INDEXES OF NITRIC OXIDE SYSTEM IN EXPERIMENTAL ANTIPHOSPHOLIPID SYNDROME

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Received: 11 November 2019; Accepted: 21 January 2020

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antibodies to negatively charged membrane phospholipids (aPL). Endothelial dysfunction is one of the most dangerous APS manifestations followed by thrombosis, placental insufficiency and often foetal death due to circulatory disorders in placenta blood vessels. It is established that synthesis and bioavailability of nitric oxide (NO) in the endothelium are impaired at APS, but the role of NO system in pregnancy failure at this pathology remains ambiguous. The aim of this research was to estimate the indexes of the nitric oxide system in animals with an experimental antiphospholipid syndrome before pregnancy and on the 18th day of pregnancy, without treatment and under treatment with nitric oxide synthesis modulators (L-arginine and aminoguanidine). In the blood serum and liver of the BALB/c mice with experimental APS, the content of eNOS and iNOS and the level of NO2− and NO3− with the use of Gris reagent were determined before pregnancy and on the 18th day of pregnancy. The data obtained indicate the relative inefficient NO production by eNOS and NO hyperproduction by iNOS in the blood serum and liver of mice in the pathogenesis of experimental APS. Thus, in mice with APS before pregnancy and on the 18th day of the pregnancy, the eNOS content and NO2− level were decreased while the iNOS content and NO3− level were increased compared to the indexes in the control animal group. L-arginine administration to the animals with APS at the follow-up periods resulted in an increased eNOS content and NO2−, NO3− levels in blood serum and liver with the simultaneous decrease in iNOS content in the liver as compared to indexes in untreated mice with APS. The combined use of L-arginine and selective iNOS inhibitor aminoguanidine caused a significant increase in eNOS content and a decrease in iNOS content followed by normalization of NO2− and NO3− levels in blood and liver of mice with experimental APS before pregnancy and on the 18th day of pregnancy compared to untreated mice with APS.

Keywords: antiphospholipid syndrome, nitric oxide, eNOS, iNOS, L-arginine, aminoguanidine.

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antibodies to negatively charged membrane phospholipids (aPL) in the blood [1-4]. Antibodies and cell membrane phospholipids interaction causes conformational and metabolic changes in membranes, cell dysfunction and blood clotting disorders. APS is manifested by vessels thrombosis of different sizes and localizations, stroke, obstetric pathology or thrombocytopenia [4-6]. In the case of gestational APS, the antibodies are formed predominantly to cardiolipin that leads to placental arterial thrombosis, which is liable to cause intrauterine foetal death or premature birth [7, 8]. Hypercoagulation in the plasmic haemostasis component, which develops under the influence of aPL, is followed by thrombosis in the microvasculature, placental insufficiency, chronic hypoxia and often foetal death due to acute circulatory disorders in placenta blood vessels [9, 10].

APS occurs in 27-42% cases among patients with refractory pregnancy loss. The foetal demise takes place in 90-95% of women with aPL without adequate treatment [10, 11].

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Practitioners still have difficulties in diagnosing and prescribing treatment for patients with APS [4]. Laboratory diagnostics for this disorder implies lupus anticoagulant testing and cardiolipin antibodies test, less often the determination of the level of specific antibodies to phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, as well as beta-2-glycoprotein-I, annexin V, prothrombin [2, 4]. Antiphospholipid antibodies are recognized not only by phospholipids but also by the blood plasma proteins bound to the anionic surface. Plasma proteins are cofactors in aPL and endothelial cell’s phospholipids interaction. The dysfunction of blood vessels endothelium is known to be followed by vasoconstriction and thrombus development [12-14]. Endothelial dysfunction combined with recurrent thrombosis is one of the main and most dangerous manifestations of APS [15, 16]. It is also established that synthesis and bioavailability of nitric oxide (NO) in the endothelium at APS are impaired [6, 12, 17].

The enzyme responsible for NO biosynthesis is the nitric oxide synthase (NOS) which catalyzes the reaction of L-arginine, NADPH and oxygen conversion to the free radical NO, L-citrulline and NADP and exists in three isoforms: endothelial (eNOS, 1.14.13.39), inducible (iNOS, 1.14.13.39), and neuronal (nNOS, 1.14.13.39) [18].

