Клінічна фізіологія та біохімія

УДК 612.616.2.015.33:616.69-008.8

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NO-Synthase Pathway of L-Arginine Transformation in Spermatozoa of Infertile Men with Different Forms of Pathospermia

Elucidation patterns of male germ cells functioning is of particular relevance in the present conditions due to increasing trends in male reproductive disorders [1]. Men infertility is caused by the effect of different pathological processes in genital organs and glands, resulting in pathospermia, which reduces fertilizing ability of sperm cells. Common methods for diagnosis of infertility including clinical and morphological, cytological, bacteriological methods do not always indicate the cause of the decrease in viability of spermatozoa and do not reveal mechanisms of the changes [13]. Over the past two decades a large amount of researches devoted to studying the role of nitric oxide and NO-synthase in the regulation of physiological functions and pathological processes appeared. However, the role of NO-synthase in sperm cells in different forms of male infertility remains poorly understood [9, 11].

The NO formation in the human body is carried out as a result of oxidation of nitrogen atom that is part of the amino acid L-arginine by enzyme – Nitric Oxide Synthase (NOS) [4, 10, 11, 20]. There are three of its isoforms: neuronal – NOS (nNOS), inducible (macrophage) – NOS II (iNOS) and endothelial – NOS III (eNOS). Constitutive isoforms of NO-synthase (nNOS, eNOS) provide a synthesis of NO in physiological conditions. iNOS is inactive in physiological conditions and is activated in response to pathogenic stimuli [3–6, 12, 20, 26]. Therefore, the functional and regulatory features of inducible isoform of NOS depend on the nature of pathological process and the affected organ [12]. Both constitutive and inducible isoforms of NOS are related to NO production in the early phase of inflammation and their pro-inflammatory effect is manifested. Late phase of inflammation is associated with local leukocyte activity and infiltration. Its development is contributed only by NO, produced by iNOS, localized in leukocytes [12]. The elucidation of changes in NOS isoforms activity in men with impaired fertility determines actuality of present work.

The aim of the research is to detect changes in the activity of NOS isoforms of sperm cells in patients with different forms of infertility.

Materials and methods. Spermatozoa preparation. Semen samples of men aged 21–44 years were used in the research. Relatively healthy donors with no reproductive disorders and infertile men were among the men under study. The control group consisted of 20 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and have 1–3 healthy children). Before turning to the study, all men had familiarized themselves with patient information leaflets and gave informed consent to participate in the research.

The studied group consisted of patients with infertility. The semen analysis and biochemical studies characterizing the state of NOS system in sperm cells were carried out. Semen analysis included the following parameters: motility, concentration and morphological characteristics of spermatozoa. The study of morphological characteristics of sperm cells was based on the following method [19]. Semen samples were received in the central laboratory of the Lviv regional hospital. Samples were collected in sterile containers by masturbation after a minimum abstinence period of 3–5 days. Semen parameters (sperm concentration, mobility, morphology and life forms percent) were estimated using light microscopy according to the WHO guidelines (2010) [31]. Terms of sample selection meet the requirements of the principles of the Helsinki Declaration on protection of human rights, Convention of Europe Council on human rights and biomedicine and the provisions of laws of Ukraine.

Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 g for 10 min in media which contained, mM: 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). The content of total protein in the samples was determined by Lowry method using a kit to determine its concentration (Simko Ltd). Determination of NO-synthase was carried out in permeabilized spermatozoa. The detergent saponin in final concentration of 0.5 % was added to sperm suspension for permeabilization of sperm membranes [1].

