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EXPERIMENTAL STUDIES / BADANIA DOŚWIADCZALNE

Experimental evaluation of Enalapril effect on protein oxidative modification, proteolytic processes and cerebral morphological changes in rats with type 2 diabetes mellitus

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Experimental evaluation of Enalapril effect on protein oxidative modification, proteolytic processes and cerebral morphological changes in rats with type 2 diabetes mellitus

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Neurodegenerative processes with type 2 diabetes mellitus in particular aggravate the course of the disease, change the usual life rhythm, and are a considerable part of high disability and lethality rates.
The aim *of the study was to examine enalapril effect on protein peroxide oxidation, activity of proteolysis and fibrinolysis enzymes and morphological state of the cerebral cortex and hippocampus under conditions of neurodegeneration in case of experimental type 2 diabetes mellitus.*
Material and methods. *Changes in the content of protein peroxide oxidation products activity of proteolysis and fibrinolysis enzymes in the cerebral cortex and hippocampus are examined under enalapril effect (1 mg/kg) in nonlinear laboratory albino male rats with neurodegeneration under conditions of type 2 diabetes mellitus simulated by streptozotocin and high-fat diet.*
Results. *After introduction of enalapril during 14 days for rats with type 2 diabetes mellitus the content of proteins of a neutral and main character, activity of proteolysis and fibrinolysis enzymes in the cerebral cortex and hippocampus decreases, which is indicative of reduced protein peroxide oxidation, intensified under conditions of diabetic neurodegeneration. Morphological picture of the cerebral cortex and hippocampus is characterized by a decreased amount of cells with karyopyknosis and increased relative density of the tigroid substance staining of neurons and lack of denudation signs of the vessels, which is indicative of a positive rebuilding of the neuron structure.*
Conclusions. *The conducted study demonstrates a protective effect of enalapril in case of central neurodegeneration caused by type 2 diabetes mellitus, one of the mechanisms of which is decrease of protein peroxide oxidation in the cerebral cortex and hippocampus inhibiting the processes of nerve cells destruction.*

Key words: *type 2 diabetes mellitus, neurodegeneration, enalapril, proteolysis, fibrinolysis*

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Eksperymentalna ocena wpływu enalaprylu na modyfikację oksydacyjną białek, procesy proteolityczne i zmiany morfologiczne mózgu u szczurów z cukrzycą typu 2

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W szczególności procesy neurodegeneracyjne występujące w cukrzycy typu 2 pogarszają przebieg choroby, zmieniają zwykły rytm życia i stanowią znaczną część wysokiego wskaźnika niepełnosprawności i śmiertelności.
Celem *pracy było zbadanie wpływu enalaprylu na utlenianie nadtlenków białek, aktywność enzymów proteolizy i fibrynolizy oraz stan morfologiczny kory mózgowej i hipokampu w warunkach neurodegeneracji w przypadku eksperymentalnej cukrzycy typu 2.*
Materiał i metody. *Zmiany zawartości produktów utleniania nadtlenków białek, aktywność enzymów proteolizy i fibrynolizy w korze mózgowej i hipokampie są badane pod wpływem enalaprylu (1 mg/kg) u nieliniowych laboratoryjnych samców szczurów, albinosów z neurodegeneracją w warunkach cukrzycy typu 2 symulowanej przez streptozotocynę i dietę wysokotłuszczową.*
Wyniki. *Po wprowadzeniu enalaprylu w ciągu 14 dni u szczurów z cukrzycą typu 2 zmniejsza się zawartość białek łącznie i o charakterze obojętnym, a także zmniejszeniu ulega aktywność enzymów proteolitycznych i fibrynolitycznych w korze mózgowej i hipokampie, co wskazuje na zmniejszone utlenianie nadtlenków białek, nasilone pod wpływem stany neurodegeneracji cukrzycowej. Obraz morfologiczny kory mózgowej i hipokampu charakteryzuje się zmniejszoną liczbą komórek z kariopyknozą i zwiększoną względną gęstością barwienia neuronów substancją tygroidową oraz brakiem objawów denudacji naczyń, co wskazuje na pozytywną odbudowę struktury neuronów.*
Wnioski. *W przeprowadzonych badaniach wykazano ochronne działanie enalaprylu w przypadku centralnej neurodegeneracji wywołanej cukrzycą typu 2, której jednym z mechanizmów jest zmniejszenie utleniania nadtlenków białek w korze mózgowej i hipokampie, hamujące procesy niszczenia komórek nerwowych.*

