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ENDOTHELINE-1'S DYNAMICS IN NON-PROLIFERATIVE DIABETIC RETINOPATHY AND ITS CORRECTION WITH L-ARGININE

Introduction. Diabetic retinopathy (DR) is diagnosed in 50% of patients with type 1 diabetes (DM) with a disease duration of 10-15 years and in 75-90% of patients with a diabetes duration of more than 15 years [1]. Vascular pathology correction is one of the important DR treatment directions. The number of patients with DM increases annually and according to the State Register of the Ministry of Healthcare of Ukraine in Ukraine is already more than 1 million people, which is slightly less than 2% of the total population [2-4]. In this case, diabetic retinopathy has a special place among the complications of type 2 diabetes [5, 6]. DR, as one of the most frequent and adverse manifestations of diabetes, remains the leading cause of significant visual impairment [2, 7 - 10]. Retina microstructural changes are detected at the DM onset, they gradually trigger the pathogenetic reactions cascade [11-14], which lead to microcirculation infringement and tissue hypoxia.

Endothelial dysfunction is one of the key links in vascular dysfunction in diabetes [15]. There are a number of clinical and experimental studies in the literature that associated with endothelial disorders and arteries elastic properties to the diabetes vascular complications development [16-19]. Endothelial dysfunction (ED) leads to impaired nitric oxide (NO) synthesis, which plays an important physiological role, having a wide range of bioregulatory effects [20 - 22]. NO is characterized by vascular tone regulation, proliferation and apoptosis, oxidative processes regulation, angioprotective properties. All this is necessary for the vascular homeostasis regulation. It is also a strong peripheral vasodilator [21]. The main substrate for NO synthesis is arginine [22]. It performs number of important functions in the body, but its main role is that it's substrate for the nitric oxide synthesis [23-26]. Therefore, for the correction of ED, in particular for DM, this amino acid from which the endothelium can synthesize the necessary substances [27].

Materials and methods The research design and clinical characteristics of the patients were as follows. The criteria for inclusion in the research were the patient voluntary informed consent to participate in the research, age over 18 years, and for the research group - type 2 diabetes presence, verified DR. Patients examination and treatment with type 2 diabetes and DR were performed according to the WMA Declaration of Helsinki, the Unified clinical protocol of primary and secondary (specialized) medical care. "Diabetes mellitus type 2" and the Order of the Ministry of Health of Ukraine dated 21.12.2012 No.1118 “On Approving and Implementation of Medical–Technological Documents on the Standardization of Medical Aids in Type 2 Diabetes”

According to the classification of the American Diabetes Association, our study involved patients who were diagnosed with a non-proliferative stage of DR [28] in the initial stage with the presence of single microaneurysms, spot hemorrhages or solid exudates. But for a more complete characteristic of step-by-step levels of change, to assess the progress of DR has been chosen criteria developed by a fundamental ETDRS study [29].

The research involved 108 patients (216 eyes) diagnosed with type 2 diabetes between the ages of 45 and 60. Of these, 56 are men and 53 are women. Most patients (95 patients - 88%) experienced emetropic refraction. In 9 patients (8.3%) was determined myopic refraction, of which: 5 had mild myopia, and 4 had moderate myopia. 4 patients (3.7%) had low grade hypermetropic refraction. All patients' visual acuity is corrected.

Research involved patients with a non-proliferative stage of low-grade DR without evidence of clinically significant macular edema. Disease severity was determined by the ETDRS Final Retinopathy Severity Scale (for Individual Eyes) and corresponded to levels 14, 15, 20 and 35 (Fundus Photographic Risk Factors for Progression of Diabetic Retinopathy ETDRS Report Number 12) [29].

Inclusion criteria: presence of single intraretinal microvascular abnormalities (IRMA), microaneurysms and/ or microhemorrhage, hard and soft exudate localized outside the macular area. These DR characteristics were determined in seven fields according to the modified Airlie House classification. According to the scheme of the specified classification, the fields localization is as follows (Fig. 1):

1 - is centered on the optic nerve disk (OND)

2 - is centered on the macula,

3 - is temporal to the macula,

4-7 – are fields tangential to the horizontal line passing between the upper and lower part of the OND and to the vertical line passing through the center of the macula [30].

