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EFECT OF METFORMIN STIMULATED ENDOTHELIAL NOS ON LYMPHOCYTIC PROLIFERATION

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Undeniable is the fact that NO is involved in the pathogenesis and control of infectious diseases, has an effect on tumors, autoimmune processes and chronic degenerative diseases. However, this transmitter has a direct dose dependence, the effects of which can be diametrically opposed. Despite a large amount of information on the effects of NOS on immune cells, the issue of the effects of certain types of it, namely eNOS, on the proliferative function of the lymphocyte cell immunity cell remains open. The study used the Metformine (Mf), which is a trigger for the production of NO, namely endothelial synthase nitrogen oxide (eNOS). This gastransponder was chosen as it has been used for therapeutic purposes for decades and has a bulky evidence base of research. Mf in the form of tablets Mefarmil 500 mg ("Kievmedpreparat") was used as the eNOS synthesis trigger. The study was conducted on white BALB mice weighing 25-30 g. All animals were divided into four groups: control group, and three experimental ones with Mf concentrations: 0,7; 1,1; 2,0 mg per individual.

The research lasted 20 days, the drug was administered at the same time. The red bone marrow was taken from the femoral bone by puncture method, blood was taken from the abdominal cavity. The smears were dyed with the Romanovsky-Gimse dye. Mice have been shown to affect the number of lymphocytes under various metformin concentrations (eNOS). Namely, a decrease in the concentration of 0,7 mg and an increase in lymphocytes at doses of 1,1 and 2,0 mg of metformin compared with control. The dose-dependent effect of eNOS on the selection of prolymphoblasts and lymphoblasts, reduction of blasts in group 2, and reduction of proliferoblasts in the group 1 and lymphoblasts of group 3, and increase of lymphoblasts in group 1 and prolymphoblasts of group 3 were established.

Keywords: *eNOS, metformin, lymphocyte, proliferation.*

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ВПЛИВ СТИМУЛЬОВАНОЇ МЕТФОРМІНОМ ЕНДОТЕЛІАЛЬНОЇ NOS НА ПРОЛІФЕРАЦІЮ ЛІМФОЦИТІВ

Безсумнівним є те, що NO відіграє визначальну роль у патогенезі та контролі інфекційних захворювань в організмі, пригнічує ріст злоякісних клітин, залучається до розвитку аутоімунних процесів та хронічних дегенеративних захворювань. Однак, цей газотрансміттер володіє властивістю дозозалежності, ефекти якого можуть бути діаметрально протилежні.

Незважаючи на досить значний обсяг інформації стосовно впливу NOS на імунні клітини, питання про вплив деяких його типів, а саме ендотеліальної синтази оксиду нітрогену (eNOS), на проліферативну функцію клітинної ланки імунітету залишається відкритим.

У дослідженні використовували препарат метформін, який є тригером для продукції NO, а саме eNOS. Цей газовий трансмітер було обрано, оскільки він

широко застосовується з терапевтичною метою протягом десятиліть і має значну доказову базу досліджень. Експеримент проводили на білих мишах лінії BALB. Всі тварини були розділені на чотири групи: контрольна та три експериментальні (яким відповідно уводили метформін у концентраціях 0,7; 1,1; 2,0 мг на особину).

Дослідження тривало 20 діб, препарат уводили в один і той самий час. Червоний кістковий мозок брали зі стегнової кістки методом пункції, кров отримували з черевної порожнини. Препарати фарбували за класичним методом Романовського-Гімзи.

Було встановлено, що метформін (у порівнянні з контролем) у різних дозах здатен спричиняти вплив на кількість лімфоцитів у змішаній крові. Виявлено зниження кількості лімфоцитів після введення метформіну в концентрації 0,7 мг та збільшення їхнього рівня після уведення високих доз: 1,1 і 2,0 мг. Встановлено дозозалежний ефект впливу метформіну (який стимулює продукцію eNOS) на дозрівання пролімфобластів і лімфобластів: зменшення бластів у групі 2, зниження кількості пролімфобластів у групі 1 та лімфобластів у препаратах кісткового мозку тварин 3-ї групи, а також збільшення лімфобластів у групі 1 і пролімфобластів у 3-й групі.

Ключові слова: *eNOS, метформін, лімфоцити, проліферація.*

During the last two decades, nitrogen oxide (NO) has recognized as one of the universal transmitters in the immune system [15]. It participates in the pathogenesis and the fight against infectious diseases, tumor protection, autoimmune processes and chronic degenerative diseases [16-19]. Because of its variety of reactions with molecules and its wide spectrum and the fact that it's activity NO is strongly dependent on its concentration, it arouses the interest of scientists [20-22].

