J Nanostruct 12(2): 343-352, Spring 2022

RESEARCH PAPER

Personalized Approaches to the Use of the Antioxidant Ethoxidol in Patients with Coronary Heart Disease

Olesya V. Romaschenko [1], Michail V. Pokrovsky [1], Sergey V. Nadezhdin [1], Vadim V. Rumbesht [1], Nina I. Zhernakova [1], Petr K. Alferov [1], Natalia D. Grischenko [1], Tatiana V. Gorbach [1], Kharkiv Kharkiv [2], Lyudmila V. Statsenko [3], Vladimir G. Kukes [4], Alexey V. Sychenko [1]*

[1] *Belgorod State University, Belgorod, Russia*

[2] *National Medical University, Kharkiv, Ukraine*

[3] *Belgorod Regional Clinical Hospital of St. Joasaph, s, Belgorod, Belgorod region, Russia*

[4] *First Moscow State Medical University, Moscow, Russia*

ARTICLE INFO

Article History:
Received 14 December 2021
Accepted 22 March 2022
Published 01 April 2022

Keywords:
Antioxidants
Coronary heart disease
In vitro research
Leukocytes,
Microscopy
Personalized pharmacotherapy

ABSTRACT

The application of nanoparticles and nanocarriers in the area of cardiology has gathered much attention due to the properties such as passive and active targeting to the cardiac tissues, improved target specificity, and sensitivity. It has reported that more than 50% of CHDs can be treated effectively through the use of nanotechnology. The personalized approach to the choice of drugs in the treatment of patients with coronary heart disease (CHD) is designated as "diamond". How this relates to the antioxidant drug ethoxidol is to be sanctified in this article. To develop a personalized approach to the use of ethoxidol in patients with CHD based on the definition of criteria for predicting the cytoprotective properties of this drug when tested in vitro. With the introduction of ethoxidol into a sample with a leukocyte suspension, a significant increase in VI_{cells} by 21% (from 41% to 62%, $p < 0.001$) was observed, which indicates the presence of a cytoprotective property in this drug. A more detailed analysis of the dynamics of the VI_{cells} index showed two variants of changes in cell viability: in 80% of patients VI_{cells} was increased, on average, by 28% (from 36% to 64%, $p < 0.001$) and in 20% of patients VI_{cells} was decreased, on average, by 10% (from 68% to 58%, $p < 0.05$). A number of conditions for the initial state of a patient with CHD were identified for the manifestation of cytoprotective properties in ethoxidol: a proatherogenic cholesterol profile (with a serum cholesterol level above 6.6 mmol/l and a high-density lipoprotein level below 1.6 mmol /l), impaired antioxidant status (low level of serum catalase activity - less than 5 μcatal/l), signs of mitochondrial dysfunction (increased serum urea levels above 8.3 mmol/l, decreased total blood protein levels below 76 g/l), depletion of the adaptation system function (a decrease in the level of lymphocytes in the blood less than 35% and an increase in the level of neutrophils more than 52%), the normal state of thrombopoiesis (the number of immature platelets is not more than 7%). The antioxidant drug ethoxidol had cytoprotective properties in patients with CHD. At the same time, there were a number of conditions for the initial somatic status of patients, which determined the advisability of a personalized approach to the use of ethoxidol as a cytoprotector.

How to cite this article
Romaschenko O V, Pokrovsky M V, Nadezhdin S V, et al. Personalized Approaches to the Use of the Antioxidant Ethoxidol in Patients with Coronary Heart Disease. J Nanostruct, 2022; 12(2):343-352. DOI: 10.22052/JNS.2022.02.011

