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EMBRYONAL ANTITUMOR MODULATOR INHIBITS *ex vivo* RELEASING OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-OXIDASES FROM ERYTHROCYTE MEMBRANES AND BLOOD SERUM EXOSOMES OF PATIENTS WITH DIABETES TYPES 1 AND 2

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ABSTRACT

The embryonal antitumor modulator (EATM) reveales a hypoglycemic effect in streptozotocin-induced diabetes in rats. However, the mechanisms, by which EATM stabilizes erythrocyte membranes and membranes of serum exosomes associated with characteristic changes of nicotinamide adenine dinucleotide phosphate-oxidases (Nox) of erythrocyte membranes and serum exosomes in patients with diabetes types 1 and 2, are yet to be elucidated.

It was shown that aerobic incubation of the aqueous mixture of erythrocyte membranes, serum of healthy donors and patients with diabetes mellitus types 1 and 2 in the presence of EATM resulted in suppression of Nox release process from erythrocyte membranes in diabetes types 2 and 1 patients and healthy donors by 45.5%, 31.6% and 25.1%, respectively. Under the same conditions reduced activity of extracellular Nox (eNox) released from the serum of patients with diabetes types 2 and 1 and blood serum of donors by 47.9%, 31.3% and 29.3%, respectively, was observed. The most effective concentration of EATM was 0.15 mg/ml.

Suppression mechanism for Nox and eNox releasing by EATM at types 1 and 2 diabetes mellitus is associated with decrease in lipid peroxidation that brings to membrane stability increase. Apparently, the latter is a novel mechanism of the membrane stabilizing effect exerted by EATM ex vivo. This allows to foresee some scientific prospects for EATM as an agent in vivo stabilizing the erythrocyte membranes and exosomes at diabetes type 1 and, especially, type 2.

Keywords: *EATM, NADPH-oxidase, releasing, serum, erythrocyte membranes, diabetes.*

INTRODUCTION

Embryonal antitumor modulator of Mkrtchyan (EATM) regulates synthesis of a number of immunocytokines, hormones, mediators, as well as the level of pro- and antioxidant metalloproteins in tissues of rats with streptozotocin-induced diabetes [*Mkrtchyan L., 2007; 2008; Aghajanova Y., Simonyan M., 2011; Vahedian V. et al., 2011; Aghajanova Y. et al., 2012*]. A decrease in the levels of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Nox) isoforms in blood serum, erythrocyte membranes (EM), and subcellular formations of animal spleen is also observed. On the other hand, EATM has a non-enzymatic activity for superoxide (O_2^-) production in the presence of NADPH (as a source of electrons); however, this activity is suppressed by Nox isoforms due to the intercepting of electrons needed for O_2^- formation [*Aghajanov M. et al., 2011*]. EATM has no direct antioxidant activity; nevertheless, it increases the level of key antioxidant enzymes: superoxide dismutase (SOD) and catalase *in vivo* due to membrane stabilizing effect [*Aghajanova Y., Simonyan M.,* 2011]. Indeed, antioxidant agents produce membrane-stabilizing effect inhibiting the transition of Nox from cell membranes into the homogeneous phase [*Simonyan R. et al., 2003*]. The aim of the current work was to determine the influence of EATM on the process of Nox releasing (at neutral pH) from EM and blood serum exosomes of patients with diabetes types 1 and 2.