Determination of total NOS activity (cNOS + iNOS). The samples aliquots that contained 300 µg protein were used to determine the total NOS activity. They were incubated for 60 min at 37 °C in a total volume of 1 ml substrate mixture (pH 7.0) of the following composition (mmol/ml): KH_2PO_4 – 50, MgCl_2 – 1, CaCl_2 – 2, NADPH (Sigma, USA) – 1, L-arginine – 2. The reaction was stopped by adding 0.3 ml of 2N HClO_4 . As a control samples that contained the full substrate mixture previously denatured by 2N NClO_4 were used. The mixture was centrifuged at 3500 g for 10 min and the non-protein supernatant mixtures were used to test L-citrulline by highly specific method for color reaction with antipyrine. Its sensitivity is 0.2 mg of L-citrulline in 1 ml, so it can be used to study the NOS activity. NOS activity was expressed as pmol citrulline/min per 1 mg of protein [8].

Determination of iNOS. The method similar to the previous one was used to determine iNOS, but 2 µM EDTA was added to the incubation mixture instead of CaCl_2 .

Calculation of cNOS activity. cNOS activity in the sperm cells was calculated as the difference between total NOS activity and iNOS activity.

Determination of citrulline. Protein-free aliquot samples were mixed with 2 ml of reagent (1 ml of 59 mM diacetyl monoxime (Sigma, USA) + 1 ml of 32 mM antipyrine (Sigma, USA) + 55 µM Ferrous (II) sulphate in 6N H_2SO_4) and boiled for 15 min in a water bath. After cooling the value of extinction was determined at 456 nm. The citrulline content was determined using a calibration graph [8].

Statistical analysis. Experimental data were processed by methods of variation statistics using software MS Office. Differences were calculated using the *t*-Student test for independent groups, assuming $p \leq 0.05$ as the minimum significance level. The results are presented as the mean $\pm$ standard deviation of the mean. Number of experiments (*n*) corresponds to the number of samples examined in each case (each time semen sample from a patient or a healthy donor was used).

Results and discussion. According to semen analysis, oligozoospermia was found in 12 patients (16.7 %), astenozoospermia was detected in 17 patients (23.6 %), oligoastenozoospermia was observed in 10 patients (13.9 %). Thirty-nine (54.2 %) infertile men had leukocytes content in the semen lower than $1.0 \times 10^6 \text{ ml}^{-1}$, only in 33 patients (45.8 %) leukocy-

tospermia was noted (the leukocytes content ranged from $1.0 \times 10^6 \text{ ml}^{-1}$ to $3.0 \times 10^6 \text{ ml}^{-1}$) which indicates inflammation in this group of men. The data of main morphological characteristics of semen samples of infertile men with different forms of pathospermia are represented in Table.

Main morphological characteristics of semen samples of infertile men with different forms of pathospermia

Investigated parameters of ejaculates	normozoospermia	infertile men			
		oligozoospermia	astenozoospermia	oligoastenozoospermia	leukocytospermia
The concentration of spermatozoa, 10^6 ml^{-1}	$50,0 \pm 6,40$	$11,95 \pm 2,35^{***}$	$44,30 \pm 5,35$	$9,95 \pm 1,65^{***}$	$46,40 \pm 6,20$
The relative number of motile sperm, %	$52,86 \pm 3,22$	$42,33 \pm 4,95$	$24,05 \pm 5,35^{***}$	$26,05 \pm 4,25^{***}$	$42,34 \pm 3,24$
The number pathological forms, %	$32,8 \pm 2,8$	$39,72 \pm 3,2$	$45,5 \pm 5,2^*$	$42,7 \pm 3,2$	$42,4 \pm 3,6$
Concentration of WBC in the ejaculate, 10^6 ml^{-1}	$0,28 \pm 0,06$	$0,46 \pm 0,08$	$0,34 \pm 0,08$	$0,44 \pm 0,09$	$1,56 \pm 0,25^{***}$

Hereinafter: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$ relative to the control group (normozoospermia).

According to current literature data, all three isoforms of NO-synthase – nNOS, eNOS and iNOS were identified in sperm flagellum and cytoplasm [22]. It was shown [28] that human sperm maturation is associated with increased activity of NO synthase during spermatogenesis.

