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Association of glycation markers with the progression of the initial stages of diabetic non-proliferative retinopathy in type 2 diabetes mellitus

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Abstract

Relevance. In the development and progression of non-proliferative diabetic retinopathy (NPDR) in type 2 diabetes mellitus (DM2), an important role belongs to the activity of the protein glycation process and the formation of advanced glycation end products (AGE).

Objective: To link glycation markers carboxymethyl lysine (AGE-CML) and circulating AGE receptor (sRAGE) to the progression of early-stage of nonproliferative diabetic retinopathy in patients with type 2 diabetes.

Material and methods. 91 patients (182 eyes) with diabetes mellitus aged 42 to 80 years were examined. The control group included 25 people of the appropriate age. At the time of the initial examination and 1 year later, the NPDR stage was established according to the modified ETDRS system of clinical signs Airlie House. The content of glycation markers was determined by enzyme-linked immunosorbent assay in blood plasma. MedStat and

MedCalc v.15.1 (MedCalc Software bvba) software packages were used for statistical research.

Results. The initial manifestations of diabetic retinal lesions occurred in 27.5% of patients, began after 7.16 ± 1.11 years and were accompanied by higher glycemia. The content of AGE-CML in diabetes mellitus was significantly increased compared to the control, which was more pronounced in the presence of initial retinal changes - was 1.3 times higher than in patients without such changes ($p = 0.015$). The content of sRAGE decreased several times, which was also associated with the presence of diabetic retinal changes - in their presence it was 2.2 times lower ($p < 0.001$). The content of AGE-CML was significantly higher (1.5 times; $p < 0.001$) in the presence of NPDR progression during 1 year of observation than without it. The content of sRAGE in the presence of progression was 1.6 times ($p < 0.001$) lower.

Conclusion. Thus, accumulation of blood AGE-CML and sRAGE reduction is connected with the occurrence and progression of NPDR.

Key words: nonproliferative diabetic retinopathy; type 2 diabetes mellitus; Advanced Glicated End Products; AGE-CML; sRAGE

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia [1, 2]. Today, diabetes is a non-infectious epidemic of the XX-XXI centuries [3]. The total number of patients with diabetes in the world has quadrupled over the past 40 years [4, 5]. According to the global report of WHO experts on diabetes, in 2018 422 million people had diabetes, and according to the International Diabetes Federation, the projected incidence of diabetes in 2045 will be 629 million people [6]. According to the available incidence statistics in Ukraine, in 2017, 1.27 million patients with diabetes were registered, and in 2019 - already 1.5 million [7]. The risk of blindness in patients with diabetes is 2.4 times higher than in people without diabetes [8]. Almost 94 million people have eye damage caused by diabetes [9].

It is known that the percentage of type 2 diabetes (CD2) has become and reaches 90% [6, 7, 9]. Diabetes mellitus is considered as a disorder of carbohydrate metabolism, the basis of which is insulin resistance and relative insufficiency of insulin or a violation of its secretion on the background of chronic hyperglycemia [5, 6]. Chronic hyperglycemia acts as a promoter of the development of micro- and macrovascular complications [8, 10].

One of the earliest and most common microvascular complications is retinal microangiopathy, which is a key factor in the development of diabetic retinopathy (DR) against the background of progressive damage to the nervous and vascular system of the eye [11, 12].

Color photographs of the fundus determine the level of DR, according to the modified ETDRS scale, during its study on the fundus camera in 7 standard fields in the system of clinical signs Airlie House [13]. Assessment of clinical signs in diabetic nonproliferative retinopathy (NPDR) includes the following unified clinical manifestations: microaneurysms (MA) and microhemorrhages (MH), intraretinal microvascular abnormalities (IRMA), retinal venous abnormalities and nonperfusion of retina. The earliest manifestations of NPDR are MA - pathological local dilation of the lumen of blood vessels, which in fluorescent angiography are registered in the form of bright areas. There are two ways of their further development: either regression of small formations, or fibrosis of more stable, with accumulation of lipids in a basal membrane. The nature and speed of this process depends on the number of advanced glycated end products (AGE) in the body, the most known of which is glycated hemoglobin [14].

