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VON WILLEBRAND FACTOR LEVEL DYNAMIC DURING THE ENDOTHELIAL DYSFUNCTION DEVELOPMENT IN EXPERIMENTAL DIABETIC RETINOPATHY

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Abstract

Aim: an analysis changes of von Willebrand factor dynamics' level during the endothelial dysfunction development in the pathogenesis of experimental diabetic retinopathy and in different ways of its correction.

Methods: the research was carried out on white Wistar line rats, weight -180-220 g. Type 2 diabetes and DR were simulated by intraperitoneal streptozotocin administration (Sigma, USA) dissolved in 0.1 M citrate buffer with a 4.5 pH. The streptozotocin dose - 55 mg / kg animal body weight - was divided into two injections. The streptozotocin administration was preceded by a high-fat diet for 28 days.

Results: was proved the structural and functional state violation of the endothelium in experimental diabetic retinopathy, as evidenced by an increase in the Von Willebrand factor level in group 2 ($p < 0.001$), most pronounced in stage 3. It was confirmed that the correction of studied diabetes mellitus complication only by a hypoglycemic drug, even with long-term administration, does not correct the endothelial dysfunction development ($p < 0.001$). Also, it was found that the addition of aflibercept and L-arginine solution - in the correction - to hypoglycemic drugs significantly ($p < 0.001$) improves the endothelium condition, but doesn't solve the problem completely. There is evidence that the correction of modeled pathological condition by reducing hyperglycemia, administration of aflibercept and bromfenac (group №5) has a positive effect on von Willebrand factor normalization ($p < 0.001$), but the effect is less pronounced than in group 4 ($p < 0.001$ on 2nd and 3rd stages of the study) and partially loses effectiveness in the 3rd stage. It was found that in rats in which diabetic retinopathy was modeled with subsequent hyperglycemia correction by aflibercept, L-carnitine and bromfenac administration (group №6), the decrease in pathologically elevated von Willebrand factor level is more pronounced compared with groups from 3 to 5, indicating the feasibility of this correction method. Moreover, it was found that the most effective correction method was in the 7th group of the experiment in which was performed hyperglycemia correction, aflibercept, L-arginine solution and citicoline administration: in the 2nd and 3rd stage of the experiment the von Willebrand factor level didn't differ statistically from the intact group.

Conclusion: 1. As a result of our research was proved the structural and functional state violation of the endothelium in experimental diabetic retinopathy, as evidenced by an increase in the Von Willebrand factor level in group 2 ($p < 0.001$), most pronounced in stage 3. 2. It was confirmed that the correction of studied diabetes mellitus complication only by a hypoglycemic drug, even with long-term administration, does not correct the endothelial dysfunction development ($p < 0.001$). 3. Also, it was found that the addition of aflibercept and L-arginine solution - in the correction - to hypoglycemic drugs significantly ($p < 0.001$) improves the endothelium condition, but doesn't solve the problem completely.

4. There is evidence that the correction of modeled pathological condition by reducing hyperglycemia, administration of aflibercept and bromfenac (group №5) has a positive effect on von Willebrand factor normalization ($p < 0.001$), but the effect is less pronounced than in group 4 ($p < 0.001$ on 2nd and 3rd stages of the study) and partially loses effectiveness in the 3rd stage. 5. It was found that in rats in which diabetic retinopathy was modeled with subsequent hyperglycemia correction by aflibercept, L-carnitine and bromfenac administration (group №6), the decrease in pathologically elevated von Willebrand factor level is more pronounced compared with groups from 3 to 5, indicating the feasibility of this correction method. 6. Moreover, it was found that the most effective correction method was in the 7th group of the experiment in which was performed hyperglycemia correction, aflibercept, L-arginine solution and citicoline administration: in the 2nd and 3rd stage of the experiment the von Willebrand factor level didn't differ statistically from the intact group.

Key words: experimental diabetic retinopathy, endothelial dysfunction, von Willebrand factor, correction, metformin, aflibercept, L-arginine, citicoline, L-carnitine, bromfenac.

Introduction

The number of people suffering from diabetes mellitus (DM) increasing every year, both in our country and in the whole world. This pathology is associated with a complex of complications, primarily diabetic retinopathy (DR), which play a key role in visual impairment [1-4], they are diagnosed in 40-85% of patients with diabetes.

DR development and its progression depends not only on hyperglycemia. Also, important factors among the causes are hypertension and the macular edema development [5-9]. Endothelial dysfunction (ED) is currently considered central link in the occurrence and progression of DR [10, 11]. The initial morphological ED signs are endothelial cell proliferation, loss of pericytes and basement membrane thinning, resulting in aneurysms, changes in the vascular capillaries diameter and hemodynamic disorders [4, 12, 13].

Endothelial cell are the one, which the first to respond to dyslipidemia, hyperglycemia, glucose toxicity and dyslipidemia by synthesizing atherogenic factors [10, 14]. There is an increase in the vascular wall permeability and weakens its elasticity, which, in turn, stimulates exudates and hemorrhages formation. During this pathological cycle, transcapillary transport is blocked, and as a result retinal ischemia develops [14].

Objective an analysis changes of von Willebrand factor (VWF) dynamics' level during the ED development in the pathogenesis of experimental DR and in different ways of its correction.