Słowa kluczowe: *cukrzyca typu 2, neurodegeneracja, enalapryl, proteoliza, fibrynoliza*

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A progressive increase of type 2 diabetes mellitus (DM) complications make this problem rather important for clinical and fundamental medicine. Neurodegenerative processes with type 2 DM in particular aggravate the course of the disease, change the usual life rhythm, and are a considerable part of high disability and lethality rates. Up-to-date tendencies in the therapy of DM patients enabled to reduce the signs of vascular complications which are the cause of diabetic encephalopathy and morphofunctional disorders of nerve cells state. Meanwhile great success in considerable improvement of DM patient's life still should be achieved.

Free radicals are known to play a leading role in pathogenesis of many diseases including DM [2,16]. Thus, glucose oxidation results in the formation of oxygen active forms which

initiate the processes of oxidation and destruction of biomolecules, nerve cells of the cerebral cortex and hippocampus in particular [12]. Free radicals cause the destruction of proteins, which can directly lead to their fragmentation or denaturation, which are the substrate for intracellular proteases [14]. The increase in the number and activity of proteolysis enzymes leads to the activation of fibrinolytic, kallikrein-kinin, renin-angiotensin-aldosterone systems and complement, which causes the development of inflammatory and destructive processes in the brain [18].

It is a recognized fact today that excessive amount of angiotensin II (AII), renin-angiotensin system (RAS) effector, in the brain induces NADPH-oxidase complex – an important intracellular source of oxygen active forms [6]. Local (tissue) RAS including brain RAS are known to be activated in case of DM and become involved into pathogenic mechanisms of complications from the side of the central nervous system (CNS) [9,10]. Respectively, a pharmacological effect on RAS activity is a target therapy of cerebral lesions in case of DM. In addition, AII is a regulator of insulin secretion by pancreatic β-cell and sensitivity of the peripheral tissues to insulin – critical factors of type 2 DM development. The cerebral microcirculatory perfusion and vascular morphology in rats with type 2 DM is found to improve in case of RAS blockade with olmesartan and enalapril [4]. Inhibition of AII formation by means of enalapril under conditions of diabetic central neurodegeneration is not excluded to be manifested by biochemical changes and at the same time by structural rebuilding of the cerebral cortex and hippocampus, responsible for cognitive functions.

The aim of the study was to examine enalapril effect on protein peroxide oxidation, activity of proteolysis and fibrinolysis enzymes and morphological state of the cerebral cortex and hippocampus under conditions of neurodegeneration in case of experimental type 2 DM.

MATERIALS AND METHODS

The experiments were conducted on male rats with the body weight of 0.18-0.20 kg kept under conditions of natural day and night change. All the experimental procedures were performed according to the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes dated 18.03.1986; Directive 86/609/EEC on the protection of animals used for scientific purposes, and the Order of the Ministry of Health of Ukraine No 690 dated 23.09.2009.

Type 2 DM was simulated experimentally by means of intraperitoneal (i/p) introduction of Streptozotocin (Stz) in the dose of 30 mg/kg for rats kept on high-fat diet for 30 days with free access to fructose solution (200 g/L) [8,11,20]. The control group of animals with a standard diet and free access to water was injected i/p with citrate buffer only (pH=4.5). On the 7th day after Stz introduction type 2 DM was confirmed by detection of glucose concentration in the blood plasma on an empty stomach. Rats with hyperglycemia lower than 10 mmole/L were excluded from the experiment.

On the 11th week after Stz introduction the rats with type 2 DM were randomized into groups: those with i/p introduction of enalapril in the dose of 1 mg/kg and those injected with physiological solution (saline). Rats from the control group received saline during the whole correction period (14 days).

Euthanasia of rats was performed under mild ether narcosis. The brain was removed cool. It was washed with cooled 0.9% NaCl solution, and the cerebral cortex and hippocampus were removed by the stereotaxic atlas [19]. Cytoplasmic fraction was isolated by means of differentiation centrifugation method of the examined structures homogenate on the refrigerator centrifuge at 1000 g 10 minutes, followed by 1400 g 10 minutes at the temperature of 4°C.