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Material and Methods

The study was carried out at "Muratsan" Hospital of the Yerevan State Medical University after M. Heratsi and the Institute of Biochemistry after G.H. Buniatyan of the National Academy of Science of the Republic of Armenia. The study included: 12 patients with type 1 diabetes and 12 patients with type 2 diabetes (in both groups post-diagnosis disease duration made 4-6 years), as well as 12 healthy volunteers. Blood samples were drawn from the cubital vein using 0.2% sodium oxalate as an anticoagulant. To isolate Nox fractions (cytochrome b_{558}) of purified EM and blood serum of volunteers and patients with type 1 and type 2 diabetes, DE-52, KM-52 cellulose ("Whatman", England) and Sephadex DEAE A-50 ("Pharmacia", Sweden) were used. To determine the O_2^--producing activity of Nox nitrotetrazolium blue (NTS), phenazine metasulfate (FMS), sodium pyrophosphate and disodium or tetrasodium salt of NADPH ("Sigma-Aldrich", USA) were used. Mono- and di-substituted potassium phosphate (analytical grade) was used for preparation of potassium phosphate buffer (KPB) solutions. To titrate pH of the solutions hydrochloric acid was used. All other reagents (sodium pyrophosphate, sodium chloride, KOH, etc.) were of analytical grade as well. To perform ion-exchange chromatography glass columns with glass filter, sizes 4×30 *cm*, 2×20 *cm*, 1×15 *cm*, were used. For EM cleaning K-24 and K-70 centrifuges ("Veb MLW Zentrifugenbaum Engelsdorf", Germany) were used. Optical absorption spectra were recorded on a "Specord UV/VIS" spectrophotometer (Germany); the optical path length was 1 *cm*. The statistical analysis was carried out by variation statistics of Student-Fisher test, with $p \leq 0.05$. The study was approved by the Ethics Committee of YSMU.

Isolation and purification of the erythrocyte membranes: Red blood cells from whole blood were precipitated by adding saline (1:20). After 5-minute centrifugation of sedimented erythrocytes at 6000 *r/min* the specified cells were subjected to hemolysis in distilled water (1:10 v/v). Then EM were precipitated by centrifugation of hemolysate at 6000 *r/min* (pH 5.6) for 15 *min*. Thereafter, precipitated EM were again mixed with water (1:200 v/v) and after centrifugation under the same conditions homogenized in 0.04 M KPB (1:1000 v/v) and centrifuged. The last purification procedure was repeated 3 times to obtain a colorless supernatant. The EM cleared of hemoglobin and other plasma proteins were washed with water to remove traces of salts and homogenized in water (at 1:10 v/v) [*Simonyan G. et al., 2001*].

Isolation of blood serum: After erythrocytes purification by centrifugation under the above mentioned conditions serum was extra-centrifuged under similar conditions for the complete removal of traces of plasma cells and red blood cells [*Simonyan G. et al., 2005*].

Experimental conditions: Serum and EM samples of the volunteers' blood and the blood of patients with diabetes mellitus type 1 and type 2 (5 *ml* each) were incubated (with 10 *ml* water) under aerobic conditions at 4°C for 4 days at pH 7.4-8.0, in the absence and in the presence of 0.15 *mg/ml* of EATM. Then isolation of Nox isoforms fractions from these biosystems was carried out, as below.

Isolation and purification of Nox from the erythrocyte membranes: After incubation of EM aqueous mixtures under the above mentioned conditions and their centrifugation (12,000 *r/min*, 15 *min*), the supernatant was diluted 30 times with water and ion exchange chromatography held on DE-52. After the removal of hemoglobin traces through washing of this column with 0.02 M KPB the Nox fraction was eluted with 0.1 M KPB [*Simonyan G. et al., 2001*].

Isolation of extracellular Nox from the serum: Traces of erythrocytes and plasma cells from the serum were removed by centrifugation at 12,000 *r/min* for 15 *min*. Blood serum (10 *ml* or more) was incubated for 4 days under aerobic conditions at 4°C with electrophoretically homogeneous ferrihemoglobin (15×10^{-6} M) of the erythrocyte cytosol. After centrifugation of the incubation solution under these conditions for 15 *min* and dilution of the supernatant with water (20 times), extracellular Nox (eNox) was initially isolated from the solution by ion exchange chromatography on Sephadex DEAE A-50 with eluting fractions of eNox by 0.03 M KPB, pH 7.4. After dilution of the eluate with water (20 times) its ion exchange chromatography was performed on a DE-52 cellulose column (equilibrated with 0.004 M KPB); the fraction of eNox was also eluted with 0.03 M KPB. The eNox amount was determined by measuring the density of the maximum absorbance of Nox solution at 530 *nm* (β-absorption

band). Specific content of eNox was determined by calculation per 1 *ml* of eNox solution obtained from 1 *ml* serum [*Simonyan G. et al., 2005*].