Elucidation of NO system implication into APS development as well as the search for effective treatment of this pathology is an urgent and social issue [3, 10, 19].

It is known that NO is involved in the regulation of the vascular tone and blood coagulation. NO synthesized by eNOS regulates numerous physiological processes, including thrombus formation, leukocyte adhesion, vascular permeability and blood cell migration [20]. The vascular effects of NO are: direct vasodilation, which depends on blood flow and is mediated by specific receptors; indirect vasodilation by inhibition of angiotensin vasoconstrictive effects and sympathetic innervation; antithrombotic activity by suppression of platelet adhesion to endothelium [21]. In addition, NO has an anti-inflammatory effect due to inhibition of leukocytes adhesion to the vascular endothelium and interception of superoxide anion and antiproliferative effect on unstriated muscle cells [22]. Vasoconstriction, thrombosis, inflammatory processes, hypertrophy and vascular stenosis occur when NO production or bioavailability is inhibited. Therefore the activity of endothelial NO synthase, which controls NO formation from L-arginine, is crucial for the vascular tone maintenance and thrombus development prevention [6, 20, 21, 23].

It is proved that aPL-induced changes in endothelial cells are crucial in cell adhesion to endothelium. In studies on the cultures of human, bull and mouse endothelial cells, it was shown that stimulation of monocyte adhesion by aPL was accompanied by the decrease of NO bioavailability [13].

A dual effect of NO on myometrium contractile activity during pregnancy was demonstrated in the model of APS induced by lipopolysaccharides. NO was shown to be a uterine relaxant when its concentration was low, but significant decrease in NO production led to abortion and premature birth. On the other hand, hyperproduction of NO mediated by iNOS increased uterine contractions and the risk of miscarriage [17]. Despite the fact that the pathogenetic aspects of APS are sufficiently studied, there are only a few studies on the involvement of NO in the biochemical mechanisms of vascular complications at APS.

The contradictory of the available information on the role of NO system in the development of pregnancy failure at APS and the data on the effectiveness of NO precursors in reducing of this pathology manifestations necessitates further study of the role of this system in obstetric APS as well as the development of the methods for correction of its complications.

The aim of the research was to estimate the indexes of nitric oxide system in the blood and liver of pregnant animals with an experimental antiphospholipid syndrome before pregnancy and on the 18th day of pregnancy, without treatment and under treatment with nitric oxide synthesis modulators (L-arginine and aminoguanidine).

**Materials and Methods**

The studies were conducted using female BALB/c mice, which were kept on a standard vivarium diet. The experiments were performed following the principles of bioethics according to “Ethical Guidelines for the Use of Animals in Research” adopted at the First National Congress on Bioethics (Kyiv, 2001) and consistent with the provisions of the “European convention for the protection of vertebrate animals used for experimental and other scientific purposes” (Strasbourg, 1986) and European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

APS was simulated by administrating cardiolipin (Sigma, USA) intramuscularly, four injections...
at 14-day intervals (30 μg per 1 injection) [24]. To improve the immune response effectiveness, cardiolipin was emulsified in 75 μl of complete Freund’s adjuvant for the first injection; the following injections were with the incomplete Freund’s adjuvant. APS was developed in 2 weeks after the last cardiolipin injection. Microprecipitation reaction with a cardiolipin antigen, using the test-system “Cardiolipin antigen for microprecipitation reaction” (Biolik, Ukraine), was carried out to confirm APS development [24].

Experimental animals (female BALB/c mice aged 2-3 months, 25-30 g weight, which were kept on a standard vivarium diet, in cages in natural light, temperature 20-22 °C; access to food and water was free) were divided into 10 groups: 1, 2 (control) – animals without APS; 3, 4 – animals with experimental APS; 5, 6 – animals with APS injected with L-arginine hydrochloride (Sigma, USA, 25 mg/kg); 7, 8 – animals with APS injected with aminoguanidine (Khimlabororreaktyv, Ukraine, 10 mg/kg); 9, 10 – animals with APS injected with L-arginine in combination with aminoguanidine. L-arginine and aminoguanidine were administered intraperitoneally once a day, repeatedly within 27 days after APS development. The identical volumes of the solvent were administered intraperitoneally to the animals of the control group.

