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DYNAMICS OF MARKERS OF ENDOTHELIAL DYSFUNCTION IN EXPERIMENTAL ANTIPHOSPHOLIPID SYNDROME

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Abstract

Analysis of the entire set of currently available factors allows us to consider antiphospholipid syndrome (APS) as a unique model of autoimmune platelet vasculopathy, the study of which is essential for determining the relationship between such fundamental pathological processes as atherosclerosis, vasculitis, blood clotting disorders and the immune system. At the same time, the diagnosis and treatment of APS is based on serological tests, and little attention is paid to hemostatic markers and endothelial dysfunction in this pathology. The aim of the study was to study the dynamics of endothelial state indicators in animals with simulated antiphospholipid syndrome and the effectiveness of their correction by introducing warfarin, immunoglobulin and L-arginine.

Research results. Disruption of the functional state of the endothelium in animals with simulated antiphospholipid syndrome was found. A pronounced increase in the von Willebrand factor level ($p < 0.001$) in the blood of rats with experimental APS was proved. It was revealed that the development of experimental antiphospholipid syndrome leads to an increase in the level of homocysteine in the blood of rats ($p < 0.001$). The most pronounced results of correction were found in the fifth group, the animals of which received complex therapy with warfarin, immunoglobulin and L-arginine.

Key words: *antiphospholipid syndrome, pathology, L-arginine, warfarin, immunoglobulin, endothelial dysfunction*

Introduction

Antiphospholipid syndrome (APS), first described by GRV Hughes and co-authors in 1986, attracts the attention of clinicians in various fields of medicine [1] The above pathology is characterized by a triad of clinical and laboratory signs: recurrent venous or arterial thrombosis localized in any part of the drunken bloodstream, obstetric pathology in the form of primary premature pregnancy and intrauterine fetal death and hematological disorders (thrombocytopenia, hemolytic anemia) [2] APS was first described in the framework of systemic lupus erythematosus (SLE), but then thrombotic disorders were established, in the absence of reliable clinical and serological criteria for this or any other leading disease. To define a new nosological form, the term was proposed - primary APS (PAPS) [3].

It has now been established that primary APS is a widespread autoimmune process, which is based on the formation of a high titer of autoantibodies to negatively charged membrane phospholipids (MF) and associated glycoproteins (P2-GP-1 and others). The main targets of antiphospholipid antibodies (APA) are: cardiolipin, phosphotidylserine, phosphotidylethanolamine, phosphotidylic acid, and from protein components-B2 glycoprotein-1, and prothrombin [4]. These AFAs block phospholipid-protein complexes of plasma, membranes of blood cells and endothelium, manifested by a decrease in their thromboresistance, activation of platelet hemostasis and imbalance in the coagulation hemostasis system.

A variety of clinical manifestations of APS from recurrent venous and arterial thrombosis, early heart attacks and strokes, to premature pregnancy, thrombocytopenia, Raynaud's syndrome, leads to a mosaic of clinical manifestations, complications and laboratory changes. Clinical and morphological studies indicate that the basis of APS is a kind of vasculopathy associated with thrombotic or occlusive vascular injury. The spectrum of clinical manifestations of antiphospholipid vasculopathy is not less diverse than with another universal form of vascular pathology - systemic vasculitis. At the same time, in contrast to vasculitis or atherosclerosis, there are no pronounced inflammatory or degenerative changes in the vascular wall, emphasizing the nosological independence of the APS [5].

In general, the analysis of the entire set of currently available factors allows us to consider the APS as a unique model of autoimmune platelet vasculopathy, the study of which is essential for deciphering the relationship between such fundamental pathological processes as atherosclerosis, vasculitis, impaired blood clotting and the immune system. At the same time, the diagnosis and treatment of APS is based on serological tests, and little attention is paid to hemostatic markers and endothelial dysfunction in this pathology.

The role of etiological factors, triggering mechanisms (including at the initial stages of the formation of APS) are not clearly defined, the biochemical markers of the disease, unformed effective treatment regimens for APS are poorly understood. By the end, the problem of treating patients with APS was solved. This is due to the heterogeneity of pathogenetic mechanisms, clinical manifestations and the lack of reliable clinical and laboratory indicators that allow predicting the recurrence of thrombotic complications [6].

Thus, there is a need to solve the above problems, which is important for a comprehensive study of patients in order to clarify the pathogenesis, more complete and early diagnosis of various APS variants and determine the optimal tests for monitoring the therapy and creating correction schemes.