State of NO-synthase system in the sperm cells of fertile men was characterized by the dominance of eNOS activity. This can be explained by the lack of factors that activate iNOS in healthy men with preserved fertility, primarily bacterial lipopolysaccharide, proinflammatory cytokines etc. iNOS activity detected at extremely low levels compared to its constitutive isoform. The obtained average NOS activities in our study in the sperm cells of men with normozoospermia are consistent with data from other researchers [33].

It is believed that that NOS-dependent synthesis of "basal" NO is realized by cNOS, whereas iNOS provides additional amounts of NO in the cell under various pathological conditions. However participation of iNOS in physiological ("basal") synthesis of NO and in response to pathological stimuli [15, 24] was confirmed.

Sperm of infertile men, as compared to fertile men, was characterized by decreased eNOS activity (Fig.). According to the obtained results, the eNOS activity of spermatozoa in patients with impaired fertility of all groups was significantly lower. The eNOS activity in spermatozoa of men with oligozoospermia was 1.5 times ($p < 0.05$), in men with astenozoospermia – 1.4 times ($p < 0.05$), in men with oligoastenozoospermia – 1.5 times ($p < 0.05$) and in men with leukocytospermia – 3.2 times ($p < 0.001$) lower than in healthy men. The maximum change (decrease) in eNOS activity was observed in patients with leukocytospermia in comparison with healthy donors.

Increased iNOS activity was observed in spermatozoa of infertile men. Men with leukocytospermia were distinguished to have the most express iNOS activity in sperm cell which exceeded the reference values in healthy donors in 56 times ($p < 0.001$). Meanwhile, in spermatozoa of other studied groups the activation of iNOS was of weaker intensity. The iNOS activity was 22.9 times ($p < 0.001$) greater in sperm cell of

men with oligozoospermia, in almost 30 times ($p < 0.001$) greater in men with asthenozoospermia and in 30.7 times ($p < 0.001$) greater in patients with combined pathology.

It is well known that leukocytes are markers of inflammation and/or the presence of infection. White blood cells negatively affect sperm cells because they stimulate the formation of reactive oxygen, induction and development of oxidative stress, thus inhibiting the sperm motility and functional activity [21]. In particular, it was shown [17] that active forms of oxygen produced by white blood cells, can cause damage to sperm DNA.

It has been shown that pathospermia is accompanied by an imbalance in the system of NO synthesis in the sperm cells. This imbalance includes the activation of the inducible isoform of NO-synthase (iNOS) and significant inhibition of its constitutive isoform. Thus, according to the obtained data in men with violations of reproductive function of different forms of pathospermia the redistribution of NO-synthase system towards Ca^{2+} -independent inducible isoform of the enzyme was detected. This indicates uncoupling of NOS in spermatozoa and its dysfunction. As a result of this uncoupling the NOS can switch from NO production to superoxide anion (O^{2-}) generation [14, 16].

It is known that cNOS produces NO at low, physiologically appropriate concentrations, whereas iNOS produces NO in extremely high concentrations [28].

Pathological activation (induction) of iNOS leads to overproduction of NO which has destructive potential, increasing the total concentration of NO metabolites and contributing to nitrative stress. Excessive NO production is more dangerous than its deficit. In high concentrations, NO is the endogenous intoxication factor that determines its cytotoxic effect and causes cells and tissue death by apoptosis and necrosis mechanisms.

In case of pathospermia iNOS causes excessive production of free radicals, including "harmful" NO, which in the interaction with oxygen free radicals (superoxide anion) creates more powerful radical with cytotoxic properties – peroxynitrite (ONOO^-). It causes the activation of processes of lipid peroxidation, protein modification, inhibition of the biosynthesis and reduction of reparative ability of DNA.

It was shown that high levels of NO in the mitochondria of cardiomyocytes lead to a sharp increase in Ca^{2+} , prevent the absorption of oxy-

gen, decrease the ATP formation, lead to depletion of energy reserves of cell [2].