The animals of groups 1, 3, 5, 7 and 9 were removed from the experiment using thiopental sodium anaesthesia (intraperitoneal injection of 1% solution, 50 mg/kg of animal weight) on the 10th day after APS confirmation. Female mice of the groups 2, 4, 6, 8, 10 were mating with male mice (in the ratio of 1 male to 3 females) in 10 days after the beginning of APS development. The identical volumes of the solvent were administered intraperitoneally to the animals of the control group.

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The results obtained comply with the data on eNOS inhibition under aPL effect that was accompanied by decreased NO synthesis, increased adhesion of leukocytes to the blood vessels endothelium and thrombosis [13].

A significant increase in inducible NOS isoforms activity that may be caused by increased production of proinflammatory cytokines IL-1β, IL-6 and TNF-α at APS was evidenced [29, 30].

In our research, it was established that in the animals of groups 3 and 4 with APS, the content of NOx decreased in blood serum (by 26 and 45%) and liver (by 13 and 68%), and the level of NO3- increased in blood serum (by 75 and 60%) and liver (by 20 and 95%), respectively as compared to the control (Table 1, 2).

The data of our study on the decrease of NO2- concentration and increase of NO3- concentration at APS comply with the data of other researches [3, 12]. In addition, it was established that at primary APS, the content of NO3- decreases depending on the aPL titres and the amount of vascular thrombosis [12].
High serum nitrate levels in the patients with APS and in the experimental models are probably caused by an increased content of iNOS-dependent NO. The increase in NO₃⁻ level at APS may be a consequence of an increase in the rate of peroxynitrite generation. According to E. Nasonov [3], increased nitrate concentration and recurrent thrombosis in the patients with APS may be evidence of endothelial dysfunction (endothelium-dependent vasodilation) that is typical for this pathology and is apparently associated with oxidative stress development. The fate of NO in biological systems is controlled by three main processes: NO diffusion and intracellular consumption, autooxidation to nitrogen (III) oxide (N₂O₃), and reaction with superoxide (O₂⁻), which yields peroxynitrite. Peroxynitrite subsequently may react with carbon dioxide and give rise to various reactive oxygen species and reactive nitrogen species: nitrogen dioxide (NO₂), carbonate radical (CO₂⁻), and hydroxyl radical (·OH) [31].

It was established that endothelial dysfunction under conditions of oxidative stress is associated with the disturbance of NO bioavailability caused by the production of O₂⁻, which rapidly binds and inactivates NO [3, 23]. On the other hand, a decrease in eNOS activity at APS is also evidenced [32] that was confirmed by the results of our research. As a result, a paradoxical situation could take place in the intrahepatic microcirculation: the flowing blood contains NO; at the same time, a rapidly increased portal flow creates additional pressure on the sinusoidal walls that requires further activation of eNOS and nitric oxide production in the sinusoidal endothelial. However, this does not happen. Apparently, the increased NO concentration in the blood, flowing by the feedback mechanism, rapidly inhibits eNOS expression in the liver. At these conditions, vasodilation deficiency, induced by eNOS, is followed by reducing of sinusoids diameter and increasing of the overall portal vascular resistance. Thus, despite NO hyperproduction, its content at the level of intrahepatic microcirculation could be insufficient [33] that was confirmed by the decrease in the NO₂⁻ content in the liver detected in our research.
Fig. 3. eNOS content in the liver of mice with APS before pregnancy and on the 18th day of pregnancy in case of L-arginine and aminoguanidine administration: 1, 2 – control; 3, 4 – antiphospholipid syndrome (APS); 5, 6 – APS + L-arginine; 7, 8 – APS + aminoguanidine; 9, 10 – APS + L-arginine + aminoguanidine. 1 ml contains 1×10^6 liver cells. *P < 0.05 compare to the control group; **P < 0.05 compare to the group of animals with APS; •P < 0.05 compare to the group of intact animals on the 18th day of pregnancy; ••P < 0.05 compare to the group of animals with APS on the 18th day of pregnancy

Fig. 4. eNOS content in the blood serum of mice with APS before pregnancy and on the 18th day of pregnancy in case of L-arginine and aminoguanidine administration: 1, 2 – control; 3, 4 – antiphospholipid syndrome (APS); 5, 6 – APS + L-arginine; 7, 8 – APS + aminoguanidine; 9, 10 – APS + L-arginine + aminoguanidine. *P < 0.05 compare to the control group; **P < 0.05 compare to the group of animals with APS; •P < 0.05 compare to the group of intact animals on the 18th day of pregnancy; ••P < 0.05 compare to the group of animals with APS on the 18th day of pregnancy.

