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TEST SYSTEM FOR EVALUATION OF THE INFLUENCE OF THE BIOLOGICAL ACTIVITY OF SUBSTANCES ON THE SIGNAL SYSTEM OF NF-κB: FOCUS ON THE DERIVATIVES OF 3-HYDROXYPYRIDINE

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Abstract

Introduction: The nuclear factor kappa B is one of the most promising targets for the development of innovative medicines in the development of modern pharmacology and the formation of the concept of targeted therapy. Potential candidates modulating the activity of this transcription factor are 3-hydroxypyridine derivatives.

Materials and methods: At the first stage of our study, 11 pharmacological agents-inhibitors of NF-κB in vitro were screened with an estimate of the activity of the p65 subunit in mononuclear cells stimulated by bacterial lipopolysaccharide. The most active substances were: mexidol, ethoxidol and the agent under the code XS-9. Further inhibitory activity against NF-κB pharmacological agents was studied on 4 models: L-NAME-induced endothelial dysfunction, staphylococcal sepsis, acute toxic liver damage and acute pancreatitis.

Main part: On L-NAME-induced endothelial dysfunction, the inhibitory activity of the compounds, was arranged in the following order: XS-9 → ethoxydol → mexidol. With staphylococcal sepsis and acute toxic liver damage, the compounds XS-9 and ethoxidol demonstrated the same effectiveness with respect to the modulation of the activity of the NF-κB signaling system, mexidol had the least effect. On the model of acute pancreatitis, the inhibitory activity of the compounds, was arranged in the following order: ethoxidol → XS-9 → mexidol.

Conclusion: Thus, 3-hydroxypyridine derivatives showed a significant activity on the NF-κB signaling system, both in the screening method and in various models leading to its pathological activation. In this connection, it is necessary to study further the mechanisms of action of these pharmacological substances and to confirm their effectiveness in the presented models in in vivo experiments.

Key words: Nuclear factor kappa B; NF-κB, 3-hydroxypyridine derivatives; pharmacological correction.

Introduction.

The derivatives of 3-hydroxypyridine belong to the simplest heterocyclic analogues of aromatic phenols and have a wide spectrum of pharmacological activity: antioxidant, antihypoxic, anti-inflammatory and anti-ischemic [1], cardio- and endothelioprotective [2, 3], antistress, neuroprotective [1] and many others. An extensive list of the types of pharmacological effects can be explained by the involvement of the signaling system of the transcription factor, the kappa B factor, in the implementation of these protective processes. In this connection, the confirmation of the hypothesis of the presence of potential inhibitory NF-κB activity in 3-hydroxypyridine derivatives is an important stage in the development of a new class of medicinal means with antioxidant properties [3, 4, 5]. It was decided to conduct a screening study so as to select the most effective 3-hydroxypyridine derivatives on mononuclear cells of intact rats [6] and further study their inhibitory effect on the NF-κB signaling system in various models.

Materials and methods.

For the purpose of screening for NF-κB inhibitors, we studied a number of drugs that were added to freshly isolated standard ficoll density gradient (ρ = 1.077, PANECO, Russia) mononuclear cells (MNCs) of blood of 6 intact Wistar rats, 3 males and 3 females.

The preparations used in the screening study at the final concentrations are presented in Table 1.

Table 1

Characteristics of the studied drugs

Name	Chemical name	The in vitro concentration, μg / ml
Mexidol	2-ethyl-6-methyl-3-hydroxypyridine succinate	35
Ethoxydol	2-Ethyl-6-methyl-3-hydroxypyridine malate	35
LHT-05-09	3-hydroxy-2-ethyl-6-methylpyridinium glutamate	35
LHT-20-16	3-hydroxy-2-ethyl-6-methylpyridinium asparaginate	35
LHT-03-15	3-hydroxy-2-ethyl-6-methylpyridinium 4-aminobenzoate	35
LHT-21-16	3-hydroxy-2-ethyl-6-methylpyridinium nicotinate	35
LHT-01-09	3-hydroxy-2-ethyl-6-methylpyridinium N-acetylaminohexanoate	35
LHT-4-97	3-hydroxy-2-ethyl-6-methylpyridinium N-acetylaminoacetate	35
LHT-02-09	3-hydroxy-2-ethyl-6-methylpyridinium N-acetylaminoglutamate	35
LHT-11-09	3-hydroxy-2-ethyl-6-methylpyridinium acetylsalicylate	35
XS-9	Beta-hydroxynicotinoylhydrazone 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine dihydrochloride	35

