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THE LEVEL OF NITRIC OXIDE AND ARGINASE ACTIVITY IN PATIENTS WITH ARTERIAL HYPERTENSION AND DIABETES MELLITUS DURING COVID-19

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The aim of this study was to assess the level of nitric oxide production and arginase activity in patients with arterial hypertension and type II diabetes mellitus during infection with SARS-CoV-2. The study groups included patients with arterial hypertension, patients with arterial hypertension combined with a severe course of COVID-19 and patients who, in addition to arterial hypertension and COVID-19, were suffering from type II diabetes mellitus. The volunteers without any clinical signs of diseases and normal blood pressure formed the control group. It has been established that arterial hypertension, combined with COVID-19 occurs along with reduced L-arginine, nitric oxide, superoxide dismutase activity and increased arginase activity. At the same time, the presence of arterial hypertension in patients with diabetes and coronavirus disease is accompanied by a decline in the content of L-arginine and arginase activity. Our study's results may help scientists find new pharmacological targets in the future treatment of coronavirus disease and comorbid disorders.

K e y w o r d s: L-arginine, arginase, nitric oxide, superoxide dismutase, hypertension, COVID-19, diabetes mellitus.

The coronavirus disease (COVID-19) triggered a crisis in public health security, dramatically worsening the course of many chronic diseases, including cardiovascular pathologies, leading to the death of approximately six million people worldwide [1].

An analysis of the factors associated with an unfavorable prognosis of COVID-19 shows that the damage to the respiratory system is most often combined with arterial hypertension (AH), coronary heart disease (CHD), chronic heart failure, diabetes mellitus (DM), chronic obstructive pulmonary diseases and liver damage [2-4]. Based on the results of a meta-analysis of a significant cohort of studies, it was established that people with hypertension (from 15% to 53.8%), type II diabetes (8%) and/or cardiovascular pathology (5%) were more likely than those without these comorbidities to get COVID and experience side effects [5]. Among patients with a severe course of COVID-19, hypertension was registered in 28.8% [5] and, when patients were admitted to the intensive care units, hypertension occurred in 53% of cases [6]. In another study, the most common comorbidities were hypertension – 49.7%, diabetes – 28.3% and coronary heart disease – 27.8% [7], and the presence of hypertension was associated with a 2.5-fold increased risk of severe COVID-19 [5, 8].

According to data from clinical laboratory and microbiological studies, it was established that the point of contact for SARS-CoV-2 is angiotensin-converting enzyme-2. This enzyme is present in the endothelium of cells of the alveolar epithelium and

blood vessels, cardiomyocytes, as well as the cells of the epithelium of the gastrointestinal tract [9, 10]. That is why, in 14–50% of cases during the early stage of the disease, coronavirus affects the epithelium of the alimentary canal, causing dyspepsia and diarrhea, while angiotensin-converting enzyme-2 in the alveolar epithelium and cardiomyocytes cause COVID pneumonia to develop [11]. Coronavirus infection is accompanied by the release of a significant amount of pro-inflammatory cytokines, which contribute to the development of endothelial dysfunction. This causes the imbalance of the renin-angiotensin-aldosterone system towards vasoconstriction, a pro-inflammatory reaction and the formation of systemic damage to the cardiovascular system [12].

L-arginine is involved in many metabolic processes and recent studies indicate that it may also play an important role in the development of the coronavirus disease caused by SARS-CoV-2 [13]. In addition to being a well-known vasodilator, nitric oxide (NO) is also an important mediator of blood coagulation, has antimicrobial properties and inhibits the replication of SARS-CoV-2 [14].

Therefore, the changes in concentration of L-arginine, activation of neutrophils, action of pro-inflammatory cytokines and chemokines (interleukin-1β,6,8,9 and 10, tumor necrosis factor-α, monocyte chemotactic protein-1) and stimulation of T- and B-cell responses are considered the main mechanisms of endothelium damage in COVID-19 [15, 16].

Considering that elderly patients with syntropic comorbid disorders are forming the main group of patients with COVID-19, it is expedient to assess the course of COVID-19 in patients with AH and type II DM. The aim of this study was to assess particular parameters of L-arginine metabolism in patients with AH and type II DM during infection with SARS-CoV-2.