Today there is no simple consolidated picture of the function NO in the immune system, the protective and toxic NO effects are often observed in parallel. Its unique inter-and intracellular signalling makes it extremely difficult to predict the effects of NOS inhibitors and NO donors, which still impede therapeutic use [23-25].

Except the vasodilating, neurotransmitter and stress-stimulating properties, undoubtedly the participation of NO in the reactions of oxidative stress, glutamate-calcium cascade and inflammation [1-3]. The properties of nitrogen oxide as an effector in various reactions of the immune-endocrine system depend on the quantity and location of the product of this compound [26]. Therefore nitrogen oxide, depending on the specific conditions, demonstrates destructive and protective functions [4-8].

Nitrogen oxide of the human body and animals may have endogenous and exogenous origin. Endogenous NO is formed from the essential amino acid of L-arginine under the action of enzymes NO-synthase (NOS). These enzymes form the family of cytochrome P-450-like hemoproteins and are divided into

two types - constitutive (calcium and calmodulin-dependent) and inducible (calcium-dependent). Constitutive are also divided into neuronal (nNOS, type I) and endothelial (eNOS, type III) isoforms [14].

Endothelial nitrogen oxide (NO) is a critical regulator of cardiovascular homeostasis. Endothelial synthase nitrogen oxide (eNOS or NOS3), derived from NO, is an endogenous vasodilator gastransducer that constantly regulates the diameter of the blood vessels and maintains an antiprolite and anti-apoptotic medium in the vessel walls. First of all, it is a simple factor regulated by calmodulin (CaM), and it is evident that eNOS has evolved to be controlled for some post-translational lipid modifications, focusing on multiple residues, and regulated protein-protein interactions [8, 10 - 11].

From the open questions of the eNOS action on cell proliferation, its role in the selection of lymphocytes in in vivo experiments remains unclear, namely the effect on the prolymphoblast in the red bone marrow. Thus, the purpose of this research was to investigate the role of endothelial synthase nitrogen oxide in the proliferation of lymphocytes in the blood of white BALB mice and their correlation with precursors in the red bone marrow.

METHODS

The research was conducted on BALB mice weighing 20-25 g. Experimental animals were under normal vivarium conditions on a standard full diet. The sick animals were not taken for the test. The work follows the general ethical principles of animals in accordance with the First National Congress of Bioethics (Kyiv, 2001) and the European Convention for the Protection of Vertebrate Animals used for Research and Other Scientific Purposes (Strasbourg, 1986).

Metformin (Mf) in the form of tablets Mefarmil 500 mg («Kievmedpreparat») was used as the eNOS synthesis trigger. Mf activates AMP-activated proteinkinasen, which leads to increased phosphorylation of endothelial synthase nitrogen oxide, which leads to an increase in NO production [12, 13]. The drug was administered orally in a glucose solution (1719,9 μmol / L) using a specific probe.All animals were divided into four groups: a control group, and three experimental ones with metformin concentrations: 0,7; 1,1; 2,0 mg per individual.

The research lasted 20 days, the Mf solution was injected at the same time. Animals were withdrawn from the experiment by laceration (diethyl ether). The red bone marrow was taken from the femoral bone by puncture method, blood was taken from the abdominal cavity. The smears were dyed with the Romanovsky-Gimse dye. Cell counting was performed using standard methods [27].

Conducted statistical and graphical analysis of the results using the program Statistica 6.0. Correlation between the data was carried out using Pearson correlation. The critical level of reliability was with $P \leq 0,05$.

RESULTS

It was established that in the research of bone marrow smears it was found that the number of lymphocytes and prolymphoblasts and lymphoblasts significantly changed and there was a direct correlation depending on the dose of Mf.

Dose dependence of lymphocyte response under metformin action. The number of lymphocytes in the blood of mice varied depending on the dose of Mf. Figure 1 shows that the dose of the first group reduced the number of lymphocytes in the blood by 26,3 ± 2,8%. The injection of Mf into the second group led to an increase in the number of lymphocytes by 10,4 ± 7,8% of the control. Individuals of the third group recorded an increase in the number of lymphocytes by 14,5 ± 3,8% of the control, which confirms the dose-dependent effect of Mf, which in turn causes the induction of eNOS and its subsequent effect on the proliferation of lymphocytes in laboratory mice.

