CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1020034

Available online at: http://www.iajps.com Research Article

PRECLINICAL STUDY OF THE DNA-DAMAGING ACTIVITY AND GENOTOXICITY OF CARBAMYLATED DARBEPOETIN

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Abstract:

The article presents the results of a potential mutagenicity studying of a new drug from the group of erythropoietin, carbamylateddarbepoetin. The study was performed using DNA-comet assay in mice, and in micronucleus test. As a positive control there were used mice which had been injected with genotoxicantmethylmethane sulfonate at a dose of 40 mg/kg and as a negative control there were mice after administration of equivalent doses of a placebo. The results showed that the mutagenic effect of the drug in single (3.59±1.02) dosing and three times (4.0±0.78) dosing had not been different from the mutagenic effect of placebo (4.39±1.34). Micronucleus test was performed using a cytogenetic damage of bone marrow cells and checking the appearance of polychromatophil cells, containing micronuclei. It was established that carbamylateddarbepoetin within stated doses according to the used test, has no a potential carcinogenic effect.

Key words: *DNA-comet assay, mutagenicity, carbamylateddarbepoetin, cytogenetic damage.*

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Please cite this article in press as Pavel D. Kolesnichenko et al., ***Preclinical Study of the DNA-Damaging Activity and Genotoxicity of Carbamylated Darbepoetin,*** *Indo Am. J. P. Sci, 2017; 4(10).*

INTRODUCTION:

A search for innovative molecules and its safety inspection are important tasks of pharmacology. Each new drug must undergo a toxicological evaluation, then there is an obligatory regard to its allergenic and carcinogenic effects.

A significant problem is natural or synthesized innovative molecules [1, 2], with potentially mutagenic properties, which are able to give them in the combined action of genotoxicants. 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]. Information about the impact of new drugs on spontaneous and induced mutagenesis obtained in studies on mammalian cells in vitro and in vivo, is always relevant [10, 11]. In several studies of non-toxic substances such as e.g. vitamins there were suddenly revealed comutagenic and mutagenic effects, explaining their autoxidation, accompanied by the initiation of free-radical oxidation and change in redox potential. Therefore, potential genotoxicity testis necessary for low-toxicity substances such as drugs of the human erythropoietin, both natural and recombinant. Some studies demonstrate antimutagenic properties of erythropoietin, which are associated with its ability to inhibit the processes of free radical-induced oxidation [12], other scientific works indicate undesirable side effects [13]. In this regard, the question of the possible influence of antioxidants on chromosomal variability remains open. Not so long ago, 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 molecule darbepoetin and carbamylated amino acid residue of alanine in the N-terminal of this protein - 9C-DEPO, which is hyperglycosylated derivant of the human recombinant erythropoietin [14]. 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. This study was performed according to the approved "Guidelines for preclinical research" [15].

The aim of this study was to evaluate the DNA-damaging activity and cytogenetic damage ofthe carbamylateddarbepoetin.

MATERIALS AND METHODS

The study was performed in BALB/cmale mice weighing 18-20 g at the age of 8-12 weeks. Each experimental group included 6 animals. Animals were randomized to either experimental and control groups after adaptation period and examination, excluding the inclusion in the experiment of sick and injured animals. The basic rules of management and care consistent with the standards outlined in the "*Guide for the care and use of laboratory animals" (National Academy press; Washington, D.C., 2011)*, the rules approved by state standard 53434 – 2009 "Principles of good laboratory practice" and the International recommendations of the European Convention for the protection of vertebrate animals using for experimental and other scientific purposes (ETSN 124, Strasbourg, 22.06.1998).

The study drug was given as a single dose subcutaneously in maximal volume of 1 ml (subtoxic dose) and multi dose deliveryfor 4 days subcutaneouslyexpressed in a human daily therapeutic dose not to exceed 50 μg/kg. The mice of the control group (negative control) were administered equivalent volume of placebo. In the micronucleus test as the positive control the animals were irradiated at a dose of 20 roentgens per hour, with known cytogenetic effect. In the DNA-comet assay as a positive control there were used the mice which had been injected intraperitoneally with genotoxicantmethylmethane sulfonate at the dose of 40 mg/kg, for a period of 3 hours.

