

Impact of nitric oxide synthesis modulators on the state of humoral immune system in experimental antiphospholipid syndrome

Nataliia Mekhno¹, Olha Yaremchuk¹

¹ Medical Biochemistry Department I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

Corresponding author: Nataliia Mekhno (mekhno_nyar@tdmu.edu.ua)

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Abstract

Background: Antiphospholipid syndrome is an autoimmune disease of multiple venous and/or arterial thrombosis and/or pregnancy loss. Oxidative stress only enhances the body's immune response. In pathological conditions, the formation of nitric oxide is disrupted, which can be manifested by vasoconstriction, increased coagulation, and endothelial dysfunction.

Objective: The aim of the research was to study the level of immunoglobulins and circulating immune complexes (CICs) in experimental antiphospholipid syndrome and its correction with L-arginine and aminoguanidine.

Materials and methods: Antiphospholipid syndrome was modeled on white female BALB/c mice. L-arginine (25 mg/kg) and aminoguanidine (10 mg/kg) were used for its correction. The content of immunoglobulins and CICs was studied.

Results: It was established that the level of immunoglobulins (Ig) and circulating immune complexes increased in the group of animals with antiphospholipid syndrome compare to the control. The levels of IgA and CICs decreased significantly, and the levels of IgM and IgG did not change in the mice with antiphospholipid syndrome and L-arginine correction. In cases of aminoguanidine administration, decreased IgM and IgG levels and no significant decrease in IgA and CICs was evidenced compare to the animals with antiphospholipid syndrome. In cases of using a combination of L-arginine and aminoguanidine agents, only IgM did not change, all other parameters decreased compare to the animals with APS.

Conclusion: The parameters of the humoral immunity in female mice with experimental antiphospholipid syndrome increase. The level of immunoglobulins and circulating immune complexes decrease depending on the chosen correction agents or their complex administration. Thus, L-arginine and aminoguanidine has a positive effect on various immunity responses by decreasing the negative impact of pathobiochemical alterations.

Keywords

immunoglobulins, circulating immune complexes, APS

Introduction

Constantly circulating autoantibodies to phospholipids are characteristic features of antiphospholipid syndrome (APS). This is a serological sign of APS. An increased con-

centration of cardiolipin antibodies is found in approximately 6–18% of cases, mainly in young women. The ratio between the incidence of primary APS in women and men is 4:1, and of secondary APS – 7:1. In 31–52% of cases an in-

creased number of antibodies to cardiolipin in the patients with APS leads to development of clinical manifestations (Dieudonné et al. 2021; Fartushok and Fartushok 2022).

This issue cannot be studied adequately due to relatively low incidence of APS. Accordingly, there is a lack of reliable information. APS develops not only in adults, but also in children: from the neonatal period to the adolescence. Catastrophic antiphospholipid syndrome with rapid progression of multiorgan thrombosis and microangiopathy occurs in childhood as well. There is no information on cases of APS in children in Ukraine, but it has been registered in Canada and Germany. A high level of antiphospholipid (aPL) antibodies in children is usually asymptomatic. It may be caused by frequent infectious diseases in childhood (Sorice et al. 2016; Dudnyk et al. 2018).

Characteristic features of APS are a high tropism of blood vessels and thrombus. Non-inflammatory thrombogenic vasculopathy of different vessel calibre is the main vascular manifestation. Systematic recurrent thrombosis is a typical in APS. The level of aPL antibodies rapidly increases with B-lymphocyte activation. Antiphospholipid antibodies are found in the blood of 70% of patients with systemic lupus erythematosus. In systemic scleroderma, Sjogren's disease and rheumatoid arthritis aPL antibodies are revealed much less frequently. Antiphospholipid antibodies are found in the blood of patients with cancer, autoimmune thrombocytopenic purpura, acute and chronic infectious processes of bacterial, parasitic or viral origin, after taking oral contraceptives and psychotropic medications. Antibodies cause organ and system dysfunction due to the associated pathobiochemical mechanisms of hemostasis and immune response (Yaremchuk and Posokhova 2019; Yaremchuk 2020; Dieudonné et al. 2021).

