Seminal plasma homocysteine, folate and cobalamin in men with obstructive and non-obstructive azoospermia

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Abstract

Purpose The aim of this study was to analyze homocysteine, folate and cobalamin in men with normozoospermia, obstructive and non-obstructive azoospermia.

Methods Analysis of plasma and seminal plasma homocysteine, folate and cobalamin in 72 azoospermic and 62 normozoospermic men. Evaluation of the azoospermic patient included testicular biopsy, endocrine, urological and ultrasound examination.

Results Homocysteine (1.2 $\mu\text{mol/l}$) and cobalamin (322.05 pmol/l) concentrations (median values) in seminal

plasma were significantly lower ($p < 0.001$) in men with azoospermia than in men with normozoospermia (2.5 $\mu\text{mol/l}$ and 579.0 pmol/l). Folate and cobalamin concentrations were significantly higher in obstructive than in non-obstructive azoospermia. Significant correlations were determined between testis volume and seminal plasma homocysteine in azoospermic men.

Conclusion Lower concentrations of homocysteine and cobalamin (but not folate) were found in azoospermic seminal plasma than normozoospermic. Folate and cobalamin were higher in seminal plasma from obstructive azoospermia than in non-obstructive azoospermia patients.

Capsule Folate and cobalamin concentrations in seminal plasma are significantly higher in obstructive than in non-obstructive azoospermia.

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Introduction

A wide range of disorders is associated with pathology of homocysteine metabolism. Folate is essential in the remethylation of homocysteine into methionine. In folate-dependent homocysteine remethylation, cobalamin (vitamin B12) is a cofactor for methionine synthetase. A deficiency of these vitamins results in hyperhomocysteinemia, which is associated with several health problems [1–3]. Evidence is increasing that homocysteine, folate and cobalamin are very important factors in human reproduction and male infertility [4–6]. Relationships between hyperhomocysteinemia and gonadal abnormalities, such as altered spermatogenesis were demonstrated. Thiol metabolism is also important for the stabilization of sperm membranes and the protection of sperm DNA against damage [7, 8]. Proliferation and differentiation of the male germ cells can be disturbed at every level. This may occur as a result of

influences that directly or indirectly affect spermatogenesis [9, 10].

Variations in sperm quality in relation to homocysteine, folate and cobalamine concentrations in blood and seminal plasma were usually investigated in men with oligozoospermia or fertile men [11, 12]. Little is known about the homocysteine metabolism in men with azoospermia (AZ). The aim of this study was to determine and compare homocysteine, folate and vitamine B12 (cobalamin) concentration in seminal plasma and also their serum levels in men with normozoospermia (NZ), non-obstructive (NOA) and obstructive azoospermia (OA).

Materials and methods

Study subjects

From January 2005 until September 2009 AZ men referred to our clinic for examination and infertility treatment were considered for participation. The selection criteria for participation were comprised of: (a) no spermatozoa could be detected on high-powered microscopic examination of a pellet after centrifugation (15 min at 3000×g) of the seminal fluid on at least two occasions; (b) history without gonadotoxic treatment, orchidectomy or malignant testicular tumour. The control group was comprised of men being screened for sperm donation during the same time period and having had at least two results of normozoospermia. Weight (kg) and height (m) were measured on the day of semen analysis and used to compute body mass index (BMI). A questionnaire was used from which the following data were extracted: smoking and use of medication for hypertension and diabetes. Informed consent was obtained from all participants. The project was approved by the Brno Faculty Hospital Scientific council and ethics commission.

Study protocol

Participants provided the ejaculates produced via masturbation in polypropylene containers in the hospital after abstinence period of 3–5 days. Semen analysis was performed according to the guidelines of the World Health Organization [13]. Sperm counts were evaluated using the Neubauer counting chamber. Before isolation of the seminal plasma the remaining liquefied ejaculate was centrifuged at 2790 g for 10 min and the samples of seminal plasma were stored at -80°C until analysis.

Venous blood samples for the determination of plasma homocysteine (Hcy) were drawn after at least 8 h of fasting into tubes containing ethylenediamine tetra-acetate (EDTA) (Monovette EDTA, Sarstedt AG & Co., Nümbrecht, Germany). Samples were kept on ice, and plasma was

separated within 1 h. Plasma concentration of Hcy was determined by high-performance liquid chromatography with fluorescence detection on Agilent 1100 (Agilent Technologies, Waldbronn, Germany). The lower limit of detection was $0.5\text{ }\mu\text{mol/l}$, inter-assay variation 7.1%.

Venous blood samples for the determination of testosterone, FSH, LH, total folate and cobalamine concentrations were drawn into vacutainer tubes (Monovette Serum Gel, Sarstedt AG & Co., Nümbrecht, Germany) and allowed to clot. After centrifugation the blood serum was collected and analyzed during routine laboratory procedure. Electrochemiluminescence assay on Modular (Roche Diagnostics, GmbH, Prague, Czech Republic) was used for folate and cobalamine analysis (inter-assay variation 11.3% and 10.3%). Chemiluminescence immunoassay on micro-particles (CMIA) on Architect i 2000 SR (Abbott Diagnostics, Prague, Czech Republic) was used for the determination of testosterone, FSH and LH (inter-assay variation 13.4%, 7.3% and 7.4%).