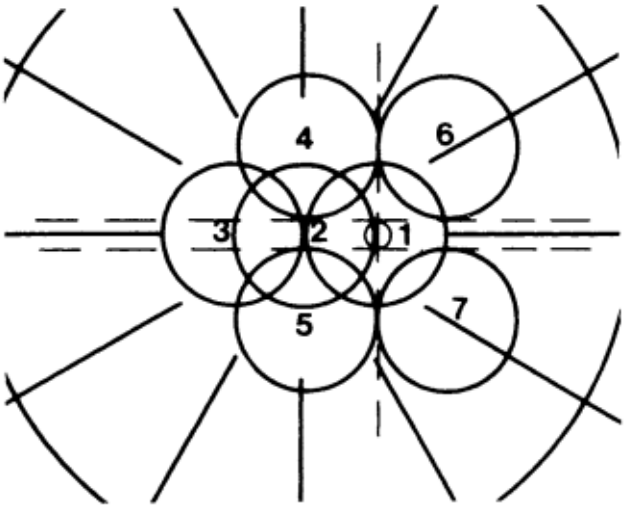

Figure 1. Field localization according to the modified Airlie House classification.

Exclusion criteria were signs of subclinical macular edema or more pronounced stage of the macula [31], more advanced DR (presence of neoplasms, proliferation, etc.), retinal vessels thrombosis or embolism, age-related or other types of macular degeneration, pre-operative eye injury, surgical injury to the eye, including any kind of laser coagulation, vitreoretinal surgery, asteroid hyalosis.

Patients were divided into main and control groups. Main group' patients were prescribed 4.2% solution of L-arginine intravenously 100 ml 1 time a day for 10 days, followed by the transition to a solution for oral administration 5 ml 3 times a day for 4 weeks [32, 33].

Based on the blood pressure and glycosylated hemoglobin studies, groups were formed so that in each of the groups 50% of patients received arginine and 50% did not receive.

At the first stage, at the research beginning, all patients underwent pre-treatment with L-arginine: visometry, tonometry, biomicroscopy, ophthalmoscopy.

Visometry was performed using Golovin-Sivtsev tables, Pole optotypes. Biomicroscopy was performed on a slit lamp PS - 615 by Topcon (Japan). A Goldman lens was used to inspect the peripheral mesh sections. Tonometry was performed with a Maklakov's applanation tonometer. For examination of the fundus was used direct and indirect binocular ophthalmoscope by Keeler (United Kingdom).

Endothelin-1 biochemical study in serum was performed by enzyme-linked immunosorbent assay (ELISA) using a set of Endothelin-1 DRG reagents (USA).

After 6 months from the research start, a was performed complete examination, including re-determination of the severity of DR by ETDRS in patients without L-arginine correction and on the background of its admission. At the same time were determined biochemical parameters of the endothelium.

The following software was used for statistical data processing: IBM SPSS 19.0 (license number O6T4PC5YWM8GFB559ANSECAJEBF66JIHZUJZJ 2CHLXHUAQJD9YYEVITSVPXWIXPJHKTREQT CKF3HWWFPGBDCQZYEE77F4C4VPHM#, Matlab 7 (the serial number 1293-0415-9995-9609-9701).

Pairwise statistical averages comparisons for the series of quantitative data, each of which didn't differ from the normal distribution, were used with the Student's t-test with two-sided critical area for independent samples. Variable value shift analysis in the same patients at different stages was performed using the Student's criterion with two-sided critical area for the dependent samples.

Pearson's chi-squared ($\chi 2$) test was used to compare the two empirical categorical data distributions. To compare qualitative dichotomous data was used the criterion "Fisher angular transformation" with Yates correction, the value of its statistics was denoted as T.

In all cases, statistical significance level was designated as “p”. Significant were differences at $p < 0.05$, high at $p < 0.01$, very high at $p < 0.001$.

Research results

Clinical trials results are presented in tables 1-3. Table 1 presents distribution of patients number who received and did not take L-arginine (2 subgroups of 54 patients) by ETDRS levels.

After 6 months, the patients status undergoing ETDRS remained unchanged. This is also confirmed by the absence of significant differences between distributions and in pairs between the patients number at the same level before and after treatment.

At the same time, the patients group who didn't take L-arginine changed the patients distribution by levels, showing the criterion "chi-square" with a high level of significance ($p = 0.005$).

Table 1

Distribution by DR severity (according to ETDRS) at the research beginning and depending on therapeutic tactics

Patients groups	Research stages	Levels «14-20»	Level «35»	Level «43»	Significant differences in distributions and frequencies (pairwise)
Patients taking L-arginine	First stage (before treatment)	12	42	0	–
	After 6 months.	12	41	1	
Patients who didn't take L-arginine	First stage	12	42	0	χ^2=10,52; p=0,005 $T_{43to-43after}$=3,38, p=0,001
	After 6 months..	7	38	9	

Also in this group noteworthy level "43", in which the patients number increased statistically significant within 6 months from 0 (0.0%) to 9 (16.7%) patients. It is worth noting that the distributions themselves are also significantly different, indicating that patients are regrouped between the ETDRS levels, moreover, towards the worsening condition. This dynamics is illustrated in fig. 1.