Screened preparations were further tested at the same concentrations on the MNCs blood of Wistar rats with L-NAME-induced endothelial dysfunction, staphylococcal sepsis, acute toxic liver damage and acute pancreatitis (6 animals in each subgroup, 3 males and 3 females).

L-NAME induced endothelial dysfunction was modeled by administration of NG-nitro-L-arginine methyl ester (L-NAME) to rats according to the conventional method [7, 8]. Blood sampling for a 5 ml study was carefully performed with aseptic and antiseptic drugs from the right ventricle of the heart on the 8th day.

Staphylococcal sepsis was modeled using Staphylococcus aureus culture as described previously [9]. Blood sampling for a 5 ml study was carefully performed with aseptic and antiseptic drugs from the right ventricle of the heart on the 3rd day.

Acute toxic liver damage was modeled by intraperitoneal injection of 3 ml / kg carbon tetrachloride as a 50% solution in olive oil, three times, after 24 hours. Blood sampling for a 5 ml study with careful observance of aseptic and antiseptic was performed from the right ventricle of the heart on the 4th day.

Acute pancreatitis was modeled by R.N. Wang in the modification of S.A. Alekhin [10]. Blood sampling for 5 ml was carefully performed with aseptic and antiseptic drugs from the right ventricle of the heart on the 5th day.

The NF-κB activity was studied in 2 ml eppendorf tubes (Gen Follower, China), where 1 ml of the rat MNC slurry, 1×10^6 cells per ml of RPMI-1640 medium (Paneco, Russia) was added with 5% serum of embryos of cows (HiClone, USA), then the test preparation dissolved in phosphate-buffered saline was added at the appropriate final concentration, in a screening experiment – bacterial lipopolysaccharide (Pyrogenal, NF Gamalei Institute, Russia) in concentration 1 μg / ml. The cells were incubated for 30 minutes at 37 ° C on a shaker, then centrifuged for 5 minutes at 200 g, after that the supernatant was removed, the MNC pellet was examined for p65 activity of the NF-κB subunit in strict accordance with the instructions for the NFkB p65 kit. TotalMultispecies InstantOne ™ ELISA Kit Invitrogen / Thermo Fisher Scientific, cat., 85-86081-11).

Briefly, the rat MNCs of 1×10^6 rats was lysed in 1 ml of the lysis buffer included in the kit, the supernatant was added to the wells of a 96-well plate from the ELISA kit. After several steps, the sandwich reaction products were determined on a Multiskan FC microplate reader (Thermo Fisher, Germany) at 450 nm.

Since there are no conventional units of NF-κB activity, and the chosen research method allows to determine the quantitative content of the p65 subunit of NF-κB in an arbitrary number of cells (in our case, 1×10^6 MNCs) in order to simplify further calculations of the relative effect of the studied substances, units of optical density, directly obtained

from a microplate reader, rounded to the 2nd decimal place. The statistical processing of the results was carried out in the MS Excel 2013 program with the Attestatv.13 module using the non-parametric statistics method – Kruskal-Wallis criterion- as the distribution of the p65 subunit of NF-κB in the cells was not normal.

Main part.

The results of the screening of candidates for the role of inhibitors of NF-κB activity (Medians – Me and 1, 3 quartiles – Q1; Q3) are presented in Table 2 and in Fig. 1.