Materials and Methods

The study included 83 patients with AH aged 28 to 84 years (mean age 59.97±1.69 years), among whom 37 were men (44.6%) and 46 were women (55.4%). All patients signed written consent to conduct a comprehensive examination by the principles of the Declaration of Helsinki and the Council of Europe Convention on Human Rights and Biomedicine. All patients were initially informed and blood samples were taken from patients upon ethical consent (Protocol No 10, 20.12.2021), with the permission of the Bio-Ethics Review Board at the Danylo Halytsky Lviv National Medical University. The patients were receiving inpatient treatment in the therapy center or intensive care in the anesthesiology department of the communal non-commercial enterprise of the First Territorial Medical Association of Lviv. To achieve the set goal, the examinees were divided into three groups: the I group included 30 patients with AH; the II group had 27 patients with AH who were hospitalized due to a severe course of COVID-19; the III group involved 26 patients who, in addition to AH and COVID-19, also had concomitant type II DM. The control group consisted of 28 volunteers without any clinical signs of diseases and normal blood pressure.

The diagnosis of AH was made in accordance with the Unified Clinical Protocol for Hypertension (2012), the Global Recommendations of the International Society of Hypertension (2020) and the European Society of Hypertension and the European Society of Cardiology for the management of patients with hypertension. The presence of COVID-19 was established using polymerase chain reaction (PCR) to detect SARS-CoV-2 RNA in the tested samples. The diagnosis of DM was established according to the consensus recommendations of the American Diabetes Association and the European Association for the Study of Diabetes, 2021.

Examination of patients and volunteers from the control group was carried out on the basis of generally accepted modern informative research methods, which included clinical (complaints, disease, life history, palpation, percussion, auscultation); somatometric; laboratory (general blood test, biochemical blood test, lipid profile, PCR to detect SARS-CoV-2 RNA); instrumental studies (echocardiography, if necessary, computed tomography of the lungs and pulse oximetry). The concentration of ferritin, procalcitonin, D-dimer and troponin were determined according to indications.

The level of L-arginine, nitrite-anion, the activity of arginase and superoxide dismutase in the blood serum of patients in all the groups were determined in the laboratory of the biochemistry department at Danylo Halytsky Lviv National Medical University. The study of the content of L-arginine in blood serum was performed using the Sakaguchi reaction [17]. First, the protein in serum samples was sedimented by the addition of an equal volume of 5% trichloroacetic acid with further centrifugation at 15,000 g for 15 min. Then 10% sodium hydroxide, 0.02% α-naphthol diluted in ethanol, 0.4% sodium hypobromite and 40% urea were added to the super-

natant and mixed well. After 20 min optical density was measured at a wavelength of 500 nm. The blank mixture contained the same quantities of reagents, but distilled water instead of supernatant. The concentration of L-arginine was measured using a calibration curve and expressed in μmol/l.

Determination of arginase activity was carried out by measuring the concentration of produced urea as described by F. Bernardi with minor modifications [18]. We performed the reaction of activation with a 10 mM manganese chloride in a 0.1 M glycine buffer (pH 9.5) for 10 min at 37°C. The next step included incubation with 0.25 M L-arginine for 30 min under the same conditions. The reaction was stopped by 10% perchloric acid, the sedimented protein was centrifugated at 12,000 g for 15 min. For the detection of urea, a mixture of concentrated sulfuric and phosphoric acids (1:3) and an equal volume of 4% isonitrosopropiophenone diluted in ethanol was added. The samples were boiled for 60 min at the temperature of 100ºC and optical density (λ = 540 nm) was measured after cooling. The blank test tube contained the same reagents and distilled water instead of supernatant. The concentration of urea was determined using a calibration curve and the activity of arginase was expressed in μmol of urea/min×mg of protein.

The concentration of nitrite anion (NO2-) (the stable product of the NO metabolism) in blood serum was determined with Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) by a method described by Kiselyk et al. [19]. For this purpose, the samples were deproteinized by the addition of 3 N perchloric acid with further centrifugation at 12,000 g for 15 min. The reaction mixture included supernatant, 3 M ammonia, 0.1 M hydrochloric acid, 10% acetic acid and Griess reagent. All reagents were prepared using distilled water. After mixing and 10 min of incubation, the optical density was measured at wavelength 550 nm. The blank solution contained the same reagents and distilled water instead of supernatant. The concentration of nitrite anion was calculated using a calibration curve and expressed in μmol/l.

