Pharmacological Screening Antihypoxic and Cytoprotective Properties of Mexidol and Analogs of Human Erythropoietin in Cultured Leukocytes of Pigs

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Abstract

The article outlines improved methodological approaches to preclinical screening of antihypoxic and cytoprotective drugs. To investigate the effect of Mexidol, carbamylated darbepoetin, darbepoetin, and placebo (isotonic solution for the substance) against the background of hypoxic and cytotoxic effects on cell culture. Get data on the cytoprotective and antihypoxic therapeutic corridor of Mexidol. A placebo-controlled screening test of mexidol, darbepoetin, and carbamylated darbepoetin was conducted on pig leukocytes cultures. Hanks solution was used as the nutrient medium. From the preparations, working solutions of a certain concentration were prepared; for Mexidol, 150 μg/ml; the remaining substances, 500 ng/ml. Data on the cytoprotective and antihypoxic therapeutic corridor of mexidol were obtained. In this experience, there are no significant differences between darbepoetin and its derivative from placebo. The technique used in the experiment, has shown its effectiveness and the possibility of its further use as a screening system. Derivatives of erythropoietin at 60-minute incubation do not have either antihypoxic or cytoprotective effects on leukocytes of pigs. Mexidol has antihypoxic effect at a concentration of 15 ng/μl and cytoprotective - at a concentration of 3 ng/μl.

Introduction

Currently, there are many ways to determine cell viability, which have high sensitivity, throughput and specificity. They range from the most routine analysis of the exclusion of trypan-blue dyes to a very complex analysis of individual cells, for example, using RAMAN microscopy. Cell viability is often defined as the number of healthy cells in a sample. Methods for assessing cell viability can be divided into those that analyze entire populations and those that include analysis of individual cells. Of course, the analysis of the whole population is quick, however, it gives a less detailed result than measurements of viability at the level of individual cells [1]. One of the first methods of evaluation Cell viability was a test for the exclusion of dye trypan blue, which is still widely used. It is based on the fact that viable cells have an intact cell membrane that does not pass the dye and thus excludes staining. Dead and damaged cells absorb the trypan blue color and, as a result, turn blue, because their membrane is no longer able to control the passage of macromolecules. There are techniques in which dyes are washed out of damaged cells with buffer solutions and in this case, viable cells remain stained [2, 3, 4].

Pathological processes at the cellular level, as well as adaptation processes, can be thought of as non-specific, one-by-one reactions that result in the formation of a non-specific response. The basis of the pathogenesis of cytotoxic and hypoxic damage to cells and tissues is always oxidative stress, which is a

cascade of reactions that activate free-radical processes.

Oxidative or oxidative stress is a consequence of an imbalance in the functioning of the pro- and antioxidant systems of the cell, which in turn leads to damage to cellular structures and individual macromolecules (proteins, lipids, DNA), cell membrane damage and, consequently, cell death [5,6]. Disruption of the structure and functions of mitochondria leads to a decrease in the level of ATP in the cell and the cessation of the functioning of ion pumps, which in turn leads to an imbalance of potassium, sodium and calcium ions inside the cell and the extracellular space. All this leads to the activation of the complex of enzyme systems - phospholipase, proteinkinase [7]. Damage to the cytoskeleton, cell membrane and changes in the intracellular concentration of ions leads to the formation of acidosis, as a result of the accumulation of metabolic products such as lactate and others. Adaptive cell reserves initially activate metabolism. However, as soon as the damaging factor damages the mitochondria, the metabolism becomes disorganized, the damage becomes critical, and the cell dies [8].

Currently, it has been proven that free radical cell damage is one of the main factors in the pathogenesis of over 100 human diseases, including ischemic disorders of the brain and cardiac circulation, acute fetal hypoxia, diabetes mellitus [9].

In the experiment, the effect of a number of substances was evaluated: darbepoetin, carbamylated darbepoetin and mexidol (ethylmethylhydroxypyridine succinate).

Mexidol (ethylmethylhydroxypyridine succinate), as stated in the official instructions to the drug, is an inhibitor of free-radical processes, membrane protector, has antihypoxic, stressprotective, nootropic, anticonvulsant and anxiolytic action. The mechanism of action of mexidol is due to its antioxidant, antihypoxic and membrane-protective action. It inhibits lipid peroxidation, increases the activity of superoxide dismutase, increases the lipid-protein ratio, reduces the viscosity of the membrane, increases its fluidity. By changing the activity of calcium-membransvyazannyh enzyme phosphodiesterase, adenylate cyclase, acetylcholinesterase) receptor complexes (benzodiazepine, GABA, acetylcholine), mexidol enhances their ability to bind to ligands, and contributes to maintaining the structural and functional organization of biological membranes, transport of neurotransmitters and improve synaptic transmission. Mexidol increases the content of dopamine in the brain. It causes activation of aerobic glycolysis and a decrease in the degree of inhibition of oxidative processes in the Krebs cycle under hypoxic conditions with an increase in ATP and creatinephosphate, activation of the energy-synthesizing functions of mitochondria, and stabilization of cell membranes. The drug improves metabolism and blood supply to the brain, improves microcirculation and rheological properties of blood, reduces platelet aggregation. Stabilizes membrane structures of blood cells (erythrocytes and platelets) during hemolysis. It has a hypolipidemic effect, reduces the content of total cholesterol and LDL.