Purpose: to increase the effectiveness of treatment by the introduction of warfarin, immunoglobulin and L-arginine in experimental antiphospholipid syndrome based on the study of disorders of the functional state of the endothelium.

Methods

The study was carried out on 100 outbred male rats weighing 180-220 g. The animals were divided into the following groups:

The first group - control - intact animals that were on the standard diet of the vivarium (n = 20).

The second group consisted of rats with simulated antiphospholipid syndrome (n = 20).

The third group (n = 20) - animals that, against the background of simulated pathology, received correction by intraperitoneal administration of

human immunoglobulin (ZAO Biopharma) at a dose of 0.5 g / kg of body weight and intragastric administration of a solution of L-arginine in 0.9% sodium chloride solution at a dose of 500 mg / kg.

The fourth group (n = 20) - animals that, against the background of simulated pathology, received correction with warfarin in doses calculated by the coefficient of compliance and intraperitoneal administration of human immunoglobulin at a dose of 0.5 g / kg of body weight.

The fifth group (n = 26) - animals that, against the background of a modeled pathology, received correction with warfarin, intraperitoneal administration of human immunoglobulin (ZAO Biopharma) at a dose of 0.5 g / kg of body weight and intragastric administration of a solution of L-arginine in 0.9% sodium chloride solution at a dose of 500 mg / kg.

Antiphospholipid syndrome was modeled by subcutaneous injection of cardiolipin antigen in a total dose of 0.2-0.4 mg to another rat every other day. within three weeks [7]

The daily dose of Warfarin for a rat weighing 200 g is 0.2 mg.

The introduction of a nitric oxide donator - a solution of L-arginine (SIMESTA, production of the PRC, quality standard USP32) was carried out by intragastric administration of a solution of L-arginine in 0.9% sodium chloride solution at a dose of 500 mg / kg through a syringe with an intragastric tube. The volume of the solution depended on the weight of the animal and did not exceed 1 ml. The drug was administered once a day before morning feeding, for 10 days.

The study lasted 8 weeks, during which the animals were monitored and / or treated.

The rats were removed from the experiment under light ether anesthesia in accordance with the "Rules for Performing Work with the Use of Experimental Animals" approved by the Order of the Ministry of Health of Ukraine No. 249 dated 01.03.2012 and the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty" (as amended on 15.12. 2009r and from 16.10.2012r).

After euthanasia, blood samples were taken from rats in the study of endothelial functioning indicators (von Willebrand factor and homocysteine content) in the blood of laboratory animals.

The von Willebrand factor level was determined by the enzyme immunoassay by ristocytin time [8, 10].

Homocysteine levels were determined using high performance liquid chromatography according to the method of S.M. Pfeiffer [9].

Results

According to modern concepts, the endothelium is, in addition to a purely anatomical inner layer of blood vessels, also an independent endocrine gland with high metabolic activity. The endothelium plays the role of a modulator of the secretion of vasoconstrictors and vasodilators, is in constant contact with the blood and at the same time receives messages from biologically active substances circulating in the vessels. Endotheliocytes play an important role in the pathogenesis of cardiovascular pathology, because they are not only target cells, but also produce substances that regulate platelet adhesion and aggregation, pro- and anticoagulant activity, maintain vascular tone and fibrinolytic activity. To date, it has been proven that during chronic damage to the endothelium, its dysfunction develops, which in turn leads to the migration of smooth muscle cells from the media to the intima with the formation of fibrous plaques, the release of growth factors and platelet adhesion. This dysfunction is defined as pathophysiological (decreased or increased) production of biologically active substances (BAS) in the endothelium. In its pathogenesis, an imbalance of vasoconstrictors (anta oxide, angiotensin, endothelin-1) and vasodilators (NO, S-NO) produced by the endothelium plays a key role. This dysregulation leads to defects in homeostatic mechanisms. The further development of processes in the wall depends on the ability of endotheliocytes to correct this imbalance [30, 38]. The degree of endothelial dysfunction can be determined by examining substances in the blood that damage the endothelium. Such risk factors include hyperhomocysteinemia, hypercholesterolemia, increased levels of cytokines - IL-1β, IL-8, TNF-α. Biologically active substances formed in the endothelium are classified as follows:

1) factors that accumulate in the endothelium and are secreted from it during stimulation - tissue plasminogen activator. P-selectin, von Willebrand factor. They can enter the bloodstream both when

the endothelium is activated and when it is damaged.

2) factors that are constantly secreted in the endothelium and released into the blood (prostacyclin, NO).