To determine the activity of superoxide dismutase (SOD) superoxide radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), which contained 78 μM β-nicotinamide adenine dinucleotide (reduced form, NADH), 50 μM nitroblue tetrazolium, 10 μM phenazine methosulfate and 50 μl of plasma sample [20]. The color reaction of superoxide radicals and nitroblue tetrazolium was detected at 560 nm. SOD activity was reported as IU.

The obtained results were processed using Microsoft Excel 2010 and Statistica® 6.0 license programs (StatSoft Inc., USA). The mean (M) and standard error of the mean (m) were estimated. The probability of differences was determined by using a one-way ANOVA, followed by Tukey's post hoc test. Differences at $P < 0.05$ were considered significant.

Results and Discussion

Demographic and laboratory characteristics of the control group, patients with AH, AH and COVID-19, AH in combination with COVID-19 and DM are presented in Table.

In our study we had found that patients with AH and COVID-19 had normal level of WBC count ($7.25 \pm 0.62 \times 10^3$/μl). At the same time, in patients with AH in combination with COVID-19 and DM the level of WBC count was slightly higher and was $9.65 \pm 1.49 \times 10^3$/μl. Normal level of WBC count can be explained by the fact that, in this study, nearly 65% of the patients with COVID-19 had normal or decreased WBC counts consistent with the main characteristic of novel coronavirus pneumonia [21, 22]. In addition, the highest serum creatinine was reported in patients with AH in combination with COVID-19 and DM, it can be linked to the pathophysiology of diabetic nephropathy.

We found significant differences in the levels of L-arginine in the blood serum of patients with AH (77.10 ± 3.34 μmol/l) compared to patients with AH and COVID-19 (65.59 ± 2.83 μmol/l) and AH in combination with diabetes during COVID-19 (61.18 ± 2.59 μmol/l) (Fig. 1). At the same time, no significant differences were found between the levels of L-arginine in the blood serum of patients with AH and COVID-19 and patients with AH in combination with COVID-19 and DM (Fig. 1).

The highest activity of arginase was observed in patients with AH and COVID-19 and constituted 10.33 ± 0.42 μmol of urea/min×mg, which was reliably higher by 26% than in the control group (7.69 ± 0.25 μmol/min×mg) and by 41% in combination of AH with DM and COVID-19 (6.14 ± 0.36 μmol/min×mg), respectively.

A significant decrease in NO_2^- content was established in all groups of examined patients in comparison with the control group. No significant differences were found when comparing NO_2^- levels in patients with AH (2.66 ± 0.09 μmol/l) and in the combination of AH with COVID-19 (2.26 ± 0.11 μmol/l). In addition, no significant dif-

Table. Demographic and laboratory characteristics of the control group, patients with AH, AH and COVID-19, AH in combination with COVID-19 and DM

Characteristics	Groups				
	Control, mean ± SD (n = 28)	AH, mean ± SD (n = 30) (1)	AH + COVID-19, mean ± SD (n = 27) (2)	AH+COVID-19+DM, mean ± SD (n = 26) (3)	P
Age (years)	48.33 ± 4.16	50.20 ± 3.03	64.12 ± 2.19	66.55 ± 2.25	$P_{C-1} > 0.05$ $P_{C-2} < 0.05$ $P_{C-3} < 0.05$ $P_{1-2} < 0.05$ $P_{1-3} < 0.05$ $P_{2-3} > 0.05$
Female	12 (42.86%)	13 (43.33%)	17 (62.96%)	12 (46.15%)	–
Male	16 (57.14%)	17 (56.67%)	10 (37.04%)	14 (53.85%)	–
WBC, $\times 10^3/\mu l$	5.66 ± 1.23	6.78 ± 0.65	7.25 ± 0.62	9.65 ± 1.49	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} < 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
RBC, $\times 10^6/\mu l$	4.66 ± 0.09	4.53 ± 0.12	4.71 ± 0.11	4.82 ± 0.17	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} > 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
ESR, mm/hr	11.02 ± 0.93	8.36 ± 0.89	31.70 ± 4.57	35.67 ± 4.83	$P_{C-1} > 0.05$ $P_{C-2} < 0.05$ $P_{C-3} < 0.05$ $P_{1-2} < 0.05$ $P_{1-3} < 0.05$ $P_{2-3} > 0.05$
Hb, g/l	146.72 ± 4.06	152.64 ± 3.18	137.53 ± 4.09	141.05 ± 4.25	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} > 0.05$ $P_{1-2} < 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
Total protein, g/l	78.94 ± 2.64	81.08 ± 2.82	74.69 ± 1.98	73.4 ± 1.88	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} > 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$

