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The level of secondary messengers and the redox state of NAD^+/NADH are associated with sperm quality in infertility

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ABSTRACT

In order to explore the interrelation of Calcium, cAMP, and redox state of pyridine nucleotides in seminal plasma and ejaculate quality in cases of idiopathic infertility we conducted an evaluation of 170 infertile males and 46 fertile males aged 20–43 years. Sperm analysis was undertaken according to WHO protocol. The content of Calcium in the seminal plasma was detected using optical emission spectrometry, cAMP levels were determined via enzymatic immunoassay. The redox state of pyridine nucleotides was evaluated from the ratio of pyruvate to lactate, determined via enzymatic method. Our results show a decrease in Calcium, cAMP, pyruvate and the oxidation-reduction potential of pyridine nucleotides in the seminal plasma of infertile males with pathospermia. This corresponds to anaerobic inversion of oxidative conversions and metabolism inadaptation. Such processes are often seen in inflammatory and autoimmune conditions. cAMP levels reliably correlated with the number of progressively mobile sperm cells, but not with the number of their pathological forms. A positive correlation between the concentration of cAMP and calcium was discovered as well. Pathospermia was characterized by the positive relation between the value of the NAD^+/NADH coefficient and the spermatozoa concentration that was not present in fertile donors. Our study shows distinct changes in the concentration of secondary messengers and redox state of pyridine nucleotides in the seminal fluid that can act as molecular predictors for the development of idiopathic infertility.

1. Introduction

Infertility is an increasingly burdening healthcare problem, being diagnosed nearly 10 % of couples, equally as often in males and females (Kashanian and Brannigan, 2015; Agarwal et al., 2015; Rehman et al., 2018). In most cases, male infertility genesis remains uncertain (Levine et al., 2017; Flannigan and Schlegel, 2017). Spermatogenesis defects are caused by a complex interaction of internal and external factors (environmental, behavioral, alimentary, hereditary) (Madeen and Williams, 2017; Sharpe, 2010; Galimova et al., 2014). This leads to a decrease of quantitative and functional values of the ejaculate: concentration, motility, sperm morphology, disturbed acrosome reaction, antisperm antibodies production. Cases of idiopathic infertility may be connected immunological pathology, genetic and epigenetic changes (Darbandi et al., 2018; Aitken, 2016). Current research suggest an important link between testicular immunology and male infertility, specifically due to germ-cell autoantigen leakage through the blood-testis barrier, promoting prolonged inflammation and pathological changes in the testis (Qu et al., 2019).

Spermoplasma has a peculiar chemical composition. It contains a

Abbreviations: NAD^+, nicotinamide adenine dinucleotide oxidized; NADH, nicotinamide adenine dinucleotide reduced; cAMP, cyclic adenosine monophosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; AOS, active oxygen species; TZI, teratozoospermia index; ELISA, enzyme-linked immunosorbent assay; LDG, lactate dehydrogenase; WHO, World Health Organization; Ca, calcium; ART, assisted reproductive technologies; ATPase, adenosine triphosphatase; SD, standard deviation; DNA, deoxyribonucleic acid; MCT, mocarboxylate transporters; ROS, reactive oxygen species.

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significant number of proteins and amino acids, lipids and carbohydrates, a number of enzymes, hormones, cytokines and other low-molecular regulatory peptides, cations, anions, metabolites, co-enzymes, nucleotides, the main function of which is to provide normal metabolism and viability of spermatozoa (Juyena and Stelletta, 2012). At the same time, insufficient research has been carried out concerning secondary mediators - calcium ions, cyclic AMP, as well as the oxidation-reduction state of pyridine nucleotides in the development of idiopathic infertility in males. In the current study we conducted an evaluation of inflammatory components, calcium, cAMP and redox state of pyridine nucleotides in seminal plasma and ejaculate quality in patients with idiopathic infertility, to evaluate their role in the pathology.

2. Materials and methods

2.1. Cohort characteristics

170 infertile males were included in this main study group. A control group consisted of 46 fertile men. The study was approved by the Local Ethics Committee at First Moscow State Medical University and was carried out in accordance with Helsinki Declaration of the World Medical Association. Informed patient consent was acquired from all patients. All patients underwent complex clinical and laboratory evaluation with a general clinical assay of their ejaculate.