High titres of lupus anticoagulant, anticardiolipin antibodies, antibodies to beta-2-glycoprotein I (β 2GPI) class IgG and/or IgM are determined in cases of APS (Álvarez-Rodríguez et al. 2018; Pérez et al. 2018; Misasi et al. 2020). However, the phospholipid-binding protein β 2GPI, as well as anionic phospholipids, phospholipid-protein complexes: vimentin, protein C, annexin II, annexin V, lysobisphosphatidic acid, sulfatides, oxidized low-density lipoproteins, etc., is the target for antiphospholipid (aPL) antibodies (Shemer et al. 2019). Such a variety of aPL antibodies leads to activation of platelets, endothelial cells and causes thrombosis and obstetric complications (Radice and Pattanaik 2018; Shemer et al. 2019; Misasi et al. 2020; Jarne-Borràs et al. 2022). Even one episode of macrothrombosis or miscarriage is enough for clinical criteria of APS. The diagnosis of APS is established according to the Sydney classification 2006 and modified by the Sapporo criteria (Fartushok TV and Fartushok NV 2022). The tests not included in these criteria are also taken into account for laboratory confirmation of APS: assessment of the level of antibodies to prothrombin, phosphatidylserine, phosphatidylserine-prothrombin complex, annexin A5 and A2, phosphatidylglycerol, phosphatidylcholine and IgA to cardiolipin and β 2GPI (Jarne-Borràs et al. 2022).

Hypercoagulation that develops after coronavirus disease has drawn the attention to formation of aPL antibodies and its clinical significance yet again (Dieudonné et al. 2021). Antibodies are produced by B-cells of the body in response to a viral trigger. However, its degree of pathogenicity has been studied insufficiently (Zuo et al. 2020). Hypoxic vascular occlusion, direct activation of immune and vascular cells, and cytokine storm are the mechanisms that lead to arterial or venous micro- and macrothrombosis in cases of severe acute respiratory syndrome (SARS-CoV-2). Lung histopathology in the patients with severe COVID-19 confirms the signs of acute respiratory distress syndrome and blockage of small blood vessels (Zuo et al. 2020; Schasfoort et al. 2021). Studies on the effectiveness of treatment for humoral immunity has not provided any positive results. It is contradictory since such treatment for other autoimmune diseases is successful (Dieudonné et al. 2021).

Nitric oxide is a free radical; it is a part of signalling pathways to the target cells; it takes part in physiological and pathological processes. It is formed from the amino acid L-arginine by enzymatic oxidation on account of NO synthase. There are several isoforms of NO: constitutive NO-synthase (cNOS), which is present in tissues; inducible NO-synthase, which is activated in some time after the impact of cytokines. There are also neuronal and endothelial forms of the constitutive type; and a macrophage form of the inducible type (Asghari et al. 2019). FAD, FMN, NADPH, heme, calmodulin and cofactor Tetrahydrobiopterin (BH4) are crucial for continuous functioning of NO synthase. Nitric oxide is produced by Ca^{2+} -independent iNOS, which in pathological conditions synthesizes it in significantly larger quantities; L-arginine in such cases is scarce as a substrate for functioning of Ca/calmodulin-dependent cNOS. As a result, superoxide radical is produced instead of NO. When nitric oxide interacts with the superoxide radical (O_2^-), the hydroxyl anion radical (OH^-) and peroxynitrite (ONOO^-) are produced. Peroxynitrite under physiological conditions is a stable compound, but in pathology it rapidly decomposes and damages DNA, inhibits enzymatic systems, and causes cell death (Strutynska et al. 2019).