* *Corresponding Author Email: Romashenko@bsu.edu.ru*

INTRODUCTION

Nanoformulation is a practical therapeutic approach to diminish the probable side effects of medications and enhance drug delivery efficiency. Encapsulation of natural products and their derivatives provides unique advantages, including reduced systemic adverse side effects, enhanced biosafety, high solubility and bioavailability of drugs, extended circulation time, and restricted accumulation in target organs. Different features of target organs can indicate the specific types of nanostructures that have to be designed to cure diseases. Diverse organic and inorganic nanostructures, including nanoparticles (NPs), have been developed to deliver phytochemicals such as curcumin, emodin, gymnemic acid, tilianin, puerarin, berberine, quercetin (QUE), scutellarin, magnolol, breviscapine, resveratrol, baicalin, naringenin, and so on. The personalized approach to the choice of drugs in the treatment of patients with coronary heart disease is designated by the ex-president of the European Society of Cardiology Robertro Ferrary as "diamond" [1]. The widespread prevalence and high mortality from CHD are aimed at finding rational combinations of drugs in the treatment of patients [2-4]. Rationalization of pharmacotherapy is important in clinical medicine [5-8]. The accepted standards for the treatment of stable angina with drugs from the groups of antiplatelet agents, anticoagulants, beta-blockers, statins, angiotensin-converting enzyme inhibitors, nitrates, calcium antagonists have a high level of evidence, but do not fully ensure the effectiveness of treatment [9]. Currently, the standard of treatment for stable angina pectoris includes a number of metabolic drugs that provide a cardiocytoprotective effect [9]. The direction of cytoprotective pharmacotherapy is traditionally considered to be of secondary importance in the treatment of hypoxic conditions associated with tissue ischemia, including angina pectoris [9-11]. The ambiguous efficacy of cytoprotectors discovered by a number of authors may indicate the need for a personalized approach to prescribing this group of drugs [12-14]. Currently, a whole area of personalized pharmacotherapy is actively developing [15]. The use of an antioxidant drug in the complex pharmacotherapy of coronary heart disease can increase the effectiveness of treatment [16], since myocardial ischemia is associated with atherosclerosis of the coronary vessels and activation of lipid peroxidation [17]. The antioxidant property of the drug pathogenetically can lead to cytoprotective and energy-saving effects [18], which would be important in case of myocardial ischemia. In this regard, it is of particular interest to study the effect of the antioxidant drug ethoxidol on cell viability in patients with CHD. What determines the manifestation of the cytoprotective properties of ethoxidol, is a personalized approach to the use of this drug required in patients with CHD? We did not find answers to these questions in the literature, and we devoted this work to this. The experimental model for studying the viability of cells was the blood leukocytes of patients, since they can reflect the internal state of the human body and are readily available material for research. These immune cells are considered as a kind of "mirror of homeostasis", which can be used to determine the nature of the process underlying the disease, its severity, prognosis and effectiveness of therapy [19]. Moreover, W. Jin, G. Deng-Feng, W. Hao et al, based on a number of their own studies, argue that the nature of mitochondrial damage in cardiomyocytes and peripheral blood leukocytes is identical, leukocytes reflect changes in cardiomyocytes, as in a mirror [20]. To develop a personalized approach to the use of ethoxidol in patients with coronary heart disease based on the determination of criteria for predicting the cytoprotective properties of this drug when tested in vitro.

MATERIALS AND METHODS

Experimental

30 patients with CHD: stable angina pectoris of I-III functional classes were examined. Patients underwent general and biochemical blood tests with determination of cholesterol profile, urea level, total protein in blood serum, catalase activity in blood serum. To determine the cytoprotective properties of ethoxidol, blood leukocytes of patients were studied in vitro by fluorescence microscopy using an Eclipse Ti-U inverted fluorescence microscope (Nikon, Japan). Living and dead cells were determined by staining leukocytes with fluorescent dyes (Calcein AM, Ethidium bromide), and the cell viability index (VI_{cells}) was calculated. The materials were processed statistically, the criteria for predicting the cytoprotective effect of ethoxidol were determined using Wald's predictive analysis. The patients with coronary heart disease: stable

angina pectoris of I-III functional classes (acute coronary syndrome was excluded from the study) were admitted to the Department of Cardiology No. 1 of the Belgorod Regional Clinical Hospital of St. Joasaph from January to June 2019. The study group included 20 women and 10 men aged from 49 to 81 years, the average age of patients was 66.0 ± 2.0 years old.