Methods

The research was carried out on white Wistar line rats, weight -180-220 g.

In accordance with the objectives animals were divided into 7 groups:

1st group – 60 intact animals;

2nd group – 60 animals in which DR was modeled without further correction;

3rd group – 60 animals in which DR was modeled with subsequent hyperglycemia correction;

4th group – 60 animals in which DR was modeled with subsequent hyperglycemia correction by aflibercept and L-arginine solution administration;

5th group – 60 animals in which DR was modeled with subsequent hyperglycemia correction by aflibercept and bromfenac administration;

6th group – 60 animals in which DR was modeled with subsequent hyperglycemia correction by aflibercept, L-carnitine and bromfenac administration;

7th group – 60 animals in which DR was modeled with subsequent hyperglycemia correction by aflibercept, L-arginine solution and citicoline

Type 2 diabetes and DR were simulated by intraperitoneal streptozotocin administration (Sigma, USA) dissolved in 0.1 M citrate buffer with a 4.5 pH [15]. The streptozotocin dose - 55 mg / kg animal body weight - was divided into two injections. The streptozotocin administration was preceded by a high-fat diet for 28 days [16].

Drug doses:

Hypoglycemic drug - metformin (Merck Sante, manufactured in France) - at a dose of 300 mg / kg body weight in drinking form [17] in 0.9% sodium chloride solution through a syringe with an intragastric tube daily.

L-arginine solution administration, which is NO donor, (SIMESTA, made in China, USP32 standard) was carried out by intragastric L-arginine solution administration in 0.9% sodium chloride solution at a dose of 500 mg / kg [18] through a syringe with intragastric tube. The volume of the solution depended on the animal weight and didn't exceed 1 ml. The drug was administered once a day before morning feeding, daily for 10 days [18].

Aflibercept (anti-VEGF therapy) administered as subconjunctival injections at a dose of 0.08 ml (25 mg / ml) [19].

Bromfenac – instillation of 0.09% eye drops solution 1 time per day [20].

L-carnitine ("Sigma", USA) was administered as aqueous solution through a syringe with an intragastric tube at a dose of 25 mg / 100 g of animal weight [21, 22].

Citicoline - 81.8 mg / kg (0.33 ml / kg) was administered intramuscularly once a day.

Animals withdrawal from the experiment was carried out in three stages:

1st stage of the study - the 30th day after the start of modeling diabetes;

2nd stage of the study - the 60th day after the start of modeling diabetes;

3rd stage of the study - the 180th day after the start of modeling diabetes.

Animals were removed from the experiment by decapitation under light ether anesthesia according to "Rules for carrying out works using experimental animals", approved by the Order of the Ministry of Health of Ukraine No. 249 of 01.03.2012 and the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruel Treatment" (as amended on December 15, 2009, and 10.16.2012)

Blood samples was drawn from the retroorbital venous plexus, which lies in orbit behind the eyeball. The puncture was performed in a circular motion with a glass pipette with an extended capillary, the tip of which is ground at an angle of 45 °. The conjunctival sac was punctured in the medial corner of the eye between the eyeball and the orbit. After puncture, the pipette was inserted to a depth of 2-4 mm behind the eyeball. Control of entry into the venous plexus - filling the pipette capillary with blood.

The VWF level was determined by enzyme-linked immunosorbent assay (ELISA) [23, 24].

Statistical processing of the obtained results.

Before using parametric, normality-based statistical distribution methods, it were used to test the series of quantitative data for normality using the Shapiro–Wilk test (Shapiro–Wilk W-test). Due to the normal distribution of digital data in the samples, was used Student's parametric criterion. Graphically, the data are presented as boxplot. For informative data presentation, an analysis of the Mann-Whitney criterion of the studied indicators was additionally performed.

Mann-Withey U test analysis was additionally carried out for more informativeness of presented data.

Results

It have been repeatedly confirmed – in modeled experiments on rats with endotoxemia and blood vessels inner wall mechanical damage that VWF level increased in the blood in combination with the severity of vascular endothelial damage, which was also proven in clinical trials[25-28]. Endotheliocytes synthesize VWF, which is normal in blood plasma at physiological concentrations [28].

The VWF has significant role, its the messenger function in platelet aggregation and in vascular-platelet interaction at the adhesion stages [29, 30]. During these reactions, VWF acts as a mediator between platelets and subendothelial structures of the damaged vascular wall [31].

The VWF is formed with a small excess. Molecules that aren't involved in the physiological functions implementation accumulate in the endothelial cells intracellular organelles - Weibel-Palade bodies (WPBs). There they are subject to post-translational modification and multimerization and can be quickly mobilized [28].

The von Willebrand factor has a high molecular weight and activates thrombosis. By promoting the platelet receptors attachment to collagen and fibronectin of the vessels, it thereby enhances platelet aggregation and adhesion. The VWF formation is on the rise by endothelial damage or by vasopressin influence. If we take into account that the secretion of vasopressin is increased during stress, we can conclude that due to the VWF synthesis activation in extreme conditions are increased vascular thrombogenicity. It should be noted that VWF also stabilizes the factor VIII molecule, prolonging its half-life and facilitating its transport to the site of hemostatic plug formation [32].