2.2. Sperm analysis

Ejaculate evaluation consisted of determination of physical and chemical properties and cellular composition. Prior to evaluation, the patients were informed of requirements to exclude thermal procedures, antibiotics and large doses of alcohol and continence. The continence period was 4 days. The ejaculate was obtained by masturbation in a sterile, non-toxic disposable plastic container. The container was marked with an individual patient number, date and the time the sperm was acquired. After collection, semen specimens were allowed to liquefy at room temperature for 30 min and then used for analysis. On microscopic examination, sperm count, percentage of motile sperm, sperm with normal morphology were objectively evaluated. Sperm count and percentage of motile sperm were evaluated according to standards set by the World Health Organization (2010). To evaluate the average number of defects per one abnormal spermatozoon, the Teratozoospermia Index (TZI) was calculated (Barratt et al., 2011). Seminal plasma was obtained by centrifuging the ejaculate at 400 rpm for 20 min. The test specimens were stored at a temperature of -80 ° C for no more than 3 months.

2.3. Biochemical assay

For determination of mineral content, samples were subjected to mineralization using microwave digestion system Anton Paar Multiwave 3000. Concentrations of Calcium (Ca) were measured by inductively coupled plasma optical emission spectrometry (Perkin Elmer Optima 2000 DV). Sperm cAMP levels were measured using direct cyclic AMP ELISA kit (Enzo Life Sciences / Biomol, Farmingdale, NY). This assay has a high sensitivity ranging from 10 mol to 1 pmol of cAMP. A standard curve was run for each assay, and the unknown cAMP concentrations were obtained by interpolation as recommended by the manufacturer. The metabolic status of sperm was defined by oxidation-reduction potential of pyridine nucleotides: the NAD^+/NADH ratio. The later was calculated according to the molar concentrations of lactate and pyruvate. The concentration of lactate in the seminal plasma was determined with a photometric method (CitricScreen, BioScreen). The measurement wavelength was 390−410 nm. The concentration of pyruvate was found in the conjugated reaction using LDH (Sigma-Aldrich).

2.4. Statistical analysis

All data are reported as means ± SD. An independent *t*-test was performed to compare the measures and mean of data parameters between the two groups. Pearson correlation criterion and linear regression analysis were used to evaluate the relationship between sperm parameters and biochemical scores. The relationship between three groups was evaluated using ANOVA and Tukey post hoc analysis. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using RStudio and Statistica 9.0 (Stat Soft, Inc., USA) software.

3. Results

3.1. Sperm analysis

Three groups of men were distinguished according to the results of spermograms (Table 1). Group I consisted of fertile donors. Infertile men were divided into two groups (II and III). Group II included 65 patients without changes in their spermogram (normospermia). Group III included 105 males with pathospermia.

The patients in Group II had standard spermogram parameters that did not differ from that of fertile donors, with the exception of spermatozoa concentration, which had a tendency to decrease. Spermatozoa concentration was significantly decreased (by 64 %) and the level of cells with morphological lesions was increased (by 2.2 times) in patients in Group II. At the same time, there were no statistically significant differences in ejaculate amount and in the content of progressively mobile cells. It was found that the TZI was increased in infertile men from Groups II, III in contrast with those from Group I (by 15 % and 19 %, respectively, $p < 0.05$). Statistically significant differences in TZI were found in all groups. The ANOVA and Tukey post hoc analysis were conducted during final verification of data. The results supported our primary findings, and suggest a significant difference in sperm quality between all groups.

3.2. Biochemial assay

Biochemical assay results showed a number of peculiarities regarding the content and distribution of the evaluated metabolites in seminal plasma (Table 2).

A significant concentration gradient of the studied analytes in fertile donors and infertile males was noted. Pathospermia was characterized by a significant calcium decrease in seminal plasma ($p<0.01$). Calcium level decrease in seminal plasma of infertile males almost completely coincided with the dynamics cAMP changes, and even surpassed them regarding the amplitude of oscillations. The levels of cAMP in cases of idiopathic infertility with pathospermia reduced significantly ($p < 0.02$) when compared to Group 1. Since the optimal concentration of calcium and cAMP acts as a trigger for the motor activity of gametes, it was of great interest to determine the correlation between these parameters of spermoplasm with the morphofunctional characteristics of the ejaculate.