The use of L-arginine as a dietary supplement promotes vasodilation. Nitric oxide activates soluble guanylate cyclase expressed in the cytosol of vascular wall smooth muscle cells (Klinger and Kadowitz 2017). However, there is an "arginine paradox": the more L-arginine is supplied, the more it is broken down into citrulline and urea by arginase (Dioguardi 2011). Instead, arginase inhibition promotes vasodilation (Klinger and Kadowitz 2017). These dual properties of L-arginine are another reason for using it in our study.

Aminoguanidine is one of the substances we used for APS correction. It is an inhibitor of inducible NO-synthase (iNOS) (Asghari et al. 2019). iNOS produces large amounts of nitric oxide in response to certain biochemical irritants. Production of nitric oxide by inducible NO-synthase is 1000 times higher than its production by

endothelial NO-synthase (Ahmad et al. 2018). iNOS is a pathological isoform of NO-synthase, which is activated mainly by pro-inflammatory cytokines (Vashchenko 2021). NO is hyperactively produced by iNOS; it is a free radical that causes destructive and inflammatory damage to cells. Aminoguanidine inhibits formation of glycation end products by reacting with the carbonyl groups of proteins; thus, it suspends body aging, prevents vessel walls thickening, reduces cataracts progression, age-related thickening and skin yellowing, and prevents immune impairment (Ahmad et al. 2018).

Characteristic feature of autoimmune diseases is production of autoantibodies that have a toxic effect on cells. To compare circulating immune complexes (CICs) have a much greater damaging effect (Álvarez-Rodríguez et al. 2018).

The parameters of humoral immunity: immunoglobulins and CICs in cases of antiphospholipid syndrome can both increase and decrease. This proves reduced immunological tolerance. Data from different sources are not consistent that causes controversy and the need for further studies.

Materials and methods

The research was conducted on female BALB/c mice, which were kept on a standard vivarium diet. The experiments were performed following the principles of bioethics according to the General Ethical Principles of Animal Experiments, adopted by the First National Congress on Bioethics (Kyiv, 2000) and comply with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986), and the European Union Directive 2010/10/63 EU on animal experiments.

APS was modeled by intramuscular injections of cardiolipin (Sigma, USA) 4 times (30 µg per injection) with a 14-day interval (Zaichenko et al. 2011). To increase the efficiency of the immune response for the first injection, it was emulsified in 75 µl of Complete Freund's Adjuvant; the other – with Incomplete Freund's Adjuvant. Antiphospholipid syndrome developed in 2 weeks after the last cardiolipin injection.

The experimental animals were divided into 5 groups: the 1st (control) group – the intact animals; the 2nd group – the animals with APS; the 3rd group – the animals administered with L-arginine hydrochloride (L-Arg) (Sigma, USA, 25 mg/kg); the 4th group – the animals administered with aminoguanidine (AG) (Khimlaborreaktyv, Ukraine, 10 mg/kg); the 5th group – the animals treated with a combination of L-arginine and AG. The doses of corrective substances for mice were calculated according to the species sensitivity coefficient (Yaremchuk and Posokhova 2019). The control group of animals was injected with identical volumes of 0.9% sodium chloride. The animals were taken out of the experiment by 10 mice in each group under thiopental-sodium anaesthesia in 10 days after APS

development (intraperitoneal injection of 1% solution at the dose of 50 mg/kg of animal weight).

The content of immunoglobulins of class A, M and G in blood serum was evaluated by the immunoenzyme analysis. Reagent kits eBioscience, Inc (USA) were used on the STAT-FAX analyser according to the manufacturer's instructions. The number of immunoglobulins was assessed in g/L.

The content of circulating immune complexes was evaluated by the method of Yu.A. Grinevich (Grinevich and Alferov 1981) via precipitation reaction with polyethylene glycol-6000.

The digital data were statistically processed by STATISTICA 10 program. The figures were compared using the Mann-Whitney U-test to determine possible differences. The differences were statistically significant at $p \leq 0.05$.