The patients underwent the entire necessary set of examinations according to the protocol for managing patients with stable angina pectoris [9], including general and biochemical blood tests with determination of cholesterol profile, urea level, total protein in blood serum, and platelet fractions. Serum catalase activity was also determined using a standard spectrophotometric method. Blood sampling was performed in the morning on an empty stomach in a vacuum tube with ethylenediaminetetraacetic acid (EDTA). A prerequisite for the selection of patients for the study was the absence of X-rays for at least 21 days before blood sampling due to the well-known destructive effect of X-rays on human leukocytes and the ability of white blood cells to completely renew the composition for 21 days with an average life expectancy of leukocytes of 7-9 days [21].

To determine the viability of blood cells, leukocytes (0.5 ml) were collected manually with a micropipette under aseptic conditions, mixed with 2 ml of RPMI-1640 culture medium with glutamine (PanEko, Russia), then placed into the wells of a 24-well plate, 20 µl of leukocyte suspension in each well. The culture medium and the drug were added in an amount necessary to create a therapeutic concentration of ethoxidol in the well of 0.68 µg / ml. We were guided by the official instructions for the medical use of ethoxidol. Then the samples were incubated for 3 hours (time sufficient for the drug to interact with the cells) in an incubator with 5% CO_2 content at a temperature of 37°C (the conditions of the human internal environment). After 3 hours of incubation, 500 µl of the supernatant was taken from each well and fluorescent dyes were added to the remaining 500 µl at a final concentration of 1nM/µl for Calcein AM (Invitrogen, USA), which stains only live cells and at a final concentration of 2nM/µl for Ethidium bromide (Sigma-Aldrich, USA), which stains only dead cells [22]. The samples were again placed in a thermostat under the same conditions for another 30 minutes (time sufficient for staining the cells). When developing the scheme of the experiment, we were guided by the tutorial of Mitroshina E.V. et al. (2015) [23].

The results were evaluated by fluorescence microscopy using an Eclipse Ti-U inverted microscope (Nikon, Japan). The data were processed using specialized software EZ-C1 FreeViewer Ver3.90 (Nikon).

The number of living and dead cells was counted, the cell viability index was calculated using the formula:

$$VI_{cells} = (Z_{living\ cells} - Z_{dead\ cells}) / (Z_{dead\ cells}) * 100 \quad (1)$$

where VI_{cells} is the cells viability index (in %), $Z_{living\ cells}$ - the number of living cells in 10 fields of view $Z_{dead\ cells}$ - the number of dead cells in 10 fields of view.

By the nature of the change in the cells viability index under the influence of the drug administered in vitro, the presence of the cytoprotective properties of ethoxidol was judged according to the method we developed [24].

A total of 12,000 cells were analyzed. The materials were processed statistically with the calculation of the arithmetic mean, the error of the mean, and the assessment of the significance of differences according to the Student's t-test. Wald's predictive analysis was also performed. The study was carried out on the basis of the laboratory of cell technologies of the Research Institute of Pharmacology of Living Systems, Belgorod State University.

RESULTS AND DISCUSSION

A surface modification with polyethylene glycol (PEG) serves as a hydrophilic shield that reduces protein absorption and undesirable non-specific interaction with MPS, which is preferable for nano-DDS in cancer. However, incorporation of nanomaterials into MPS itself is one of the intended mechanisms of drug delivery, especially when targeting inflammatory diseases including atherosclerosis. On the other hand, accumulation of nanomaterials depends on the permeability of target lesions. In tumor blood vessels, inflammatory atherosclerotic lesions, and ischemic myocardium, nanomaterials extravasate from blood vessels due to enhanced permeability. Tumors lack functional lymphatic vessels in their tumor microenvironment, which enhances the accumulation of nanomaterials. These phenomena

are referred to as “enhanced permeability and retention effects” or “passive-targeting” (Fig. 1). Targeting can be either active or passive. Targeting applies to the way in which delivery is made, either passively or actively, to the target of interest, such as a specific microenvironment, biological material, or cell surface modifier. This also enables an agent to be multifunctional in therapeutics, diagnostics, or theranostics, depending on the modifications (Fig. 2).