The levels of cAMP reliably correlated with the number of progressively mobile spermatozoa ($p < 0.05$), but not with the number of their pathological forms. A positive relation between the concentration of cAMP and Ca ($p < 0.05$) was found in all sperm samples. The level of calcium was also associated with the amount of ejaculate ($p < 0.001$), but only in infertile males with pathospermia. The fact of such a connection in infertile males may be conditioned by the high variability of ejaculate amount in this category of patients. In all fertile males the ejaculate amount was within the normal range (>1.5 mL).

Lactate/pyruvate ration analysis showed lactate increase in infertile males, especially in cases of pathospermia, whereas pyruvate, an important energy substrate in sperm cells decreased. The lactate/pyruvate ratio may indirectly reveal the redox state of cells, certain cellular compartments and biological fluids. All infertile patients had a statistically significant decrease in the value of the NAD^+/NADH indicator

Table 1
Semen parameters of subjects in the study groups. Values are presented as means ± SD. Note: *statistically significant results at $p < 0.05$; **$p < 0,05$ as compared with fertile donors; ***$p<0,05$ as compared with normospermia.

Parameter	Fertile men	Infertile men		p-value (ANOVA)	Tukey post hoc analysis		
	Group I (n = 46)	**Group II (n = 65)**	**Group III (n = 105)**		**Group 1 vs Group II**	**Group I vs Group III**	**Group II vs Group III**
Volume (mL)	3,6 ± 0,2	3,4 ± 0,3	3,5 ± 0,2	**<0.001***	**<0.001***	**0.044***	**0.020***
Sperm count (×10^6 mL)	69,6 ± 3,9	59,2 ± 5,1	17,4 ± 2,0**	**<0.001***	**<0.001***		
Abnormal forms (%)	34,2 ± 2,2	39,1 ± 2,4	67,1 ± 5,1***	**<0.001***	**<0.001***		
Progressive motility (%)	49,3 ± 2,5	47,0 ± 3,2	28,8 ± 1,9***	**<0.001***	**<0.001***		
Teratozoospermia index	1,45 ± 0,03	1,67 ± 0,03**	1,73 ± 0,04***	**<0.001***	**<0.001***		

Table 2
The content of some metabolites and the redox state of pyridine nucleotides in the seminal plasma of the examined males. Values are presented as means ± SD. Note: *statistically significant results at $p < 0.05$; **$p < 0,05$ as compared with fertile donors; ***$p<0,05$ as compared with normospermia.

Parameter	Fertile men	Infertile men		p-value (ANOVA)	Tukey post hoc analysis		
	Group I (n = 46)	**Group II (n = 65)**	**Group III (n = 105)**		**Group 1 vs Group II**	**Group I vs Group III**	**Group II vs Group III**
Calcium, mM/l	5,15 ± 0,42	4,36 ± 0,29	2,28 ± 0,18	**<0.001***	**<0.001***		
cAMP, mM/l	27,9 ± 2,2	24,6 ± 2,2	14,6 ± 1,8	**<0.001***	**<0.001***		
Lactate, mM/l	4,51 ± 0,32	6,38 ± 0,43	9,04 ± 0,67	**<0.001***	**<0.001***		
Pyruvate, mM/l	2,14 ± 0,11	2,02 ± 0,12	1,26 ± 0,09	**<0.001***	**<0.001***		
[NAD$^+$]/[NADH]	4317 ± 38	2922 ± 25*	1394 ± 15*,**	**<0.001***	**<0.001***		

(nearly 32–67 % below Group I). Pathospermia was characterized by the positive relation between the value of the NAD$^+$/NADH coefficient and the spermatozoa concentration ($p < 0.01$), this was not present in fertile donors.