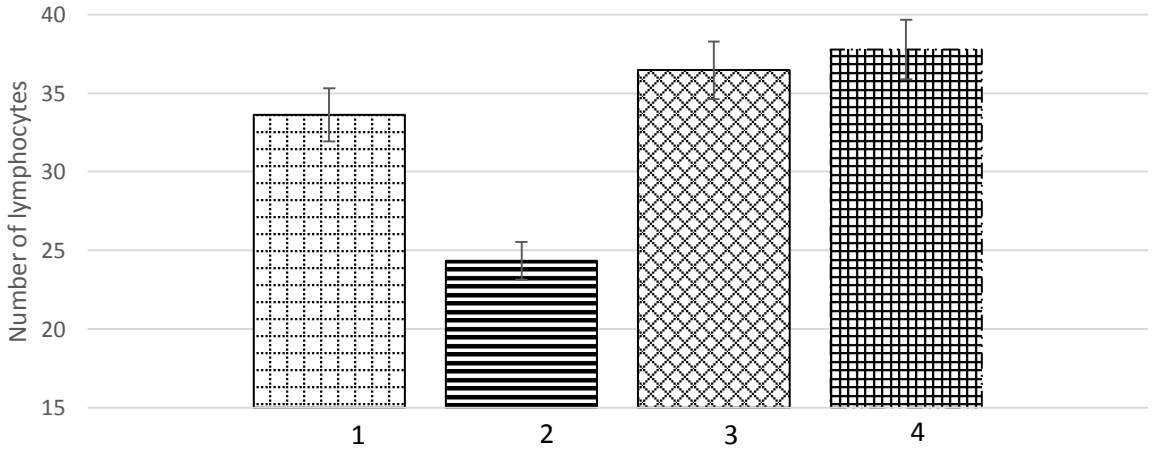
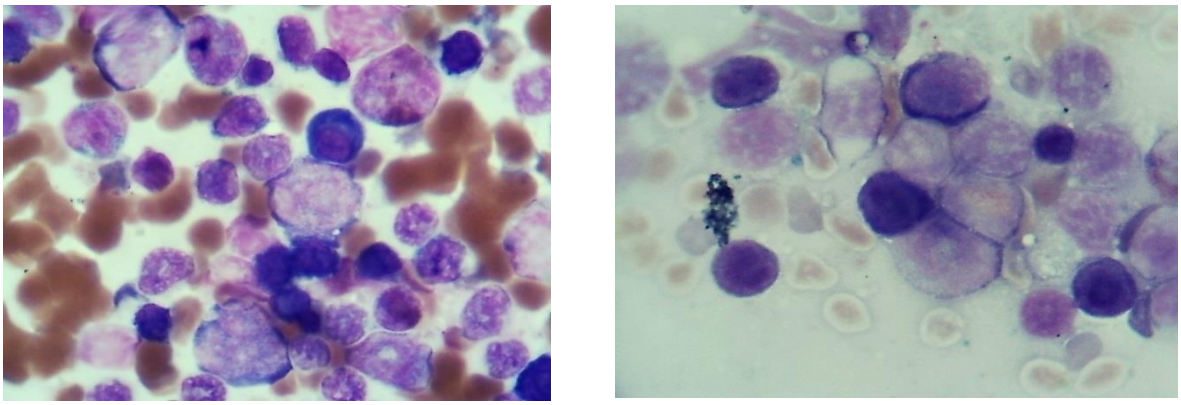


Fig. 1. Dependence of lymphocyte number on metformin concentration.

Comment: 1– control group; 2 – first group (0,7 mg Mf); 3 – second group (1,1 mg Mf); 4 – third group (2,0 mg Mf).

Results of metformin effects on blastocytes. In investigating the smears of red bone marrow of mice, a reliable quantitative change in the parameters of blast cells was found (Fig. 2).

At concentration of 1,1 mg Mf, a decrease in proliferation of 45 ± 4,4% for prolymphoblasts and 17,3±4,4% for lymphoblast was found to be different from control.



A B

Fig. 2. Microphotography of thered bone marrow smear inthe control group (A) and 3 group (B) (Mf=2,0 mg) where the increase level of prolimphoblasts is visible (×100).

In groups 1 and 3 with low and high Mf concentrations, the reverse effect of high and low doses was observed. So in group 1 (0,7 mg Mf) the number of prolymphoblases is reduced by 38,8 ± 3,1% in contrast to control, and the number of lymphoblasts increases by 44,8 ± 3,1% (Fig. 3).

Conversely, in group 3 (2,0 mg Mf), the number of lymphoblasts decreased by 6,9 ± 0,07% from control, and by more than 50 ± 0,07% by prolymphoblasts. This confirms the role of eNOS in the proliferation of lymphocyte precursors, and the dose-dependent effect of the transmitter on the cell [9].