Results and discussion

Antibodies are multifunctional regarding elimination of pathogens from the body. They distinguish and capture the antigen, help lymphocytes and macrophages in its presentation, cause damage to mast cells, split the cells with specific antigenic substances, facilitate opsonization through the complement system (Pérez et al. 2018). Their interaction with the antigenic determinant is crucial as well as their part in complement retention, implementation of opsonization, induction of cytotoxicity and immunoregulation, etc. Antibodies accelerate extinguishing of foreign invaders through its engulfment by enzyme systems after linking of an antibody to an antigen (Dieudonné et al. 2021).

Immunoglobulins are produced by plasma cells (mature B cells) in response to antigens of various geneses. Plasma cells produce antibodies that neutralize foreign antigens during its initial entry into the body (Demkovych et al. 2021, 2022). The immune system is able to recognize and remember antigens so that in the future the immune response is accelerated and production of antibodies is increased. This reduces the chance of reinfection and disease progression (Mozaffari et al. 2018).

Increased formation of immunoglobulins was established in the animals with APS compare to the controls.

In our research the content of IgA in the group of animals with APS increased by 167% compare to the control group (Fig. 1). In the animals treated with L-arginine, the amount of immunoglobulin A decreased by 66%; in the group of animals treated with aminoguanidine – by 10%; and in the group of L-arginine + aminoguanidine it decreased by 37% compare to the APS group.

Immunoglobulins of IgA class are crucial in the immune protection of mucous membranes. There are secretory immunoglobulin A (sIgA) and serum immunoglobulin A (IgA). The level of sIgA formed on the mucous membranes prevails among all other types of antibodies. It is significantly less in blood serum. Mostly, immunoglobulins of M and G class (IgM and IgG) circulate there (Bayram et al. 2019).

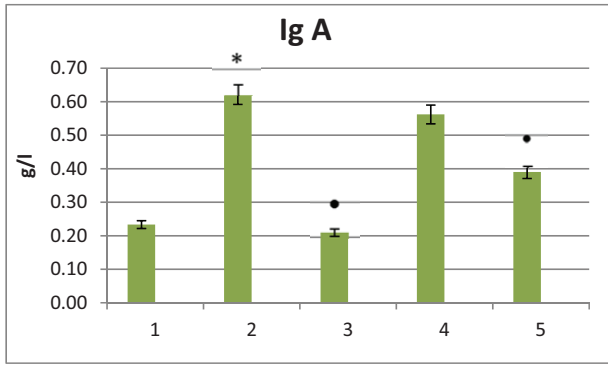


Figure 1. The level of Ig A in the blood serum of mice with APS and using correction agents. **Notes:** group 1 – control group; 2 – APS; group 3 – APS + L-arginine; group 4 – APS + aminoguanidine; group 5 – APS + L-arginine + aminoguanidine. * $P < 0.05$ compare to the control group; • $P < 0.05$ compare to the group of animals with APS.

IgA level decreases the most in the group of L-arginine correction since it is a substrate for synthesis of nitric oxide (NO) that effects regulation of immune processes and some pro- and anti-inflammatory factors (Yaremchuk 2020). Defense of respiratory tract, genitourinary system and gastrointestinal organs from infections is the main function of IgA. Its level also increases in cases of autoimmune diseases. Besides, IgA has the highest rate of synthesis in the body among other globulins (Mozaffari et al 2018).

In our research the level of immunoglobulin M increased sharply in the animals with APS in more than 2 times (211%) compare to the control (Fig. 2) as IgM was an acute phase protein that indicated to oxidative stress, apoptosis and necrosis of cells as a consequence of APS development. This parameter increased within 1 week. In the groups of animals with L-arginine and aminoguanidine administration immunoglobulins M slightly decreased (by 23% and 37%) compare to the group of animals with APS. In the group of animals administered with combined corrective substances there were no significant changes compare to the animals with APS.