Table 1. NO_2^- and NO_3^- content in the blood serum and liver of mice with APS in case of L-arginine and aminoguanidine administration (M ± m, n = 10)

| Group of animals (experiment series) | Index | Blood serum | Liver |
|-------------------------------------|-------|-------------|-------|
|                                     |       | NO_2^-, μmol/l | NO_3^-, μmol/l | NO_2^-, μmol/kg | NO_3^-, μmol/kg |
| Control                             |       | 2.23 ± 0.10  | 11.22 ± 0.48 | 1.55 ± 0.05  | 9.17 ± 0.35  |
| APS                                 |       | 1.65± 0.15   | 19.65 ± 0.97 | 1.35 ± 0.05  | 11.01 ± 0.51 |
|                                     |       | *P < 0.05    | *P < 0.001   | *P < 0.05    | *P < 0.05    |
| APS + L-arginine                    |       | 2.35 ± 0.09  | 30.50 ± 2.12 | 1.88 ± 0.05  | 15.96 ± 0.83 |
|                                     |       | *P < 0.01    | *P < 0.005   | *P < 0.001   | *P < 0.005   |
| APS + aminoguanidine                |       | 1.84± 0.10   | 8.63 ± 0.30  | 1.42 ± 0.03  | 9.46 ± 0.28  |
|                                     |       | *P > 0.05    | *P < 0.001   | *P > 0.05    | *P < 0.005   |
| APS + L-arginine + aminoguanidine   |       | 2.06± 0.05   | 12.82 ± 1.04 | 1.69 ± 0.02  | 10.20 ± 0.36 |
|                                     |       | *P < 0.05    | *P < 0.005   | *P < 0.005   | *P > 0.05    |

Notes: P – statistically significant differences compare to the control group; P_i – statistically significant differences compare to the group of animals with APS.
In our opinion, the increase in NO\textsubscript{3}– level in the blood serum, proved in our studies, is caused by increased iNOS expression in the liver. In the case of reduced substrate content, iNOS can also produce reactive oxygen intermediates and intensify destructive processes in the liver [34].

The involvement of the NO system into the pathogenesis of liver damage at APS allows suggesting the effectiveness of NO-synthases activity modulation to prevent and remove the manifestations of this pathology.

L-arginine, as the precursor in NO synthesis, is known to form H-bond networks with the heme group and amino acid residues inside NOS catalytic site, this ligand-receptor interaction profile is similar in all enzyme isoforms [18, 35].

We proved that L-arginine introduction into the animals with APS before pregnancy and on the 18th day of pregnancy was followed by the increase in the eNOS content in the blood (by 54% and 92%) (Fig. 2) and in the liver (in 1.9 and 3.7 times) (Fig. 1); iNOS content in the blood did not changed significantly \((P > 0.05)\), while in the liver it was decreased (by 21 and 26%, respectively) (Fig. 3). In animals with APS injected with L-arginine (groups 5 and 6), the increase in NO\textsubscript{2}– level in the blood serum (by 42 and 30%) and in liver (by 39 and 92%), as well as the increase in NO\textsubscript{3}– level in blood serum (by 55 and 60%) and in liver (by 45 and 19%), was detected respectively, compared to the mice with APS of groups 3 and 4 (Table 1, 2).

Aminoguanidine is structurally similar to L-arginine, it inhibits iNOS selectively and thus reduces NO formation. Aminoguanidine is shown to have antioxidant activity as a scavenger of hydrogen peroxide, hydroxyl radical and superoxide [36].

No significant changes in eNOS content in the blood and liver of mice with APS injected with aminoguanidine before pregnancy and on the 18th day of the pregnancy were observed as compared to the indexes in animals of groups 3 and 4, respectively (Fig. 1, 2).

