactic reaction, the carbamylated darbepoetin, solution for injection (OOO "Pharmapark", Russia) were used at single and tenfold daily therapeutic dose. The single daily therapeutic dose of the investigated drug for Guinea pigs-albinos weighing 300 ± 30 g was $50 \mu\text{g}/\text{kg}/\text{day}$, the tenfold daily therapeutic dose was $500 \mu\text{g}/\text{kg}/\text{day}$. The range of injection volumes was from 0.1 ml to 1.2 ml,

depending on the weight of the animal and frequency of the dose. The drug was injected subcutaneously in dorsal area. Data obtained in animals after injection of a corresponding volume of placebo, buffer for the carbamylated darbepoetin were used as a control [15].

For the reaction of active cutaneous anaphylaxis the animals were divided in the following groups (10 in group, 5 females and 5 males): I – placebo, II – the carbamylated darbepoetin at the dose of $50 \mu\text{g}/\text{kg}$; III – the carbamylated darbepoetin at the dose of $500 \text{mg}/\text{kg}$. Sensitization of the Guinea pigs-albinos by the drug was performed by subcutaneous injection at an effective therapeutic dose ($50 \mu\text{g}/\text{kg}$) and at the dose of 10 times its excess ($500 \mu\text{g}/\text{kg}$). The first injection was subcutaneous; two subsequent injections were alternate day in the femur area. The shocking dose was chosen experimentally in such a way as not to cause irritation of the injection site. To determine the shocking dose there were used 5 males and 5 females Guinea pigs-albinos. Each Guinea pig at different points in the dorsal area due to amicrospace was injected subcutaneously by the solution of the carbamylated darbepoetin in a volume of 0.05 ml in concentrations of $14 \mu\text{g}/\text{ml}$, $27.5 \mu\text{g}/\text{ml}$, $55 \mu\text{g}/\text{ml}$ and $110 \mu\text{g}/\text{ml}$. Then we were watching the injection site for three hours to determine possible local irritating action. There was found that subcuticular injection of the carbamylated darbepoetin solution at the maximum concentration $110 \mu\text{g}/\text{ml}$ has minor signs of a local irritative effect. The drug at the concentrations of $14 \mu\text{g}/\text{ml}$, $27.5 \mu\text{g}/\text{ml}$ and $55 \mu\text{g}/\text{ml}$ did not have the local irritative effect. Thus, further experiments there was used the maximum concentration of the drug ($110 \mu\text{g}/\text{kg}$) which has no a strong local irritative effect after subcuticular injection. On the 14th day after sensitization the sites of Guinea pigs' dorsal area with clipped skin were injected subcuticularly by two-fold drug dilutions, at concentrations that do not cause skin irritation. After 20 minutes, the Guinea pigs were injected intravenously by 0.5 ml of 1% Evans blue solution. After 30 min the animals were euthanized with ether and measured the size of the blue spots on the inner side of the skin at the site of injection (in case of positive reaction the spot diameter should be not less than 6 mm). To control the reactivity of the skin the same animal on a different area with clipped skin was administered with 0.05 ml of diluent (sterile isotonic sodium chloride solution). The shocking dose injections and Evans blue was also administered control animals, which in the days of sensitization of the animal groups receiving the drug, were administered only a placebo. Such animals at the site of subcuticular

injection of the drug was measured by the diameter of the colored spots.

In the study of the systemic anaphylactic reaction Guinea pigs-albinos were administered the study drug in a daily therapeutic dose (50 µg/kg) in a dose of 10 times its excess (500 µg/kg). The first injection was subcutaneous; two subsequent injections were alternate day in the femur area. The animals were divided in the following groups (10 in group, 5 females and 5 males): IV – control; V – carbamylated darbepoetin at the dose of 50 µg/kg/day; VI – carbamylated darbepoetin at the dose of 500 µg/kg per day. The shocking dose injection was given intravenously at the 14th day after the sensitizing injection (18-th day of the experiment). The shocking dose was equal to the total sensitizing dose; it was 150 mg/kg for animal group receiving the carbamylated darbepoetin at the dose of 50 mg/kg, and 1500 mg/kg for animal group receiving the carbamylated darbepoetin at the dose of 500 µg/kg (table 1). It was planned to evaluate the intensity of anaphylactic shock in the Weigle indices.