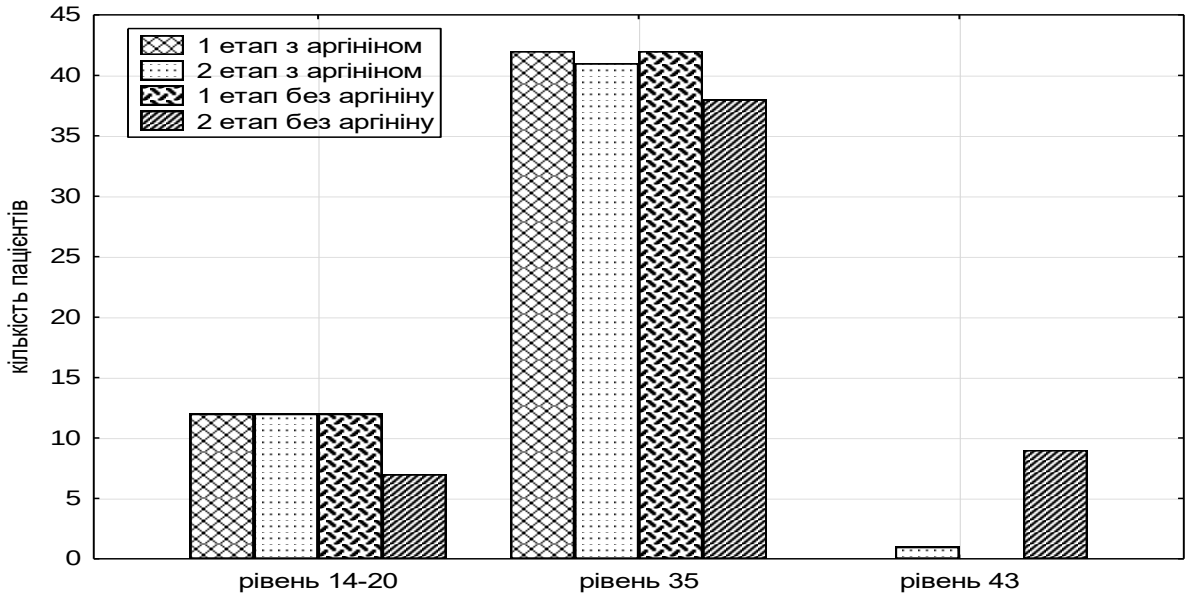

Fig. 1. Distribution by DR severity (according to ETDRS) at the research beginning and depending on therapeutic tactics

Endothelin-1 determination was made at the research beginning (Table 2, Fig. 2) and after 6 months (Table 3, Fig. 3). Comparison of endothelial indicators functional status in patients with different levels of DR severity.

Table 2

Endothelin-1 level research results in the first stage of the study. Comparison between levels of DR severity (according to ETDRS)

	Levels «14-20» 24 patients	Level «35» 84 patients	Statistical significance of differences
endothelin-1	0,268±0,010	0,382±0,007	<0,001
eNO- synthase	0,728±0,013	0,584±0,006	<0,001

It's detected that with the progression of DR is the pathological vasoconstriction development, which can be judged by a significant increase in endothelin-1 in patients with 35 levels of diabetic retinopathy (according to the classification of ETDRS).