The content of protein oxidation modification (POM) in homogenates was determined by the amount of their oxidation modification products by means of spectrophotometry method with the wave length of 370 and 430 nm. The method is based on the reaction of interaction of oxidized amino acid protein residues with 2.4-dinitrophenylhydrazine with the formation of its derivatives, which optic density was determined by means of spectrophotometry. Aldehyde- or ketone- derivatives of a neutral or the main character possessing different ranges of absorption spectrum are known to be formed resulting from protein oxidation depending on amino acids of a neutral (valine, leucine, isoleucine, etc.) or main (lysine, arginine, etc.) character prevailing in their molecules. With λ=370 nm ketone dinitrophenylhydrazones of a neutral character are determined, with λ=430 nm – aldehyde dinitrophenylhydrazones of the main character [13]. POM content was expressed in the units per gram (u/g) of the tissue.

The state of proteolytic activity was determined on the basis of reaction with albumin (low molecular weight protein), collagen and casein (high molecular weight protein) associated with orange azo dyes, which gives a bright color in the alkaline environment. Studies of fibrinolytic activity were performed to assess the degree of staining of the solution due to the formation of plasmin in the presence of ε-aminocaproic acid (non-enzymatic fibrinolytic activity (NFA)) or without it (total fibrinolytic activity (TFA)). Enzymatic fibrinolytic activity (EFA) was determined by the difference between total and non-enzymatic tissue activity. Evaluated proteolysis and fibrinolysis at E440/(h×mg of tissue), where E440 is the extinction index for activity [7,17].

Brain samples for histological examination were fixed in 10% neutral formalin solution, and after the standard histological chemical treatment the tissue was placed into paraffin. Paraffin histological tissue sections of the cerebral cortex 5 mcm thick were made by means of the sliding microtome MC-2. After deparaffination (dewaxing) certain sections were stained with hematoxylin and eosin, and others – with neutral red according to Nissl method in order to find tigroid substance [22]. Microslides were examined under the light microscope. Digital copies of optic images were obtained with the digital camera Olympus SP550UZ and analyzed by means of a special computer program for histological examinations ImageJ (1.48v, free license, W. Rasband, National Institute of Health, USA, 2015) [5].

The results of the study were statistically processed by means of *Student* criterion. To confirm reliability of the conclusions *Mann-Whitney* nonparametric criterion of comparison was used as well. It demonstrated similar results of calculations made by means of *Student* criterion concerning p value. Differences were considered statistically valuable with $p \leq 0.05$.

RESULTS

The experiments determined (fig. 1) that in comparison with the control group, rats with the model of diabetic neurodegeneration demonstrated 1.5 times increase of proteins of a neutral character in the cerebral cortex and 1.6 times increase – in the hippocampus. The content of proteins of the main character in both examined structures increased as well – 1.5 times and 1.4 times respectively. Thus, proteins of a neutral character experienced greater damage.

After enalapril administration protein peroxide oxidation in the cerebral cortex and hippocampus, registered at λ=370 nm, 1.2 times and 1.3 times decreased in comparison with rats suffering from type 2 DM. At λ=430 nm POM in the cerebral cortex and hippocampus 1.3 times and 1.2 times decreased respectively.

Studies have shown (tab. 1) that under conditions of diabetic neurodegeneration, the proteolytic activity of the cerebral cortex was characterized by an increase in the enzymatic cleavage of azoalbumin by 22.0%. The lysis of macromolecular proteins by lysis of azocasein was also detected: by 6.7% in the cortex and by 26.8% in the hippocampus. At the same time, the rates of azocol degradation in both studied structures increased by an average of 66.5%.

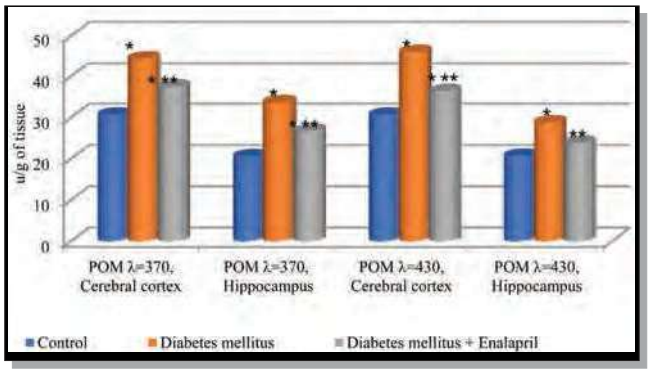

Figure 1. Enalapril effect on protein peroxide oxidation in the cytosolic fraction of the cerebral cortex and hippocampus of rats with type 2 diabetes mellitus, M±m, n=7
Rycina 1. Wpływ enalaprylu na utlenianie nadtlenków białek we frakcji cytozolowej kory mózgowej i hipokampu szczurów z cukrzycą typu 2, M±m, n=7