There are more than 20 forms of AGE, which are divided into two groups: fluorescent and non-fluorescent. The most important representatives of the first group include carboxymethyl-lysine, carboxyethyl-lysine and pyrraline. The second group is represented by a dimer of methylglyoxal-lysine and pentosidine [14]. The presence of lysine in the molecular structure is a common feature for all AGEs. Condensation of the amino group of lysine with glucose leads to rearrangement of Amadori products with their oxidation. The reaction product is carboxymethyl-lysine (AGE-CML) - the most common AGE in vivo, its level in the blood serves as a specific marker of AGE accumulation [15].

The effect of AGE on cell structures initiates signaling cascades that increase the expression of NF-kB, VEGF with the activation of intercellular adhesion molecules, cytokines, MARK, increasing the activity of NADP oxidase. Against the background of reduced bioavailability of nitric oxide, modified proteins damage the vascular endothelium; contribute to apoptosis of retinal pericytes, which are the primary risk factors for the development of NPDR [16].

The AGE receptor (RAGE) is a polyligand transmembrane protein that belongs to the superfamily of immunoglobulin receptors. In the structure of the eye, RAGE is expressed on microglia, pericytes, Mueller cells, pigment epithelium, which becomes the basis for the development of chronic inflammation [17]. Its action can be described as follows: the

transmembrane domain adheres directly to the cell membrane, the intracellular domain binds to RAGE ligands, and the cytosolic tail provides signal transduction [18, 19]. There are also circulating soluble RAGE isoforms: esRAGE - products of alternative splicing, and cRAGE, generated by proteolysis associated with the RAGE membrane (FL-RAGE). Together, esRAGE and cRAGE form a soluble receptor (sRAGE), which competes with RAGE and prevents the binding of FL-RAGE / ligands [20].

Objective: To establish the link of glycation markers - carboxymethyl lysine (AGE-CML) and circulating AGE receptor (sRAGE) with the progression of early-stage nonproliferative diabetic retinopathy in patients with type 2 diabetes.

RESEARCH DESIGN. MATERIAL AND METHODS

The study was prospective, cohort, case-control.

91 patients (182 eyes) with diabetes mellitus aged 42 to 80 years were examined. The control group included 25 people aged 45 to 79 years. Patients in the control group did not have diabetes and underwent routine ophthalmological examination. All subjects received informed consent to participate in the study.

At the time of the initial examination and after 1 year, all patients underwent conventional ophthalmological examinations, which included visometry, refractometry, tonometry, static perimetry, gonioscopy, biomicroscopy, and ophthalmoscopy. Ophthalmoscopy was performed using an aspherical Volk Super / Field lens (NC USA) and a Goldman three-mirror contact lens. In addition, all patients underwent spectral domain optical coherence tomography (OCT) on the device Optical Coherence Tomography 3D OCT-1000 (Retina3D protocol, RetinaRaster); also used OCT in the "Angio" mode (RetinaAngio protocol, wide 6x6 mm). Examination of the fundus was performed on a fundus camera, if necessary - with photography in 7 standard fields in accordance with the modified ETDRS system of clinical signs Airlie House [13]. The photographs examined the same unified clinical signs of DR: MA and MG, IRMA, retinal venous abnormalities and retinal non-perfusion.

The content of glycation markers was determined by enzyme-linked immunosorbent assay using R&D System (US) reagent kits in blood plasma. Blood sampling was performed from the ulnar vein on an empty stomach in an amount of 3 ml once at the time of the initial examination. The content of glycation markers (AGE-CML and sRAGE) in blood plasma was expressed in ng / ml.

MedStat and MedCalc v.15.1 (MedCalc Software bvba) software packages were used for statistical research. The mean (M) and its standard deviation (SD) were calculated.

