



# Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)  
ISSN 2520-2588 (Online)  
Regul. Mech. Biosyst., 9(1), 80–84  
doi: 10.15421/021811

## The kinetic properties of arginase in sperm cells of infertile men

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### Article info

Received 27.01.2018

Received in revised form  
16.02.2018

Accepted 18.02.2018

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**Fafula, R. V., Iefremova, U. P., Onufrovyh, O. K., Maksymyuk, H. V., Vorobets, D. Z., & Vorobets, Z. D. (2018). The kinetic properties of arginase in sperm cells of infertile men. *Regulatory Mechanisms in Biosystems*, 9(1), 80–84. doi: 10.15421/021811**

Nowadays the role of NO in the development of male infertility is actively studied. Arginase (EC 3.5.3.1) is a manganese metalloenzyme which converts L-arginine to L-ornithine and urea and reciprocally regulates NO production. Although arginase activity has usually been detected in the reproductive tract, including spermatozoa, no data relating to the kinetic properties of the enzyme in ejaculated spermatozoa has been reported. This study was designed to study the kinetic parameters of arginase of spermatozoa of infertile men. Spermatozoa arginase activity was measured by determining levels of urea production. Kinetic analysis of the enzyme reaction was performed in a standard incubation system with modified physical and chemical characteristics or the respective components (the substrate concentration,  $Mn^{2+}$  concentration, incubation time and protein content). Pathobiochemical and kinetic properties of sperm arginase obtained from human normozoospermic and pathospermic samples were compared. The maximum rate of L-arginine hydrolysis (determined by L-arginine) for arginase of spermatozoa obtained from men with preserved fertility was 2.0, 1.8 and 1.9 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for L-arginine was not significantly different between fertile and infertile men. The maximum rate of L-arginine hydrolysis (determined by  $Mn^{2+}$ ) for arginase of spermatozoa obtained from men with preserved fertility was 1.6, 1.7 and 1.7 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for  $Mn^{2+}$  were not significantly different between fertile and infertile men. In the whole range of time, the urea production by arginase in sperm cells obtained from oligozoospermic samples is much lower compared to value in healthy donors. The results of kinetic analysis indicate that urea production by arginase is much more intense in the control group than in patients with various forms of pathospermia. The initial (instantaneous) reaction rate of arginase reaction was lower for oligozoospermic samples compared to normozoospermic samples. It has been found that inhibition of arginase activity in sperm cells of infertile men occurs by non-competitive type and was related to marked decrease in maximum reaction rate while affinity of arginase to L-arginine and  $Mn^{2+}$  was unaffected.

**Keywords:** arginase activity; L-arginine; enzyme inhibition; spermatozoa; pathospermia

### Introduction

Infertility is an important worldwide socio-demographic and medical-biological problem for most developed countries. It affects 10–15% of couples globally and approximately up to 40–50% of infertility is caused by the male factor (Agarwal et al., 2015; Luc et al., 2015). According to the trends observed, the problem of male infertility is predicted to increase (Winters et al., 2014). Nowadays the role of NO in the development of male infertility is actively studied. It is a crucial biological messenger involved in a variety of physiological and pathophysiological processes in different cells, including spermatozoa. Disturbances in the NO-signaling system are considered as key pathogenetic links in the development of male infertility (Kullisaar et al., 2013).

Arginase (EC 3.5.3.1) is a manganese metalloenzyme which converts L-arginine to L-ornithine and urea and reciprocally regulates NO production by competing with NO-synthase for common substrate (L-arginine) (Vanhouthe et al., 2008; Venkatakrisnan et al., 2010). Two isoforms of arginase exist in mammals. Arginase I (cytosolic form) is expressed in hepatocytes and is thought to be primarily involved in ureagenesis, whereas arginase II (mitochondrial form) is expressed extrahepatic tissues. Type II arginase plays an important role in regulating the nitric oxide production and is involved in the biosynthesis of polyamines and aminoacids. Also, arginase plays an important role in

regulating the cellular synthesis of NO and modifies its biological effects (Mori et al., 2007).