Perhaps the nature of these changes is universal for all cell types, including sperm cells. The depletion of energy reserves of cell, including macroergs will affect the sperm motility.

NO in concentrations above physiological norm can have genotoxic effects, damaging chemical structure of sperm DNA [7]. It was shown that NO hyperproduction affect the formation and maturation of sperm cells, their mobility and morphology [25].

It was established that NO concentration is positively correlated with spermatograms parameters in men with oligozoospermia with normal or subnormal sperm motility. At the same time, it was found an inverse correlation between spermatograms parameters and NO concentration in men with normo-, asteno-, terato-, leukocyto- and leukoastenospermia. Therefore, it is suggested that disruption of the sperm functions is associated with cytotoxic effects of excessive NO production [27].

The obtained data indicate violations of NO-homeostasis, hyperproduction of "harmful" NO by iNOS and activation of nitric stress in the spermatozoa of men with various forms of pathospermia, which will result in disruption of their functions, in particular the fertilizing ability.

Our data are consistent with results from other authors [34] who found increased NOS activity in sperm of infertile men.

Hadwan MH et al. [18] also showed that NOS activities in sperm cells and seminal plasma of men with astenozoospermia were higher than men with preserved fertility. It was shown that oral zinc supplementation develops sperm count, motility and the physical characteristics of sperm in animals and in some groups of infertile men.

Our data also are confirmed by immunohistochemical studies [30], which confirmed that constitutive expression of NOS isoforms are higher in isolated spermatozoa from fertile donor with normozoospermia. Instead, iNOS expression was higher in samples obtained from patients with idiopathic astenozoospermia.

However, other researchers [35] found no statistically significant difference in activity of Ca^{2+} -independent NO-synthase in seminal plasma of fertile and infertile men.

It was established that under conditions of hyperthermia due to scrotal heat stress the activity of NO-synthase and NO content in semen plasma increases [32].

Therefore, determination of the dynamics of the activity of NOS isoforms may be an additional prognostic criterion used for confirmation of infertility and for the evaluation of effectiveness of treatment.

Conclusions. 1. "Uncoupling" of NO-synthase in sperm cells in infertile men, redistribution of activities in the NO-synthase system with their shift toward Ca^{2+} -independent inducible isoform, which indicates dismetabolic changes in NO synthesis, namely its hyperproduction have been found.

2. Among the different forms of pathospermia the most expressed violations of NO-synthase pathway of L-arginine is observed in patients with leukocytospermia because white blood cells stimulate the formation of reactive oxygen, induction and development of oxidative and nitrative stress in sperm cells.

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Стаття надійшла до редколегії 11.08.2016

Функціонування NO-синтазного шляху метаболізму L-аргініну в сперматозоїдах неплідних чоловіків із різними формами патоспермій

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Вивчено особливості NO-синтазного шляху метаболізму L-аргініну в сперматозоїдах неплідних чоловіків із різними формами патоспермій. У чоловіків із порушенням фертильності відбувається роз'єднання (uncoupling) NO-синтази сперматозоїдів, перерозподіл активності у системі NO-синтаз із їх зрушенням у бік Ca^{2+} -незалежної індукційної ізоформи, що свідчить про дисметаболичні зміни в системі синтезу NO, а саме, його гіперпродукцію. Серед різних форм патоспермій в неплідних чоловіків найбільш виражені порушення NO-синтазного шляху метаболізму L-аргініну спостерігаються у пацієнтів із лейкоцитоспермією, адже лейкоцити стимулюють утворення реактивних форм Оксигену, індукцію та розвиток оксидативного та нітразивного стресу в сперматозоїдах. Отримані нами дані вказують на порушення NO-гомеостазу, гіперпродукцію “шкідливого” NO за участю iNOS та активацію нітрозивного стресу в сперматозоїдах чоловіків із різними формами патоспермій, що призводить до порушення їх функцій, зокрема, запліднювальної здатності.

Ключові слова: NO-синтаза, оксид азоту, сперматозоїди, неплідність.