Frequency (%) and its standard error (SE,%) were used for qualitative characteristics. In all cases, the differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

According to the classification of the American Academy of Ophthalmology (2002) in all patients at the time of the first examination, DR was not detected (stage I - no retinopathy). In the majority of patients (72.5%), the level of DR on the final ETDRS scale was 10 in both eyes. In the remaining patients (27.5%), the level of ETDRS was 10 in one eye, and in the other eye, there were isolated changes in vascular caliber, varicose veins and tortuosity, IRMA or MH, which corresponded to the level of ETDRS 14, 15.

According to this observation, patients were divided into two groups (Table 1): 1st - in which at the beginning of the study DR was not in both eyes and 2nd - in which one eye had no DR, and the other were noted single vascular changes (ETDRS level was14 or 15).

Table 1 - Distribution of patients by groups

A group of patients	Age, years	Sex		Duration of the disease, years
		Men	Women	
Control	65,12±9,02	40,0±9,8%	60,0±9,8%	-
1st	68,41±7,78	30,3±5,7%	69,7±5,7%	4,20±2,23
2nd	66,76±7,39	32,0±9,3%	68,0±9,3%	7,16±1,11
Intergroup comparisons	F=1,62; p=0,201	$\chi2$=0,78; p=0,676		t=6,35; p<0,001

Notes: data display format: quantitative - M ± SD; nominal -% ± SE; F - Fisher's criterion for analysis of variance (ANOVA); $\chi2$ - Pearson's criterion for comparing data distribution frequencies; t - Student's criterion for independent samples; p - the probability of differences (taken at p <0,05).

There was no difference in age and sex between control group and other groups of patients. The duration of diabetes mellitus was longer in patients of group 2: these patients, on average, were ill for three years longer than those who did not have diabetic changes in the fundus ($p < 0.001$). The analysis of the state of carbohydrate metabolism showed worse indicators in patients of the 2nd group who had a higher level of glycaemia (9.34 ± 1.86 mmol / l against 7.87 ± 2.15 mmol / l in the 1st group; $p = 0.016$). Thus, the initial manifestations of diabetic retinal lesions occurred in 27.5% of patients, began after 7.16 ± 1.11 years and were accompanied by higher glycaemia.

Based on these data, it seemed reasonable to determine the state of pathological glycation and to establish its connection with the progression of DR.

The content of AGE-CML in DM2 was significantly increased compared to control (Table 2): 1.9 times in patients without retinal changes and 2.4 times in patients with initial manifestations of diabetic retinal lesions ($p < 0.001$). The difference between the last was also significant - in the 2nd group the content of AGE-CML exceeded that in the 1st in 1.3 times ($p = 0.015$).

Table 2 - AGE-CML and sRAGE content by patient groups (M ± SD)

Indicators	Control	Groups of patients		Comparison between groups
		1st group	2nd group	
AGE-CML, ng/ml	363,9±74,00	685,0±267,4	875,7±263,2	F=29,78; p<0,001
Post-hoc	Control	p<0,001	p<0,001	
	1st group		p=0,015	
sRAGE, ng/ml	1,322±0,298	0,513±0,188	0,233±0,128	F=196,72; p<0,001
Post-hoc	Control	p<0,001	p<0,001	
	1st group		p<0,001	

Notes: data display format - M ± SD; F - Fisher's criterion for analysis of variance (ANOVA); the Post-hoc lines shows the probability of differences for paired (a posteriori) comparisons between these groups according to the Tukey test (Tukey HSD) for unequal in size samples; p - the probability of difference (assumed at $p < 0.05$).

In contrast, the content of sRAGE in diabetes mellitus decreased several times, which was also associated with the presence of diabetic retinal changes. Thus, in patients of the 1st group it was 2.6 times less than the control, and in the 2nd group - 5.7 times ($p < 0.001$). The difference between the groups was significant: in the 2nd group the sRAGE content was 2.2 times lower than in the 1st ($p < 0.001$).

The results that have been established are clearly demonstrated in Figs. 1, which clearly shows the dependence of the presence of diabetic retinal damage on the content of glycation markers in the blood.