Data regarding arginase activity (expression) in the sperm cells of infertile men are limited (Hadwan et al., 2014). Arginase II-deficient mice show a reduction in fertility (Stephen et al., 2004). We have demonstrated previously inhibition of the arginase pathway of the L-arginine metabolism, which was not significantly dependent on the type of disruption of spermatogenesis (Fafula et al., 2016). Arginase activity was detected not only in spermatozoa, but also in seminal plasma. A positive correlation has been shown between arginase activity and semen volume, semen mass activity, sperm motility and sperm concentration (Gür et al., 2012). It was found that plasma arginase activity was significantly lower than in the non-stress situation and during stress there was a negative correlation between the percentage of rapid progressive motility and arginase activity (Eskioçak et al., 2006). Similar relationships between the plasma arginase activity and the sperm concentration and sperm motility were detected in sheep and bulls (Gür et al., 2012; Türk et al., 2011). Although arginase activity has usually been detected in the reproductive tract, including spermatozoa, no data relating to the kinetic properties of enzyme in ejaculated spermatozoa has been reported. Therefore, this study was designed to study the kinetic parameters of arginase of spermatozoa of infertile men.

## Materials and methods

**Subjects.** This study involved 16 infertile men with different forms of pathospermia. A detailed medical history was compiled for all studied cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility lasting over 10 years, azoospermia, testicular varicocele, genital infection, chronic illness and serious systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal reactive oxygen species levels and decreased antioxidant activity (Atig et al., 2012).

Ejaculates from a 16 infertile and 10 fertile healthy individuals were obtained. Infertile men were age-matched to fertile control cases. Subjects were classified into three groups as having different forms of pathospermia (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). Semen samples of fertile men represent the control group, which consisted of 10 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and have healthy 1–3 children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction at 37 °C with 5% CO<sub>2</sub> in air, semen samples were examined for volume, sperm concentration, pH, morphology and motility according to the World Health Organization guidelines (WHO Laboratory Manual for the Examination and processing of Human Semen, 2010).

**Ethical approval.** Before becoming involved in the study, all the men were made aware of patient information leaflets and gave informed consent to participate in research. Terms of sample selection meet the requirements of the principles of Helsinki Declaration on Protection of Human Rights, Convention of Europe Council on Human Rights and Biomedicine and the provisions of laws of Ukraine. Approval for the study was taken from the Ethics Committee of Danylo Halytsky Lviv National Medical University. All patients and healthy donors gave written informed consent to participate in the research (Ethical Committee Approval, protocol No 6 from March 29, 2017).

**Cell preparation.** 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 (Lowry et al., 1951) using a kit to determine its concentration ("Simko Ltd"). Determination of arginase activities was carried out in permeabilized spermatozoa. The detergent saponin in a final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes.

**Arginase activity assay.** Spermatozoa arginase activity was measured by determining levels of urea production. Briefly, incubation media of the following composition (mmol/ml): L-arginine – 100, MnCl<sub>2</sub> – 2, Tris-HCl – 20 (pH 9.5) was used. The protein concentration usually did not exceed 50–100 mg. The mixture was incubated at 37 °C for 90 min, and the reaction was stopped by adding 1 ml 50% trichloroacetic acid. After centrifugation, the urea was determined in the supernatant spectrophotometrically by measuring absorbance at 520 nm according to the assay kit "Simko Ltd". Arginase activity was expressed as nmol urea per min per mg protein.

**Kinetic analysis.** Kinetic analysis of the enzyme reaction was performed in a standard incubation system (as described above) with modified physical and chemical characteristics or the respective components (the substrate concentration, Mn<sup>2+</sup> concentration, incubation time and protein content). The apparent affinity constant for L-arginine (KL-Arg) and maximum reaction rate ( $V_{max}$ ) were determined by Lineweaver-Burk plot  $\{1/V; 1/[S]\}$ . The dynamics of urea production in arginase reaction in sperm cells of fertile and infertile men were determined according to the paper (Kosterin et al., 1987). The kinetic parameters characterizing arginase reaction – the initial (instantaneous) reaction rate ( $V_0$ ), maximum amount of the reaction product ( $P_{max}$ ) and characteristic reaction time (time half saturation)  $\tau$  were determined in coordinates  $\{P/t; P\}$ .