Sampling of the bone marrow (micronucleus test) and blood (DNA-comet assay) was performed 24 hours after the last administration of the test substances.

The DNA-comet assay. For analysis we used peripheral blood of mice obtained by incising the tip of the tail. The blood aliquots sampling (10 μl) of each animal was performed no later than 24 hours after the completion of the drug administration. Whole blood was mixed with an equal volume of RPMI-1640 medium (without L-glutamine) and carefully layered on a gradient mixture of fikoll solution with a density of 1.077 and centrifuged at 400 g for 40 min. The ring of mononuclear cells formed on the phase interface was carefully taken with a pipette and washed twice with RPMI-1640mediumand centrifugation at 400 g for 10 min. After the second washing, the precipitate had been diluted with RPMI-1640 medium to a cell concentration of $1\text{-}5x10^5$/cc and placed until use in a refrigerator at 4ºC.

The cell suspension in a volume of 60 μl was added to the test tube with 240 μl of 0.9% solution of low melting agarose (melt temperature<420C) in phosphate saline buffer (FSB), heated to 42ºC (microthermostat "Termite", Russia) and resuspended. Then 60 μl agarose solution with cells was applied to pre-coated with 1% universal agarose glass slides, covered with cover glass and placed on ice. Further, all operations were performed in a darkened room under yellow light. After solidification of the agarose (about 10 minutes) cover glasses were carefully removed, microscope slides were placed in a staining cuvette (Schifferdecker

type), filled in pre-cooled to + 4ºC lysing buffer (10 mMTris-HCl [pH 10], 2.5 MNaCl, 100 mMEDTA-Na2, 1 % TritonX-100, 10% DMSO) and incubated for at least 1 hour. After lysis ending the microscope slides were transferred into the electrophoresis chamber (SubCell GT, "Bio-Rad"). The chamber was filled with electrophoresis buffer (300 mMNaOH, 1 mM EDTA-Na2, pH>13) and the microscope slides were incubated for 20 minutes to implement the alkali-labile sites and alkaline denaturation of DNA. Electrophoresis was performed for 20 minutes at a field strength of 1V/cm and a current of ~300 mA. After electrophoresis, the microscope slides were transferred to the staining cuvette and fixed in 70% ethanol solution for 15 minutes. After fixing the microscope slides were dried and stored at room temperature before analysis.

Immediately prior to microscopy, the microscope slides were stained with the fluorescent dye SYBR Green I (1:10000 in TE buffer with 50% glycerol) for 20 minutes in a dark. Analysis was performed on epifluorescence microscope Eclipse Ti-S (Nikon, Japan) combined with a digital high resolution camera DS-Fi2 (Nikon, Japan) at x200 power. The received DNA comets images were analyzed using the software CASP 1.2.2. As an indicator of DNA damage there was used the percentage of DNA in DNA comet tail (%of DNA in tail). With each microscope slide there were analyzed at least 100 cells. Obtained data were compared with the data for the negative (solvent) and positive (methylmethane sulfonate) controls [16, 17].

The micronucleus test. There was evaluated the cytogenetic damage of bone marrow cells by the appearance of polychromatophil cells containing the micronuclei. Euthanasia of mice was performed by decapitation 24 h after administration of the test substance or radiation. According to the standard method there were prepared cell films of the bone marrow. From astragalus there was removed the bone marrow in 24-well plate for cell cultures, each well was added with 200 µl DMEM medium with 10% fetal calf serum. The resulting suspension was pipetted on microscope glasses and smeared. The microscope slides were stained with the Romanowsky-Giemsa dye, allowing to differentiate polychromatophil cells and normochromatic erythrocytes. In each experimental group from one mouse there were analyzed 3 microscope slides, counting 1000 polychromatophil cells from each slide.