In the second phase of the study, the relationship between the progression of diabetic retinal changes during 1 year of follow-up and the content of glycation markers was analyzed. Progression was defined as a change in the fundus pattern toward deterioration with the development of diabetic vascular changes where they did not occur, or initial (ETDRS 20) or moderate (ETDRS 35, 43, 47) NPDR. The total number of patients with progression of retinal changes was 56 (61.5%). In the 1st group of patients progression was observed in 50.0% of patients, while in the 2nd group - in 92.0% ($p < 0.001$), which indicated a significant tendency to progression in the presence of previous retinal damage (Table. 3).

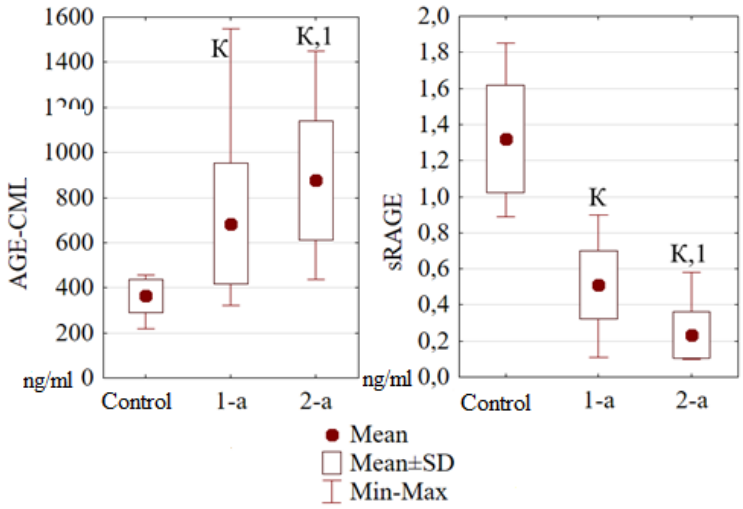

Fig. 1. The content of glycation markers in the blood of patients by groups. Probable differences are marked above the columns of the diagrams: K - with the control; 1 - with the 1st group (in all cases p <0,02)

Table 3 - DR progression in groups of patients after 1 year

DR progression	Group of patients		Comparison between groups
	1st	2nd	
positive (n=56)	50,00±6,15 %	92,00±5,43 %	$\chi 2$=13,52; p<0,001
absent (n=35)	50,00±6,15 %	8,00±5,43 %	

Notes: data display format:% ± SE; $\chi 2$ - Pearson's criterion for comparing data distribution frequencies; p - the probability of differences (taken at p <0,05)

According to this distribution, it was found the connection of the initial content of glycation products in the blood with the progression of DR (Table 4).

Table 4 - Content of glycation markers in the presence of progression of diabetic retinal changes within 1 year (M ± SD)

Indicators	Control	Progression		Comparison between groups
		positive	absent	
AGE-CML, ng/ml	363,9±74,00	863,7±300,2	608,2±176,4	F=86,00; p<0,001
Post-hoc	control	p<0,001	p<0,001	
	positive		p<0,001	
sRAGE, ng/ml	1,322±0,298	0,336±0,208	0,538±0,166	F=351,4; p<0,001
Post-hoc	control	p<0,001	p<0,001	
	positive		p<0,001	

Notes: data display format - M ± SD; F - Fisher's criterion for analysis of variance (ANOVA); the Post-hoc lines shows the probability of differences for paired (a posteriori) comparisons between these groups according to the Tukey test (Tukey HSD) for unequal in size samples; p - the probability of difference (assumed at p <0.05).

The content of AGE-CML was significantly higher (1.5 times; p <0.001) in the presence of progression of diabetic retinal changes than without them. The content of sRAGE in the presence of progression was 1.6 times (p <0.001) lower than without it. Figure 2 shows a significant difference between the groups.