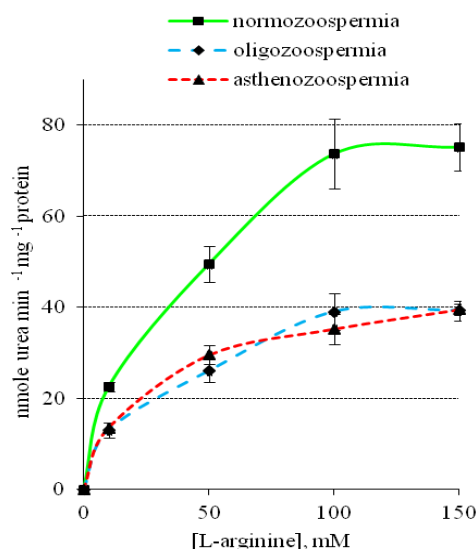
**Statistical analyses.** The data are expressed as means  $\pm$  standard error ( $M \pm SE$ ). One-way ANOVA was performed to detect statistical

significance. Differences with  $P < 0.05$  were considered as significant. Kinetic and statistical calculations were carried out using the software MS Office computer programs. The equation of the straight line that approximates the experimental data the best was calculated by method of least squares. The absolute value of the correlation coefficient  $r$  was from 0.80 to 0.95.

## Results

Pathobiochemical and kinetic properties of sperm arginase obtained from human normozoospermic and pathospermic samples were compared. Different methodological approaches (studies on purified enzymes, isolated subcellular structures, on whole cells or on homogenates) are used for studying arginase activity. Enzymes might be in latent state and inaccessible to substrates in whole cells. Therefore, testing their activities is possible after prior disturbance of integrality of sperm membranes. This can be achieved by introducing a substance leading to perforation of plasma membranes (detergent) in the incubation medium. Using a suspension cells pretreated with detergent (saponin) is an adequate model for correct testing of arginase activity. Under these conditions the natural interrelation of intracellular structures is obeyed.

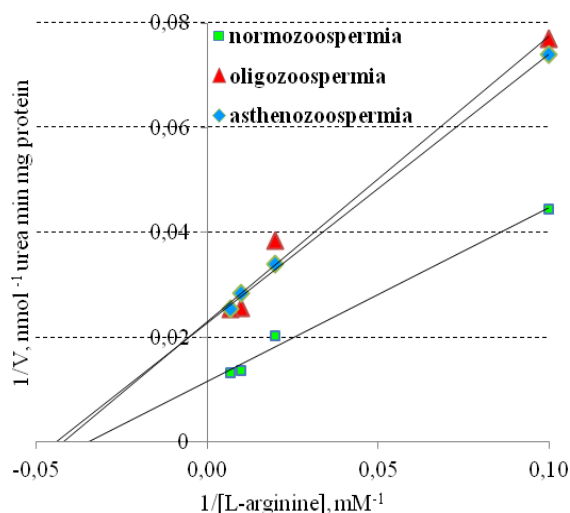
**Kinetic analysis of arginase activity on L-arginine concentration.** Since arginase is an enzyme that hydrolyzes L-arginine, changes in its concentration in the incubation medium affect the rate of arginase reaction. The dependence of the arginase activity on the substrate concentration in the incubation medium was determined by the apparent affinity constant to the substrate KL-Arg. For its determination L-arginine was added to the incubation medium in concentrations ranging from 10 to 150 mM (at constant concentration of MnCl<sub>2</sub> – 2 mM). We observed a monotonic increase in the enzyme activity of sperm cells obtained from both normo- and pathozoospermic samples reaching a plateau at 100 mM (Fig. 1). As can be seen from Fig. 1 the arginase activity in pathozoospermic samples was reduced in comparison with normozoospermic samples in the whole range of L-arginine concentrations. However, the maximal arginase activity was observed in presence of 100 mM L-arginine in incubation medium for both normo- and pathozoospermic samples.