Table 2

The result of a screening study of theinhibitory NF-κB activity of various 3-hydroxypyridine derivatives

Group	Me	Q1; Q3	Group	Me	Q1; Q3
	Unit of optical density			Unit of optical density	
Intact	0.17	0.16; 0.18	LHT-21-16	1.05	1.02; 1.09
Control of LPS	2.22	1.79; 2.46	LHT-01-09	0.95	0.92; 0.99
Mexidol	0.78	0.68; 0.93	LHT-4-97	1.93	1.87; 1.97
Ethoxydol	0.79	0.68; 0.92	LHT-02-09	1.51	1.48; 1.55
LHT-05-09	2.01	1.95; 2.07	LHT-11-09	0.83	0.81; 0.86
LHT-20-16	0.80	0.78; 0.85	XS-9	0.65	0.62; 0.73
LHT-03-15	1.14	1.11; 1.18			

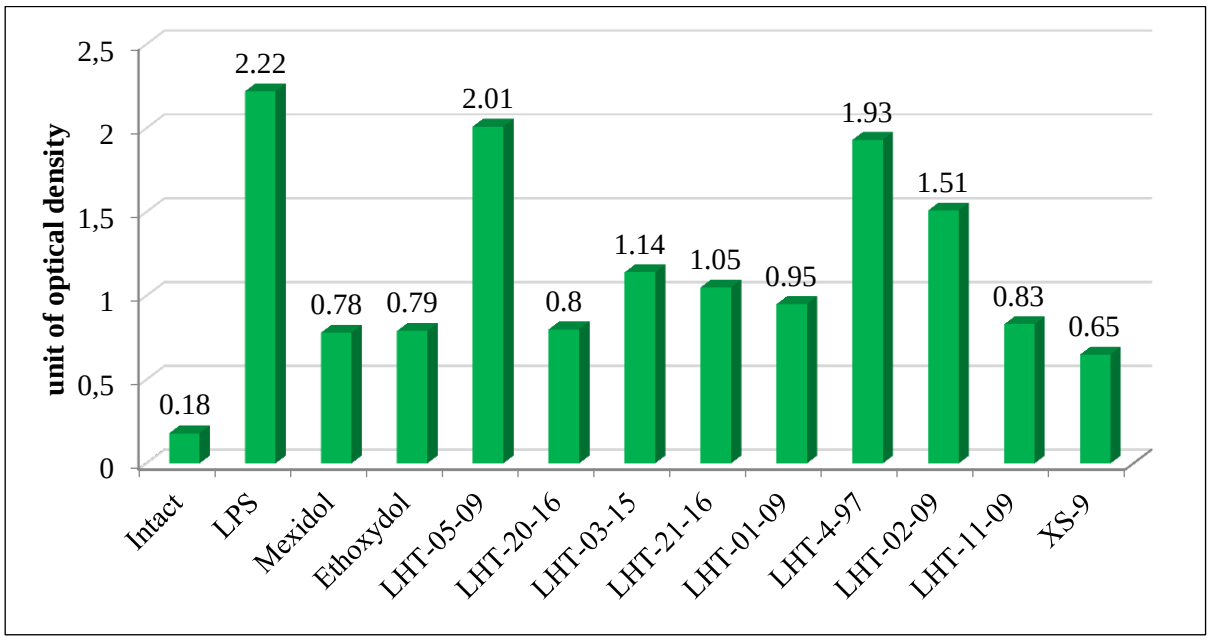

Fig. 1. Effect of the study drugs on LPS-induced NF-κB activity in mononuclear cells of intact rats

As can be seen from Fig. 1, mexidol, ethoxydol and XS-9 were the most inhibitory in the LPS-induced expression of p65 NF-κB in MNCs. Therefore, these substances were chosen for further studies in experimental models.

One possible pathology, where inhibition of NF-κB activity is pathogenetically justified, the solution of which will reduce the stimulating effect of the produced pro-inflammatory cytokines on the endothelium, is endothelial dysfunction. To study the inhibitory potential of the drugs studied, the latter was chosen in the model of chronic vascular pathology, reproduced by L-NAME induction as well as in the variant of acute dysfunction inherent in septic shock and reproduced by the introduction of endotoxin and live staphylococcus.