For drug delivery purposes, the most commonly studied nanocarriers are crystal nanoparticles, liposomes, micelles, polymeric nanoparticles, solid lipid nanoparticles, superparamagnetic iron oxide nanoparticles and dendrimers. All of these nanocarriers are formulated for natural product based drug delivery. For applications in cancer treatment, Gupta et al. synthesized chitosan based nanoparticles loaded with Paclitaxel (Taxol) derived from Taxus brevifolia, and utilized them for treatment of diferent kinds of cancer. The authors concluded that the nanoparticle loaded drug exhibited better activity with sustained release, high cell uptake and reduced hemolytic toxicity compared with pure Paclitaxel. Berberine is an alkaloid from the barberry plant. Chang et al. created a heparin/berberine conjugate to increase the suppressive Helicobacter pylori growth and at the same time to reduce cytotoxic efects in infected cells which is depicted in Fig. 3.

Fig. 4 presents the cell therapy approach performance that demonstrated great potential in retrieving cardiac tissue during heart failure. Theoretically, the therapeutic cells can integrate into the damaged cardiac cells and release their therapeutic paracrine factors to regenerate and heal the stunned part of myocardium. However, preclinical and clinical trials of cell therapy approaches thus far have found low retention and engraftment to the host cardiac tissue.

Fig. 5a and 5b show micrographs of living and dead cells in the fields of view without

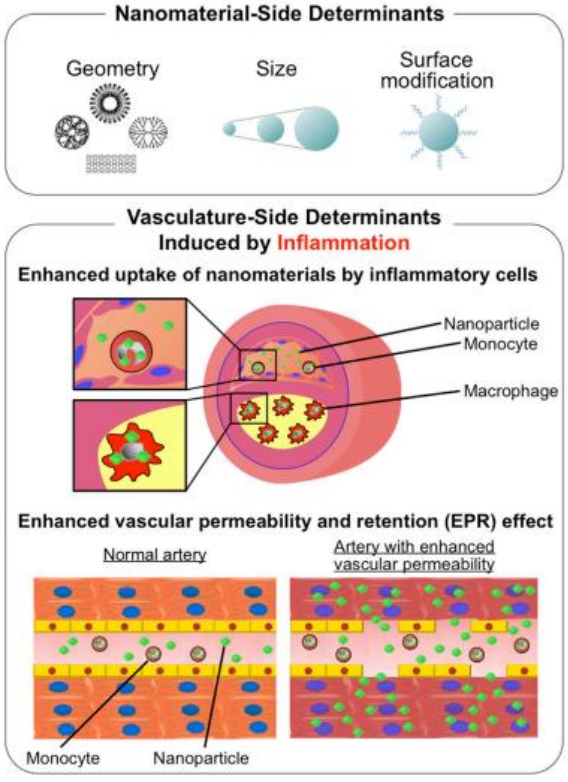

Fig. 1. Schematic description of determinants of physiological behavior of nanomaterials. (Upper panel) Nanomaterial-side determinants: geometry, size, and surface modification. (Lower panel) Vasculature-side determinants induced by inflammation: enhanced uptake of nanomaterials by inflammatory cells and enhanced vascular permeability and retention (EPR) effect.