T a b l e . (Continuation)

Characteristics	Groups				
	Control, mean ± SD (n = 28)	AH, mean ± SD (n = 30) (1)	AH + COVID-19, mean ± SD (n = 27) (2)	AH+COVID-19+DM, mean ± SD (n = 26) (3)	P
Sugar, mmol/l	4.65 ± 0.32	4.67 ± 0.24	5.97 ± 0.34	11.18 ± 0.94	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} < 0.05$ $P_{1-2} > 0.05$ $P_{1-3} < 0.05$ $P_{2-3} < 0.05$
AST (U/l)	24.51 ± 2.96	45.53 ± 9.03	33.53 ± 5.92	30.21 ± 3.20	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} > 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
ALT (U/l)	16.76 ± 1.86	46.77 ± 8.66	45.03 ± 9.18	28.91 ± 4.91	$P_{C-1} < 0.05$ $P_{C-2} < 0.05$ $P_{C-3} > 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
Creatinine, μmol/l	78.26 ± 2.76	100.40 ± 6.18	96.61 ± 4.41	123.72 ± 18.39	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} < 0.05$ $P_{1-2} > 0.05$ $P_{1-3} > 0.05$ $P_{2-3} > 0.05$
Urea, mmol/l	4.68 ± 0.76	5.88 ± 0.59	7.60 ± 0.81	10.09 ± 1.39	$P_{C-1} > 0.05$ $P_{C-2} > 0.05$ $P_{C-3} < 0.05$ $P_{1-2} > 0.05$ $P_{1-3} < 0.05$ $P_{2-3} > 0.05$

ferences were found when comparing NO_2^- indicators in the group of patients with AH in combination with DM and COVID-19 (2.59 ± 0.14 μmol/l) and patients who only had AH (2.66 ± 0.09 μmol/l) or AH and COVID-19 (2.26 ± 0.11 μmol/l).

The highest SOD activity was observed in the group of patients with AH, which was significantly 21 and 17% more than in patients with AH combined with COVID-19 and AH with COVID-19 and DM, respectively. Whereas no significant changes were found when comparing the SOD indicators of the studied groups with AH and COVID-19 or AH in combination with COVID-19 and DM.

Numerous clinical studies have shown that the level of L-arginine in the blood serum is reduced in patients with COVID-19 compared to healthy individuals [23]. It might lead to a decrease in the production of NO, the main endothelium-relaxing factor. Along with this, in patients with a severe course of COVID-19, an increase in the level of NO is noted