Through our changes investigation in the level of the studied indicator, the following results were obtained (Table 1).

In the group in which diabetic retinopathy was modeled without further correction (group № 2) already at the 1st stage were identified an increase in the VWF level by 19.66% compared to the data of intact animals ($p < 0.001$). In the second stage, VWF was higher by 22.69% compared with the data of the 1st group and by 3.77% compared with the data of its group in the previous stage ($p < 0.01$). The results of the third stage confirm the ED progression: compared with the intact group, the VWF level is higher by 25.09% ($p < 0.001$). Analyzing the dynamics of VWF in group №2, it was found that at the 3rd stage its level was higher by 6.77% ($p < 0.001$) compared to the first stage, and by 3.12% compared to the second.

The 3rd group results, in which the animals received only a hypoglycemic drug were as follows. At the first stage was detected significant increase

in the ED factor level - by 17.78% compared with intact animals (p <0.001), its level is only 2.35% lower than the results of the group without correction (no statistical significance). In the second stage in this group the VWF level increased by 2.3% and is by 19.66% higher compared to group №1 (<0.001). Compared with the group without correction revealed its decrease by 3.92% (p <0,01). At the 3rd stage, the studied marker level in the 3rd group decreased slightly: by 0.89% compared with the first stage and by 3, 26% (p <0.05) compared with the second one. At the same time, its level is 3.26% lower than the data of second group (without correction) and is higher by 17.05% compared to intact animals, which indicates a pronounced progression of endothelial dysfunction.

In the 4th group, on the 30th research day, the pathological increase in the von Willebrand factor level is less pronounced compared with the previous groups at this stage: the VWF level was 10.79% (p <0.01) less than in the groups without correction, as well as 11% (p <0.01) higher compared to intact animals. The level of the studied marker in this group is 8.25% (p <0.01) lower than in third group, which indicates that adding nitric oxide to the donor in corrective therapy at an early stage is effective. On the 60th day, there is significant tendency to normalize the ED marker level in this group: the VWF is 21.41% (p <0.01) less pathologically elevated than in second group, which simulated DR without further correction. Compared with the intact group data, its level increased by 6.14% (p <0.1). Compared with the data of group №3,we can say that the correction applied in fourth group contributed to the VWF normalization by 16.84% (p <0.01). Compared with the first stage, the results of the 4th group at the 2nd stage are better by 5.47% (p <0.01). At the 3rd stage (180th day) there is a positive trend to normalize the ED studied marker level: its level is by 29.35% (p <0.001) lower than in the group without DR correction. It was detected pathological increase in comparison with intact animals by only 3.11% (p <0.05). Compared with the data of the third group, which used hypoglycemic therapy, the NO use is more effective by 16.81% (p <0.001). Analyzing the gradual dynamics of VWF level in the 4th group, it was found that in the 3rd stage it is 8.87% (p <0.001) lower compared to the first, and 3.23% (p <0.05) lower compared to the second stage.

Also, in analyzing the fifth group data VWF level is 13.11% (p <0.001) higher than in the intact animals group. Compared with second group, the ED marker value is lower by 8.16% (p <0.001), compared with group №3 data, the use of bromfenac in corrective therapy is 5.68% (p <0.001)) more effective, and in comparison with group №4 (in which a nitric oxide donor was added), in group 5 the normalization of the VWF level is less pronounced by 2.38%. The second research stage was revealed negative dynamics in the form of a partial increase in the VWF amount: compared to the first group it is higher by 13.65% (p <0.001), compared with the fifth group data at the previous stage by 0.62%. Compared with the second group data, in which the modeled DR wasn't corrected, the VWF level is less significantly increased by 11.7% (p <0.001), and compared with the data of the third group by 7.49% (p <0.001). Compared with the group №4 data, the VWF level is pathologically higher by 8% (p <0.001). In the third stage, it was found that the NSAIDs use does not inhibit the ED development in experimental DR. The VWF level is by 14.17% (p <0.001) higher than intact animals data. Compared with the significantly increased level of this marker in the group without correction, in the 3rd stage, in the 5th group it is lower by 14.58% (p <0.001). Compared with the group №3 data, the results are better by 3.47%. And in comparison with group №4, corrective therapy of fifth group was less effective by 11.42% (p <0.001). Analyzing step-by-step the VWF increasing level dynamic in group №5, it was found that in the third stage it is higher by 1.23%, and in comparison with the second stage by 0.62%.

The following results were obtained in group №6, which received a complex correction with metformin, aflibercept, L-carnitine and bromfenac (that is, compared with group №5, therapy was improved by the L –carnitine administration). The VWF level in comparison with group №2 is lower by 13.55% (p <0.001), and in comparison with intact animals its level is increased by 8.78% (p <0.001). Compared with the group №3 data was detected a decrease in the studied marker by 10.95% (p <0.001), and in comparison with the fourth group by 2.5%. Compared with the data of group №5, the VWF level is lower by 4.99% (p <0.05). On the 30th day it was found that VWF level is by 20.74% (p <0.001) lower than the value in the second group. Compared with