In the case of aminoguanidine administration, the content of iNOS decreased in the blood (in 2.4 and 4.0 times) (Fig. 4) as well as in the liver (in 2.1 and 3.2 times) (Fig. 3) of animals with APS before pregnancy and on the 18th day of pregnancy, respectively. The level of NO\textsubscript{2}– did not change significantly both in blood serum \((P > 0.05)\) and in the liver of animals with APS before pregnancy, but on the 18th day of pregnancy, it decreased by 21% (Table 1, 2). The level of NO\textsubscript{3}– was also decreased both in blood serum (by 56% and 50%) and in the liver (by 14 and 44%), respectively (Table 1, 2).

Combined administration of L-arginine with aminoguanidine into the animals with APS at the follow-up periods caused a significant increase in eNOS content in blood serum (in 1.4 and 2.2 times) (Fig. 2) and in the liver (in 2.4 and 4.1 times) (Fig. 1) and a significant decrease in iNOS content in blood serum (in 3.2 and 3.8 times) (Fig. 4) and in the liver (in 2.3 and 3.3 times) (Fig. 3). These changes were

### Table 2. NO\textsubscript{2}– and NO\textsubscript{3}– content in the serum and liver of mice with APS on the 18th day of pregnancy in case of L-arginine and aminoguanidine administration \((M \pm m, n = 10)\)

| Group of animals                        | Blood serum | Liver           |
|----------------------------------------|-------------|-----------------|
|                                        | NO\textsubscript{2}–, μmol/l | NO\textsubscript{3}–, μmol/l | NO\textsubscript{2}–, μmol/kg | NO\textsubscript{3}–, μmol/kg |
| Control                                | 1.99 ± 0.08 | 9.57 ± 0.58     | 2.11 ± 0.08 | 9.72 ± 0.31 |
| APS                                    | 1.10 ± 0.07 | 15.29 ± 0.66    | 0.68 ± 0.04 | 18.94 ± 0.78 |
| \(P < 0.001\)                          |             | \(P < 0.005\)   |             | \(P < 0.001\) |
| APS + L-arginine                       | 1.43 ± 0.10 | 24.44 ± 1.51    | 1.30 ± 0.09 | 22.44 ± 0.97 |
| \(P_1 < 0.05\)                         |             | \(P_1 < 0.005\) |             | \(P_1 < 0.05\) |
| APS + aminoguanidine                   | 1.04 ± 0.04 | 7.65 ± 0.37     | 0.82 ± 0.03 | 10.55 ± 0.22 |
| \(P_1 > 0.05\)                         |             | \(P_1 < 0.001\) |             | \(P_1 < 0.001\) |
| APS + L-arginine + aminoguanidine      | 1.51 ± 0.11 | 10.82 ± 0.53    | 1.94 ± 0.11 | 11.41 ± 0.54 |
| \(P_1 < 0.05\)                         |             | \(P_1 < 0.005\) |             | \(P_1 < 0.001\) |

Notes: \(P\) – statistically significant differences compare to the control group; \(P_1\) – statistically significant differences compare to the group of animals with APS.

In our opinion, the increase in NO\textsubscript{3}– level in the blood serum, proved in our studies, is caused by increased iNOS expression in the liver. In the case of reduced substrate content, iNOS can also produce reactive oxygen intermediates and intensify destructive processes in the liver [34].

The involvement of the NO system into the pathogenesis of liver damage at APS allows suggesting the effectiveness of NO-synthases activity modulation to prevent and remove the manifestations of this pathology.

L-arginine, as the precursor in NO synthesis, is known to form H-bond networks with the heme group and amino acid residues inside NOS catalytic site, this ligand-receptor interaction profile is similar in all enzyme isoforms [18, 35].