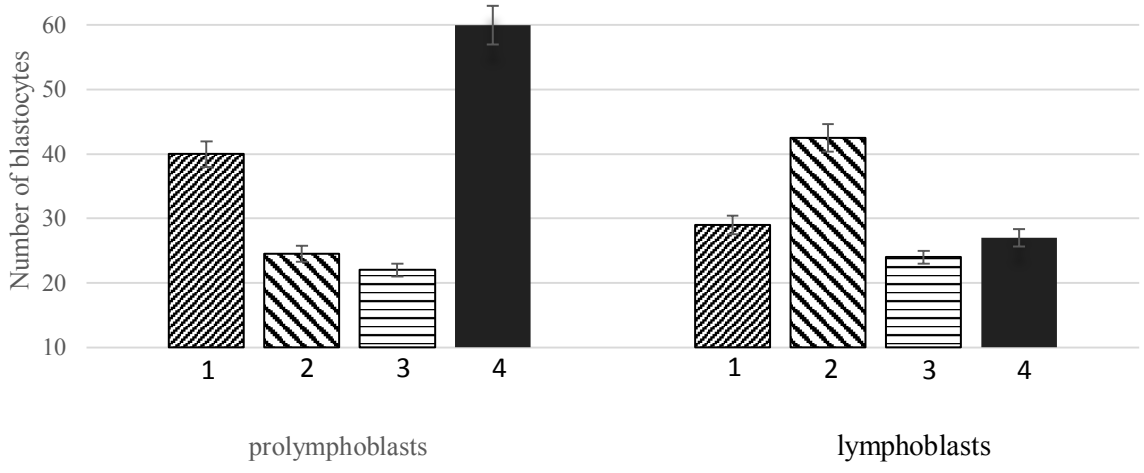


Fig. 3. Dependence of the number blastocytes on concentration Mf.

Comment: 1– control group; 2 – first group (0,7 mg Mf); 3 – second group (1,1 mg Mf); 4 – third group (2,0 mg Mf).

Parson correlation between prolymphoblasts, lymphoblasts in the red bone marrow and lymphocytes in the blood is high (r = 0.84), which suggests the role of eNOS for blast proliferation, and the possibility of using this gastransponder

for therapeutic purposes, namely, the proliferative activity of cells of the lymphocytic type.

DISCUSSION

Physiologically, eNOS affects laminar shear stress. Through G proteins (Gs), it can lead to changes in cellular blood elements, such as lymphocytes [28]. Also, the direct dependence of eNOS and CaM (Calmodulin), leads to an increase in the level of cytoplasmic Ca^{2+}, as well as Akt-mediated phosphorylation and diacylglycerol (DAG) [29-33]. The response of lymphocytes to antigens depends on the signaling network connected to the TCR. Although NO is involved in regulating the expression of cytokines and proliferation of lymphocytes, through mechanisms of which it carries out its functional activity, this process is not yet fully investigated [34]. Especially, the processes of selection of blastocytes in the red bone marrow.

The experiment shows that stimulation of eNOS by metformin results in a change in the proliferative activity of lymphocytes and their precursors, as evidenced by an experiment with an increase in the expression of eNOS and an increase in primary T cells [35]. The activation of endothelial NO leads to the nitrosylation of caspase-3 and caspase-8, which leads to the inhibition of processes of cell apoptosis [36]. In mice and human cells, eNOS is localized either on the Golgi apparatus, or in caveol, this also applies to lymphocytes [8]. In the process of synthesis NO, the gastransponder activates ERK (extracellular signal-regulated kinase) and, at some concentrations, suppresses the proliferation of T cells [37].

This provides new insights into the role of eNOS in early signaling events and its effects on the proliferation of lymphocyte precursors, as well as the dose-dependent NO effect on physiological mechanisms in the initial phase of adaptive immune responses.

CONCLUSIONS

It was shown that in mice, the number of lymphocytes changes with varying concentrations of metformin (eNOS). Namely, a decrease in the concentration of 0,7 mg and an increase in lymphocytes at doses of 1,1 and 2,0 mg of metformin compared with control. In addition, the dose-dependent effect of eNOS on the selection of prolymphoblasts and lymphoblasts, reduction of blasts in group 2, and reduction of proliferoblasts in the group 1 and lymphoblasts of group 3, and increase of lymphoblasts in group 1 and prolymfoblasts of group 3 were established.

The variation of the action of the metformin (eNOS) drug on the proliferation and selection of the lymphatic link in the red bone marrow and blood of mice was revealed. The correlation of bone marrow blisters and lymphocytes with different concentrations of metformin is also observed, which confirms the assumption of the effect on the proliferative function of endothelial

synthase nitrogen oxide, as well as the possibility of using this drug as an immunomodulator.

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