The results are shown in Fig. 2 and 3.

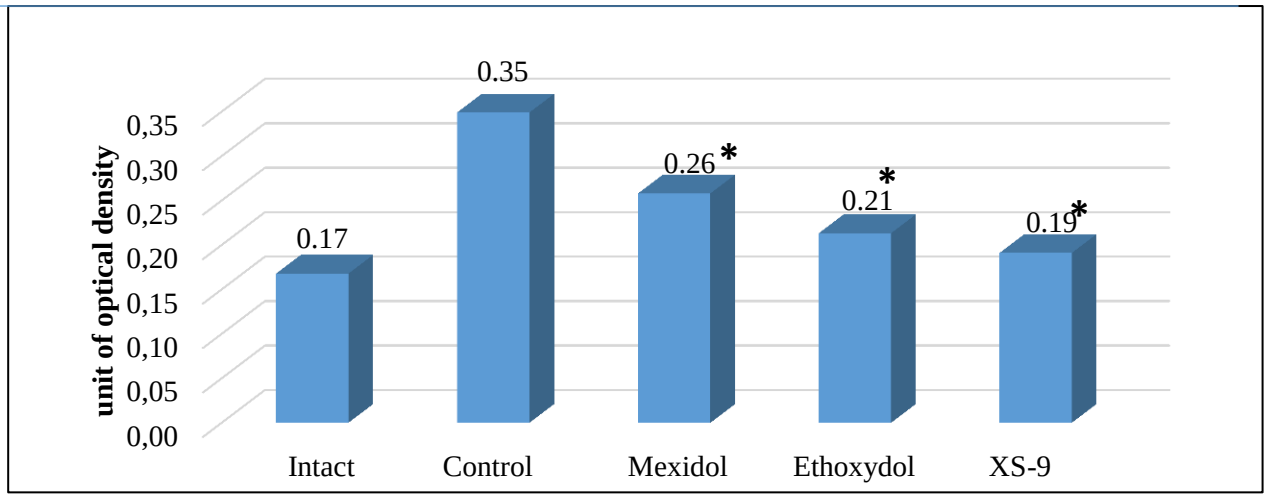

Fig. 2. Effect of the study drugs on the activity of NF-κB in mononuclear blood cells of rats with L-NAME-induced endothelial dysfunction.
Note: * – p <0.05 compared with the control