Determination of O_2^--producing activity of Nox and eNox: O_2^--producing activity of Nox isoforms was determined by nitrotetrazolium blue (NTB) method calculating the percentage of formazan forming stimulation (at 560 *nm*) as a result of NTB reduction by superoxide radicals. The amount of protein, which stimulates the formation of formazan by 50%, was considered as unit of O_2^--producing activity. NADPH-dependent O_2^--producing activity of Nox was determined by adding the NADP-Na_4 (10^{-4} M) to the reaction mixture. In this mixture (3 *ml*) the value of Nox absorption density (at 530 *nm*) was 0.03 [*Löehneysen K. et al., 2010*].

Determination of the ferriHb-reducing activity of Nox and eNox isoforms: The principle of determining Nox isoforms ferrihemoglobin (ferriHb)-reducing activity is that in aerobic incubation of Nox with ferriHb *in vitro* ferriHb is restored to ferroHb. We used the electrophoretically homogeneous ferriHb of erythrocytes. At density of the optical absorption band of β-matrix solution of Nox isoforms (A_{530}=0.3) 0.1 *ml* Nox was added to 3 *ml* of ferriHb (A_{565}=0.9). After Nox adding directly to the ferriHb solution without stirring the reaction mixture was placed in a thermostat at 36°C and incubated 5-6 hours. Then the reaction mixture was placed in 1 *cm* glass cuvettes, and the kinetics of lowering the density of α-absorption (at 565 *nm*) of ferriHb during incubation for 4 hours at 20°C was recorded. Gradual reduction of ferriHb to ferroHb was accompanied by lowering in density of ferriHb α-absorption. This decline is in proportion to ferroHb formation (at 555 *nm*). The amount of Nox (or cytochrome b_{558}) causing decrease in density of ferriHb α-absorption to 0.05 in 30 *min* at 20°C was considered as unit of ferriHb-reducing activity [*Löehneysen K. et al., 2010*].

Results

It is shown that in 4-day aerobic incubation of the EM of volunteers' blood and the blood of patients with type 1 diabetes and type 2 diabetes under conditions close to physiological pH (pH=8), varying degrees of releasing of acidic-type isoforms of NADPH-oxidase (Nox) in the homogeneous phase (in solution) was observed. The shapes of the optical spectra of the released Nox in the absence or presence of EATM were virtually identical (Figure 1).

As obvious from Figure 1, the shape of the optical spectra of the released Nox (the optical spectral index – A_{412}/A_{560}, characteristic peaks of optical absorption in the visible region of the spectrum) are almost identical for EM obtained from donor blood serum and the blood of patients with types 1 and 2 diabetes mellitus, in the absence and presence of EATM (Figure 1). This indicates that EATM has no denaturating effect on the human EM Nox. Herewith, the degree of the Nox releasing from EM at types 2 and 1 diabetes is higher by 83.3±7.4% (p=0.01; n=12) and 58.4±6.5% (p=0.003; n=12), respectively, compared with the Nox releasing from EM of donor blood. Considering that a certain amount of Nox is localized on the surface of the cell membranes of various modifications [*Tang X. et al., 2007; Löehneysen K., 2010*], it can be assumed that in these conditions Nox releasing from the EM is not complete, but partial. Thus, only the Nox localized on the surface of EM is getting released, while the complete cleavage of Nox from EM occurs in extreme conditions [*Cho N., Morré D., 2009*]. The EATM suppresses Nox releasing from EM of blood samples drawn from types 2 and 1 diabetes patients and those of donor blood by 45.5±6.2%, 31.6±4.4% and 25.1±3.7% (p=0.001; n=12), respectively.

Thus, inhibition of EATM-induced Nox releasing from EM was most pronounced in type 2 diabetes. This indicates that the stability of EM in insulin-independent diabetes was compromised to a greater extent than in insulin-dependent diabetes.