For the delayed type reaction, the animals were divided in the following groups (10 in group, 5 females and 5 males): I – placebo, II – carbamylated darbepoetin. Mice were sensitized once by subcuticular injection at the tail base of 60 µl of the

drug emulsion in Freund's complete adjuvant (CFA) (1 ml of lanolin, 3 ml of paraffin oil, 5 mg of inactive BCG vaccine, 50 µL of tween-20, 0.5 ml of distilled water), dose is equivalent to a 10 mM solution in CFA in a 1:1 ratio. To prepare such emulsion there was used the Hanks solution with a pH of 7.5. For the detection of sensitization after 5 days, mice were injected with 40 µl of 10 mM solution of the test drug in Hanks solution in the pad of the hind paw. Through 6-22-24 h after the test there was measured edema with engineering micrometer MK-0-25. The difference in thickness of both paws shows the size of edema which characterizes the intensity of the delayed type reaction. Control animals were sensitized by the CFA emulsion with the Hanks solution in the same way that in the animal group treated with the study drug. Analysis of the reaction was performed after 48-72 hours [16].

RESEARCH RESULTS:

In the course of the study of the active cutaneous anaphylaxis of the carbamylated darbepoetin, solution for injection (OOO "Pharmapark", Russia) it was found that the study drug at single and tenfold daily therapeutic doses did not have allergenic properties (table 1). The results are presented in table 1.

Table 1:

The results of the study of allergenic properties of the carbamylated darbepoetin, solution for injection (OOO "Pharmapark", Russia) in the reaction of active cutaneous anaphylaxis. The spot size (M ± m), mm on the inner side of the skin at the drug injection site

It was found that in the sensitized by the drug Guinea pigs the spot sizes of the exudate formed at the sites of the shocking dose injections, reached a diameter of 3.5 mm and on average did not exceed the spot sizes of the exudate in the control sites and the control animals. Erythema or infiltration and ulceration at the injection site as the shocking dose of the drug, and the control of reactivity, were absent.

The study of the allergenic properties of the carbamylated darbepoetin, solution for injection (OOO

Number of the groups	Administrated substances	The spot size, mm
I	Control (placebo), the shocking dose of the drug	2.74±0.10
	Control (placebo), the control of the reactivity	2.54±0.13
II	Carbamylated darbepoetin, the shocking dose of the drug	2.85±0.18
	Carbamylated darbepoetin, 50 µg/kg, the control of the reactivity	2.58±0.18
III	Carbamylated darbepoetin 500 µg/kg, the shocking dose of the drug	2.87±0.17
	Carbamylated darbepoetin 50 µg/kg, the control of the reactivity	2.60±0.11

Table 2: The results of the study of allergenic properties of the carbamylated darbepoetin, solution for injection (OOO "Pharmapark", Russia) in the delayed type reaction in the mice after 6, 12 and 24 hours

Number of the groups	Administrated substances	The difference in thickness of the paws with the drug(right) from the intact (left), mm		
		6 hours	12 hours	24 hours
I	The time of measurement after administration	6 hours	12 hours	24 hours
	The control, CFA emulsion with Hanks solution	0.18±0.04	0.18±0.04	0.18±0.04
II	The time of measurement after administration	6 hours	12 hours	24 hours
	Experiment, CFA emulsion with carbamylated darbepoetin	0.19±0.04	0.17±0.04	0.16±0.03
pin comparison with the control		0.593	0.856	0.703

"Pharmapark", Russia) in the model of systemic anaphylactic reaction discovered that the test drug in single and tenfold (50 µg/kg 500 µg/kg, respectively) therapeutic doses did not have allergenic properties (table 4 of the supplement). Symptoms of allergization were not observed, so show the results in the Weigle indices was not possible. The animals of all experimental groups did not show the symptoms of allergization, such as scratching, ruffling of hair, decrease of body temperature, sneezing, spasmodic cough, lateral position of the animal, feces output and urine passage, spasm of airways, convulsive jumping, convulsions, death of the animal.

The study of allergenic properties of the carbamylated darbepoetin in the delayed type reaction in the mice found that the study drug at the concentration of 110 µg/ml did not have allergenic properties (table 2).

Erythema or infiltration and ulceration at the injection site as the shocking dose of the drug, and the control of reactivity were observed none of the animal involved in the experiment. The relative size of the paw, which was administered the drug did not differ from mass of the control paw, that indicated a lack of the edema.

CONCLUSION:

Thus set our self-one of the main task of preclinical studies of studying the allergenic action of potential drugs was to prove or exclude the possibility of the development of allergenic reactions caused by pharmacological agent or its metabolites in the experiment on animals. The results of the reaction of active cutaneous anaphylaxis in Guinea pigs-albinos showed the absence of the allergenic properties of the carbamylated darbepoetin at the single and tenfold daily therapeutic doses (50 µg/kg and 500 µg/kg,

respectively). The systemic anaphylactic reaction in Guinea pigs-albinos confirmed the absence of sensitizing properties of the studied drug at the single and tenfold daily therapeutic doses (50 µg/kg and 500 µg/kg, respectively).

On the strength of all the research results we can conclude that the carbamylated darbepoetin does not have allergenic properties and can be recommended for further preclinical studies.

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