3) factors that are produced and accumulated in the endothelium (t-RA), or endothelial receptors - protein C receptor, thrombomodulin.

4) factors that are NOT produced under physiological conditions, but the synthesis of which sharply increases with endothelial activity - endothelin-1, E-selectin.

Often, endothelial dysfunction is the main cause of circulatory disorders in the organ, as it leads to angiospasm or vascular thrombosis. On the other hand, pathophysiological changes in peripheral circulation, such as venous congestion and ischemia, can lead to endothelial dysfunction. Endothelial dysfunction markers are of great importance for the prevention and early diagnosis of cardiovascular pathology, as indicators of vascular intimal damage at the initial stage.

1. Study of the dynamics of von Willebrand factor in experimental antiphospholipid syndrome

One of the key markers of activation of endothelial dysfunction is von Willebrand factor. This sulfitation of glucoproteins is synthesized in megakaryocytes and endothelium and by ensuring the adhesion of platelets to the collagen of the vascular wall takes part in vascular-platelet hemostasis. In adhesion and aggregation of platelets, von Willebrand factor plays a key role under conditions of high blood flow velocities, at which the blood current significantly prevents the formation of a hemostatic plug, and other adhesion mechanisms cannot provide platelet fixation. Also important is the role of von Willebrand factor in thrombus formation in small arteries. The von Willebrand factor at the site of damage to the vascular wall causes platelet adhesion to the vascular wall and is one of the initial mechanisms of platelet plug formation. Under physiological conditions, RF does not bind platelets. When the subendocardial matrix of the vascular wall is damaged, von Willebrand factor binds to the primary matrix component. As a result, platelet aggregation is stimulated, and the formation of platelet plugs. This marker interacts with microfibrils of the subendothelium and collagen, which leads to the attachment of platelets to glucoprotein 1b. In this case, VWF connects the exposed subendothelial layer and platelets. This combination with platelet receptors stimulates platelet complexes IIb / IIIa, which plays a significant role in atherosclerotic changes in the arteries. In this regard, an increase in the concentration of von Willebrand factor in blood plasma, as well as an increase in fibrinogen, can be considered as the main marker of hypercoagulability. Increased activation of the von Willebrand factor complex and glycoprotein IIb-IIIa in patients with coronary heart disease has been proven. Also revealed the relationship between the activity of this complex and the degree of myocardial ischemia, which increases with the transition from angina pectoris to acute myocardial infarction, reaching the maximum in acute coronary syndrome.

An increase in von Willebrand factor in patients with stable exertional angina has been proven, and a relationship between the level of EF and the severity of angina has been revealed. In patients with acute coronary syndrome, a twofold increase in the level of this marker was determined. The relationship between the level of von Willebrand factor in the blood and cardiovascular pathology has been proven. The following pattern was noted - a significant increase in the concentration of von Willebrand factor in acute coronary syndrome is a harbinger of a negative prognosis. A correlation has been established between the quality of life in patients with coronary heart disease and an increase in markers of endothelial dysfunction, in particular von Willebrand factor. The severe course of acute and chronic heart failure was associated with increased levels of von Willebrand factor and IL-6 in the blood serum of coronary artery disease.

According to the literature, it is known that the relationship between an increase in the concentration of VWF in the blood and the degree of vascular endothelial impression has been repeatedly proved in a number of model experiments in rats with endotoxemia and mechanical damage to the endothelium, as well as in a number of clinical observations.

The von Willebrand factor is synthesized by endothelial cells and circulates in blood plasma at a concentration of about 10 pg / ml. The most significant role of VWF is the function of a mediator

in vascular-platelet interaction at the stages of adhesion and platelet aggregation. In these reactions, VWF acts as a bridge between the subendothelial structures of the damaged vascular wall and platelets, as well as between individual platelets.

It is known that von Willebrand factor is synthesized with a certain "reserve", VWF molecules that do not take part in the implementation of physiological functions accumulate in the intracellular organelles of endotheliocytes - Weibel-Palade bodies, where they are subject to multimerization and post-translational modification and from where they can be quickly mobilized.

Considering the above, the study of von Willebrand factor in experimental antiphospholipid syndrome is informative. Table 1 presents the results of the analysis of this marker of endothelial dysfunction obtained by us during the experiment.

In the study of the von Willebrand factor level, very significant differences were found when comparing the data of group No. 2, in which antiphospholipid syndrome and group No. 1 were not corrected. (intact animals). Differences were found at the level of significance p <0.001.