4. Discussion

Calcium plays an essential role in chemotaxis, acrosomal reaction and capacitation (Correia et al., 2015; Alasmari et al., 2013). A calcium decrease in infertile males may be due to a disturbance in the mechanisms of sperm cell Ca transportation in cases of idiopathic infertility. The revealed close correlation between the Ca and cAMP secondary mediators and sperm motility indicate the interconnection and interdependence of the gamete motor apparatus dysfunction mechanisms with the processes of intercellular signals transmitting and their energy supply.

The results of our research show a greater value of the NAD$^+$/NADH spermoplasm coefficient in fertile males, which is calculated based on lactate and pyruvate molar concentrations, when compared with other liver or myocardium metabolic states - more than 4000 in contrast to 500–700 (Lamb et al., 2008). The decrease in the redox potential of pyridine nucleotides and the conversion of this system as the main interface of metabolic processes to the hyper-reduced state corresponds to the anaerobic inversion of oxidative conversions and metabolic disadaptation in general (Oka et al., 2012).

Calcium and cAMP act as synergists that trigger a cascade of phosphorylation reactions in flagellar proteins during spermatozoa maturation. Along with ATP they are directly or indirectly involved in managing apoptosis in epididymal and ejaculate gametes (Mendoza et al., 2011). The deficiency of ATP, regardless of its cause, leads to necrosis activation and apoptosis inhibition, similar to the effect of abnormal concentrations of free radicals or lactate (Erkkila et al., 2006). In turn, ATP metabolism is closely connected with formation and transformations of cyclic nucleotides, in particular, cAMP, which is directly involved in most physiological processes determining fertility. Cyclic AMP in spermatozoa increases the hexose utilization rate, and serves as a positive inductor of protein biosynthesis, initiates motor activity at the stage of maturation in the epididymis, stimulates and prolongs progressive mobility, inhibits premature capacitation, regulates the supply of Ca^{2+} and Na^+ ions and the proton-ATPase activity during acrosomal reaction (Muratori et al., 2009; Wertheimer et al., 2013). Therefore, the deficiency of cAMP in seminal fluid that develops in cases of idiopathic infertility may contribute to reproductive dysfunction. cAMP has been shown to have previously unknown autocrine and paracrine functions in the extracellular space as an intercellular communication agent (Osycka-Salut et al., 2014; Alonso et al., 2017).

The NAD$^+$/NADH ratio is considered to be the integral index of metabolic and energy states in cells and tissues. In fertile males the ratio is high, which indicates a normal aerobic state. Our research shown NAD$^+$/NADH ratio decrease in idiopathic infertility. This entails many negative consequences, since the redox state of pyridine nucleotides is an important metabolic homeostasis indicator, as well as a regulator of intracellular signals in response to stress stimuli (Christensen et al., 2014). Oscillations of NAD$^+$/NADH are associated with monitoring of the quantity and quality of mitochondria, the activity of ion channels, the expression of DNA repair enzymes, and the degree of protein acetylation/deacetylation, which in turn are associated with such biological processes as oxidative stress, aging and apoptosis (Jang et al., 2012; Kilfoil et al., 2013).

NAD$^+$ is also used as a substrate by certain enzymes, including poly (ADP-ribose)-polymerases and sirtuins, the targets and final products of which regulate the growth and differentiation cells (Marcu et al., 2014; Anderson et al., 2017). Low NAD$^+$ levels are associated with disruption of histone acetylation and their replacement by protamines during spermiogenesis, which leads to the increase of abnormal spermatozoa (Bell et al., 2014). Sirtuins act as modulators of metabolic and stress-induced reactions (Srivastava, 2016). Thus, the shifts in the content of pyridine nucleotides may affect the survival rate and forms of spermatozoa cell death via several closely related mechanisms. As such, the metabolism of NAD$^+$ may be considered as a perspective molecular target for various pathologies (Mouchiroud et al., 2013), including infertility.