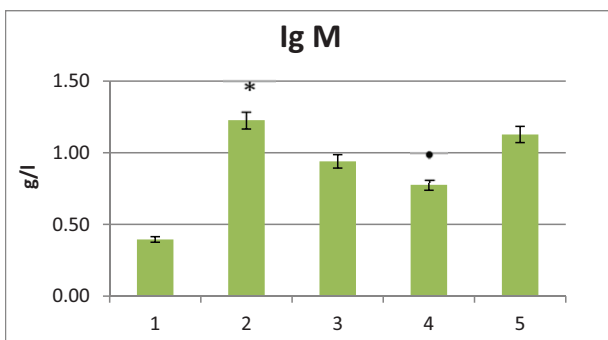


Figure 2. The level of immunoglobulin M in the blood of mice. **Notes:** group 1 – control; group 2 – APS; group 3 – APS + L-arginine; group 4 – APS + aminoguanidine; group 5 – APS + L-arginine + aminoguanidine. * $P < 0.05$ compare to the control group; • $P < 0.05$ compare to the group of animals with APS.

Serum IgM in norm is the first to provide body immune response to damaging factors. IgM binds to the complement the most efficiently contributing to neutralization of viruses and agglutination of bacteria. Immunoglobulins of this class are produced and increase during the first weeks after the impact of pathogenic factors; then they gradually decrease and are replaced by IgG (Schasfoort et al. 2021).

Increased levels of IgM and IgA are evidence of phagocytosis activation. Increased IgM level in systemic disease indicates the onset of reagin syndrome with subsequent development of hypersensitivity vasculitis (Gaiduchok 2020).

In our research IgG initiated synthesizing with disease progress. They were revealed in the blood on the 14th-16th day from the antigenic activation of B cells and reached the peak on the 21st-24th day. The level of IgG increased by 129% in the group of animals with APS compare to the control (Fig. 3); the level of IgG in the group of animals with APS + L-arginine did not change compare to the group of animals with APS. In the group of animals administered with aminoguanidine and the combination of L-arginine + aminoguanidine, the level of IgG decreased by 42% and 64% compare to the APS group.

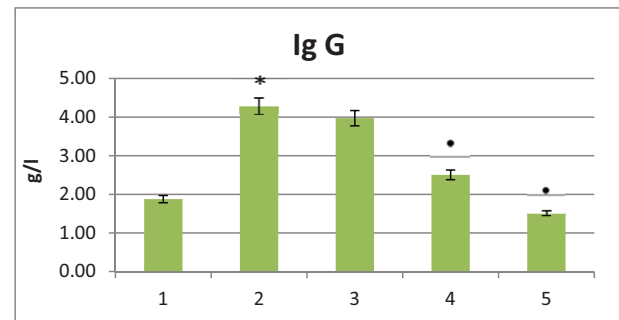


Figure 3. Level of Ig G in blood serum of mice. **Notes:** group 1 – control; group 2 – APS; group 3 – APS + L-arginine; group 4 – APS + aminoguanidine; group 5 – APS + L-arginine + aminoguanidine. * $P < 0.05$ compare to the control group; • $P < 0.05$ compare to the group of animals with APS.

Such changes in the IgG levels is the evidence of a reduced destructive effect of peroxynitrites due to blocking of inducible NO synthase. IgG make up almost 80% of all immunoglobulins in circulating blood. Due to a low molecular weight they can penetrate into the extravascular space where they provide defensive function (for example, it penetrates the blood-brain barrier and provides passive immune defense of the fetus and the newborn). IgG are in the circulatory system both in a free state and in the “antigen-antibody” complex binding to the defective cells. IgG provide long-term immunity after illnesses (Vashchenko 2021).

Thus, it has been established that activated B-lymphocytes produce a large number of immunoglobulins. In mice body the antibodies are produced in the spleen, which is responsible for a specific immune response to antigens circulating in the blood (Boichuk et al. 2020).

Antigens or autoantigens neutralization is one of the important tasks of immunoglobulins. As a result, CICs are

formed; they are subsequently eliminated from the bloodstream to support immunobiological homeostasis. However, occasionally, CICs adhere to the blood vessel walls and the basement membranes of internal organs. The products of oxidative reaction and proteases produced by activated neutrophils and resident macrophages cause secondary tissue damage in response to subendothelial accumulation of immune complexes. Therefore, evaluation of CICs in the blood serum of mice with antiphospholipid syndrome is important (Krynytska 2013).