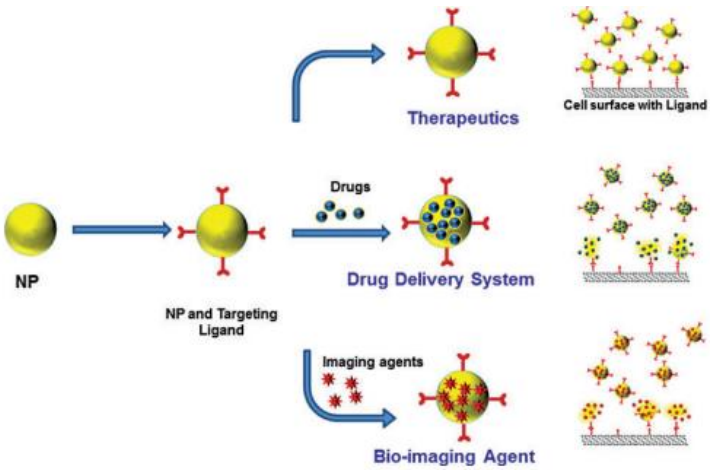

Fig. 2. Multifunctional properties of targeted nanoparticles. Nanoparticles can be used as therapeutics, drug delivery systems, or diagnostic bioimaging agents.

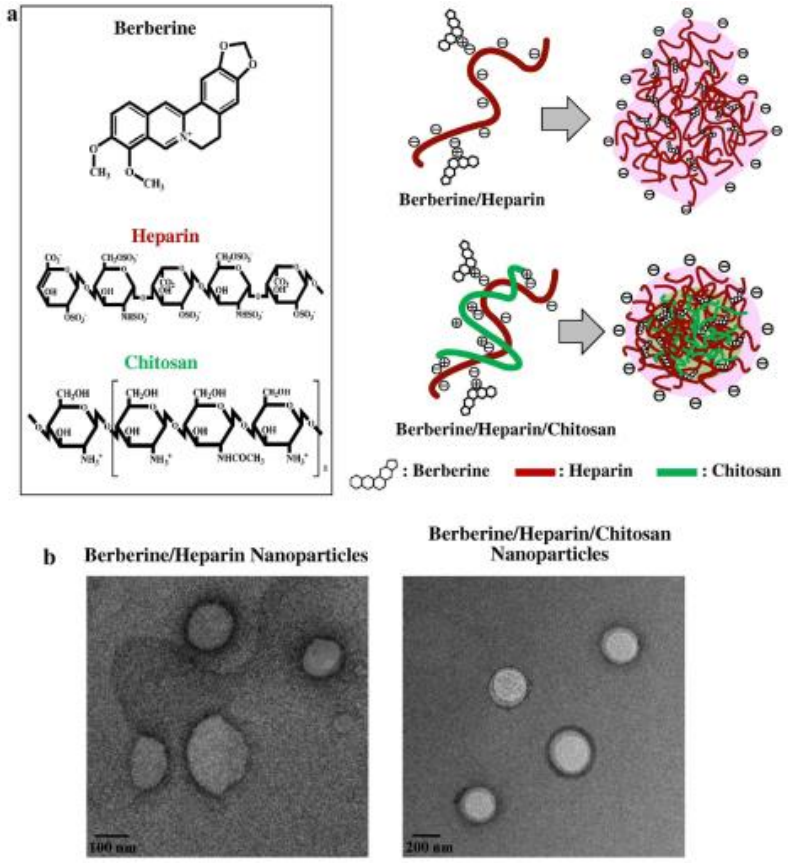

Fig. 3 a Structure of berberine/heparin based nanoparticles and berberine/heparin/chitosan nanoparticles. b TEM images of the berberine/ heparin nanoparticles and berberine/heparin/ chitosan nanoparticles.

the addition of ethoxidol. The VI $_{cells}$ in patients with CHD, on average in the group (without the introduction of ethoxidol), was 41%. Fig. 6a and 6b show photomicrographs of living and dead cells in the field of view of the wells, where ethoxidol was added at a therapeutic concentration. The VI