It was found that under the specified conditions suppression of EATM-induced Nox release process in EM occurred in a dose dependent manner (Figure 2). The most effective concentration of EATM to inhibit Nox releasing from EM *ex vivo* was 0.15 *mg/ml*.

A similar phenomenon was observed with eNox of donor serum and serum of patients with types 1 and 2 diabetes. During aerobic incubation of donor serum and serum of patients with types 1 and 2 diabetes with 1.5×10^{-5}M of hemoglobin from donor blood erythrocytes cytosol, with and without EATM, there was different release of eNox, appar-

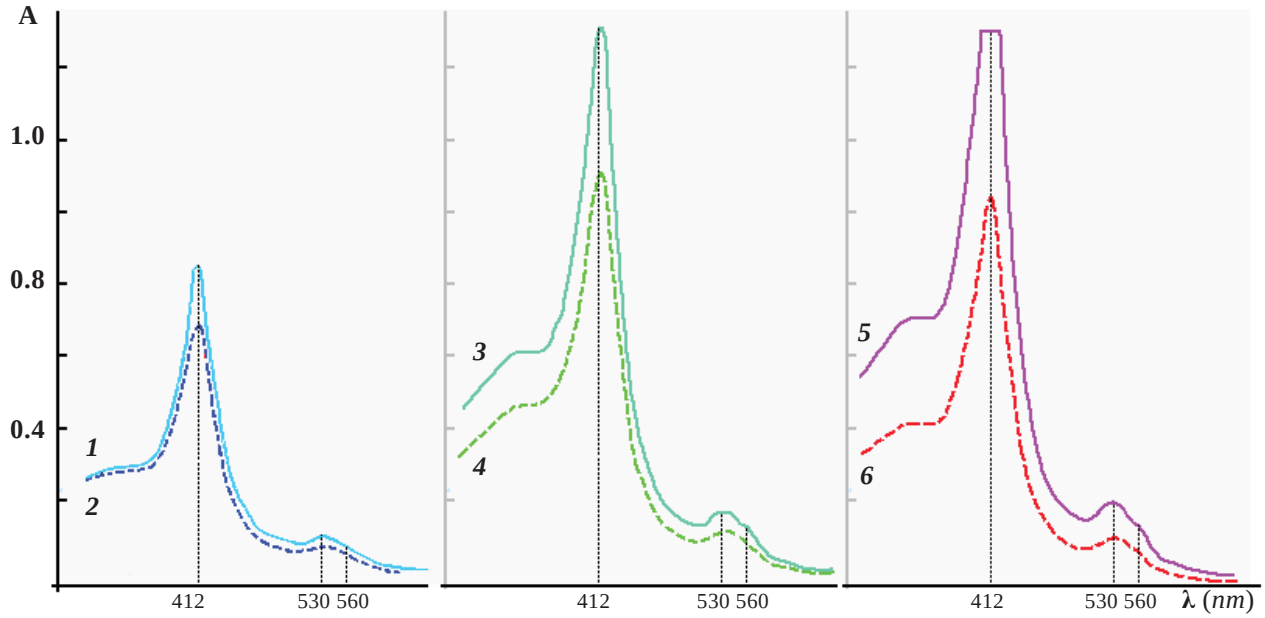

FIGURE 1. *Optical absorption spectra of Nox released from EM obtained from blood of patients with types 1 and 2 diabetes and healthy donors after 4 days of aerobic incubation of aqueous mixtures at 4°C and pH 8 in the absence (solid lines) and presence (dashed lines) of 0.15 mg/ml of EATM. The absorption spectra of Nox: EM of donor blood (1, 2); EM of blood samples drawn from type 1 (3, 4) and type 2 diabetes (5, 6) patients. Maximum optical absorptions for Nox at 560, 530 and 412 nm (oxidized) are shown to be similar to the rest of the Nox spectra.*

ently from exosomes present in serum [*Berridge M., Tan A., 2000; Duijvest D. et al., 2011; Rupp A. et al., 2011*]. The shapes of the optical spectra of the released eNox in donor serum and serum of patients with types 1 and 2 diabetes, with and without EATM exposure, were almost similar (Figure 3).

