Changes in the asymmetric dimethylarginine and endothelial nitric oxide synthase levels in pathogenesis of experimental diabetic retinopathy

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Endothelial dysfunction associated with impaired nitric oxide excretion plays an important role in the onset and progression of diabetic retinopathy. It has been proven that a decrease in the activity of endothelial NO-synthase (еNО-S), the inhibitor of which is asymmetric dimethylarginine (ADMA), plays an important role in this. Objective: to study the level of asymmetric dimethylarginine and endothelial nitric oxide synthase at different stages of development of diabetic retinopathy in the experiment. The study was conducted in Wistar white rats of 180-200 g weight. According to the tasks, the animals were separated into 2 groups as follows: group 1 - 60 intact animals, group 2 - 60 animals with simulated diabetic retinopathy without further correction. Type 2 diabetes mellitus and diabetic retinopathy were simulated through intraperitoneal administration of Streptozotocin (Sigma, USA) diluted in 0.1M citrate buffer with pH=4.5. Streptozotocin dose of 55 mg per kg of animal weight was divided into two administrations. The streptozotocin intake was preceded by a 28-day high-fat diet. Our study showed impaired endothelial function in diabetic retinopathy, as evidenced by an increased ADMA level (p<0.001). We have determined a stepwise increase of asymmetric dimethylarginine level in blood of rats with simulated pathology which is apparent in its highest at phase 3. Pathogenetic effect of increased ADMA on еNО-S activity was verified at all experimental stages, Impairment of physiological nitric oxide synthesis in simulated pathology has been proved as evidenced by reduced activity of endothelial NO-synthase yet on the 30th day with further negative dynamics up to the 180th day (p<0.001 compared with the intact group findings).

Keywords: diabetic retinopathy, endothelial dysfunction, asymmetric dimethylarginine, endothelial NO-synthase, comparative description.
involvement of endothelial NO-synthase (eNOS). ADMA has been determined to be an endogenous competitive inhibitor of the endothelial NO-S [8]. Considering the above, ADMA level and endothelial nitric oxide synthase in vascular complications of diabetes mellitus, in particular in diabetic retinopathies, appear to be relevant for investigators.

**Study purpose:** to study the level of asymmetric dimethylarginine and endothelial nitric oxide synthase at different stages of development of diabetic retinopathy in the experiment.

**Materials and methods**

The study was conducted in Wistar white rats of 180-200 g weight. According to the tasks, the animals were separated into 2 groups as follows: Group 1 - 60 intact animals, and Group 2 - 60 animals with simulated diabetic retinopathy without further correction.

Type 2 diabetes mellitus and diabetic retinopathy were simulated by means if intraperitoneal administration of Streptozotocin (Sigma, USA) diluted in 0.1M citrate buffer with pH 4.5 [22]. Streptozotocin dose of 55 mg per kg of animal weight was divided into two administrations. Streptozotocin administration was preceded by a 28-day diet rich in fats [15].

The animals were withdrawn from the experiment by means of decapitation under brief ether anaesthesia according to the Rules to Perform Works with Use of Experimental Animals approved with Decree of the Ministry of Health of Ukraine No.249 of 01.03.2012 and the Law of Ukraine No.3447-IV, About Animal Protection Against Cruelty (as amended on 15.12.2009 and 16.10.2012).

The animals were withdrawn from the experiment in three stages: Study stage 1: 30th day after commencement of diabetes mellitus simulation; Study stage 2: 60th day after commencement of diabetes mellitus simulation; Study stage 3: 180th day after commencement of diabetes mellitus simulation. The endothelial synthase (μmol/L/hour) and ADMA level (μmol/L) were determined with spectrofluorometric technique in blood of the experimental rats.

Prior to application of parametric methods that are based on normality of statistical distribution, we have verified the arrays of numeric data being studied for normality with Shapiro-Wilk's W test. As the numeric data in the samples were normally distributed, we have used Student’s parametric criterion. The data obtained are presented in the figures in the form of box diagrams.