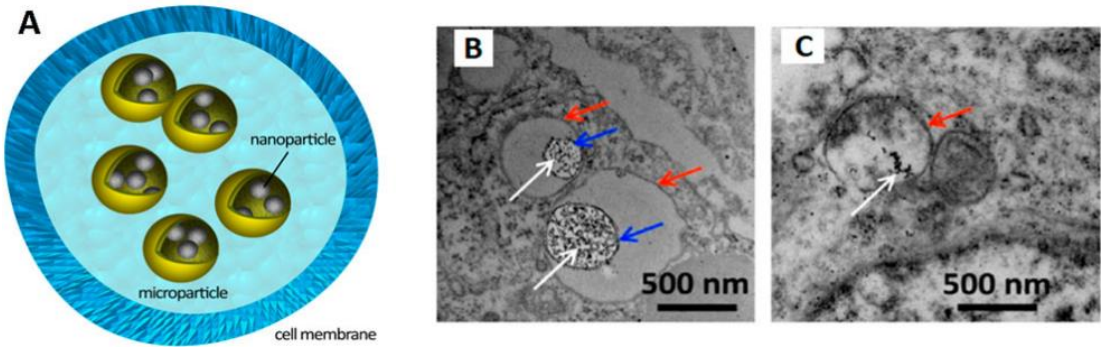

Fig. 4. A) Schematic showing high-density and safe loading of iron oxide nanoparticles in therapeutic cells using biodegradable poly(lactide-coglycolide) microparticles. Transmission electron microscopy images of the labeled cells with (B) magnetic nanoparticles embedded in microcapsules (PLGA-MPs) and (C) magnetic nanoparticles alone (IO-NPs) are shown. White, blue, and red arrows show the locations of IO-NPs, PLGA-MPs, and membrane of intracellular compartment, respectively.

$_{cells}$ of leukocytes in patients with CHD, on average in the group, after the introduction of ethoxidol increased by 21% (from 41% to 62%, p <0.001), which indicates the presence of a cytoprotective property in this drug [24].

A more detailed analysis of the dynamics of the VI $_{cells}$ index showed two variants of changes in cell viability: in 80% of patients, VI $_{cells}$ increased, on average, by 28% (from 36% to 64%, p <0.001) and in 20% of patients, VI $_{cells}$ decreased, on average, by 10% (from 68% to 58%, p <0.05).

To elucidate the reasons for the discovered phenomenon of variability in changes in cell viability after the introduction of ethoxidol into samples with leukocyte suspension of patients with CHD and to determine prognostic criteria for the cytoprotective effect of ethoxidol, we performed Wald's statistical prognostic analysis. A number of the most significant parameters of the initial state of the patient were obtained, which make it possible to predict the reaction of the cells (blood leukocytes) of the patient to the introduction of ethoxidol (Table 1.)

According to the results of our study, the cytoprotective property of ethoxidol depends on a number of parameters of the patient's initial state:

1. Antioxidant status of the patient: ethoxidol exhibits cytoprotective properties under the condition of low activity of its own antioxidant system (low level of catalase activity in blood serum - less than 5 µcatal/l).
2. Cholesterol profile: ethoxidol exhibits cytoprotective properties mainly in patients with a proatherogenic cholesterol profile - high cholesterol levels (above 6.6 mmol / l) and low levels of high density lipoproteins (below 1.6 mmol / l).
3. Mitochondrial dysfunction: ethoxidol exhibits cytoprotective properties in the presence of signs of mitochondrial dysfunction (indirect