intact animals, an increase was found at 6.7% (p <0.001). Compared with the third group, the level of Willebrand factor is lower by 16.19% (p <0.001). Compared with the data of the fourth group, the level of the Willebrand factor is slightly higher - by 0.56%. Compared with the group №5 results, the VWF level was normalized by 8.1% (p <0.01). Compared with the data of the first stage, the increase in the studied indicator level is less expressed by 2.33%, and in the third stage of the experiment in the sixth group it was found that the level of Willebrand factor is lower than in group №2 by 28.9% (p <0.001) , and with the third group by 16.4% (p <0,001). There is an increase in the VWF level by 0.35% in the second stage, which indicates the need to involve NO-donors in the complex correction. Compared with group №5, the level of the studied marker is lower by 12.5% (p <0.001). Compared with the intact group data, the VWF level is higher by 3.45% (p <0.05). It was found that the VWF level – while examining the dynamics in the sixth group – in the third stage lower by 5.85% (p <0.01) compared with the first stage and 3.45% (p <0.05) compared with the second.

We found out that in seventh group at the first stage the VWF level by 16.32% (p <0.001) lower than the second group data. Its level by 6.55% (p <0.01) higher than in intact animals. The functional endothelium state normalization in comparison with the third group was proved by 13.66% (p <0.001), in comparison with the fourth group by 5% (p <0.05), with the fifth group by 7.55% (p <0.001) and in comparison with the sixth group by 2.45%. The above indicates that corrective therapy, which consists of medformin, aflibercept, citicoline and L-arginine is most effective at 30th day of the research. At 60th day of the investigation, it was found that the VWF level in comparison with the group №2 by 26.19% (p <0.001) is close to the control values, its level is only 2.44% higher than the intact animals value. The ED studied marker concentration is lower by 21.44% (p <0.001) compared with group №3, by 3.94% (p <0.05) compared with the group №4, by 12.98% 0.001) compared with group №5, 4.52% (p <0.05) less than group №6. There is a positive dynamics in group №7 in comparison with the previous stage where the VWF level was lower by 4.41% (p <0.05). On the 180th day the VWF concentration is 32.87% (p <0.001) lower than group №2 indicators. Note that here is the maximum normalization of VWF level its only 1.48% higher than the intact group data. The ED level of this indicator is 19.98% (p <0.001) lower compared to the third group, by 2.72% compared to the fourth group, by 15.96% (p <0.001) compared to group №5 and 3,08% compared with the sixth group. At this stage, the WF level is lower by 6.51% (p <0.01) compared with the data of the first stage and 2.01% lower than the results of the second. The obtained data show that the proposed correction method effectiveness is detected already on the 30th day and gradually increases, contributing to the endothelium functional state normalization.

It should be noted that ED is characterized by a endothelium-dependent violation of blood vessels vasodilation, and increased adhesion of the endothelial lining [32]. Therefore, it can be emphasized that Willebrand factor is an important diagnostic marker of endothelial adhesion degree. Because endotheliocyte damage is accompanied by increased synthesis of Willebrand factor, this indicator can be considered as an important diagnostic test for the degree of vascular endothelial damage. [33, 35]. During the experiment, we confirmed the importance Willebrand factor determining for the DR prognosis. Against the background of the simulated DR development have been established objective changes and it has been found that the VWF significantly exceeds the physiological limits during this pathology. Proof of the selected model effectiveness and the VWF level analysis informativeness as a marker of changes in the studied pathology can be noted a significant difference in the indicators of the first (intact group) and indicators in the 2nd-7th groups. It was also advisable to compare in detail the groups results in which the treatment was carried out with the control group and with the group in which was experimental diabetic retinopathy without correction. We found a statistically significant difference between the groups where the treatment was performed and the group without correction of the modeled pathological process. It was found that the improvement in groups from 3 to 7 exponentially differed from the 2nd group. And if the indicators of the 3rd group didn't differ significantly from the 2nd, then in all others there

was a significant VWF level normalization. Comparing the improvement degree in different groups, it should be noted that the improvement is most pronounced in the 7th group of the experiment. Gradually, the dynamics of the results are clearly presented in Fig.1-Fig.4.

Discussion

1. As a result of our research was proved the structural and functional state violation of the endothelium in experimental diabetic retinopathy, as evidenced by an increase in the Von Willebrand factor level in group 2 ($p < 0.001$), most pronounced in stage 3.

2. It was confirmed that the correction of studied diabetes mellitus complication only by a hypoglycemic drug, even with long-term administration, does not correct the endothelial dysfunction development ($p < 0.001$).

3. Also, it was found that the addition of aflibercept and L-arginine solution - in the correction - to hypoglycemic drugs significantly ($p < 0.001$) improves the endothelium condition, but doesn't solve the problem completely.

4. There is evidence that the correction of modeled pathological condition by reducing hyperglycemia, administration of aflibercept and bromfenac (group №5) has a positive effect on von Willebrand factor normalization ($p < 0.001$), but the effect is less pronounced than in group 4 ($p < 0.001$ on 2nd and 3rd stages of the study) and partially loses effectiveness in the 3rd stage.

5. It was found that in rats in which diabetic retinopathy was modeled with subsequent hyperglycemia correction by aflibercept, L-carnitine and bromfenac administration (group №6), the decrease in pathologically elevated von Willebrand factor level is more pronounced compared with groups from 3 to 5, indicating the feasibility of this correction method.