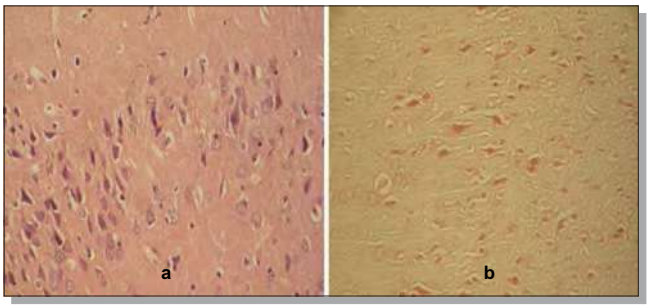

Figure 2. Cerebral cortex of the control group of rats (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 2. Kora mózgowa grupy kontrolnej szczurów (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

In comparison with the control group the percentage of neurons with the signs of karyopyknosis in the cerebral cortex was found to be 4.2±0.15 (Fig. 4a) against the ground of type 2 DM,

Table 1. The effect of enalapril on proteolysis in the cerebral cortex and hippocampus of rats with type 2 diabetes mellitus (M±m, n=7)
Tabela 1. Wpływ enalaprylu na proteolizę w korze mózgowej i hipokampie szczurów z cukrzycą typu 2 (M±m, n=7)

Indices	Brain structures	Control	Diabetes mellitus	Diabetes mellitus + Enalapril
Lysis azoalbumin, E440/(h×mg of tissue)	Cerebral cortex	102.46±1.47	125.03±1.85*	113.00±1.35* **
	Hippocampus	81.49±1.17	84.896±1.61	82.70±1.71
Lysis azocasein, E440/(h×mg of tissue)	Cerebral cortex	104.37±1.19	111.31±2.44*	105.39±0.74**
	Hippocampus	83.91±0.89	106.43±1.81*	94.11±2.03* **
Lysis azocollagen, E440/(h×mg of tissue)	Cerebral cortex	2.49±0.14	4.12±0.10*	3.41±0.21* **
	Hippocampus	2.12±0.07	3.55±0.22*	2.87±0.13* **

* – reliable difference in comparison with the control group of rats, ** – reliable difference in comparison with the group of rats with diabetes mellitus

Compared with diabetic neurodegeneration after enalapril administration, azoalbumin lysis decreased by 9.6% in the cortex. Enzymatic cleavage of azocasein was reduced by 9.6% in the cortex and by 11.6% in the hippocampus. Collagen lysis, according to azoloc degradation, decreased in both the cortex and hippocampus by 5.3% and 19.1%, respectively.

In the cerebral cortex of rats with diabetic neurodegeneration, an increase in total fibrinolysis due to enzymatic fibrinolysis was detected (tab. 2). Thus, the TFA increased by 9.9% and the EFA by 14.2% relative to the data of the control group. In the hippocampus, only EFA increased by 26.7%.

Administration of enalapril to rats with diabetes mellitus contributed to 21.4% reduction in EFA in the hippocampus alone. At the same time, there was a tendency to decrease the TFA and NFA in both the cortex and hippocampus.

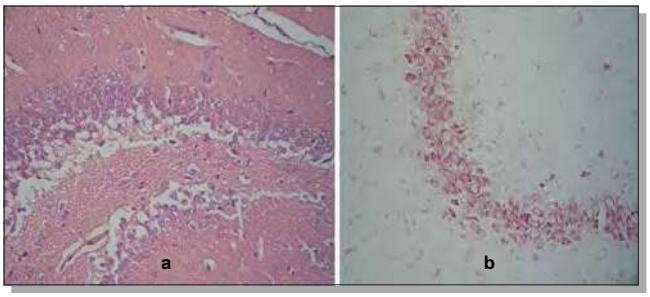

Figure 3. Hippocampus of rats from the control group (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 3. Hipokamp szczurów z grupy kontrolnej (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

Table 2. The effect of enalapril on fibrinolysis in the cerebral cortex and hippocampus of rats with type 2 diabetes mellitus (M±m, n=7)
Tabela 2. Wpływ enalaprylu na fibrynolizę w korze mózgowej i hipokampie szczurów z cukrzycą typu 2 (M±m, n=7)