As shown in Figure 3, the shape of the absorption spectra of serum released eNox (the value of the optical spectral index – A_{412}/A_{560}), characteristic peaks of the optical absorption in the visible region of the spectrum, including the absorption at 485 *nm*, which is characteristic only to eNox, are almost identical for the spectra of eNox of donor blood serum and blood serum of patients with types 1 and 2 diabetes, in the absence and presence of EATM. These data also demonstrate the absence of any disturbing effect of EATM to eNox from human blood serum. The increase of eNox releasing from the blood serum in diabetes types 2 and 1 is higher by 109.2±8.7% (p=0.02; n=12) and 45.4±5.5% (p=0.001; n=12), respectively, compared with eNox indices for donor serum. Under the influence of EATM the inhibition (in %) of eNox releasing from serum of patients with diabetes types 2 and 1 and donor blood was respectively 47.9±4.4%, 31.3±2.9% and 29.3±3.2% (p=0.001; n=12). Obviously, the greatest suppression of eNox release by EATM was also observed in the serum of patients with type 2 diabetes.

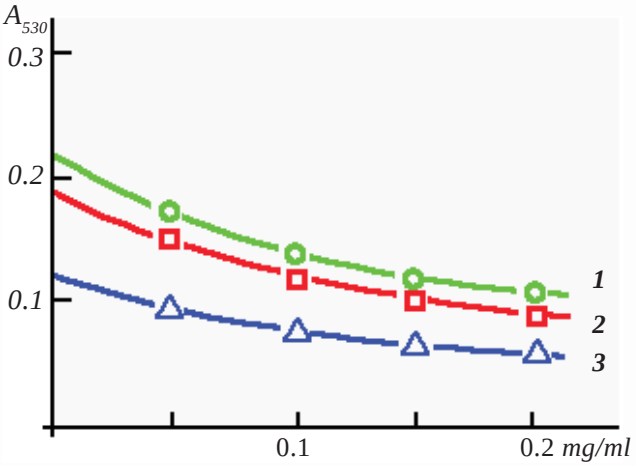

FIGURE 2. *Changes in density of maximum optical absorption of Nox (ß-absorption band) from the blood of patients with types 1 and 2 diabetes and healthy donors at 530 nm under the influence of EATM various doses. The density of EM Nox optical absorption in type 2 diabetes (1), type 1 diabetes (2) and donor blood (3) is demonstrated.*

The study shows that the stability of EM and exosomes in insulin-independent diabetes weakened to a greater extent than in insulin-dependent diabetes. In parallel, EATM reveals no negative impact not only on the Nox from the EM, but also eNox of human serum. Suppression of the eNox