**Fig. 1.** Dose-dependent effect of extracellular L-arginine concentration on arginase activity in sperm cells of fertile and infertile men ( $x \pm SE$ ,  $n = 6-8$ )

In order to elucidate the possible mechanism of change in arginase activity in patients with oligo- and asthenozoospermia, the main kinetic parameters of L-arginine hydrolysis were determined in Lineweaver-Burk plot (Fig. 2).

As can be seen, the concentration curves  $\{1/[S]; 1/[V]\}$  differ by angle of inclination for normo- and pathozoospermic patients. The main kinetic parameters of arginase of sperm cells of fertile and infertile men are presented in Table 1.



**Fig. 2.** Linearization of concentration curves represented on Fig. 1 in Lineweaver-Burk plot ( $n = 6-8$ ;  $r > 0.80$ )

**Table 1**

Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by L-arginine ( $x \pm SE$ ,  $n = 6-8$ )

Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
$K_{L-Arg}$ , mM	$21.0 \pm 1.8$	$20.8 \pm 2.6$	$23.8 \pm 1.5$	$24.2 \pm 2.0$
$V_{max}$ , nmol urea per min per mg protein	$74.6 \pm 5.2$	$37.7 \pm 3.6^*$	$41.6 \pm 3.9^*$	$40.2 \pm 4.2^*$

Note: \* -  $P < 0.001$  compared to normozoospermic men (with preserved fertility).

The data in Table 1 show that the maximum rate of L-arginine hydrolysis for arginase of spermatozoa obtained from men with preserved fertility was 2.0, 1.8 and 1.9 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for L-arginine were not significantly different between fertile and infertile men. Thus, in patients with oligo-, astheno- and oligoasthenozoospermia, the inhibition of arginase activity in sperm cells occurs by noncompetitive type, by reducing the reaction rate (value of  $V_{max}$  decreases).

#### Kinetic analysis of arginase activity on $Mn^{2+}$ concentration.

Since arginase activity is  $Mn^{2+}$ -dependent enzyme, the changes in the  $Mn^{2+}$  concentration in the incubation medium affect the rate of arginase reaction. To determine the influence of  $Mn^{2+}$  ions on arginase activity,  $Mn^{2+}$  ions at varying concentrations (at constant concentration of L-arginine - 100 mM) were added during preincubation (Fig. 3). While preincubation with a  $Mn^{2+}$  concentration of 3-4 mM fully activated sperm arginase of human pathospermic samples, a  $Mn^{2+}$  concentration of 2 mM fully activated that of normozoospermic samples.

In order to elucidate the possible mechanism of change in arginase activity in patients with oligo- and asthenozoospermia the main kinetic parameters of L-arginine hydrolysis were determined in Lineweaver-Burk plot (Fig. 4).

The main kinetic parameters arginase of sperm cells of fertile and infertile men are presented in Table 2.

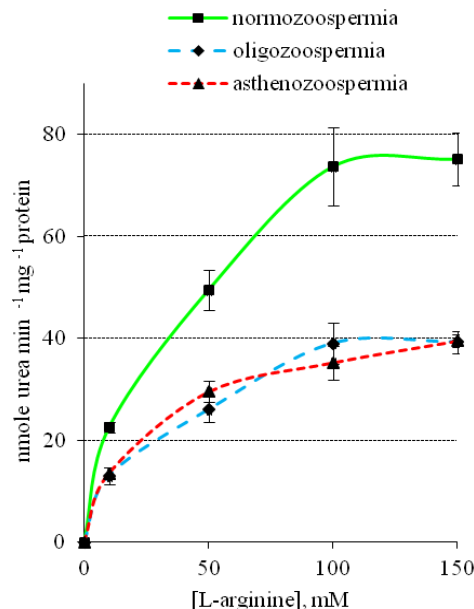
**Table 2**

Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by  $Mn^{2+}$  ( $x \pm SE$ ,  $n = 6-8$ )