We proved that L-arginine introduction into the animals with APS before pregnancy and on the 18th day of pregnancy was followed by the increase in the eNOS content in the blood (by 54% and 92%) (Fig. 2) and in the liver (in 1.9 and 3.7 times) (Fig. 1); iNOS content in the blood did not changed significantly \((P > 0.05)\), while in the liver it was decreased (by 21 and 26%, respectively) (Fig. 3). In animals with APS injected with L-arginine (groups 5 and 6), the increase in NO\textsubscript{2}– level in the blood serum (by 42 and 30%) and in liver (by 39 and 92%), as well as the increase in NO\textsubscript{3}– level in blood serum (by 55 and 60%) and in liver (by 45 and 19%), was detected respectively, compared to the mice with APS of groups 3 and 4 (Table 1, 2).

Aminoguanidine is structurally similar to L-arginine, it inhibits iNOS selectively and thus reduces NO formation. Aminoguanidine is shown to have antioxidant activity as a scavenger of hydrogen peroxide, hydroxyl radical and superoxide [36].

No significant changes in eNOS content in the blood and liver of mice with APS injected with aminoguanidine before pregnancy and on the 18th day of the pregnancy were observed as compared to the indexes in animals of groups 3 and 4, respectively (Fig. 1, 2).

In the case of aminoguanidine administration, the content of iNOS decreased in the blood (in 2.4 and 4.0 times) (Fig. 4) as well as in the liver (in 2.1 and 3.2 times) (Fig. 3) of animals with APS before pregnancy and on the 18th day of pregnancy, respectively. The level of NO\textsubscript{2}– did not change significantly both in blood serum \((P > 0.05)\) and in the liver of animals with APS before pregnancy, but on the 18th day of pregnancy, it decreased by 21% (Table 1, 2). The level of NO\textsubscript{3}– was also decreased both in blood serum (by 56% and 50%) and in the liver (by 14 and 44%), respectively (Table 1, 2).

Combined administration of L-arginine with aminoguanidine into the animals with APS at the follow-up periods caused a significant increase in eNOS content in blood serum (in 1.4 and 2.2 times) (Fig. 2) and in the liver (in 2.4 and 4.1 times) (Fig. 1) and a significant decrease in iNOS content in blood serum (in 3.2 and 3.8 times) (Fig. 4) and in the liver (in 2.3 and 3.3 times) (Fig. 3). These changes were
followed by normalization of $\text{NO}_2^-$ and $\text{NO}_3^-$ content both in the blood and in the liver in animals with APS before pregnancy and on the 18th day of pregnancy as compared to the mice with APS in groups 3 and 4 (Table 1, 2).

As it was shown in [37], L-arginine as the precursor of NO synthesis led to positive changes in foetalplacental perfusion in the patients with threatened premature birth that is beneficial for preserving foetal viability. The preterm birth initiation was also observed at NO synthesis inhibition by non-selective NO-synthase suppressor L-NAME. This effect was reduced by using the drugs of the progestin group, which activate inducible NO synthase and thereby increase NO synthesis compensatory [38].

Thus, the results of our research indicate the relative inefficient NO production by eNOS and NO hyperproduction by iNOS in animals with experimental APS. In the case of L-arginine introduction into the animals with APS, the increase in the eNOS, $\text{NO}_2^-$, $\text{NO}_3^-$ content in blood serum and liver and the decrease in iNOS content in the liver before pregnancy and on the 18th day of pregnancy were evidenced in comparison with animals with APS only. At aminoguanidine introduction, iNOS content and of $\text{NO}_3^-$ level in blood serum and liver decreased as compared to indexes in animals with APS. Combined administration of L-arginine and aminoguanidine caused the increase in eNOS content and the decrease in iNOS content in blood serum and liver that was followed by normalisation of $\text{NO}_2^-$ and $\text{NO}_3^-$ content in animals with experimental APS before pregnancy and on the 18th day of pregnancy as compared to untreated mice with APS.

Conflicts of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbioc hemjournal.org/wp-content/uploads/2018/12/ coi_disclosure.pdf and declare no conflict of interest.
NO$_3^–$ у сироватці крові та печінці в досліджувані терміни відносно показників групи миші з АФС. За комбінованого введення тваринам L-аргініну та селективного інгібітора іNOS аміногуаніду в сироватці крові та печінці миші з АФС до вагітності та на 18-й день вагітності порівняно з показниками групи мишей з АФС.

Ключові слова: антифосфоліпідний синдром, оксид азоту, еNOS, іNOS, L-аргінін, аміногуанідин.

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