6. Moreover, it was found that the most effective correction method was in the 7th group of the experiment in which was performed hyperglycemia correction, aflibercept, L-arginine solution and citicoline administration: in the 2nd and 3rd stage of the experiment the von Willebrand factor level didn't differ statistically from the intact group.

Conflict of interest

The authors declsre that there are no conflicts of interest.

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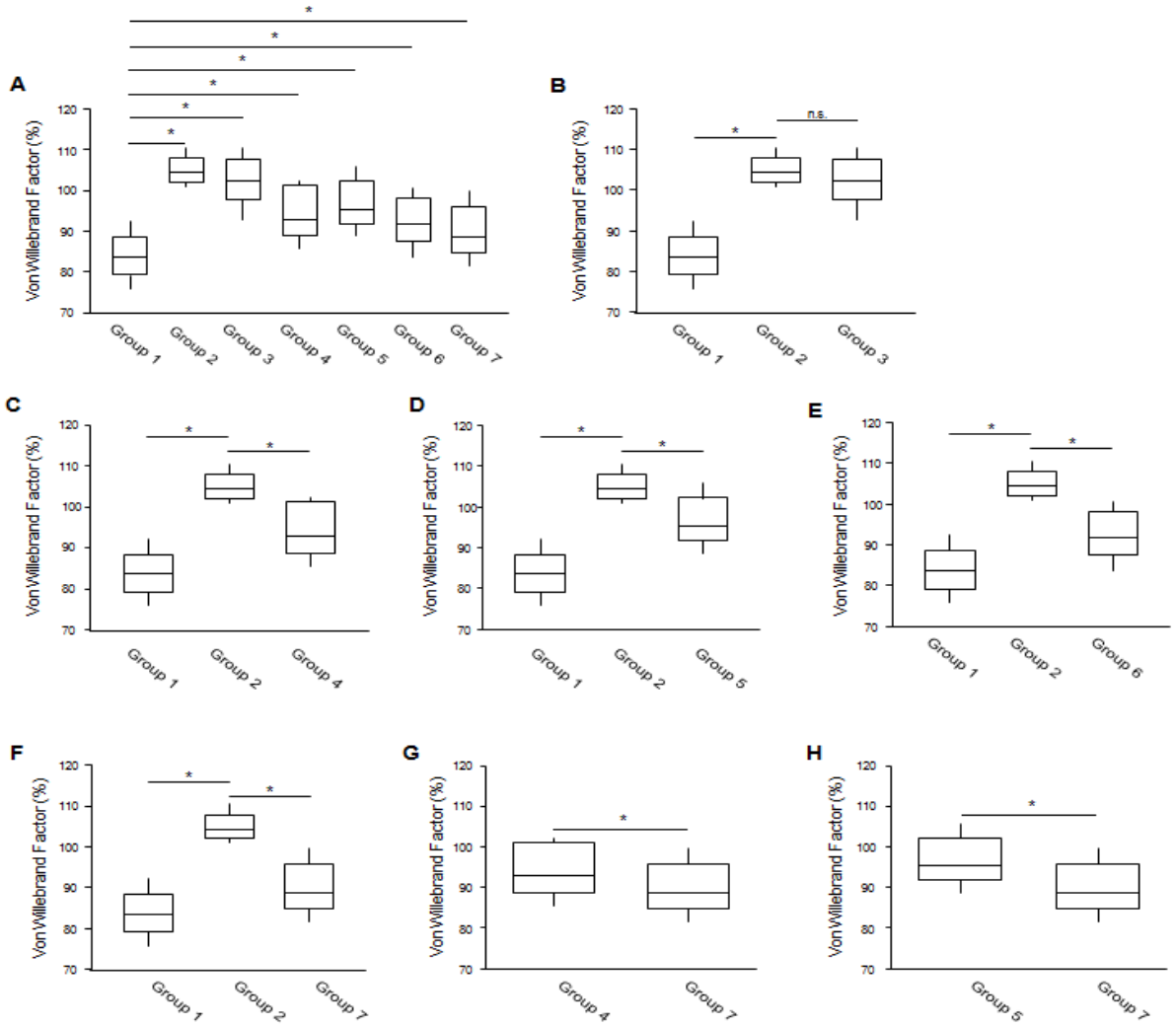
Table 1. The VWF level dynamic in the blood of experimental animals with simulated diabetic retinopathy and with different methods of its correction on the 30th, 60th and 180th day (M ± m), (%).