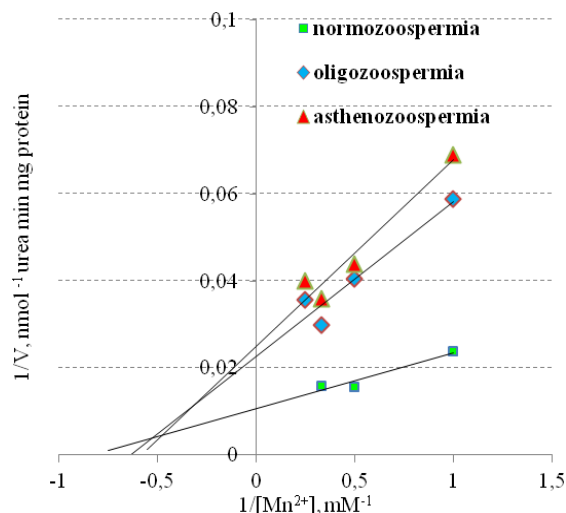
Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
$K_{Mn^{2+}}$ , mM	$1.3 \pm 0.4$	$2.5 \pm 0.8$	$1.5 \pm 0.5$	$2.1 \pm 0.7$
$V_{max}$ , nmol urea per min per mg protein	$88.2 \pm 10.5$	$55.0 \pm 5.8^*$	$52.3 \pm 9.2^*$	$53.5 \pm 6.2^*$

Note: see Table 1.

Data in Table 2 show that the maximum rate of L-arginine hydrolysis for arginase of spermatozoa obtained from men with preserved fertility was 1.6, 1.7 and 1.7 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for  $Mn^{2+}$  were not significantly different between fertile and infertile men. Thus, in patients with oligo- and asthenozoospermia, the inhibition of arginase activity in sperm cells occurs by noncompetitive type, by reducing the reaction rate (value of  $V_{max}$  decreases significantly).



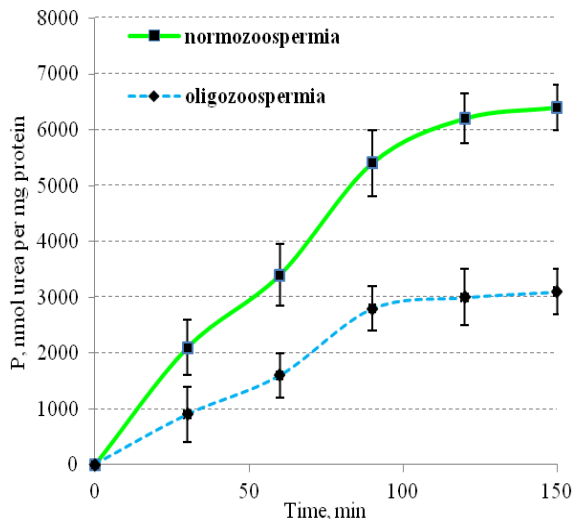
**Fig. 3.** Dose-dependent effect of extracellular  $Mn^{2+}$  concentration on arginase activity in sperm cells of fertile and infertile men ( $x \pm SE$ ,  $n = 6-8$ )



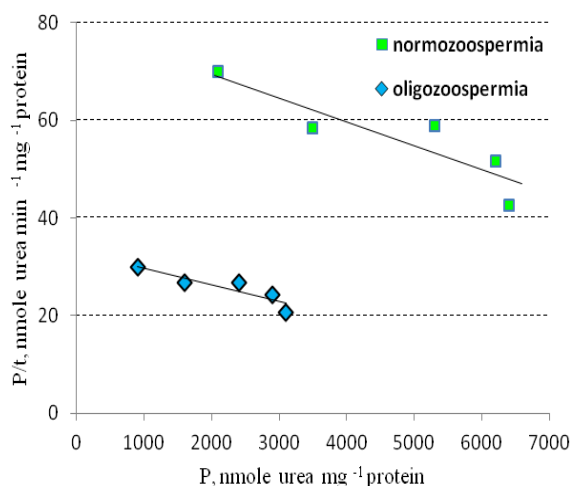
**Fig. 4.** Linearization of concentration curves represented on Fig. 3 in Lineweaver-Burk plot ( $n = 6-8$ ;  $r > 0.85$ )

**Kinetic analysis of arginase reaction on time.** The kinetics of urea production in arginase reaction was examined. Suspension of spermatozoa was incubated in the standard incubation medium (concentrations (at constant concentration of L-arginine - 100 mM and  $MnCl_2$  - 2 mM) for different periods of time (0-150 min). These experiments show that kinetics of arginase reaction by saponin-permeabilized spermatozoa is reflected by curves which tend to saturation (Fig. 5). Analysis of the results shows that kinetics of urea production by arginase is consistent with the first-order reaction in the range 0-60 min. In this time interval the dependence of urea production on the incubation period is almost linear.