**Results**

In testing ADMA level at the first stage of our study we have determined its significant (by 65.67%) enhancement in the group of simulated diabetic retinopathy (p<0.001). The following findings were obtained at the second stage: an increased level of the studied endothelial dysfunction marker was registered: increase by 71.97% versus the intact animals (p<0.001) and by 18.6% versus the experimental uncorrected group (p<0.001). On the 180th day, even more apparent increase in ADMA was revealed in the rats with simulated diabetic retinopathy: compared with findings of Group 1, its level was pathologically increased by 75.85% (p<0.001). Compared with Stage I findings, ADMA level was higher by 29.85% (p<0.001), and with Stage II findings, an increase in concentration of endothelial NO-synthase by

| Groups | Stages |  
|--------|--------|  
|        | I      | II     | III    |
| Group 1| 20.81±1.28 | 20.80±0.86 | 20.82±1.25 |
| Group 2| 60.40±1.16 | 74.21±1.36 | 86.13±1.08 |

Table 1. Dynamics of ADMA (μmol/L) level in the blood of experimental animals with simulated diabetic retinopathy at various study stages (М±m).

**Fig. 1.** Comparative indicators of the level of ADMA content in the blood of experimental animals of both groups. A - ADMA concentration (μmol/L) after 30 days; B - after 60 days and C - 180 days after the induction of diabetic retinopathy. * - statistically significant difference between the indicators of the control and experimental groups.
13.83% (p<0.001) was revealed (Table 1, Fig. 1).

As to NO-synthase activity, we have determined on the 30th day its notable decrease (by 56.25%) in the blood of rats with simulated diabetic retinopathy (statistically distinctions at significance level p<0.001). On the 60th day (Stage II), we have found out that activity of endothelial nitric oxide synthase lowered even more, by 11.63% compared with findings of the same group at the previous stage (p<0.05), and by 56.25% than findings of the intact rats (p<0.001). On the 180th day, eNO-S activity deceased by 87.50% compared with findings of Group 1.

Having reviewed the activity dynamics for the studied enzyme, we have found that it was lower by 20.0% (p<0.001) compared with the first stage findings and by 7.5% compared with the second stage (Table 2, Fig. 2).

To summarize the above, we can affirm that our experiment has proved the development of endothelial dysfunction subsequent to investigational diabetic retinopathy as evidenced by apparent increase in ADMA level at each of the study stages. Additionally, we have confirmed the inhibiting effect of asymmetric dimethylarginine on the endothelial NO-synthase activity. As mentioned above, eNO-S activity is a marker of the nitric oxide physiological synthase, and decrease in this appears to be another indicator of the endothelial functional condition.

### Table 2. Dynamics of eNO-S (μmol/L/hour) activity in the blood of experimental animals with simulated diabetic retinopathy at various study stages (М±m).

| Groups   | Stages |       |       |       |
|----------|--------|-------|-------|-------|
|          | I      | II    | III   |       |
| Group 1  | 0.752±0.030 | 0.751±0.023 | 0.750±0.031 |       |
| Group 2  | 0.480±0.021 | 0.433±0.020 | 0.401±0.022 |       |

The key role in regulation of the endothelial function is played by nitric oxide the synthesis of which requires for L-arginine amino acid. As mentioned above, asymmetric dimethylarginine is deemed to be an important NO-synthase inhibitor to block connection of L-arginine with the enzyme. Concentration of this inhibitor in blood is variable and depends on multiple reactions both on cellular and tissular levels [13]. Arginine residues in proteins which are catalysed with S-adenosyl methionine-dependent methyltransferases undergo post-translational methylation and are essential for metabolism of the said enzymes. Since the methylation is irreversible, it can be affected only through proteolysis, therefore methylated proteins are highly metabolic [13, 32]. Free ADMA is expressed under proteolysis being induced by intracellular dimethylarginine dimethylaminohydrolase affected by a few factors, mainly the glycated proteins [18].