Stages / Groups	I stage			II stage			III stage		
1 group	84,2±1,28			84,2±1,28			84,2±0,95		
2 group	104,8±0,87			108,9±1,16			112,4±1,18		
3 group	102,4±1,36			104,8±1,19			101,5±1,18		
4 group	94,6±1,4			89,7±1,35			86,9±1,14		
	1-4	p<0,001	+11,00%	I-II	p<0,01	-5,47%	I-III	p<0,001	-8,87%
	2-4	p<0,001	-10,79%	1-4	p<0,01	+6,14%	II-III	p<0,05	-3,23%
	3-4	p<0,001	-8,25%	2-4	p<0,001	-21,41%	1-4	p<0,05	+3,11%
				3-4	p<0,001	-16,84%	2-4	p<0,001	-29,35%
							3-4	p<0,001	-16,81%
5 group	96,9±1,34			97,5±1,2			98,1±1,24		
	1-5	p<0,001	+13,11%	I-II	p>0,05	+0,62%	I-III	p>0,05	+1,23%
	2-5	p<0,001	-8,16%	1-5	p<0,001	+13,65%	II-III	p>0,05	+0,62%
	3-5	p<0,01	-5,68%	2-5	p<0,001	-11,7%	1-5	p<0,001	+14,17%
	4-5	p>0,05	+2,38%	3-5	p<0,001	-7,49%	2-5	p<0,001	-14,58%
				4-5	p<0,001	+8,00%	3-5	p>0,05	-3,47%
							4-5	p<0,001	+11,42%
6 group	92,3±1,35			90,2±1,19			87,2±1,3		
	1-6	p<0,001	+8,78%	I-II	p>0,005	-2,33%	I-III	p<0,01	-5,85%
	2-6	p<0,001	-13,55%	1-6	p<0,001	+6,66%	II-III	p<0,05	-3,45%
	3-6	p<0,001	-10,95%	2-6	p<0,001	-20,74%	1-6	p<0,05	+3,45%
	4-6	p>0,05	-2,50%	3-6	p<0,001	-16,19%	2-6	p<0,001	-28,90%
	5-6	p<0,05	-4,99%	4-6	p>0,05	+0,56%	3-6	p<0,001	-16,40%
				5-6	p<0,01	-8,10%	4-6	p>0,05	+0,35%
							5-6	p<0,001	-12,50%
7 group	90,1±1,42			86,3±1,48			84,6±1,52		
	1-7	p<0,01	+6,55%	I-II	p<0,05	-4,41%	I-III	p<0,01	-6,51%
	2-7	p<0,001	-16,32%	1-7	p>0,05	+2,44%	II-III	p>0,05	-2,01%
	3-7	p<0,001	-13,66%	2-7	p<0,001	-26,19%	1-7	p>0,05	+0,48%
	4-7	p<0,05	-5,00%	3-7	p<0,001	-21,44%	2-7	p<0,001	-32,87%
	5-7	p<0,001	-7,55%	4-7	p<0,05	-3,94%	3-7	p<0,001	-19,98%
	6-7	p>0,05	-2,45%	5-7	p<0,001	-12,98%	4-7	p>0,05	-2,72%
				6-7	p<0,05	-4,52%	5-7	p<0,001	-15,96%
							6-7	p>0,05	-3,08%

Figure 1. Changes in the VWF level 30 days after diabetic retinopathy induction



(A) Box plots illustrate the distribution of Willebrand factor levels in each study group. The symbol * indicates the presence of a statistically significant difference between the results obtained in the control group (n = 20 rats) and experimental groups 2-7 (n = 20 animals in each group); Mann-Whitney Test, $p < 0.05$.

(B) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 3.

The difference between the results obtained in groups 1 and group 2 is statistically significant (Mann-Whitney Test, $p < 0.05$). The difference between the results obtained in groups 2 and group 3 isn't statistically significant (Mann-Whitney Test, $p > 0.05$).

(C) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 4. The difference between the results obtained in groups 2 and group 4 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(D) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 5. The difference between the results obtained in groups 2 and group 5 is statistically significant (Mann-Whitney Test, $p < 0.05$).