In group No. 3, an increase in the von Willebrand factor level was also found, but less pronounced than in group No. 2 (r_{2-3}<0.001). At the same time, the differences in the values of this indicator in the blood of experimental animals of this group are higher than the results of intact animals also at the level of significance p <0.001.

It is noteworthy that the level of the studied marker of endothelial dysfunction in the fourth group is higher compared to the results of the third group (p <0.001), which indicates the better efficiency of the correction of the modeled pathology in the third group of rats. Also, characterizing the results of the fourth group, it should be noted that its level is very significantly lower compared to the data of animals in which the modeled APS was not corrected (p <0.001) and pathologically increased compared to the results obtained in intact rats (p <0.001).

The most pronounced positive effect of the correction of the experimental antiphospholipid syndrome was found in group No. 5, in which animals, against the background of a modeled pathology, received correction with warfarin, immunoglobulin and L-arginine.

In the case of the group No. 2 and No. 4, the FV level in the group No. 5 is the lowest on the level of significance p <0.001, in the case of the group No. 3 in the third group, the level of the indicator is the lowest (the most significant рівні p <0.01. According to the data of intact creatures, the visibility of statistically significant indications of the results in group No. 5 was established, as well as to indicate the normalization of the level of the pre-juvenile marker in the blood of the patients in the group No. 5.

The given results are shown in Fig. 1.

2. Investigation of homocysteine levels in experimental antiphospholipid syndrome

Homocysteine is an amino acid that differs from cysteine by one methylene group. This amino acid enters the human body with proteins of animal origin in the form of methionine. If an excess of homocysteine is formed in the body, it can be transformed in the opposite direction into methionine. The metabolism of HC depends on the derivatives of vitamins - riboflavin, cyanocobalamin, folic acid and pyridoxine. Their insufficiency leads to hyperhomocysteinemia. High levels of homocysteine increase apoptosis. Accelerates the aging of endothelial cells and leads to their dysfunction. HC is characterized by a procoagulant effect, during which it inhibits the activity of heparin and antithrombin III, resulting in increased thrombin activity, and, consequently, increases the risk of thrombosis and atherosclerotic plaque rupture. Proaggregated properties of the specified amino acid in high concentrations are proved, activation of fibrinolysis processes and suppression of activity of anticoagulants is established. Homocysteine affects the synthesis of nitric oxide, reduces the sensitivity of tissues to nitric oxide, and inhibits its effect. This explains the decrease in the vasodilating effect of NO-containing drugs used in cardiac practice. Homocysteine also inhibits the progression of atherosclerosis by enhancing the proliferation of vascular smooth muscle cells. In an experiment on mice with hyperhomocysteinemia on the background of a normal diet for 60 days fibrous plaques are formed, and after 60 days hypercholesterolemia is observed. The same phenomena are observed in a shorter time and more significant vascular damage, if methionine is

added to the diet and B vitamins are removed.

The thickness of the vascular layer of the intima of the media directly depends on the concentration of homocysteine in the blood. There is a direct relationship between the mortality of patients with coronary artery disease and the level of HC in the blood. This fact is confirmed by the high% of coronary artery restenosis after angioplasty in patients with hyperhomocysteinemia, and the appointment of folic acid and vitamins B6 and B12 significantly reduces this figure. Given the above, we can say that homocysteine is an informative marker of endothelial dysfunction. In the study of homocysteine levels in our study, the following results were obtained (Table 2).

In the second group, in which the simulated APS was not corrected, a pronounced increase in homocysteine in the blood of laboratory animals (p <0.001) compared with data from intact animals. In the third group, the level of homocysteine is increased in comparison with the results of group №1 at the level of significance p <0.01 and is significantly lower than in group 2 (p <0.001), which indicates a positive effect of the correction of the 3rd group. Analyzing the data of group №4 it was determined that the correction applied in this group was less effective in the analysis of this marker than in the third (p4-3 <0,001), ie the level of the studied marker was higher (differences between the data of the 4th group and group without correction are statistically insignificant). Differences in comparison with data from intact animals are very highly significant (p <0.001).

The following results were obtained by examining the level of homocysteine in the blood of rats of the fifth group. Compared with the data of the group without correction (№2) and group №4, a decrease (normalization) of the studied marker at the level of significance p <0.001. Also, the results of the 5th group are better than the data of the group №3 (p <0,01). The absence of statistical differences in the level of homocysteine in the blood of rats of the fifth group and the first (intact) indicates the most pronounced positive effect of a complex three-component correction of pathologically elevated HC levels in the simulated antiphospholipid syndrome. Visually, these results are presented in Fig.2.