Count of the polychromatophil cells with micronuclei was carried out using theoptical microscope Eclipse E100 (Nikon, Japan) with immersion lens at 1000 power. The criterion of cytogenetic damage level was the percentage of the cells with micronuclei.

The results were processed using Microsoft Office Excel 2016. There were calculated mean, standard error of the mean, statistical significance between groups was assessed by Student t-test.

RESEARCH RESULTS:

During the study with DNA-comet assay there was processed dataset, which showed no DNA damage in the animal groups which had been administrated with carbamylateddarbepoetin (single dose subcutaneously in maximal volume of 1 ml (subtoxic dose) and multi dose delivery for 4 days subcutaneously at the dose of 50 µg/kg). The animals of the negative control group that were administered with the placebo, there are no DNA damage. Pair-wise comparison of the obtained data with the negative control did not reveal any statistically significant differences (Fig. 1, 2, 3 and table 1). In the group of positive control methylmethane sulfonate administration led to a statistically significant increase in DNA damage ($p<0.001$) in all the investigated samples in comparison with the negative control (Fig. 4 and table 1).

Table 1: The influence of carbamylateddarbepoetin on the DNA damage level in mononuclear blood cells of the mice, negative and positive controls

Group	%of DNA in tail
The negative control, placebo	4.39±1.34 *
The drug, 1 ml	3.59±1.02 *
The drug,50 µg/kg	4.0±0.78 *
The positive control, methylmethane sulfonate	21.41±1.88

** - p<0.001 in comparison with the positive control*

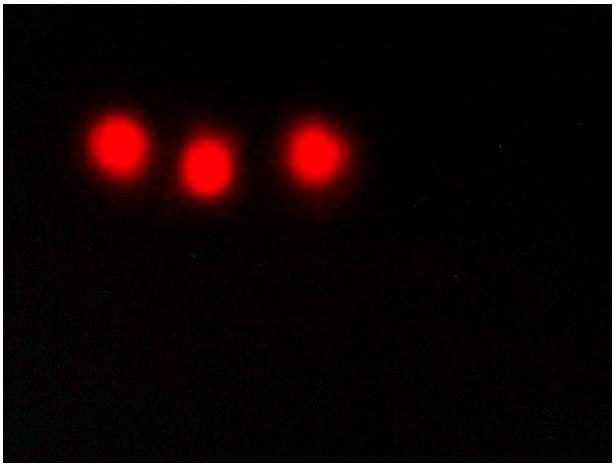

Fig 1. Photomicrography of acellnucleotid of the animal administrated with 1 ml of the drug (%TDNA=3.59); ×200.

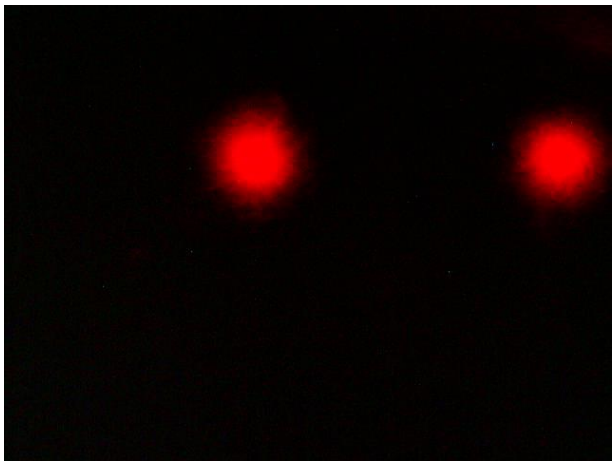

Fig 2. Photomicrography of acellnucleotid of the animal administrated with 50 µg/kg of the drug (%TDNA=4.00); ×200.