Inhibition of dimethylarginine dimethylaminohydrolase occurs with depression of NO synthesis and increase in ADMA content [13, 19]. Once in the blood, approximately 10% of the total amount of ADMA are partially hydrolysed in cells and partially excreted by kidneys. ADMA affects cells negatively contributing to oxidising stress, shortening telomeres, inhibiting NO release, increasing secretion of Interleukin-8 and monocyte chemotactic factor 1 [2]. Its effect involves the entire body: increased blood pressure, enhanced pulmonary and total peripheral vascular resistances, decreased cardiac output [13, 16]. Such atherogenesis processes like expression of inflammatory and chemotactic cytokines, monocyte adhesion [6, 9, 13], and accumulation of oxidised low-density lipoproteins activate ADMA in macrophages [21]. Patients with the atherosclerosis risk factors such as diabetes mellitus [21,
ADMA which is a structural analogue of L-arginine depresses activity of all isoforms of NO-synthases thus inhibiting formation of nitric oxide in tissues and blood plasma. It has been proved that ADMA significantly depresses NO synthesis [27, 36]. There is a strong correlation between the levels of nitric oxide in plasma under physiologic conditions which, once broken, result in vascular pathologies [7]. The studies showed that L-arginine activates vasomotor reactions in vivo [3, 4, 10]. And this is despite that endogenous L-arginine is 30 times higher in physiologic concentrations in blood plasma than Michaelis-Menten Constant for L-arginine in a NO-synthase catalysed reaction [10, 24, 27]. At first, the effect of L-arginine on vascular tone appeared to be a little paradoxical since NO-synthase was totally saturated with a substrate and additional effect of exogenous arginine could not affect intensity of the nitric oxide synthesis [3]. A little later, we have found such endogenous analogues of L-arginine like N-monomethyl-L-arginine (NMMA), asymmetric N,N-dimethyl-L-arginine (ADMA), and symmetric N,N-dimethyl-L-arginine (ADMA) [17, 27]. Two of them, ADMA and NMMA are able to inhibit NO-synthase activity [12, 25]. This allows to explain the "L-arginine paradox" since higher concentration of the substrate is required for NO-synthase activation with its endogenous inhibitors. ADMA under physiologic conditions is a stronger inhibitor than NMMA as its concentration in blood plasma is 5 times higher [20, 27, 35]. Considering the above, further analysis of the nitric oxide physiological synthesis markers and use of L-arginine for correction of pathological conditions caused by a sharply increased ADMA level in blood is enlightening.

**Conclusion**

1. Our study showed impairment of the endothelial functional condition in investigational diabetic retinopathy as evidenced by increased level of ADMA (p<0.001).

2. We have determined a stepwise increase of asymmetric dimethylarginine level in blood of rats with simulated pathology which was apparent in its highest at phase 3.

3. Pathogenetic effect of increased ADMA on eNO-S activity was verified at all experimental stages.

4. Impairment of physiological nitric oxide synthesis in simulated pathology has been proved as evidenced by reduced activity of endothelial NO-synthase yet on the 30th day with further negative dynamics up to the 180th day (p<0.001 compared with the intact group findings).

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ЗМІНА РІВНЯ АСИМЕТРИЧНОГО ДИМЕТИЛАРГІНІНА І ЕНДОТЕЛІАЛЬНОЇ СИНТАЗИ ОКСИДУ АЗОТУ В ПАТОГЕНЕЗІ ЕКСПЕРИМЕНТАЛЬНОЇ ДІАБЕТИЧНОЇ РЕТИНОПАТІЇ

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У виникненні та прогресуванні діабетичної ретинопатії важливу роль відіграє ендотеліальна дисфункція, пов'язана з порушенням виділення оксиду азоту. Доведено, що важлива роль при цьому відігрває зниження активності ендотеліальної NO-синтази (eNOS), інгібітором якої є асиметричний диметиларгінін (ADMA). Мета дослідження: вивчити рівень
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асиметричного диметиларгініна і ендотеліальної синтази оксиду азоту на різних етапах розвитку діабетичної ретинопатії в експерименті. Дослідження проводили на білих щурах лінії Вістар масою 180-200 г. Відповідно до завдання дослідження тварини були розподілені на 2 групи: 1 група - 60 інтактних тварин, 2 група - 60 тварин з моделюванням діабетичної ретинопатії без подальшої корекції. Цукровий діабет 2 типу та діабетичну ретинопатію моделювали внутрішньоочеревним введенням стрептозотоцину (Sigma, США), розведеного в 0,1М цитратному буфері з pH=4,5. Дозу стрептозотоцину 55 мг на кг ваги тварини поділяли на 2 прийоми. Прийому стрептозотоцину передувала 28-добова дієта, багата жирами. Наше дослідження показало порушення функціонального стану ендотелію при досліджуваній діабетичній ретинопатії, про що свідчить підвищення рівня ADMA (p<0,001). Ми визначили ступеневе підвищення рівня асиметричного диметиларгініна в крові щурів із модульованою патологією, максимальний прояв якого спостерігався у третьій фазі. Патогенетичний ефект підвищеного ADMA на активність eNO-S підтверджений на всіх етапах експерименту. Порушення фізіологічного синтезу оксиду азоту при патології підтверджується зниженням активності ендотеліальної NO-синтази ще на 30 добу з подальшою негативною динамікою до 180 дня (p<0,001 у порівнянні з даними інтактної групи).

Ключові слова: діабетична ретинопатія, ендотеліальна дисфункція, асиметричний диметиларгінін, ендотеліальна NO-синтаза, порівняльний опис.