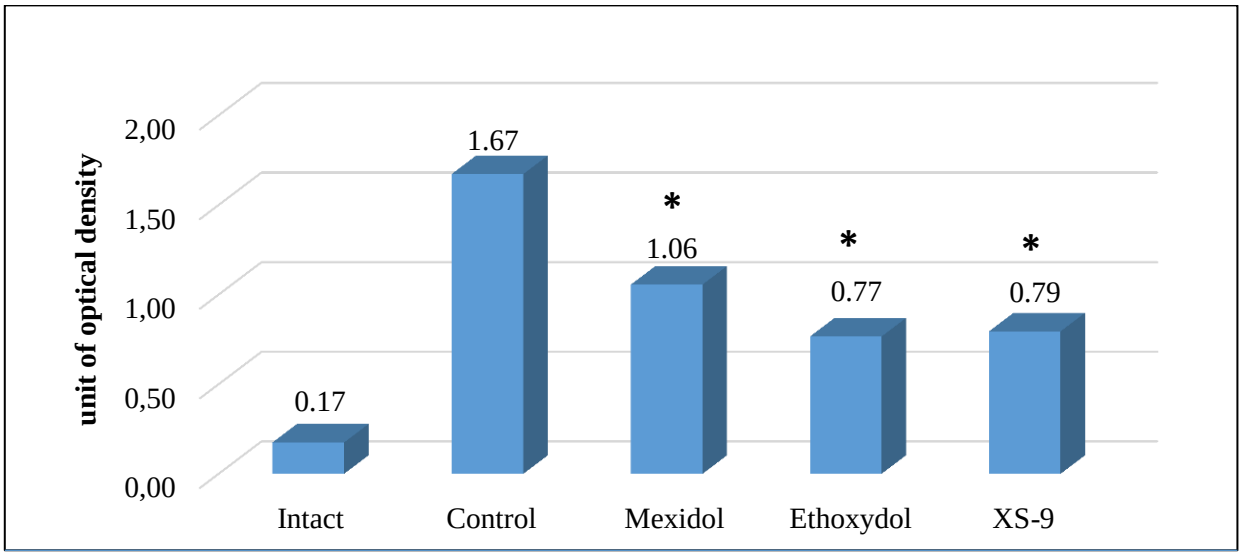

Fig. 3. Effect of the study drugs on the activity of NF-κB in mononuclear blood cells of rats with staphylococcal sepsis.
Note: * – p <0.05 compared with the control

As can be seen from the data obtained, all 3 of the studied drugs significantly decreased the NF-κB activity in the rat MNCs with both types of experimental endothelial dysfunction, although the severity of the inhibitory capacity depended on the degree of initial activity: in the septic process, NF-κB activity under the influence of ethoxydol and XS-9 decreased more than 2-fold, and in the case of L-NAME-induced endothelial dysfunction, this decrease was less pronounced, and there was also a tendency for greater inhibitory activity of these two drugs, not reaching the reliable difference with mexidol.

The inhibitory effect of ethoxidol and XS-9 on NF-κB activity was significantly higher in comparison with mexidol in MNCs obtained in animals with experimental hepatitis where these two drugs significantly suppressed NF-κB, while mexidol did not exert any actions.

On the model of acute toxic damage of the liver we observed the smallest increase in the activity of NF-κB in the MNCs of animals as compared to other models, and ethoxydol and XS-9 returned this activity to the norm observed in intact animals.

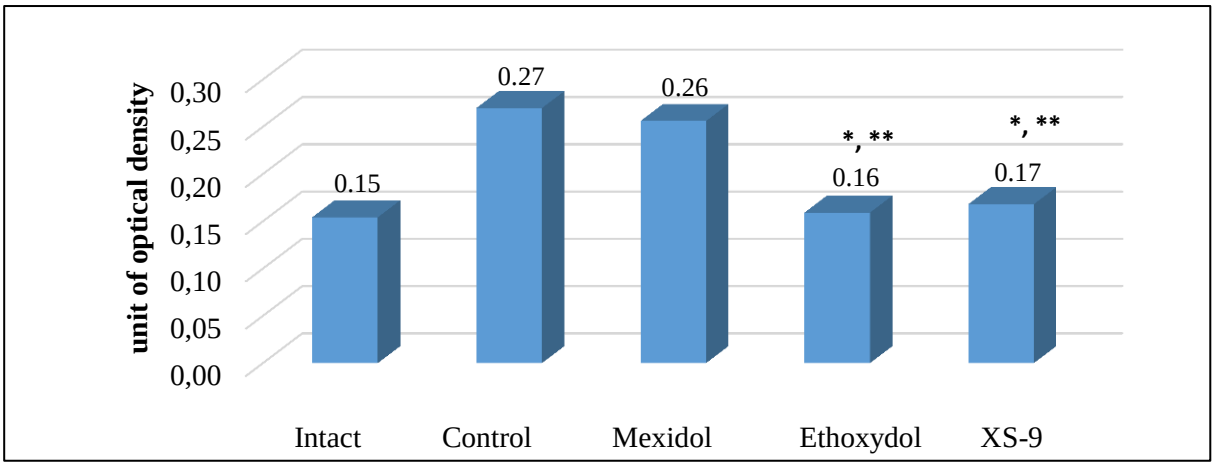

Fig. 4. Influence of the study drugs on the activity of NF-κB in mononuclear blood cells of rats with acute toxic liver damage.
Note: * – p <0.05 compared with the control; ** – p <0.05 in comparison with mexidol

Acute experimental pancreatitis is accompanied by severe systemic inflammation, which is naturally reflected in a significant increase in the activity of NF-κB in the rat MNCs (Figure 5). All the drugs, studied by us, suppressed the activity of this factor, but with varying degrees of severity. Reducing the concentration of p65 NF-κB under the influence of mexidol did not reach statistical significance at all, while ethoxidol had a significantly more pronounced effect not only in comparison with mexidol but also in comparison with XS-9.