In our research the level of CICs in the animals with APS increased in nearly 3.5 times compare to the control (Fig. 4). The level of CICs in the animals administered with aminoguanidine decreased insignificantly compare to the animals with APS. It proved to have no impact of aminoguanidine on the CICs level. In the groups of animals administered with L-arginine and L-arginine + aminoguanidine the level of circulating immune complexes decreased almost to the level of intact animals on account of L-arginine – by 74% and 76%, respectively, compare to the APS group.

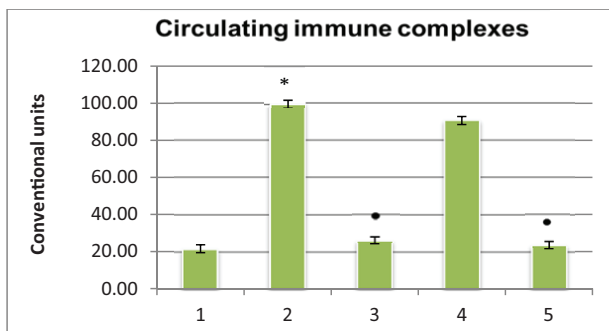


Figure 4. The level of circulating immune complexes in the blood serum of mice (%). **Notes:** group 1 – control, group 2 – APS; group 3 – APS + L-arginine; group 4 – APS + aminoguanidine; group 5 – APS + L-arginine + aminoguanidine. *P < 0.05 compare to the control group; •P < 0.05 compare to the group of animals with APS.

Circulating immune complexes (CICs) are an “antigen-antibody” complex in combination with complement components C3, C4, C1q. CICs are usually destroyed in the bloodstream under normal course of immune response. However, in an excessive number of antigens, IgM and part of the C1q complement, CICs may be accumulated in the tissues that causes increased adhesion and aggregation of platelets in the microcirculatory bed with

subsequent disturbance of blood circulation and necrosis (Levchuk 2015). Such pathological responses take place when the level of CICs prevails over its destruction or in phagocytic disturbance.

The CICs level is one of the indicators of the immune system and autoimmune symptoms. The study has proved that the level of CICs increases consequently with the level of immunoglobulins. This proves development of a humoral response to antigens and progression of autoimmune disease with development of vascular, pulmonary or renal complications (Gaiduchok 2020). The increased level of CICs is caused not only by its increased production but also by its impaired elimination. Phagocytic system is responsible for neutralization and extraction of immune complexes. In normal functioning of mononuclear and tissue phagocytes, increased level of CICs does not cause its accumulation in the organs and bloodstream (Schasfoort et al. 2021). However, any failure in phagocytosis leads to severe outcomes. Absorption of O₂, glucose consumption and lactic acid accumulation increase during phagocytosis of immune complexes. This is accompanied by oxidative stress and wasting a large amount of energy. Medium CICs are the most pathogenic because when circulating in the bloodstream for a long time they activate a sequence of damaging processes. When interacting with neutrophils and lymphocytes, medium CICs change their functional state, block Fc receptors of B cells that causes decreased formation of antibodies (Mozaffari et al 2018).

Conclusions

According to the studies of the humoral immunity of female BALB/c mice with modeled experimental antiphospholipid syndrome, an increase in immunoglobulins and circulating immune complexes has been established. Possibly, it is caused by formation of autoantibodies, progression of pro-oxidant reactions, activation of phagocytosis system with further apoptosis or tissue ischemia development. A decrease in the antibodies production is evidenced in some experimental groups of animals in cases of L-arginine administration as a donor of nitric oxide synthesis, and aminoguanidine, an inducible synthase blocker. These effects are presumably caused by positive impact of the nitric oxide, which is produced mostly on account of constitutive synthase, as well as additional substrate for its synthesis.

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