Indices	Brain structures	Control	Diabetes mellitus	Diabetes mellitus + Enalapril
TFA, E440/(h×mg of tissue)	Cerebral cortex	86.11±1.43	94.67±2.38*	92.26±1.41*
	Hippocampus	52.94±1.59	59.96±3.02	54.66±1.60
NFA, E440/(h×mg of tissue)	Cerebral cortex	57.34±1.04	61.83±1.96	61.80±0.81
	Hippocampus	31.77±1.87	32.73±2.67	32.06±1.01
EFA, E440/(h×mg of tissue)	Cerebral cortex	28.77±0.77	32.84±0.84*	29.46±1.28
	Hippocampus	21.49±0.89	27.23±1.06*	21.41±1.93**

* – reliable difference in comparison with the control group of rats, ** – reliable difference in comparison with the group of rats with diabetes mellitus.

Results of morphological studies showed that in histological specimens of the cerebral cortex and hippocampus nerve cells with the signs of karyopyknosis were not found in the control group of rats. The index of a relative density of the tigroid substance staining of the nerve cells in the cerebral cortex was 0.214±0.0013, and in the hippocampus – 0.219±0.0014 (fig. 2a, 2b, 3a, 3b).

relative density of the tigroid substance staining of neurons was 0.115±0.0010 (fig. 4b). Morphological picture of the hippocampus of rats with DM was similar to those structural changes in the cerebral cortex.

Thus, the percentage of neurons with the signs of karyopyknosis was (fig. 5a), and relative density of the tigroid substance staining – 0.119±0.0011 (Fig. 5b). The obtained results are

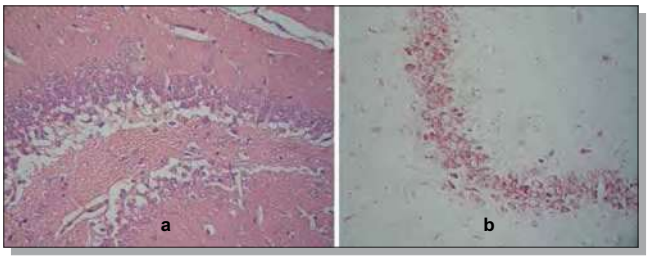

Figure 4. Cerebral cortex of the rats with type 2 DM (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 4. Kora mózgowa szczurów z typem 2 DM (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

indicative of the development of neurodegenerative changes in the examined structures. Moreover, the signs of partial denudation of vessels (stripping of the vascular endothelium) are found in histological specimens of the cerebral cortex and hippocampus of rats with type 2 DM.

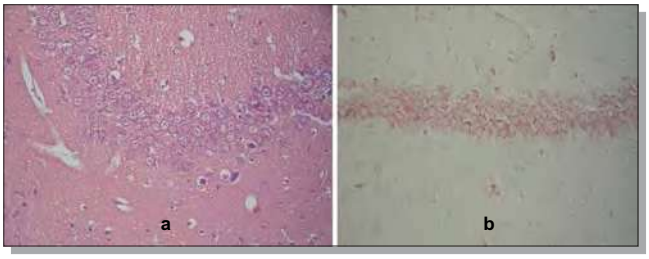

Figure 5. Hippocampus of rats with type 2 DM (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 5. Hipokamp szczurów z typem 2 DM (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

After the course of enalapril administered, the percentage of cells with karyopyknosis in the cerebral cortex and hippocampus decreased to 3.5±0.16 (Fig. 6a) and 3.9±0.16 (fig. 7a), relative density of the tigroid substance staining of the neurons increased to 0.122±0.0012 (Fig. 6b) and 0.121±0.0012 (Fig. 7b) respectively.

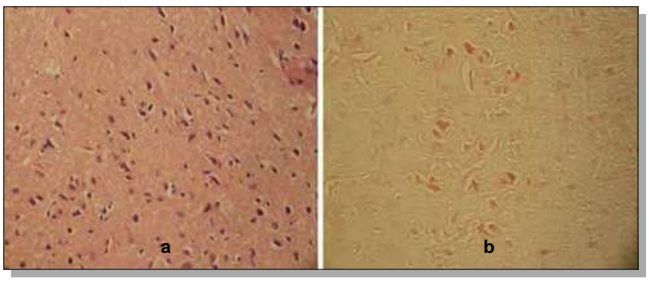

Figure 6. Cerebral cortex of the rats with type 2 DM after enalapril administration (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 6. Kora mózgowa szczurów z cukrzycą typu 2 po podaniu enalaprylu (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