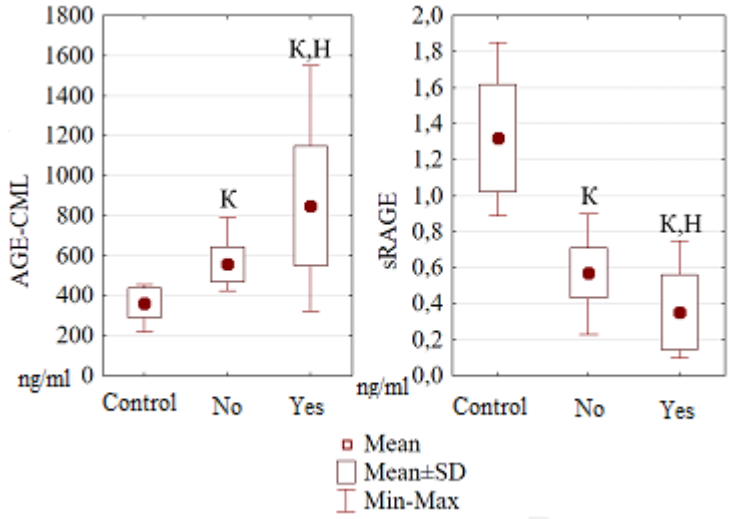

Fig. 2. The content of glycation markers in the blood of patients in the control group (control) and in patients with absence ("No") and the presence ("Yes") progression. Probable differences are marked above the columns of the diagrams: K - with the control group; H - with patients without progression (in all cases p <0,001)

Based on the data obtained from the distribution of patients who had or did not have progression, a natural question arose: is the content of glycation markers associated with the progression of diabetic retinal changes in individual eyes? To do this, the cases were grouped according to ocular changes. Our study involved 91 patients - 182 eyes, which were divided into three subgroups. The 1st included paired eyes without any signs of DR (n = 132), the 2nd - eyes without DR (n = 25), paired eyes with the presence of vascular abnormalities. The latter were included in the 3rd subgroup (n = 25). Accordingly, the 1st subgroup of observation included 132 paired eyes with ETDRS level 10; the 2nd subgroup included 25 eyes with ETDRS level 10 and the 3rd - 25 paired eyes with ETDRS levels 14, 15.

After 1 year of follow-up, the number of eyes with the progression of DR was 92 (50.5%). By subgroups, they were distributed as follows: in the 1st subgroup of eyes with progression was 55 (59.8%), in the 2nd - 22 (23.9%) and in the 3rd - 15 (16.3%). The ratio of the number of eyes with progression to the eyes without such is shown in table 5.

Table 5 - Progression of diabetic retinal changes in the eyes after 1 year

Progression:	Subgroup of the eyes			P(FEM)
	1-a (n=132)	2-a (n=25)	3-я (n=25)	
positive (n=92)	41,7±4,3% (n=55)	88,0±6,5% (n=22)	60,0±9,8% (n=15)	P1-2<0,001 P1-3=0,124 P2-3=0,051
absent (n=90)	58,3±4,3% (n=77)	12,0±6,5% (n=3)	40,0±9,8% (n=10)	
$\chi 2$=19,08; p<0,001				

Notes: data display format -% ± SE; $\chi 2$ - Pearson's criterion for comparing data distribution frequencies; P (TMF) - the probability of differences by two-way Fisher's exact method: P1-2, P1-3, P2-3 - the probability of differences in pairwise comparison of samples in the respective groups; p - the probability of difference (assumed at p <0.05).

A significant difference in the distribution of eyes in the presence of progression in the subgroups that was identified was significant (p <0.001) only when comparing the 1st subgroup with the 2nd. In the 2nd subgroup, progression was determined on the vast majority of eyes - 88.0%. Their number was in 2.1 times higher than that in the 1st subgroup (88.0% vs. 41.7%, respectively; p <0.001).

Thus, eyes that were paired with eyes with initial vascular abnormalities had the highest level of progression during the 1 year of follow-up, allegedly catching up with an eye that had already undergone changes. It should be noted that the content of glycation markers in subgroups 2 and 3 was in fact the same, because they were the same patients (corresponds to group 2 in table 3). This reasoning may justify the importance of the glycation process for the progression of DR: increasing its activity leads to the development of diabetic retinal damage first in one eye, and during the year - in most paired eyes.