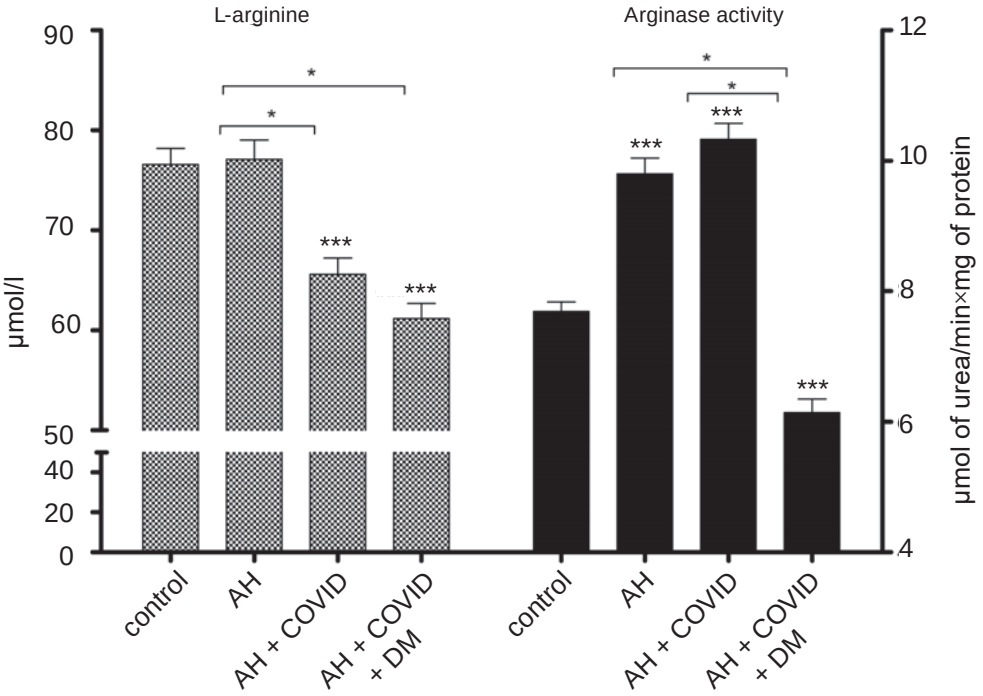

*Fig. 1. The level of L-arginine and arginase activity in the blood serum of patients in control group, and in group of patients with arterial hypertension (AH), arterial hypertension and COVID-19, arterial hypertension in combination with COVID-19 and diabetes mellitus (DM). *P < 0.05, ***statistically significant compared to control group*

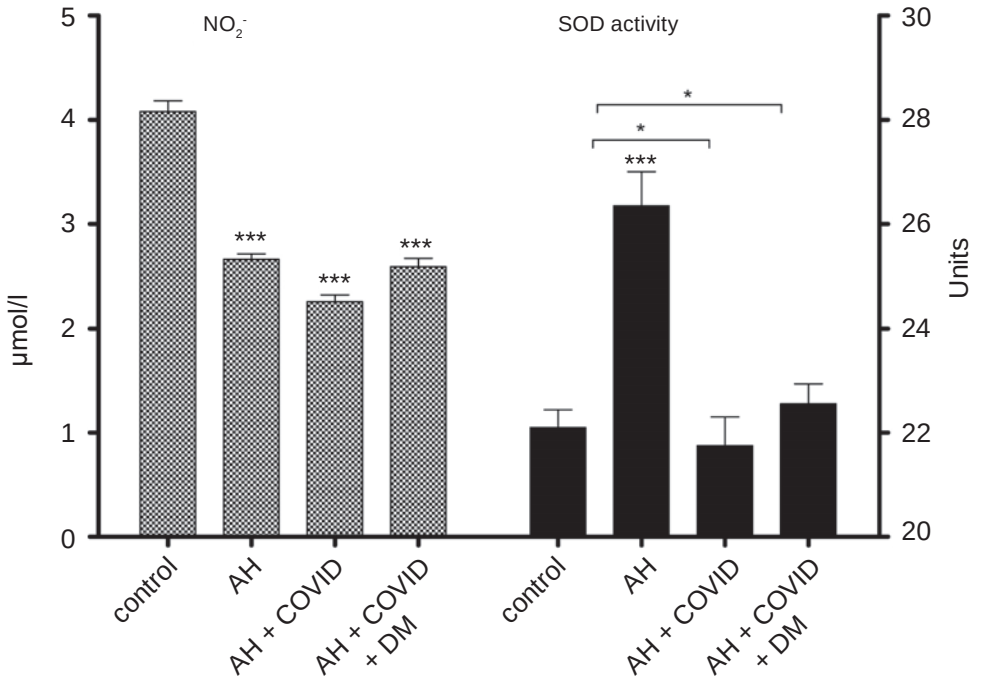

*Fig. 2. The level of nitrite anion and superoxide dismutase (SOD) activity in the blood serum of patients in control group, and in group of patients with arterial hypertension (AH), arterial hypertension and COVID-19, arterial hypertension in combination with COVID-19 and diabetes mellitus (DM). *P < 0.05, ***statistically significant compared to control group*

[24]. The reason for changes in L-arginine concentration is considered to be fluctuations in the arginase activity, which, together with NO-synthase, competes for a common substrate – L-arginine [25-27]. Although the affinity of NO-synthase for L-arginine is much higher than that of arginase, the affinity of the latter to the L-arginine is a thousand times faster than that of NO-synthase [27, 28]. The predominance of the activity of one of these two enzymes determines the function of the vascular endothelium, where NO-synthase maintains the physiological tone of the vessels, while the increase in the activity of arginase contributes to the dysfunction of the vascular endothelium [29, 30].

With COVID-19, the expression of arginase increases, which can contribute to a more severe course of the disease and a concomitant risk of respiratory and vascular complications [23]. Similarly, we expected an increasing arginase activity in patients with AH in combination with COVID-19 and DM, however, we obtained, on the contrary, a decrease in arginase activity in these patients, which may be associated with the usage of insulin for the treatment of diabetes. After all, it is known that the administration of insulin leads to the normalization of metabolic factors in patients with diabetes and a decrease in arginase activity [31]. Under the conditions of COVID-19 and DM, decreased L-arginine levels are observed, which is probably associated with endothelial dysfunction [23, 30]. A strategy of L-arginine depletion in COVID-19 has been recently proposed, based on the assumption that some stages of the SARS-CoV-2 life cycle may depend on L-arginine residues [32].