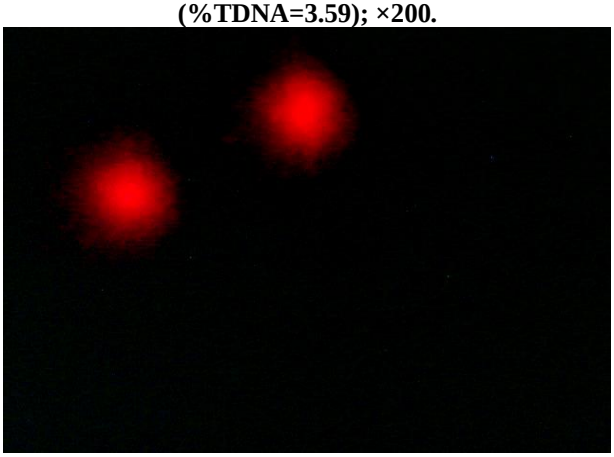

Fig 3. Photomicrography of acellnucleotid of the animal administrated with the placebo (%TDNA=4.39); ×200.

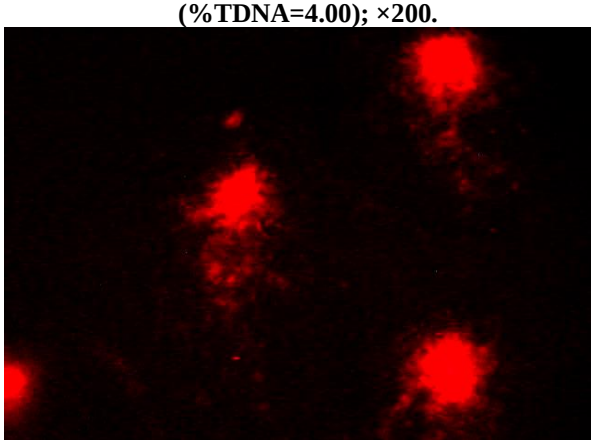

Fig 4. Photomicrography of acellnucleotid of the animal administrated with methylmethane sulfonate(%TDNA=21.41); ×200.

Analysis of the micronucleus test results showed that the carbamylateddarbepoetin in the studied concentrations (single dose subcutaneously in maximal volume of 1 ml (subtoxic dose) and multi dose delivery for 4 days subcutaneously at the dose of 50 µg/kg) and negative control did not cause the cytogenetic damage in the bone marrow cells. In the group of positive control, the radiation of the animals resulted in the cytogenetic damage of the bone marrow cells, which there were identified the polychromatophil cells with micronuclei (table 2).

Table 2: The influence of the drug on release of the polychromatophil cells with micronuclei in the bone marrow of the mice

Variants	Number of the mice	Number of the analyzed polychromatophil cells	Number of the polychromatophil cells with micronuclei	The polychromatophil cells with micronuclei,%
The drug, 1 ml	6	3000	16.67±5.61	0.55±0.19*
The drug,50 µg/kg	6	3000	17.3±6.41	0.58±0.22*
The negative control, placebo	6	3000	18.0±4.43	0.60±0.14*
The positive control, X-ray	6	3000	151.5±11.31	5.05±0.38

**p ≤ 0.01 in comparison with the positive control.*

CONCLUSION:

Mutagens can be various factors that cause changes in the chromosome structure. Biological substances and drugs on their basis are rarely mutagenic; however, some products of metabolism can be mutagens (e.g., oxidation products of lipids). To correct the primary genetic structure damage caused by mutagens in cells there are a number of system recovery, or reparation of the genetic damage. However, during the reparation a part of the primary damage can be saved and lead to mutations, which clinically are very rare. So there was set one of the most important task of a preclinical study to evaluate the potential mutagenic effect. The carbamylateddarbepoetin in the studied concentrations (single dose subcutaneously in maximal volume of 1 ml (subtoxic dose) and multi dose delivery for 4 days subcutaneously at the dose of 50 μg/kg) did not cause the genotoxic effect in the bone marrow cells and blood. Micronucleus test revealed that, the drug in the studied concentrations (single dose subcutaneously in maximal volume of 1 ml (subtoxic dose) and multi dose delivery for 4 days subcutaneously at the dose of 50 μg/kg) did not cause the cytogenetic damage in the bone marrow cells. Thus, the aggregate of the conducted researches we can conclude that the drug carbamylateddarbepoetin has no mutagenic properties.

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