Erythropoietins are cytokines that are produced in the human body, and this product is a genetically determined process. Synthesis of erythropoietin is one of the responses to a decrease in the oxygen content in cells. Hypoxia leads to the production of hypoxia-induced factor-1 (HIF-1), followed by the production of mRNA and, in fact, erythropoietin [11, 12]. Darbepoetin (DPO) is a genetically modified erythropoietin that has all the properties of its ancestor. Due to its improved structure, DPO has a large molecular weight (37.1 kDa, not 30.4 kDa) and due to which there is an increase in its half-life and, consequently, the frequency of use of the drug decreases [1, 13]. Also known form of darbepoetin, which contains carbamylated group - carbamylated darbepoetin (KDPO). KDPO does not affect hematopoiesis, but has all the same cytoprotective properties, and also has a longer half-life, which makes it promising as a drug for the treatment of hypoxic disorders [13].

It is worth noting the insufficient amount of information in the modern literature on the cytoprotective and antihypoxic properties of darbepoetin and carbamylated darbepoetin, as well as a small amount of preclinical studies of these substances, which makes them promising for further study.

Research Tasks

To investigate the effect of Mexidol, carbamylated darbepoetin, darbepoetin, and placebo (isotonic solution for the substance) against the background of hypoxic and cytotoxic effects on cell culture. Get data on the cytoprotective and antihypoxic therapeutic corridor of Mexidol.

Methods

The study was performed on a culture of neutrophil cells isolated from fresh blood of the pig. Neutrophils were isolated by collecting 30 ml of blood in tubes with EDTA (2.7% 1:10), followed by four times centrifugation (1000 rpm for 5 min) and dilution of the cell sediment in 5, and then 30 ml of phosphate-buffered saline solution. Then the cells were counted in the Goryaev chamber and the cell number was adjusted to 2 million / ml with the nutrient medium. 100 µl of cell suspension was introduced into each hole of a 96-well plate and incubation was performed for 1 hour at 37° C.

Hanks solution was used as the nutrient medium. In the experiment, the effect of Mexidol, carbamylated darbepoetin, darbepoetin, and placebo (isotonic solution for the substance) against the background of hypoxic and cytotoxic effects on cell culture was studied. From the preparations, working solutions of a certain concentration were prepared; for Mexidol, 150 µg / ml; the remaining substances, 500 ng/ml. In every two adjacent holes 1-10 of a 96-well plate, a certain amount of Hanks solution was added, respectively,

100, 90, 88, 61 and 129 µl, and 132 µl of the drug were added to the 11th and 12th holes of the tablet, and no Hanks solution was added thus creating a concentration gradient from zero to theoretically toxic. Then, the solution was transferred sequentially from 2 consecutive holes to the previous two, so that 100 µl of solution remained in each hole. With this method of introducing liquids, the volumes in all the holes were the same, and the exact concentrations in the rows of the holes are presented in Table.1.

Concentrations were selected on the basis of their therapeutic concentrations indicated in the instructions for preparations, taking into account interspecific recalculation and pharmacokinetic data [14]. As a simulation of hypoxia, oils in a volume of 50 µl were injected into the A-D series of a standard 96-well plate, and a formalin solution of 5 µl 0.4% was added to create a cytotoxic effect. The cells were fixed with 96% alcohol, and 1% crystal violet, 100 µl each, was used as a dye.

Table 1. The concentration of the tested substances in the rows of holes on the tablet

Number of row holes in the tablet	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12
Mexidol	0	0.298137 ng/µl	2.98137 ng/µl	14.9069 ng/ µl	29.8137 ng/µl	150 ng/µl
SDPO, DPO, placebo	0	0.00099 ng/µl	0.009938 ng/µl	0.04969 ng/µl	0.09938 ng/µl	0.5 ng/µl

After removing the dye, phosphate-buffered saline was washed three times [15]. The calculation of the optical density characterizing the survival of cells was carried out at 650/492 nm on Multiskan FC of the company ThermoFisher Scientific Inc., Finland. Descriptive statistics were applied to all data: the data was checked for normal distribution. The type of distribution was determined by the criterion of Shapiro-Wilk. In the case of a normal distribution, the mean and standard error of the mean were calculated. In cases of abnormal distribution, the median and quartile range were calculated. Intergroup differences were analyzed by parametric (Student's t-test) or non-parametric (Mann-Whitney test) methods, depending on the type of distribution [16]. Statistical analysis was performed using Statistica 10.0 software.