Evaluation of the azoospermic patient

Evaluation of the AZ patients included endocrine (total testosterone, follicle-stimulating hormone – FSH, luteinizing hormone – LH), urological and ultrasound examination [14]. Testicular biopsies were performed to confirm diagnosis of testicular AZ (results from previous biopsies were accepted). Bouin's solution was used for sample fixation and modified score count for evaluation of spermatogenesis [9]. The causes of AZ were divided into non-obstructive (Holstein score count ≤ 8) and obstructive (Holstein score count 10 and 9, dilated tubules of the epididymis with spermatozoa).

A MEDATA ATL HDI 5000 sonoCT (Sweden) was used for ultrasound examination and standard software was used for the ultrasonic volume of each testis calculation [15]. The mean testicular volume was calculated for each AZ men. The presence of multiple veins $>3\text{ mm}$, with concomitant reversal of flow on ultrasound colour Doppler examination was used as the criterium for varicocele diagnosis [16, 17].

High-performance liquid chromatography with fluorescence detection (HPLC-FD) and preceding derivatization was used for the determination of the total homocysteine concentration in the seminal fluid. We used $25\text{ }\mu\text{l}$ of 0.5 mmol/l N-Acetyl-L-cysteine (Fluka, Switzerland) as an internal standard, which was added in the first step to $100\text{ }\mu\text{l}$ of each sample. Next, $25\text{ }\mu\text{l}$ of 10% solution of Tributylphosphine (TBF; Sigma Aldrich, Canada) in N,N-dimethylformamide (DMF; Sigma Aldrich, Germany) was added. After 30 min reduction at -4°C , samples were deproteinized by $100\text{ }\mu\text{l}$ of 70% (v/v) perchloric acid (PCA; Fluka, Switzerland) -0.6 mol/l PCA containing 1 mmol/l

Table 1 Characteristics and basic parameters in men with non-obstructive azoospermia (NOA), obstructive azoospermia (OA), and normozoospermia (NZ)

Parameter ^a	NOA (<i>N</i> =55)	OA (<i>N</i> =17)	NOA + OA (<i>N</i> =72)	NZ (<i>N</i> =62)	<i>p</i> -value ^b
Patients characteristics					
Age	26 (21–36)	29 (23–40)	27 (21–38)	27 (23–32)	0.411
BMI	23.6 (20.1–29.1)	23.0 (19.2–26.9)	23.4 (19.4–27.8)	21.5 (18.2–25.6)	0.002*
Smoking (%)	18.6	20.5	19.4	16.1	0.617
Medication (%)	14.2	10.4	12.5	0	0.001*
Testis parameters					
Testis volume (ml)	11.3 (5.4–18.8)	15.6 (9.3–23.5)	12.5 (5.6–20.2)	19.2 (15.4–24.4)	<0.001*
Varicocele (%)	21.8	24.9	23.6	14.5	0.181
Semen parameters					
Semen volume (ml)	2.0 (1.1–3.4)	2.5 (1.6–4.0)	2.2 (1.1–3.6)	3.0 (2.2–4.1)	<0.001*
Sperm concentration (10 ⁶ /ml)	–	–	–	65.0 (40.0–100.0)	–
Total sperm number (10 ⁶)	–	–	–	228.0 (104.0–313.5)	–
Progressive movement	–	–	–	40.0 (40.0–55.0)	–
Normal sperm morphology	–	–	–	55.0 (50.0–64.0)	–
Biochemical parameters					
Testosterone (nmol/l)	12.2 (6.0–18.3)	19.5 (7.5–28.6)	15.4 (6.3–26.3)	23.8 (19.4–33.2)	<0.001*
FSH (IU/l)	7.0 (4.0–11.6)	5.7 (2.6–8.1)	6.2 (3.3–9.4)	5.4 (2.5–7.3)	0.001*
LH (IU/l)	6.7 (3.9–9.1)	4.8 (2.6–7.0)	5.8 (3.2–8.2)	3.6 (2.3–6.1)	<0.001*
Cobalamin in serum (pmol/l)	370.1 (212–540.1)	367.6 (208.6–535.4)	369.0 (212.0–536.0)	367.0 (196.0–531.0)	0.472
Folate in serum (nmol/l)	17.5 (9.6–28.2)	17.8 (9.9–28.8)	17.6 (9.8–28.4)	14.6 (10.1–31.7)	0.054
Hcy in plasma (μmol/l)	12.2 (8.4–17.2)	12.0 (8.1–16.9)	12.1 (8.3–17.0)	12.8 (7.8–16.5)	0.970
Cobalamin in seminal plasma (pmol/l)	286.0 (151.0–611.0)	411.0 (176.0–634)	322.0 (151.0–615.0)	579.0 (360.0–827.0)	<0.001*
Folate in seminal plasma (nmol/l)	20.7 (11.6–34.3)	31.5 (19.5–41.2)	22.0 (12.6–35.2)	23.5 (17.6–38.4)	0.094
Hcy in seminal plasma (μmol/l)	1.1 (0.1–3.4)	1.5 (0.4–2.9)	1.2 (0.1–2.9)	2.5 (1.6–3.8)	<0.001*