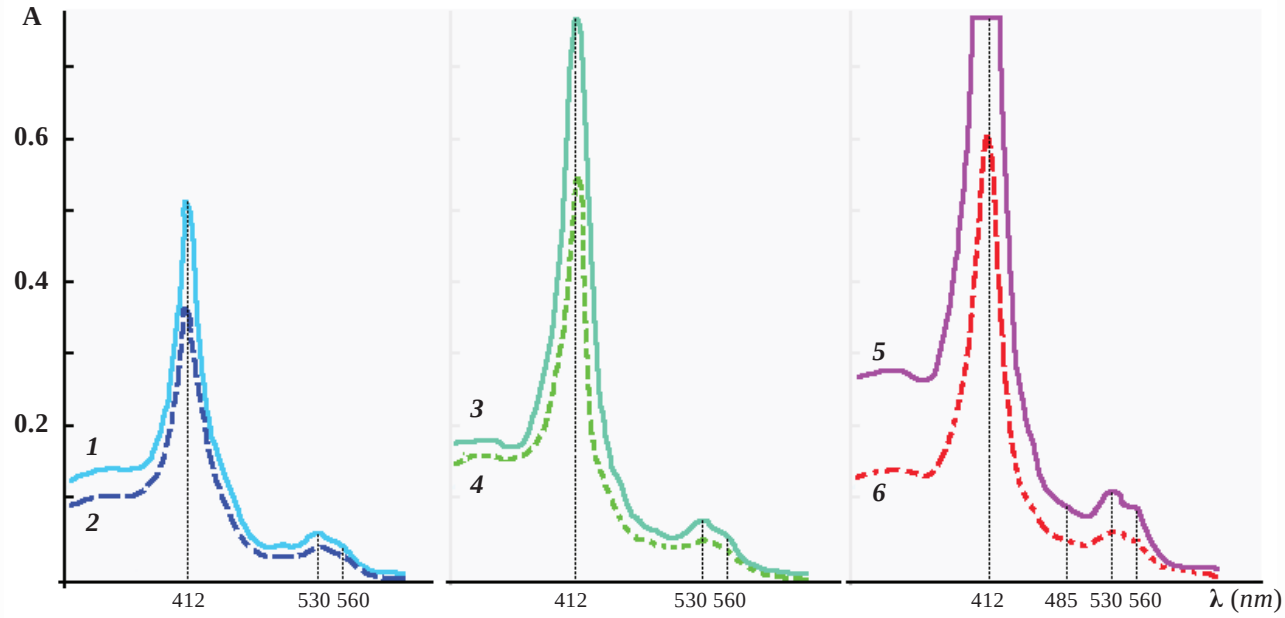

Figure 3. *Optical absorption spectra of the released eNox from serum of patients with diabetes types 1 and 2, as well as healthy donors after 4-day aerobic incubation at 4°C and pH 8 in the absence (solid lines) and presence (dashed lines) of 0.15 mg/ml EATM. Absorption spectra of eNox from serum of patients with type 2 diabetes (5, 6) and type 1 (3, 4) and donor blood (1, 2). The example of the spectrum 1 of eNox shows the characteristic maximum optical absorption at 560, 530, 485 and 412 nm (oxidized), which are similar to the rest of eNox spectra.*

release process was designed in dose dependent manner (Figure 4), the effective concentration of EATM to inhibit releasing of eNox from the blood serum *ex vivo* making 0.15 *mg/ml*.

Discussion

Our study was aimed to investigate the effects of EATM on *ex vivo* release of Nox from erythrocyte membranes and serum of patients with types 1 and type 2 diabetes. It showed that the shapes of the optical absorption spectra of the released Nox and eNox did not change significantly in patients with types 1 or type 2 diabetes, with or without EATM, compared to donor blood indices. We found that the specific content of released Nox and eNox increased not only in streptozotocin-induced diabetes in rats [*Aghajanova Y., 2011; Aghajanova Y., Simonyan M., 2012*], but also in diabetes types 1 and 2 in humans. EATM inhibits the cleavage of oxidases, providing a stabilizing effect on the EM and exosomes. Exosomes are a kind of microvesicles, which are fragments of plasma membrane ranging from 50 *nm* to 1000 *nm* shed from almost all cell types [*Grant R. et al., 2011*; *Fang D. et al., 2013*]. Exosomes play a role in intercellular communication, transport of mRNA, miRNA, and proteins between cells [*Fevrier B., Raposo G., 2004*; *Bang C., Thum T., 2012*]. Particularly, they facilitate transmission of miRNAs between cells: one of the key ways of interaction between stem cells and their microenvironment, the so-called niches [*Redis R. et al., 2012*], the regulatory link between the cells [*Lander A. et al., 2012*]. They are implicated in the process of tumor immune suppression, metastases spread, tumor-stroma interactions and