Results and Discussion

Analysis of the optical coefficient characterizing the average number of cells in 1 and 2 wells of the plate (control, without drugs) with photometry with a wavelength of 620 nm in comparison with photometry with a wavelength of 492 nm showed that the dispersion is higher when using a wavelength of 620 nm. Analysis of the dynamics of the number of cells in 1 and 2 wells of the plate with photometry of 620 nm showed that when exposed to hypoxia, cell survival was 0.2932 ± 0.145, with cytotoxic effects of formalin - 0.3336 ± 0.124. Photometry 492 nm, when exposed to hypoxia - 0.1155 ± 0.0363, cytotoxic damage - 0.1244 ± 0.0362. Considering this stability, we decided to use only photometry data obtained at 492 nm in the analysis. The results are presented in tables 2 and 3. As can be seen from table 2, only Mexidol at a concentration of 15 ng/µl has a slight antihypoxic effect. Mexidol also has a cytoprotective effect at a concentration of 3 ng/ml (Table 3). With a decrease and increase in concentrations of Mexidol does not cause a significantly significant therapeutic effect. This makes it possible to predict a therapeutic corridor in the range of indicated concentrations. Carbamylated darbepoetin and darbepoetin do not cause significant antihypoxic effects when compared with placebo. However, the average values of tables 2 and 3 can be traced to a parabolic curve, the peak of which is in the middle of the concentration gradient.

Table 2. The results of photometry of the painted environment of neutrophil blood of pigs after hypoxic exposure for 1 hour

SDPO		DPO		Placebo		Mexidol	
Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl
0.129± 0.033	0.5	0.122± 0.026	0.5	0.123± 0.028	0.5	0.123± 0.035	150
0.178± 0.031	0.1	0.171± 0.034	0.1	0.166± 0.033	0.1	0.167± 0.030	30
0.147± 0.032	0.05	0.131± 0.041	0.05	0.126±0. 031	0.05	0.153±0.02 9*	15
0.163±0.0 30	0.01	0.145± 0.031	0.01	0.141± 0.027	0.01	0.140± 0.033	3
0.135± 0.029	0.001	0.138± 0.0296	0.001	0.147± 0.034	0.001	0.152± 0.032	0.3
0.116± 0.036	0	0.116± 0.036	0	0.116± 0.036	0	0.116± 0.036	0

* -p <0.05 when compared with the Placebo group.

Table 3. The results of photometry of the painted environment of neutrophil blood of pigs after cytotoxic exposure for 1 hour with formalin solution

SDPO		DPO		Placebo		Mexidol	
Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl	Optical density	Concentration, ng/µl
0.129± 0.035	0.5	0.145± 0.033	0.5	0.146± 0.031	0.5	0.142± 0.031	150
0.142± 0.033	0.1	0.179± 0.025	0.1	0.179± 0.029	0.1	0.179± 0.035	30
0.159± 0.031	0.05	0.189± 0.034	0.05	0.175± 0.034	0.05	0.199± 0.032	15
0.180± 0.028	0.01	0.192± 0.031	0.01	0.167±0 .036	0.01	0.203± 0.029*	3
0.134± 0.031	0.001	0.151± 0.030	0.001	0.159± 0.033	0.001	0.161± 0.037	0.3
0.124± 0.036	0	0.124± 0.0362	0	0.124± 0.036	0	0.124± 0.036	0

* -p <0.05 when compared with the Placebo group

This fact confirms the correctness of the selected and recalculated concentrations, the extreme values of which do not have pharmacological efficacy. In this experiment, no significant differences in carbamylated darbepoetin and darbepoetin from placebo were found, which may have several explanations. According to previously published papers [11], not all cells have receptors for poets and their derivatives, and poets have an antihypoxic effect through secondary mediators in a living organism over time. It is possible that the hour incubation in our experiment was not enough. In addition, we do not exclude the controversial and so far unexplained phenomenon of "placebo" [12].

Conclusion

1. The technique used in the experiment, has shown its effectiveness and the possibility of its further use as a screening system.

2. Derivatives of erythropoietin at 60-minute incubation do not have either antihypoxic or cytoprotective effects on leukocytes of pigs.

3. Mexidol has an antihypoxic effect at a concentration of 15 ng/µl and cytoprotective - at a concentration of 3 ng/µl.

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