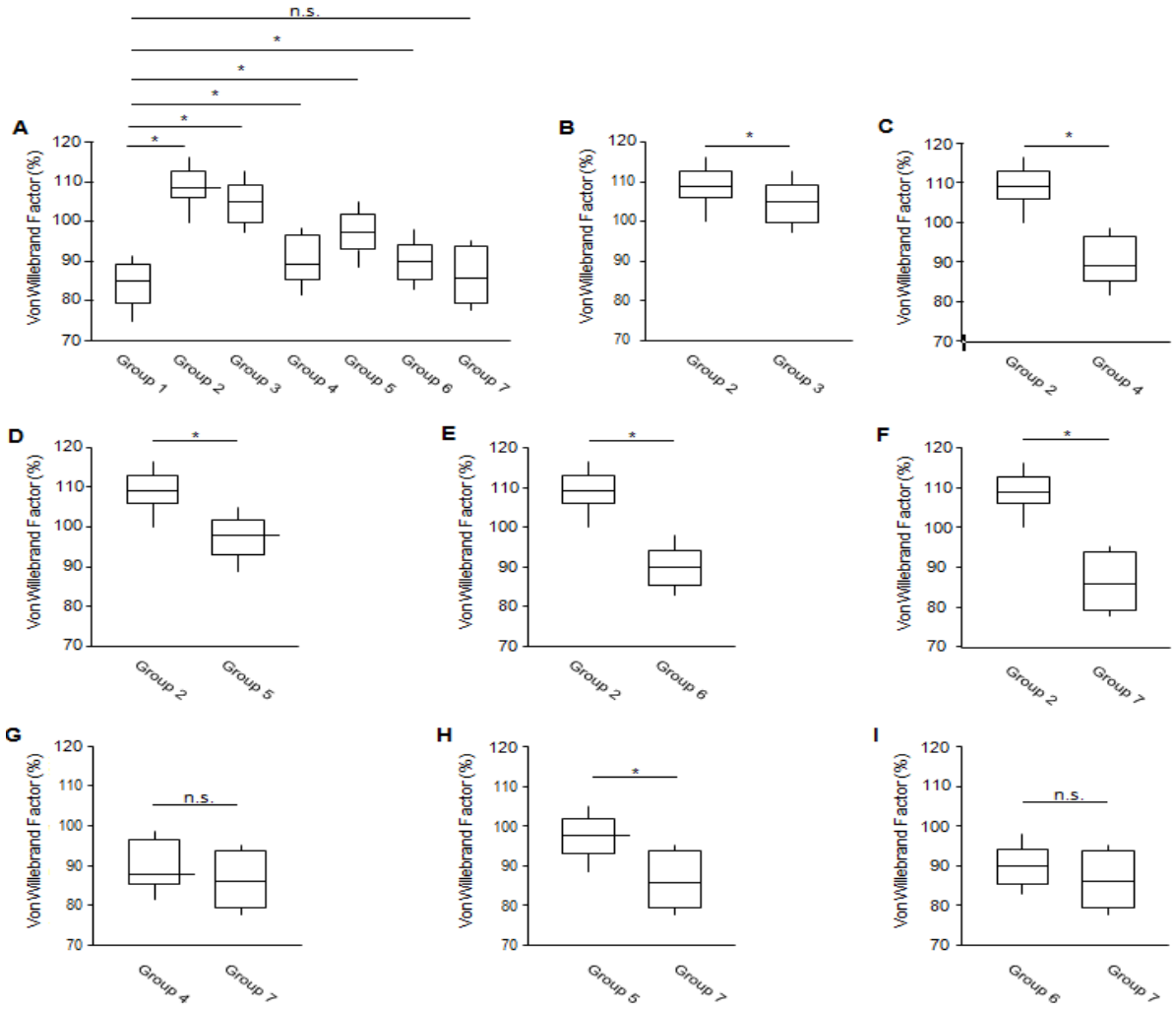
(E) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 6. The difference between the results obtained in groups 2 and group 6 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(F) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 7. The difference between the results obtained in groups 2 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(G) Box plots illustrate the distribution of Willebrand factor levels in control group, group 4 and group 7. The difference between the results obtained in groups 4 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(H) Box plots illustrate the distribution of Willebrand factor levels in group 5 and group 7. The difference between the results obtained in groups 5 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

Figure 2. Changes in the VWF level 60 days after diabetic retinopathy induction



(A) Box plots illustrate the distribution of Willebrand factor levels in each study group. The symbol * indicates the presence of a statistically significant difference between the results obtained in the control group (n = 20 rats) and experimental groups 2-7 (n = 20 animals in each group); Mann-Whitney Test, $p < 0.05$.

(B) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 3.

The difference between the results obtained in groups 2 and group 3 is statistically significant (Mann-Whitney Test, $p > 0.05$).

(C) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 4. The difference between the results obtained in groups 2 and group 4 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(D) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 5. The difference between the results obtained in groups 2 and group 5 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(E) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 6. The difference between the results obtained in groups 2 and group 6 is statistically significant (Mann-Whitney Test, $p < 0.05$).

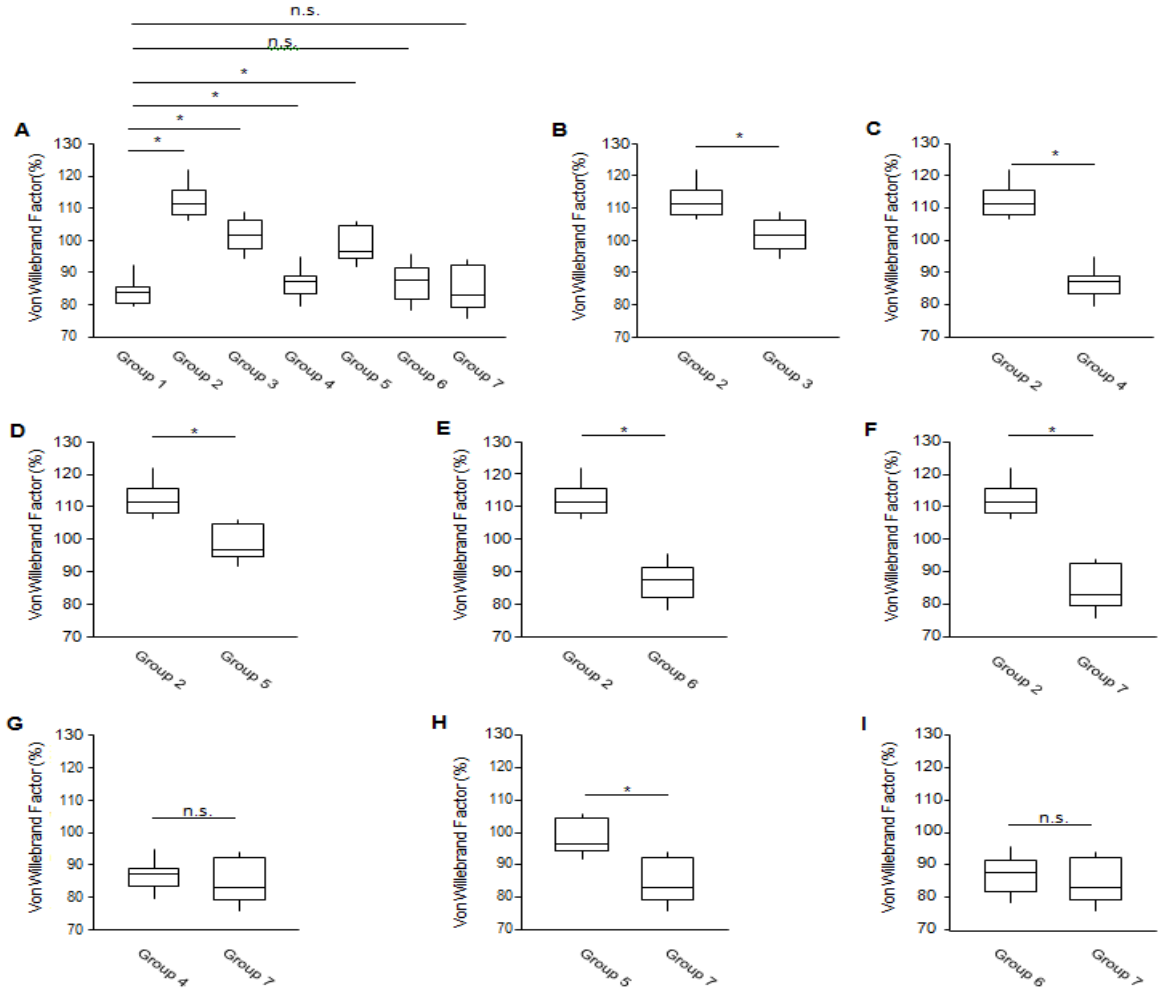
(F) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 7. The difference between the results obtained in groups 2 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(G) Box plots illustrate the distribution of Willebrand factor levels in group 4 and group 7. The difference between the results obtained in groups 4 and group 7 isn't statistically significant (Mann-Whitney Test, $p < 0.05$).