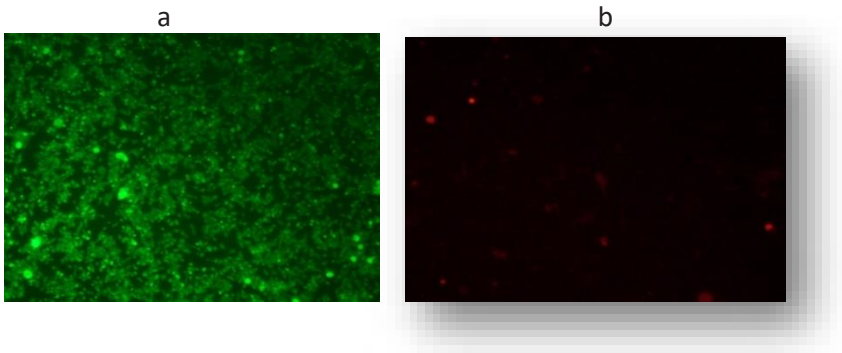

Fig. 5. a) Luminescence of membrane structures of living white blood cells (dye - Calcein AM). Fluorescence microscopy, magnification X200, without ethoxidol b) Luminescence of dead leukocytes (dye - Ethidium bromide). Fluorescence microscopy, magnification X200, without ethoxidol.

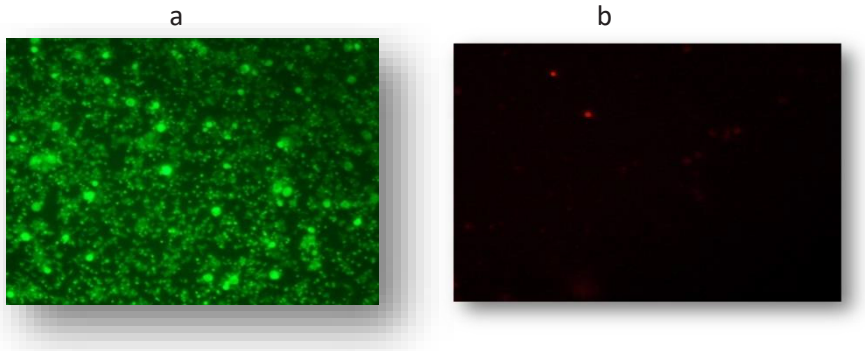

Fig. 6. a) Luminescence of membrane structures of living white blood cells (dye - Calcein AM). Fluorescence microscopy, magnification X200, with ethoxidol (0.68 μg / ml) b) Luminescence of dead leukocytes (dye - Ethidium bromide). Fluorescence microscopy, magnification X200, with ethoxidol (0.68 μg / ml).

indicators of which are higher levels of urea in the blood - over 8.3 mmol / L, and lower levels of total protein in the blood - below 76 g / L).

4. The state of depletion of the functional adaptation system, which is determined by the ratio of blood cells - a decrease in the level of lymphocytes in the blood of less than 35% and an increase in the level of neutrophils by more than 52% [25].

5. State of thrombopoietic function of blood: ethoxidol exhibits cytoprotective properties with an initially normal number of immature platelets (less than 7%).

The pathogenetic substantiation of the validity of our results and conclusions is the following theoretical provisions.

The main cause of myocardial ischemia is coronary atherosclerosis [26]. At the initial stages of atherosclerosis, atherogenic hyperlipoproteinemia is observed [27]. In the presence of vascular endothelial dysfunction (increased endothelial permeability due to activation of lipid peroxidation), atherogenic low density lipoproteins enter the vascular wall. An excess of lipids in the cell promotes further activation of lipid peroxidation, as a result of which cholesterol becomes foreign to the cell and immune mechanisms of atherosclerosis progression are triggered [28].

Lipid peroxidation is a branched chain reaction involving reactive oxygen species (free radicals). A free radical is a highly reactive molecular particle that has an unpaired electron in its outer orbital. Lipid peroxidation of membranes leads to disruption of cellular homeostasis: a decrease in the synthesis of ATP, DNA, RNA, activation of proteolytic enzymes, cytolysis, and, ultimately, cell death [29].

The antioxidant, due to the presence of unpaired electrons in its molecule, is able to capture electrons of reactive oxygen species and neutralize them, thus preventing damage to cell membranes and other structures - mitochondria, DNA molecules, RNA, maintaining normal ATP production and cell viability [30].

According to the official instructions for medical use and literature data, ethoxidol has a number of pleiotropic effects, including hypolipidemic, antiplatelet, antiischemic, membrane stabilizing, endothelioprotective, etc. Many research works are devoted to the pharmacological correction of endothelial dysfunction; endothelioprotection is an important pharmacodynamic target [31-35]. Apparently, all these pleiotropic effects are a consequence of the direct antioxidant action of ethoxidol.