Furthermore, it has been proven that a decrease in the bioavailability of L-arginine leads to an increased susceptibility to infections [33, 34]. In addition, during COVID-19 oxidative stress and endothelial dysfunction affect circulating levels of metabolites of the L-arginine pathway through arginase activity [35, 36].

An increase in the growth of arginase activity is known to result in the consumption of L-arginine by endothelial NO-synthase (NOS) for NO production [29]. At the same time, numerous studies have proven that increased arginase activity is associated with endothelial dysfunction [36], particularly in hypertension and COVID-19 [37]. On the other hand, an increase in arginase activity leads to a decline in the bioavailability of L-arginine for NOS, thereby reducing NO production. This mechanism is an important underlying factor for endothelial dysfunction [38].

A significant decrease in NO_2^- indicators in all groups of patients is probably due to the development of endothelial dysfunction, chronic inflammation combined with hypertension, oxidative stress and the progression of viral infection, which reduces the formation of NO [38]. In addition, chronically elevated glucose reduces nitric oxide production from arginine in endothelial cells in patients with DM and renal disease [39]. It was confirmed that insulin administration in patients with DM initiates the production of NO [40]. Thus, stimulation of NO synthesis is an alternative and potentially practical approach for the regulation of vascular hemostasis.

Minor changes in SOD indicators in patients infected with COVID-19 compared to healthy volunteers can be explained by the rapid development of the coronavirus disease and the inclusion of compensatory mechanisms aimed at maintaining the level of enzymes close to normal values. [41]. In addition, respiratory viral infections are usually associated with high cytokine secretion, inflammation, apoptosis and other pathophysiological processes that may be associated with redox imbalance or oxidative stress [42]. In patients with comorbidities such as AH and DM, oxidative stress and infection with COVID-19 increase this oxidative stress, with decreased SOD [43]. Consequently, the decrease in the activity of SOD in patients with COVID-19 indicates a deficiency of antioxidant protective mechanisms that progress in patients with AH and DM combined with coronavirus disease.

Conclusions. It has been established that arterial hypertension combined with COVID-19 occurs along with reduced L-arginine, nitric oxide, superoxide dismutase activity and increased arginase activity. At the same time, the presence of arterial hypertension in patients with diabetes and coronavirus disease is accompanied by a decline in the content of L-arginine and arginase activity, and a slight growth of the nitric oxide concentration and superoxide dismutase activity. The results of our study may help scientists to find new pharmacological targets in the treatment of coronavirus disease and comorbid disorders in the future.

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Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

РІВЕНЬ ОКСИДУ АЗОТУ І АКТИВНІСТЬ АРГІНАЗИ У ХВОРИХ НА АРТЕРІАЛЬНУ ГІПЕРТЕНЗІЮ ТА ЦУКРОВИЙ ДІАБЕТ НА ФОНІ COVID-19

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Метою даного дослідження було оцінити рівень продукції оксиду азоту і активність аргінази у пацієнтів з артеріальною гіпертензією та цукровим діабетом ІІ типу за інфікування SARS-CoV-2. До дослідницьких груп увійшли пацієнти з артеріальною гіпертензією (АГ), хворі з АГ і з тяжким перебігом COVID-19 та пацієнти, які, окрім АГ та COVID-19 мали ще й цукровий діабет ІІ типу (ЦД ІІ). Контрольну групу становили добровольці без будь-яких клінічних ознак захворювань і з нормальними показниками артеріального тиску. Встановлено, що АГ в поєднанні з COVID-19 перебігає на фоні зниження показників L-аргініну, оксиду азоту, супероксиддисмутази та підвищення активності аргінази. Водночас наявність АГ у хворих на ЦД ІІ та коронавірусну хворобу супроводжується зниженням вмісту L-аргініну та активності аргінази. Результати нашого дослідження можуть допомогти вченим у майбутньому знайти нові фармакологічні мішені для лікування коронавірусної хвороби та супутніх захворювань.

Ключові слова: L-аргінін, аргіназа, оксид азоту, супероксиддисмутаза, гіпертонія, COVID-19, цукровий діабет.

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