As can be seen from Fig. 5 in the whole range of time, the urea production by arginase in sperm cells obtained from oligozoospermic samples is much lower compared to the value in healthy donors. From linearization of the curves in the coordinates {P/t; P} it can be seen that maximum amount of urea production by arginase in normozoospermic samples exceeds this value in infertile men (Fig. 6). The dynamics of urea production in arginase reaction and its linearization in the coordinates {P/t; P} for astheno- and oligoasthenozoospermic patients had an identical character (not represented in this article).



**Fig. 5.** Dynamics of urea production in arginase reaction in sperm cells of fertile and infertile men ( $\bar{x} \pm SE$ ,  $n = 6-8$ )



**Fig. 6.** Linearization of concentration curves represented on Fig. 5 in coordinates [P/t; P] ( $n = 6-8$ ;  $r > 0.80$ )

By linearization of the data in the coordinates P/t on P the main kinetic characteristics of arginase reaction in sperm cells of fertile and infertile men were calculated (Table 3).

**Table 3**

Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by time of incubation ( $\bar{x} \pm SE$ ,  $n = 6-8$ )

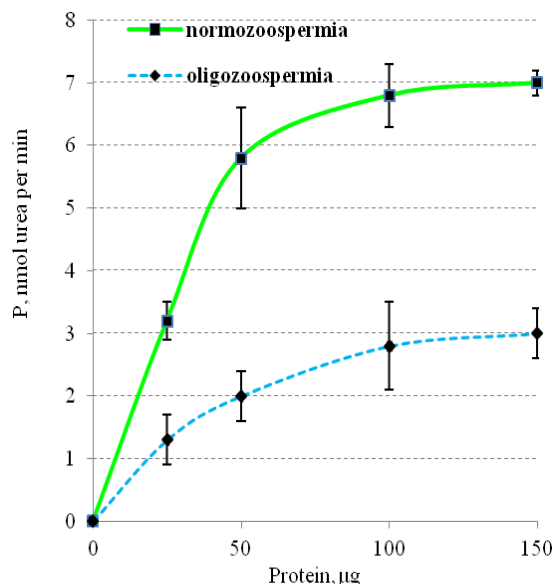
Kinetic parameters	Normozoo	Pathozoospermic men		
	spermic men	oligozoo-spermia	astheno-zoo-spermia	oligoastheno-zoospermia
$V_0$ , nmol urea per min per mg protein	$77.4 \pm 1.9$	$35.5 \pm 1.9^{***}$	$37.5 \pm 4.9^{**}$	$33.8 \pm 6.0^{**}$
$P_{max}$ , nmol urea per mg protein	$15995 \pm 928$	$10408 \pm 1047^*$	$11544 \pm 1156^*$	$11250 \pm 960^*$
$\tau$ , min	$207.4 \pm 16.8$	$291.7 \pm 15.8^*$	$322.6 \pm 61.8$	$298.5 \pm 16.0^*$

Note: \* –  $P < 0.05$ , \*\* –  $P < 0.01$ , \*\*\* –  $P < 0.001$  compared to normozoospermic men (with preserved fertility).

The maximum instantaneous rate of arginase reaction for spermatozoa obtained from men with preserved fertility was 2.2, 2.0 and 2.3 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. Maximum amount of reaction product (urea) in the control group exceeds this value in patients with all forms of pathospermia by 1.4–1.5 times. The results of kinetic analysis indicate that urea production by arginase is much more intense in the control group than in patients with various forms of pathospermia. The characteristic reaction time (time half saturation) of arginase reaction for spermatozoa obtained from men with preserved fertility was 1.4–1.5 times lower than this value for pathospermic samples.