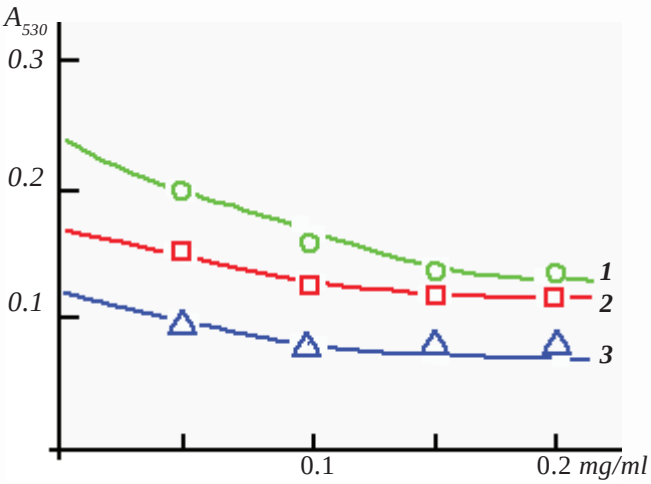

Figure 4. *Changes in density of maximal optical absorption of eNox (ß-absorption band) from blood serum of patients with diabetes type 1 and 2, as well as healthy donors, at 530 nm and different doses of EATM. Density of optical absorption of eNox from blood serum in diabetes type 2 (1), type 1 (2) and donor blood (3).*

angiogenesis along with taking part in tissue regeneration. They originate directly from the plasma membrane of the cell and reflect the antigenic content of cells, from which they originate. They remove misfolded proteins, cytotoxic agents and metabolic waste from the cell [*Redis R. et al., 2012*]. Being nanoparticles, exosomes can pass from cells into the blood stream under various pathological conditions, including diabetes [*Berridge M., Tan A., 2000; Duijvest D. et al., 2011; Rupp A. et al., 2011*].

Elevated blood levels of malone dialdehyde (MDA) in diabetes [*Aghajanova Y., 2011*] reflect the adequate increase in lipid peroxidation, including that in exosomes. The increase in MDA levels in biological membranes (EM, exosomes) results in an adequate increase in fluidity of these membranes and facilitates the process of heterogeneous phase Nox and eNox releasing into a homogeneous phase (solution). Although EATM by itself has no antioxidant activity, it increases the activity of key antioxidant enzymes, which are located not only in the cytosol, but also in cell membranes. Thus, EATM exerts membrane stabilizing effect mediated by the suppression of lipid peroxidation [*Vahedian V., 2011*]. Due to these properties EATM resembles galarmin, which produces antioxidant and antistressor activity by trapping highly toxic hydroxyl radicals. Nox isoforms being receptors of galarmin, play an immunostimulatory role and exert membrane stabilizing effect by inhibiting lipid peroxidation of biological membranes [*Simonyan G. et al., 2008*]. According to our preliminary data, Nox isoforms might also serve as insulin receptor [*Aghajanova Y., Simonyan M., 2012*]. If we accept this assumption as the basis, it becomes clear that Nox releasing in diabetes patients reduces the concentration of functionally active receptors in cell membranes, including beta cells. This, in turn, suppresses the level of signal transduction, activation of intracellular tyrosine kinases and, finally, the activation of hexokinase (glucokinase), one of the key enzymes of glycolysis [*Porat Sh. et al., 2011*]. Glucokinase determines the level of glucose metabolism in beta cells as a central regulator of glucose-stimulated insulin secretion. The absence of glucokinase would reduce the flow of glucose in beta cells and, consequently, result in reduced insulin secretion and increased blood glucose levels [*Magnuson M., Kahn C., 2000*]. EATM suppresses Nox release from different membrane structures and restores the mechanism of insulin signal transduction, thus maintaining the euglycemia. Nox isoforms are important structural and functional components of the EM, exosomes, and membranes of the immune system cells [*Cho N., Morré D., 2009*; *Löhneysen K. et al., 2010*]. An increase in their cleavage with increasing lipid peroxidation (in type 1 and, particularly, type 2 diabetes) adequately reduces stability of these membranes, which is a novel mechanism of biological membranes destabilization. Suppression of the Nox isoforms releasing process, both from EM and exosomes, in diabetes types 1 and 2 patients under the influence of EATM is a novel mechanism of its membrane-stabilizing effect *ex vivo*. This sets the ground for therapeutic application of EATM for *in vivo* stabilizing the EM and exosomes in patients with diabetes type 1 and especially type 2.

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