The system of pyridine nucleotides is closely connected with LDH, by means of which a stable glucose flow in glycolysis and stationary levels of lactate and pyruvate are maintained. Our research showed that the

imbalance of this system happens in both infertility cases and is conditioned by lactate growth. The metabolism of lactate correlates with the metabolism of pyruvate, which is restored in the LDH reaction. This process, especially under anaerobic conditions, ensures the formation of cytosolic NAD^+, required for normal glycolysis. LDH has the primary role in the reactions of glucose oxidation and ATP biosynthesis in spermatozoa. The decrease in the activity of this enzyme may be a sign of lactate energy restriction in gametes. In addition, the suppression of LDH is accompanied by glycolysis blockade due to the disturbance in the negative feedback mechanism and inhibition of NAD^+ regeneration. Sperm-specific LDH(C) is associated with 27 proteins, most of which are involved in the production, transportation and consumption of ATP, meaning this enzyme is a part of a complex that controls energy homeostasis (Odet et al., 2011).

Pyruvate combined with glucose accelerates glycolysis and stimulates the synthesis of ATP independently from mitochondrial respiration, facilitating the capacitation of human spermatozoa (Hereng et al., 2011). Pyruvate oxidation in mitochondria is activated by malate, however, the suppression of oxidation and reduction processes and pyruvate decrease in cases of infertility restricts the implementation of this scenario. As a dynamic metabolite, lactate is capable of moving among cells, tissues and organs, where it can be rapidly oxidized to final products, or converted into pyruvate and re-utilized into glucose (Philp and Baar, 2012). Lactate penetrates through biological membranes due to specific transportation systems, the so-called monocarboxylate transporters - MCT (Halestrap and Wilson, 2011). Low affinity isoforms (MCT 1 and MCT 4) are detected in the head of immature gametes, whereas a high affinity isoform (MCT 2) is expressed in the tail of mature and immature spermatozoa, which emphasizes the role of the lactate shuttle in energy supply of spermatogenesis and ejaculated cells (Brauchi et al., 2005).

Lactate initiates respiration in spermatozoa and supports their progressive mobility by moving to mitochondria, where it is oxidized with the help of mitochondrial isoform MCT 1, LDH, basigin and cytochrome oxidase (Hashimoto and Brooks, 2008). Hence there is no surprise that lactic acid is a preferable substrate for spermatozoa mitochondria even in the presence of pyruvate, glucose and fructose (Miki, 2007). It is noteworthy that in normal conditions seminiferous tubules contain a large amount of lactate secreted by Sertoli cells as a target product, which is used for energy supply of germ cells and has a clear anti-apoptotic effect (Rato et al., 2012). Aside from controlling these processes, lactate is connected with other vital functions of male gametal cells when forming reactive oxygen species (ROS) in the reaction catalyzed by NOX4 oxidase, whose products, like secondary messengers, participate in the transmission of intra- and intercellular signals, as well as in spermatozoa capacitation and hyperactivation (Galardo et al., 2014).

Thus, an increase in lactic acid content in seminal plasma in cases of idiopathic infertility is the influencing result of a variety of systemic and local factors, and may reflect its outflow from the testes, where it stimulates oxidative phosphorylation, or is involved in controlling the amount of spermatozoa with the help of apoptosis or their quality by means of ROS. At a certain stage in infertility development, the accumulation of lactate loses its adaptive potential and acquires a damaging character.

The essential issue of reproductive medicine is the low prognostic value of traditional ejaculate analysis (Leushuis et al., 2014). This problem is primarily conditioned by a wide range of physiological fluctuations in the quantitative and qualitative indices of the spermogram. It is also due to the descriptive character of morphological pathology, that gives minimal information on the cause of subfertility and cannot predict natural or artificial fertilization. Our current study provides valuable insight into the underlying pathophysiological mechanisms in idiopathic infertility, which may contribute to solving outstanding problems in immunology of male infertility.

5. Conclusion

The results of our research indicate that a key role in the development of male idiopathic infertility is due to changes in seminal plasma and spermatozoa metabolic activity. Spermatozoa metabolic profiles in cases of idiopathic infertility show distinct changes in the concentration of Calcium and cAMP, a decrease in pyruvate levels and lactate excess. The later represents a hyper-reduced state of pyridine nucleotides that can act as molecular predictor of infertility with unknown etiology. The study of spermoplasm biochemical composition enables us to evaluate the reproductive status of patients and delineate new potential treatment modalities.

Other disclaimers

None.

Declaration of Competing Interest

None.

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