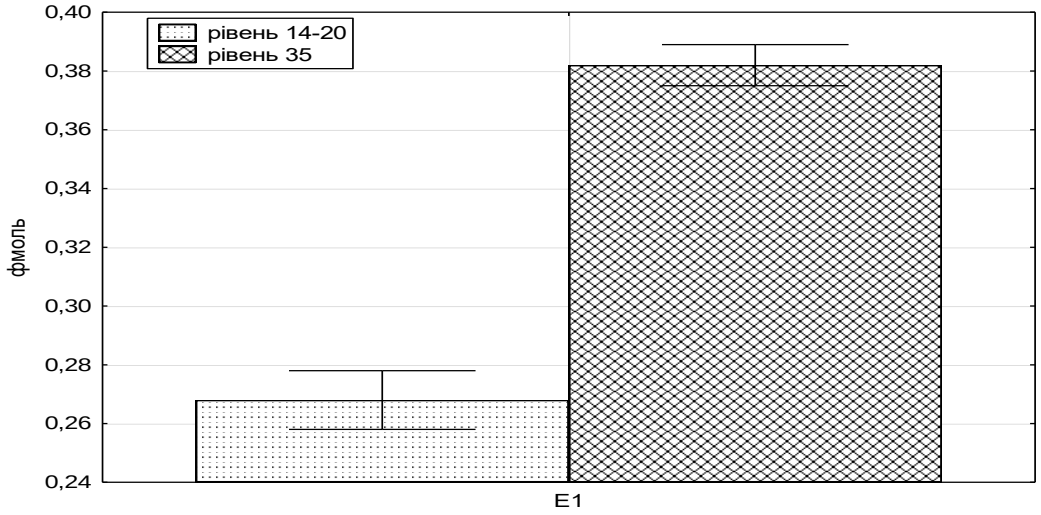

Fig. 2. Analysis results of endothelin-1 content in the blood of patients at the first research stage. Comparison between levels of DR severity (respectively ETDRS).

Table 3

Analysis results of endothelin-1 content in the blood of patients at the first research stage. Comparison between levels of DR severity (respectively ETDRS).. Second stage.

	1	2	3	4	5	6
	Stage 2 without L-arginine - levels 14-20 7 patients	Stage 2 with L-arginine - levels 14-20 12 patients	Stage 2 without L-arginine - level "35" 38 patients	Stage 2 with L-arginine - level "35" 41 patient	Stage 2 without L-arginine – level "43" 9 patients	Stage 2 with L-arginine - Level 43 1 patient
Endothelin-1	0,466±0,020	0,211±0,021 p_{12}***	0,679±0,006	0,314±0,008 p_{12}***	0,762±0,009	0,70

Footnote: *** - p<0,001

In Fig. 3 shows the tendency to normalize vasoconstrictor potential in patients receiving L-arginine, a positive therapeutic effect was found in patients in all study groups.

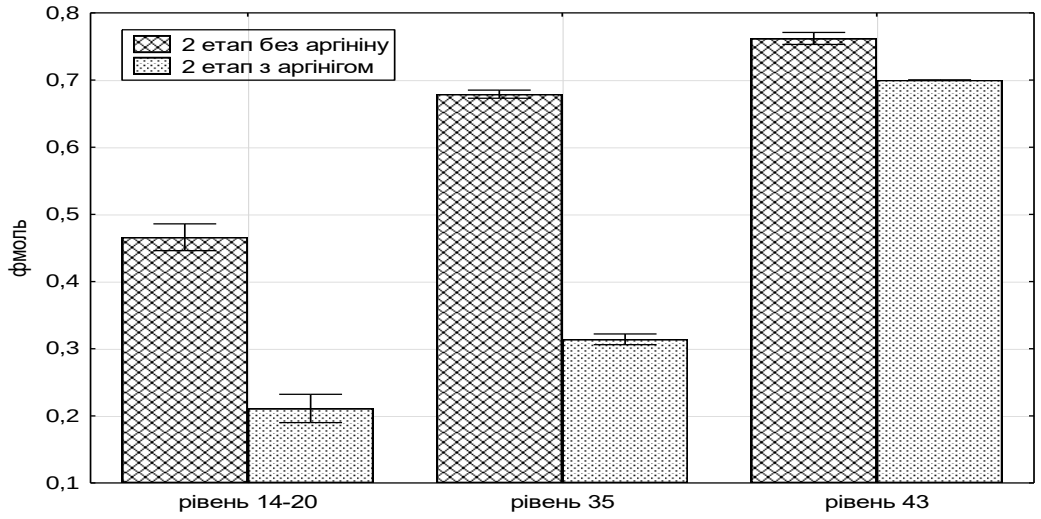

Fig. 3. Analysis results of endothelin-1 content in the blood of patients at the second research stage. Comparison between levels of DR severity (respectively ETDRS).

Research result discussion

As noted above, at the first stage of treatment, the main manifestation of DR in the patients included in the research was the microaneurysms / microhemorrhages presence or hard exudates in no more than 1-3 fields according to the modified Airlie House classification [34]. Patients with levels 14, 15, 20 had single manifestations, with only one of the signs: either microaneurysms / microcurrents, or hard or soft exudates.

In patients with more pronounced fundus changes (level 35) was observed more than one of the listed signs in one or more fields (up to three). However, it is important to emphasize that they were localized outside the macular area and, accordingly, patients didn't note macular edema and impaired visual acuity. Thus, the average visual acuity at the first and final stage of the study remained an average of 1.0 ± 0.1.