The most pronounced positive results of the fifth group can be explained as follows. Nitric oxide (NO) is formed from L-arginine by endothelial NO-synthase, which provides its secretion for normal vascular function. L-arginine is not only a donor of NO synthesis, but also corrects endothelial dysfunction by stimulating eNOS activity, interfering with the oxidation of the main cofactor NOS. This amino acid also competes with asymmetric dimethyl L-arginine, which is an eNOS inhibitor.

Conclusions

1. Established dysfunction of the endothelium in animals with simulated antiphospholipid syndrome.
2. Proven marked increase in the level of Willebrand factor (p <0.001) in the blood of rats with experimental APS.
3. It was found that the development of experimental antiphospholipid syndrome leads to an increase in homocysteine levels in the blood of rats (p <0,001).
4. The use of immunoglobulin and L-arginine solution in the treatment of rats in simulated antiphospholipid syndrome has a corrective effect on the functional state of the vascular endothelium and leads to a decrease in the level of Willebrand factor and homocysteine.
5. The use of immunoglobulin and warfarin in the treatment of rats, in a simulated antiphospholipid syndrome, affects the functional state of the endothelium, but to a lesser extent.
6. The most significant effect on vascular endothelial function in rats was observed with the combined use of warfarin, immunoglobulin and L-arginine solution.
7. In the fifth group, whose animals received complex therapy, normalization of the level of Willebrand factor and homocysteine was observed.

Acknowledgments

The authors declare that there are no conflicts of interest.

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Table 1. Results of the study of the von Willebrand factor level in the blood of experimental rats with simulated antiphospholipid syndrome and its correction (M ± m)

	first group	second group	third group	fourth group	fifth group
EF	84.3 ± 1.33	108.4 ± 1, 41	92.3 ± 1.65	101.2 ± 1.49	84.2 ± 1.89

Figure 1. The results of the study of the level of Willebrand factor in the blood of experimental rats with simulated antiphospholipid syndrome and its correction

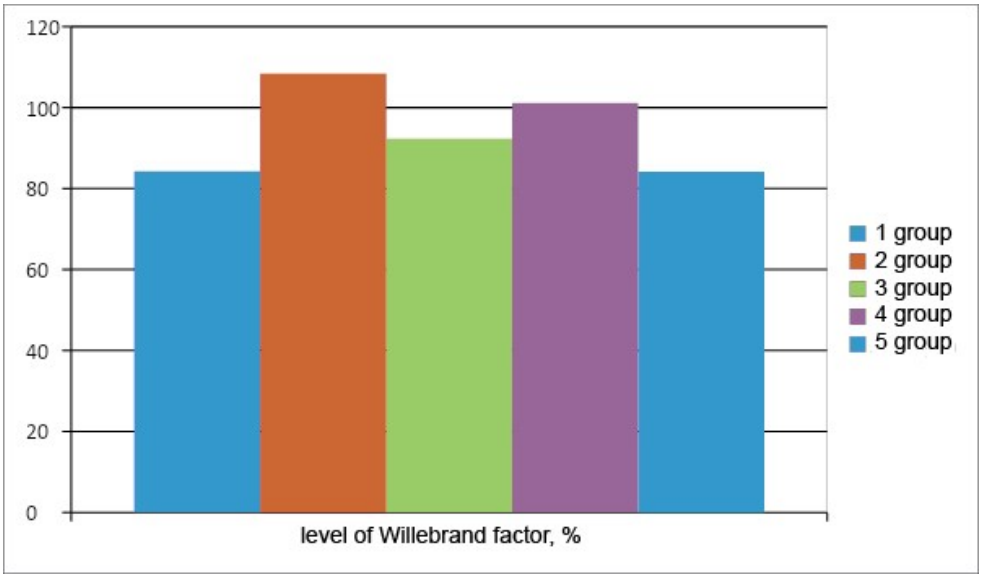

Table 2. The results of the study of the level of homocysteine in the blood of experimental rats with simulated antiphospholipid syndrome and its correction (M ± m)

	Group 1	Group 2	Group 3	Group 4	Group 5
Homocysteine	8,26 ± 0.62	14.14 ± 0.39	10.21 ± 0.41	13.46 ± 0.48	8.42 ± 0.47

Figure 2. The results of the study of the level of homocysteine in the blood of experimental rats with simulated antiphospholipid syndrome and its correction