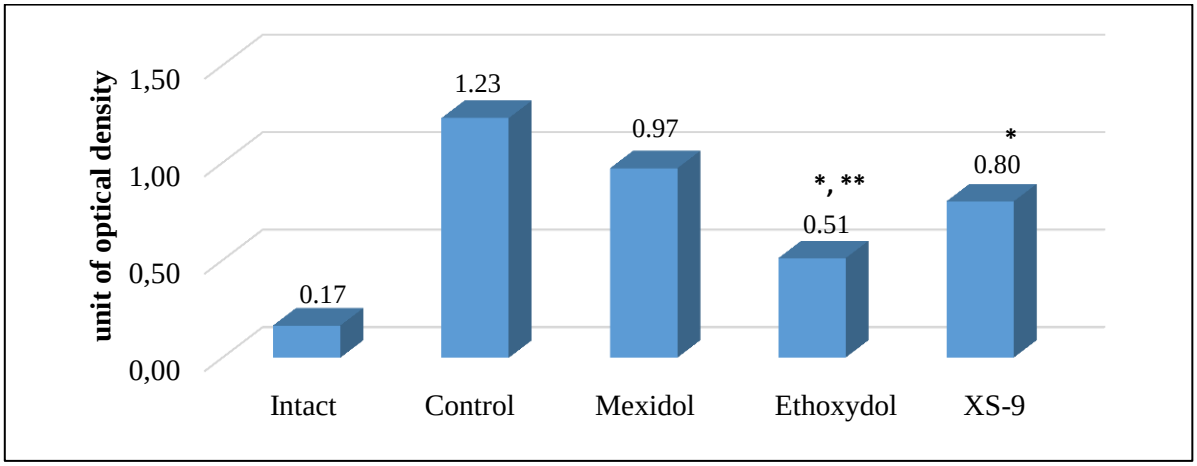

Fig. 5. Effect of the study drugs on the activity of NF-κB in mononuclear blood cells of rats with experimental pancreatitis.
Note: * – p <0.05 compared with the control; ** – p <0.05 in comparison with mexidol and XS-9.

In Table 3 all the experiments are summarized.

Table 3

The optical density in all experiments is median; 1st and 3rd quartiles

Group	L-NAME		Sepsis		ATLD		Pancreatitis	
	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3
Intact	0.17	0.14; 0.17	0.17	0.16; 0.21	0.15	0.09; 0.17	0.17	0.10; 0.17
Control	0.35	0.32; 0.38	1.67	1.48; 1.81	0.27	0.21; 0.37	1.23	1.20; 1.27
Mexidol	0.26	0.21; 0.28	1.06	1.02; 1.09	0.26	0.25; 0.26	0.97	0.94; 1.00
Ethoxydol	0.21	0.19; 0.23	0.77	0.74; 0.79	0.16	0.15; 0.20	0.51	0.45; 0.58
XS-9	0.19	0.18; 0.21	0.79	0.77; 0.83	0.17	0.16; 0.17	0.80	0.73; 0.82

Note: ATLD – acute toxic liver damage

Discussion. The family of the transcription factor NF-κB plays an important role in the life cycle of the cell, regulating the processes of proliferation, apoptosis, inflammation, cell migration, angiogenesis, immune response and others [11, 12, 13, 14]. On the other hand, the NF-κB signal system with inadequate stimulation leads to the formation of vicious circles and key pathogenetic links in the development of cardiovascular

diseases including endothelial dysfunction [15, 16], liver diseases [17, 18], pancreas [19 , 20], oncological diseases [21, 22] and comorbidity.