(H) Box plots illustrate the distribution of Willebrand factor levels in group 5 and group 7. The difference between the results obtained in groups 5 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(I) Box plots illustrate the distribution of Willebrand factor levels in group 6 and group 7. The difference between the results obtained in groups 6 and group 7 isn't statistically significant (Mann-Whitney Test, $p < 0.05$).

Figure 3. Changes in the VWF level 180 days after diabetic retinopathy induction



(A) Box plots illustrate the distribution of Willebrand factor levels in each study group. The symbol * indicates the presence of a statistically significant difference between the results obtained in the control group (n = 20 rats) and experimental groups 2-7 (n = 20 animals in each group); Mann-Whitney Test, $p < 0.05$.

(B) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 3.

The difference between the results obtained in groups 2 and group 3 is statistically significant (Mann-Whitney Test, $p > 0.05$).

(C) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 4. The difference between the results obtained in groups 2 and group 4 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(D) Box plots illustrate the distribution of Willebrand factor levels in control group, group 2 and group 5. The difference between the results obtained in groups 2 and group 5 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(E) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 6. The difference between the results obtained in groups 2 and group 6 is statistically significant (Mann-Whitney Test, $p < 0.05$).

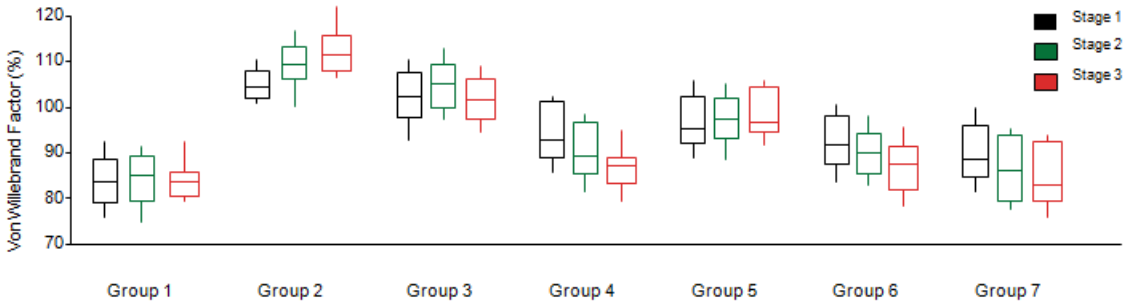
(F) Box plots illustrate the distribution of Willebrand factor levels in group 2 and group 7. The difference between the results obtained in groups 2 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(G) Box plots illustrate the distribution of Willebrand factor levels in group 4 and group 7. The difference between the results obtained in groups 4 and group 7 isn't statistically significant (Mann-Whitney Test, $p < 0.05$).

(H) Box plots illustrate the distribution of Willebrand factor levels in group 5 and group 7. The difference between the results obtained in groups 5 and group 7 is statistically significant (Mann-Whitney Test, $p < 0.05$).

(I) Box plots illustrate the distribution of Willebrand factor levels in group 6 and group 7. The difference between the results obtained in groups 6 and group 7 isn't statistically significant (Mann-Whitney Test, $p < 0.05$).

Figure 4. Changes in the VWF level 30, 60 and 180 days after diabetic retinopathy induction



Box plots illustrates the distribution of Willibrand factor level in the respective groups at each research stage (n = 20 animals in each group).