#### Kinetic analysis of arginase activity on protein concentration.

Taking into account that enzyme activity depends on the protein content in incubation medium, the arginase reaction was initiated by adding protein with concentrations ranging from 25 to 150  $\mu\text{g/ml}$  in suspension of sperm cells (Fig. 7). It was found that a gradual increase in sperm protein concentration in the incubation medium led to an increase in  $V_0$  of arginase reaction. The  $V_0$  of arginase reaction was lower for oligozoospermic samples compared to normozoospermic samples. The dependence of the urea production on the protein content in incubation medium has the same character for astheno- and oligoasthenozoospermic samples (not represented in this article).



**Fig. 7.** Dependence of the initial rate of arginase reaction in sperm cells of fertile and infertile men on protein content ( $\bar{x} \pm SE$ ,  $n = 6-8$ )

## Discussion

Nitric oxide is a biological molecule which is involved in many physiological functions and pathophysiological outcomes (Bonavida et al., 2006). Several data suggest a crucial role of NO in sperm cell physiology. Altered NO production has been implicated in the pathogenesis of the male infertility (Kullisaar et al., 2013). Overproduction of NO can lead to oxidative and nitrosative stress and may have a potential pathogenetic implication in the reduction of sperm motility (Balercia et al., 2004).

Since arginase is an arginine-depleting enzyme, it affects NOS activity and regulates NO production. This regulatory mechanism is realized by reducing L-arginine availability for NOS to produce NO (Racke et al., 2010). The proper balance between NOS and arginase activity (expression) is essential for maintenance of NO homeostasis (Porro et al., 2014). Therefore, study of arginase activity and its kinetic properties may have important clinical significance.

Previously, the arginase activity in sperm cells obtained from fertile and infertile men has been reported by our laboratory. We have found an inhibition of arginase pathway of L-arginine metabolism, which was not significantly dependent on the type of disruption of spermatogenesis

(Fafula et al., 2016). We found an inhibition of arginase pathway of L-arginine metabolism, which is not significantly dependent on the type of disruption of spermatogenesis (Kullisaar et al., 2013). We also have shown that in patients with decreased fertility potential the arginase / NOS ratio was shifted towards predominance of iNOS-derived NO production (Fafula et al., 2018). In this study, we investigated the kinetic parameters of arginase of spermatozoa of infertile men. Obtained values of affinity constant were in millimolar range which in agreement with other studies (Dillon et al., 2002). However, kinetic parameters of enzyme were determined in a closed system with isolated enzymes and do not take into account enzyme coupling, non-freely diffusible substrate pools, intracellular localization of the enzymes and substrate transporter expression and activity, diffusion gradients, and potential sequestration (Shen et al., 2005; Topal et al., 2006; Jiang et al., 2011). In the present study we used permeabilized sperm cells in which functioning of enzyme correspond to intact cells.

There are some limitations in the present study. Firstly, our control group (normozoospermic men with proven fertility) and pathospermic patients contained a highly heterogeneous population, with large variations in spermogram parameters and infertility histories. Secondly, it is therefore essential to validate our findings with greater sample sizes and to determine the disease specificity (secretory or excretory infertility, varicocele or others) by comparing spermogram parameters. Nevertheless, the present study extends previous work and provides further evidence of altered L-arginine metabolism in sperm cells in pathospermia.

## Conclusions

It has been found that inhibition of arginase activity in sperm cells of infertile men occurred by non-competitive type and was related to marked decrease in maximum reaction rate while affinity of arginase to L-arginine and  $Mn^{2+}$  was unaffected.

The publication contains the results of studies conducted under the President of Ukraine grant for competitive projects (project No Ф63/97-2016 from 10.08.2016 “Molecular biological regulatory mechanisms of disturbance of fertilizing ability spermatozoa and the development of new immuno-biochemical diagnostic methods of fertility in men” of the State Fund for Fundamental Research (The President's Order No 97/2016-pri dated: April 13, 2016).

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