At the last stage of the study, the dependence of the degree of progression of diabetic retinal changes on the content of glycation markers at the beginning of the examination was analyzed. To do this, the data were grouped as follows (Table 6). After 1 year of follow-up, among all cases with progression of retinal vascular changes (n = 92), eyes with single vascular abnormalities (EDTRS 14/15) were 22 (23.9% of the number of eyes with progression), eyes with initial NPDR (EDTRS 20) - 23 (25.0%) and eyes with moderate NPDR (ETDRS 35, 43, 47) - 47 (51.1%).

The content of AGE-CML and sRAGE in the first three groups of patients, in the presence of already established tendencies of changes, did not differ statistically significantly (p> 0.33). In patients with moderate NPDR, the content of AGE-CML was significantly

higher (1.4-1.7 times; p <0.001), and the content of sRAGE was significantly lower (1.8-2.3 times; p <0.005), than in other groups.

Table 6 - The content of glycation markers in the presence of progression of diabetic retinal changes in the eyes after 1 year of observation (M ± SD)

Indicators		No diabetic changes (n=90)	Diabetic changes present (n=92): Single vascular anomalies (n=22)	Initial NPDR (n=23)	Moderate NPDR (n=47)	Comparison between groups
		1	2	3	4	
AGE-CML, ng/ml		608,2±176,4	677,4±221,1	716,6±271,5	1022,9±259,7	F=37,75; p<0,001
Post-hoc	1		p=0,721	p=0,335	p<0,001	
	2			p=0,866	p<0,001	
	3				p<0,001	
sRAGE, ng/ml		0,538±0,166	0,417±0,214	0,463±0,210	0,236±0,147	F=31,11; p<0,001
Post-hoc	1		p=0,099	p=0,460	p<0,001	
	2			p=0,687	p=0,004	
	3				p<0,001	

Notes: data display format - M ± SD; F - Fisher's criterion for analysis of variance (ANOVA); the Post-hoc lines show the probability of differences for paired (a posteriori) comparisons between these groups (1, 2, 3, 4) according to the Tukey test (Turkey HSD) for unequal in size samples; p - the probability of differences (taken at p <0,05).

Thus, stratification by the degree of NPDR after 1 year of follow-up showed a certain dependence of the severity of diabetic changes on the initial content of glycation products - the largest changes were observed in moderate NPDR and in these cases the increase in AGE-CML was maximum and sRAGE reached minimum values.

The effect of AGE on proteins is manifested in their covalent crosslinking with biochemical modification, which leads to disruption of cell structure and function. This process also affects neighboring cells by altering the structure of protein function and indirectly activating the RAGE signaling pathway [21]. Specific AGE-binding proteins in the intercellular matrix are able to recognize and capture the modified proteins. Its level also determines the degree of endothelial dysfunction [22].

Therefore, the process of pathological glycation of proteins and activation of the AGE-RAGE pathway is one of the first pathological factors of cell damage in chronic hyperglycemia. According to our study, the content of AGE in diabetes mellitus was significantly increased both in patients without diabetic retinal changes and in their presence.

In this case, the content of AGE in patients of the 2nd group (in the presence of primary diabetic retinal changes in one eye), who had a disease experience of 7.16 ± 1.11 years was 1.3 times higher than in the 1st group (absence changes in both eyes) with experience of 4.2 ± 2.23 years. In both groups, the content of the marker significantly exceeded the control (1.9 and 2.4 times, respectively).

From this comparison it follows that glycation activity increases before diabetic retinal damage begins, possibly immediately after the onset of the disease, but its effect on the development of DR begins, on average, 4 years after the disease, and after 7 years covers 27, 5% of patients. After 1 year of follow-up, retinal changes were observed in 50.5% of patients, with the maximum rate of progression in the eyes, even to those who had already undergone diabetic changes.