At the final research stage (after 6 months), some patients (10 patients) had pathological process dynamics of DR, which manifested itself in an increase in the number of microanurysms / microhemorrhages (the main criteria for deterioration), already diagnosed in 4 or 5 fields of view, which corresponded to level 43, according to the severity score on the ETDRS Final Retinopathy Severity Scale (for Individual Eyes). The clinical changes described are based on a cascade of pathophysiological disorders, part of which is the biochemical parameters that were determined in our study.

As repeatedly noted earlier, one of the key links in vascular system disorders in diabetes is endothelial dysfunction [35-39]. It leads to the disruption of nitric oxide (NO) synthesis, which plays an important physiological role, having a wide range of bioregulatory effects [40, 41]. NO is a strong peripheral vasodilator [41]. Accordingly, with decreasing its production is observed pathological vasodilation, the marker of which is endothelin-1.

Our research found an increase in endothelin-1 (E-1), which is also a marker of ED [42]. These changes indicate a decrease in vasodilation and a vasoconstrictor potentiation of vascular tone [43] in patients with DR.

As noted in the literature, endothelin is a biologically active broad-spectrum bicyclic polypeptide and one of the most significant regulators of vascular endothelium the functional state [44].

Endothelin-1 concentration level in the blood is a determining factor in what effect (vasoconstriction or vasodilation) will be realized [45]. At low concentrations endothelin autocrine-paracrine method acts on endothelial cells, releasing relaxation factors, and increasing the concentration in a paracrine way activates receptors on smooth muscle cells, which leads to vascular spasm [46]. Endothelin-1 is mainly regarded as a marker and predictor of cardiovascular diseases severity such as myocardial infarction and coronary heart disease in general [47]. It is believed that endothelin-1 plays a significant role in the pathogenesis of pulmonary hypertension, atherosclerosis, postpartum vascular lesions, glomerulonephritis, ischemic brain damage and the development of diabetes and its complications [48-50].

The correction choice of these pathological changes was justified by the following. It is known that the main pathway for the nitric oxide (NO) synthesis from L-arginine using the enzyme endothelial NO synthase, which ensures its production in the optimal amount for the normal functioning of blood vessels.

At the same time, it is known from literature that L-arginine can potentiate NO synthesis and reduce the endothelial dysfunction manifestations by restoring eNOS activity [51]. It interferes the main NOS co-factor oxidation [52]. This amino acid also prevent inhibition of endothelial nitric oxide synthase by competing with asymmetric dimethyl-L-arginine [53]. The data obtained by us show that the processes of endothelial dysfunction are progressing in the early stages of DR And an important aspect is the metabolic correction of ED at these stages.

It should be noted that the level of endothelin-1 factor in the group of patients with 35th level of ETDRS is significantly higher than in patients with 14, 15 and 20 levels of ETDRS. This indicates that the above levels haven't only their ophthalmic features, which are basic for ETDRS classification, but also differ in the content and activity of endothelial function markers.

Thus, patients with different levels of ETDRS, depending on the level, also found unequal degree of endothelial dysfunction. The results obtained about the endothelial dysfunction characteristic for each endothelial dysfunction state can be considered as an informative characteristic of different levels of DR severity.

Performed metabolic correction with the use of L-arginine gave positive results. In patients after treatment were objectively determined stabilized DR state.

In patients who were prescribed metabolic correction with L-arginine was observed normalization of the vasoconstriction marker, there was a decrease in endothelin-1 level. The differences found are statistically significant at the significance level $p < 0.001$.

Prevention of vascular disorders further development, protection and maintenance of endothelial functions is one of the most important tasks of the DR treatment, in the early stages, during which the use of L-arginine is an effective remedy.

Conclusion:

1. The use of L-arginine allowed to stabilize the clinical course of diabetic retinopathy. After 6 months, the patients status treated with arginine as a part of complex therapy remained unchanged.
2. In the patients group who didn't use L-arginine draws attention to the level of "43", in which the number of patients increased significantly within 6 months. Patients were regrouped between the ETDRS levels in the direction of worsening.
3. It were determined differences between endothelin-1 indices depending on the severity of ETDRS severity (14, 15, 20 and 35 levels).
4. As a result of L-arginine therapy was observed normalization of the vasoconstriction marker in the patients who participated in the research.
5. It is proved that arginine is the main substrate for NO synthesis. Consequently, for the endothelial dysfunction correction, in particular in diabetic retinopathy, this amino acid is needed. This is acid from which the endothelium will be able to synthesize the necessary substances.

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