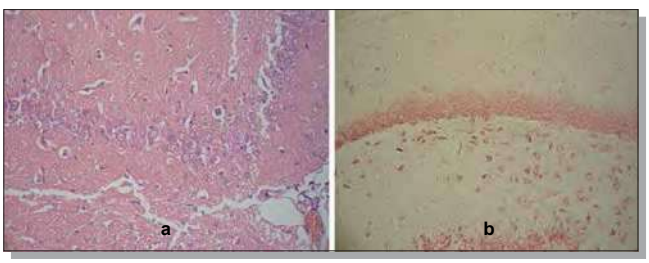

Figure 7. Hippocampus of rats with type 2 DM after enalapril administration (x200): a – hematoxylin-eosin, b – neutral red according to Nissl method
Rycina 7. Hipokamp szczurów z typem 2 DM po podaniu enalaprylu (x200): a – hematoksylina-eozyna, b – obojętna czerwień według metody Nissla

DISCUSSION

Thus, the results of our experimental studies confirm literary data concerning the fact that under conditions of type 2 DM the processes of POM in the cerebral cortex and hippocampus are intensified, and they are pathogenic mechanisms promoting disorders of neuron cytoarchitectonics. The increase in the activity of proteolytic and fibrinolytic enzymes indicates the fact of neurodegenerative processes of diabetic origin, which occur due to excessive formation of free radicals [2]. Administration of enalapril as RAS blocker during 14 days (the course of treatment) decreases POM degree, which is one of the mechanisms to improve morphological condition of the cerebral cortex and hippocampus.

Considering the fact of vasoconstrictor effect domination in patients with type 2 DM, intensification of blood circulation in the brain is primary in enalapril mechanisms due to dilation of the cerebral vessels, increase of the blood circulation volume and neuron oxygenation [1,21]. Moreover, blockade of cerebral AII promotes decrease of oxygen active forms, and thus inhibits POM, fibrinolysis and proteolysis processes in the examined structures of the brain [2]. Undoubtedly, local RAS plays an important role in improvement of microcirculation of the brain. Though, its systemic action is not excluded for obtaining changes by means of improvement of general blood supply. These processes promote reduction of oxygen active forms, inhibit processes of protein peroxidation and promote increased resistance of susceptibility to neurodegenerative changes associated with diabetes. It is worth noting that the effect of RAS blockers is realized through peroxisome proliferator-activated receptor-γ (PPARγ receptors), which are the central regulation of insulin and glucose metabolism, which suppresses the progression of neurodegeneration, mediated by hyperphosphorylation and tau phosphorylation is a mechanism of the neuroprotective action of enalapril [15].

Of course, the role of local RAS plays an important role in improving the microcirculation of brain, but the importance of systemic action for the resulting changes due to improved overall blood supply is not excluded. These processes help reduce the generation of reactive oxygen species, inhibit the processes of peroxidation and proteolysis of proteins, which reduces damage to cell membranes. As a result, the neurodegeneration processes associated with diabetes are slowed down. In fact, the reduction of fibrinolysis processes with the introduction of enalapril in rats with diabetes is another confirmation of this assumption. Because, according to the literature, fibrin is a major factor in neuroinflammation and neurodegeneration [3].

Thus, the obtained results are indicative of the participation of RAS in the formation of structural changes in the CNS in case of type 2 DM and protective properties of enalapril under conditions of diabetic neurodegeneration.

CONCLUSIONS

After 14 days of enalapril in rats with type 2 diabetes mellitus in the cerebral cortex and hippocampus, the processes of fibrinolysis and proteolysis are reduced, as well as the content of neutral and basic proteins, which indicates a decrease in increased in conditions of diabetic neurodegeneration, peroxide oxidation. Under enalapril effect in rats with type 2 diabetes mellitus morphological picture of the cerebral cortex and hippocampus is characterized by a decreased amount of cells with karyopyknosis and increased relative density of the tigroid substance staining of neurons and lack of denudation signs of the vessels, which is indicative of a positive rebuilding of the neuron structure. The conducted study demonstrates a protective effect of enalapril in case of central neurodegeneration caused by type 2 diabetes mellitus, one of the mechanisms of which is decrease of protein peroxide oxidation in the cerebral cortex and hippocampus inhibiting the processes of nerve cells destruction.

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