Another reason for the activation of the AGE-RAGE pathway is a decrease in the pool of circulating receptors - sRAGE. Most of them are generated by proteolysis associated with the RAGE membrane [20]. sRAGE functions as bait receptors and prevents AGE membrane binding. Our studies found a decrease in the content of the circulating receptor pool, which was also associated with the degree of progression of NPDR.

Regarding the established faster progression of DR after 1 year in paired to damaged eyes, it can be noted that the activation of the AGE-RAGE pathway is closely related to other factors in the pathogenesis of DR. An important place in the maintenance of chronic inflammation is the nuclear factor NF-κB, the activator of which is RAGE. Under conditions of increased number of the last, the effect of NF-κB has a positive feedback, which causes the chronic nature of inflammation [23]. Thus, having already begun, the pathological process acquires the character of self-support and progression by the type of "wrong" circle.

Based on these data, you can also explain the result with stratification at the stage of DR after 1 year. The biggest diabetic manifestations with ETDRS 35, 43, 47, which corresponded to moderate NPDR, were determined in patients with the maximum initial content of AGE-CML and the minimum - sRAGE. Enhancement of the AGE-RAGE pathway removes the inhibitory signal for endothelial cell proliferation, activates VEGF formation and facilitates angiogenesis, leading to pericyte death, which are crucial factors in the development of vascular abnormalities in NPDR [24].

The pathological effect of AGE in the structures of the eye is realized indirectly through sorbitol [25]. The last is a 6-atom hydrophilic alcohol that accumulates in the cytoplasm and leads to the development of hyperosmolar state. It damages the endothelium of small vessels with a thickening of the basement membrane; in the retina, the loss of pericytes

and retinal pigment epithelium increases [26, 27]. In our previous studies, a link between sexual shunt activation and DR development was established [28].

The accumulation of AGE-RAGE in pericytes is mediated through the effect on the mechanisms of oxidative stress, which induces the accumulation of reactive oxygen species in the retina [14]. Parallel activation of the phosphotidyl-choline phospholipase C-sphingomyelinase complex - tumor necrosis factor-α (TNF-α) leads to the transfer of NF-κB to the nucleus, which creates conditions for the activation of proapoptotic proteins [29]. Due to nuclear activation of NF-κB, a stable α-subunit modulates gene transcription directly in the cell nucleus, resulting in pericyte apoptosis [30].

Thus, non-enzymatic glycation under the conditions of DM2 leads to the synthesis of AGE, which have a high glycation ability, cumulative effect, deteriorating the microcirculation, the state of the endothelium and pericytes in the structures of the eye. The state of chronic hyperglycemia, oxidative stress, chronic inflammation creates all the conditions for the interaction of AGE with specific receptors - RAGE. Indirectly, the AGE-RAGE complex triggers a pathological mechanism, resulting in the accumulation of TNF-α, NF-κβ with activation of proapoptotic proteins. These processes determine the established dynamics of growth of diabetic vessels changes and the development of the initial stages of NPDR.

CONCLUSIONS

1. The initial manifestations of diabetic retinal lesions appeared in 27.5% of patients began after 7.16 ± 1.11 years and were accompanied by higher glycaemia.

2. The content of AGE-CML in diabetes mellitus was significantly increased compared to the control, which was more pronounced in the presence of initial changes in the retina (was 1.3 times greater than in patients without such changes; $p = 0.015$). The content of sRAGE decreased several times, which was also associated with the presence of diabetic retinal changes (in their presence it was 2.2 times lower; $p < 0.001$).

3. The progression of diabetic retinal changes was associated with the initial content of glycation markers during 1 year of follow-up. The content of AGE-CML was significantly higher (1.5 times; $p < 0.001$) in the presence of DR progression than without it. The content of sRAGE in the presence of progression was 1.6 times ($p < 0.001$) lower than without it.

4. Stratification at the stage of DR after 1 year of observation showed the dependence of the severity of diabetic changes on the initial content of glycation products - the largest changes were observed in moderate NPDR. In these cases, the increase in AGE-CML was maximum, and sRAGE reached minimum values.

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