It can be supposed that in those patients who have their own antioxidant system working well enough, the cholesterol profile is normal, the functional adaptation system copes with its task and inhibits the progression of the disease, the antioxidant drug ethoxidol turns out to be unnecessary, as if there is no personal “pharmacodynamic target” for the specific patient. And, on the contrary, in those patients in whom their own antioxidant system is depleted, the level of cholesterol is significantly increased, the lipid profile becomes proatherogenic, the functional adaptation system has exhausted its resources and is unable to restrain the progression

Table 1. Predictive model of the manifestation of the cytoprotective properties of ethoxidol in patients with coronary heart disease (according to in vitro drug testing)

No.	Feature	Range	Predictive coefficient	Informativeness ratio (private)	Informativeness ratio (general)
1	Blood catalase, µcatal / l	<5	3	0.42	0.80
		from 5	-3	0.39	
2	Total blood protein, g / l	< 76	2	0.26	0.54
		from 76	-2	0.28	
3	Complete blood count: neutrophils,%	< 52	-4	1.00	2.50
		from 52	6	1.50	
4	Complete blood count: lymphocytes,%	< 35	6	1.50	2.50
		from 35	-4	1.00	
5	lipid profile: total cholesterol, mmol / l	< 4	1	0.04	0.09
		from 4 to 5.5	-1	0.05	
		from 5.5 to 6.6	-2	0.39	
		more than 6.6	4	0.58	
7	lipid profile: high density lipoproteins, mmol / l	< 1.6	2	0.24	0.68
		from 1.6	-3	0.43	
8	Blood urea, mmol / l	<=8.3	-1	0.08	0.33
		more than 8.3	3	0.25	
9	Immature platelets in the blood,%	< 7	4	0.93	2.25
		>=7	-5	1.32	

Note. A positive predictive coefficient indicates the prediction of the manifestation of the cytoprotective effect of ethoxidol in a patient, a negative predictive coefficient indicates the prediction of the absence of the manifestation of the cytoprotective effect of ethoxidol.

of the disease, the antioxidant drug ethoxidol turns out to be very necessary and useful, capable of exhibiting and antioxidant, and cytoprotective, and hypolipidemic, and, possibly, all other pleiotropic effects.

CONCLUSION

Thus, according to the results of the in vitro study, the antioxidant drug ethoxidol reliably possesses cytoprotective properties when used in patients with coronary heart disease; however, there are a number of criteria for the personalized selection of patients for whom ethoxidol will be prognostically most useful and effective as a cytoprotector. With the introduction of ethoxidol into a sample with a leukocyte suspension, a significant increase in the viability index (VI_{cells}) by 21% (from 41% to 62%, $p < 0.001$) was observed, which indicates the presence of a cytoprotective property in this drug. A detailed analysis of the dynamics of the viability index showed two variants of changes in cell viability: in 80% of patients, increased, on average, by 28% (from 36% to 64%, $p < 0.001$) and in 20% of patients, VI_{cells} decreased, on average, by 10% (from 68% to 58%, $p < 0.05$). A number of conditions of the initial state of a patient with coronary heart disease were identified for the manifestation of cytoprotective properties in ethoxidol: proatherogenic cholesterol profile (with serum cholesterol levels above 6.6 mmol / L and high density lipoprotein levels below 1.6 mmol / L), impaired antioxidant status (low level of serum catalase activity - less than 5 µcatal / l), signs of mitochondrial dysfunction (increased serum

urea levels above 8.3 mmol / l, decrease in total blood protein levels below 76 g / l), depletion of functional adaptation systems (a decrease in the level of lymphocytes in the blood less than 35% and an increase in the level of neutrophils more than 52%), normal thrombopoietic function (the number of immature platelets is less than 7%).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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