Thus, some potential target genes under the transcriptional control of NF-κB contribute to the development of endothelial dysfunction of the "proatherogenic" phenotype. These genes encode the synthesis of such pro-inflammatory molecules as interleukin-6, tumor necrosis factor α, the receptor for the final glycosylation products (RAGE) [13, 14]. This, in turn, activates chemotaxis, adhesion and oxidative stress, supports chronic inflammation and endothelial dysfunction.

Both normal and pathological conditions, activation of NF-κB can be carried out in two ways: canonical and noncanonical [23, 24]. The canonical pathway of activation is triggered by the action of tumor necrosis factor-α, interleukin-1β, or ligands of Toll-like receptors of various types (eg, bacterial lipopolysaccharides). The non-canonical pathway is realized through the receptors of the TNF family, including the receptor of a factor activating B-lymphocytes and a CD40 ligand [24].

In unstimulated cells the nuclear factor Kappa B dimers are combined in the cytoplasm with inhibitory proteins – IκBs. Stimulus-mediated activation of IκB kinase leads to the degradation of IκBs for the release and activation of NF-κB [25]. The family of inhibitory proteins include IκBα, IκBβ, IκBγ / p105, IκBδ / p100, IκBε and factor-3 B-cell lymphoma (Bcl-3). They all consist of 30-33 amino acids that bind to NF-κB, which preserves this factor in the cytoplasm [11]. When the signaling pathway is activated, IκBs undergo phosphorylation and ubiquitinization. The best studied is IκBα. Stimulation of cells leads to its phosphorylation and binding of ubiquitin, which causes proteolysis in the system of the 26S-proteasome complex and results in the release of NF-κB, which after additional phosphorylation is allowed to migrate to the site of its action-into the nucleus of the cell. Transcriptional activity of NF-κB appears within a few minutes after stimulation [11, 25]. That is, the degradation of IκBα alters the balance between cytoplasmic and nuclear localization of NF-κB in favor of the latter, which allows dimers to accumulate in the nucleus and activate gene transcription. IκBα itself, which can penetrate the nucleus, displaces NF-κB from the DNA bond and transports it back to the cytoplasm, realizing the principle of negative feedback [23].

One of the pathological links caused by stimulation-mediated activation of NF-κB is the formation of chronic inflammation of low gradation – a typical, multi-syndrome, pathological process that develops during systemic damage and is characterized by total inflammatory reactivity of endotheliocytes, plasma and cellular factors of blood, connective tissue and on the final stages by microcirculatory disorders in vital organs and tissues [26].

Thus, blocking the pathological activation of the nuclear factor kappa B and preventing the formation of vicious circles is a key issue in modern pharmacotherapy aimed at timely prevention and treatment of interrelated pathology: endothelial dysfunction, liver and pancreas diseases.

Conclusion.

Thus, a study of the activity of NF-κB in vitro in MNCs of rats with various experimental pathologies showed the promise of using Mexidol, ethoxydol, a compound with the laboratory cipher XS-9, with an anti-inflammatory purpose, which can later be used in in vivo studies as well as in cultures of other cells, in particular, endotheliocytes.

Corollaries.

1. The presented procedure for the evaluation of the activity of NF-κB is easily reproducible and is adequate for the initial screening of drugs – potential inhibitors of NF-κB.
2. The inhibitory activity of 3-hydroxypyridine compounds is different, but the most active ones are mexidol, ethoxydol and XS-9.
3. In the L-NAME-induced endothelial dysfunction, the inhibitory activity of the compounds was arranged in the following order: XS-9 → ethoxydol → mexidol.
4. In the case of staphylococcal sepsis and acute toxic liver damage, the compounds XS-9 and ethoxidol showed the same effectiveness with respect to the modulation of the activity of the NF-κB signaling system, mexidol had the least effect. At the same time, on the model of acute toxic liver damage, the results of the positive effects of cholesterol-9 and ethoxydol significantly differed from mexidol.
5. On the model of acute pancreatitis the inhibitory activity of the compounds was arranged in the following order: ethoxydol → XS-9 → mexidol.

Conflicts of Interest: The authors have no conflict of interest to declare.

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