^a Continuous parameters are described by median and 5th and 95th percentile, categorical parameters by percentages of categories

^b Statistical significance (NZ vs. NOA + OA) was tested by means of Mann-Whitney *U* test for continuous parameters and by means of maximum likelihood chi-square test for categorical parameters; statistically significant results are given in bold and denoted by *

1 EDTA • Na₂ • 2 H₂O (Fluka, Switzerland) for 10 min and centrifuged at 21 910 g for 15 min. Subsequently, 25 μl of the deproteinized sample was derivatized with 7-fluorobenzofurazane-4-sulphonic acid (SBD-F; Fluka, Switzerland) for 1 h at 60°C by adding 50 μl of borate buffer (125 mmol/l Na₂B₄O₇ • 10 H₂O - Fluka, Germany- and 4 mmol/l EDTA • Na₂ • 2 H₂O, pH 10.1) and 25 μl of SBD-F solution (1 mg/ml of borate buffer; 125 mmol/l Na₂B₄O₇ • 10 H₂O and 4 mmol/l EDTA • Na₂ • 2 H₂O, pH 9.5). The derivatization was stopped by an ice-bath and the samples were stored at 4°C until the next day HPLC analysis. Twenty μl of each derivatized sample was injected into the column. We used a gradient elution at flow rate of 1 ml/min: 0.1 mol/l phosphate buffer, pH 3.0 : acetonitrile (ACN; Scharlau, Spain) 100 : 3 (v/v) for 3.17 min, 0.1 mol/l phosphate buffer, pH 3.0 : ACN 100 : 7 (v/v) for minutes 3.17–10.35, and 0.1 mol/l phosphate buffer, pH 3.0 : ACN 100 : 3 (v/v) for minutes 10.35–12.00.

We applied a solvent delivery system (UFLC prominence liquid chromatograph LC-20AD, Shimadzu, Duisburg, Ger-

many) and fluorescence detector (RF 10AXL, Shimadzu, Duisburg, Germany) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The separating column was Supelcosil LC-18, 150×4.6 mm, 5 μm particle size (Supelco- Analytical, Prague, Czech Republic) and guard column C18, 4×3 mm (Phenomenex, Torrance, California, USA). Data obtained were analysed with the Clarity chromatography data system (DataApex, Prague, Czech Republic). Concentrations of homocysteine were calculated using a 5-point calibration curve, which was obtained from homocysteine standard solutions (Sigma Aldrich, England) of concentrations 1.5625 μmol/l, 3.125 μmol/l, 6.25 μmol/l, 12.5 μmol/l, and 25 μmol/l. The limit of detection (signal/noise 5 : 1) was 0.2 μmol/l. All samples were analysed in triplicate in the same run.

Statistical analysis

The study groups were described using standard descriptive statistics. Continuous parameters were described by median

Table 2 Correlations of biochemical parameters in non-obstructive azoospermia ($N=55$), obstructive azoospermia ($N=17$) and normozoospermia groups ($N=62$)

Spearman correlation ^a	Cobalamin in seminal plasma ($\mu\text{mol/l}$)	Folate in seminal plasma (nmol/l)	Hcy in seminal plasma (mmol/l)
Non-obstructive azoospermia			
Cobalamin in serum (pmol/l)	0.086 ($p=0.473$)	0.084 ($p=0.482$)	0.069 ($p=0.562$)
Folate in serum (nmol/l)	-0.173 ($p=0.146$)	0.334 ($p=0.004^*$)	0.155 ($p=0.194$)
Hcy in serum ($\mu\text{mol/l}$)	0.097 ($p=0.418$)	-0.244 ($p=0.059$)	-0.206 ($p=0.082$)
Cobalamin in seminal plasma (pmol/l)		0.073 ($p=0.543$)	0.099 ($p=0.410$)
Folate in seminal plasma (nmol/l)			0.097 ($p=0.419$)
Obstructive azoospermia			
Cobalamin in serum (pmol/l)	0.425 ($p<0.001^*$)	0.242 ($p=0.057$)	0.067 ($p=0.573$)
Folate in serum (nmol/l)	0.294 ($p=0.018^*$)	0.437 ($p<0.001^*$)	-0.239 ($p=0.063$)
Hcy in serum ($\mu\text{mol/l}$)	0.099 ($p=0.410$)	-0.280 ($p=0.027^*$)	0.098 ($p=0.417$)
Cobalamin in seminal plasma (pmol/l)		0.318 ($p=0.012^*$)	-0.082 ($p=0.492$)
Folate in seminal plasma (nmol/l)			-0.075 ($p=0.532$)
Normozoospermia			
Cobalamin in serum (pmol/l)	0.925 ($p<0.001^*$)	0.442 ($p<0.001^*$)	-0.245 ($p=0.055$)
Folate in serum (nmol/l)	0.563 ($p<0.001^*$)	0.837 ($p<0.001^*$)	-0.324 ($p=0.010^*$)
Hcy in serum ($\mu\text{mol/l}$)	-0.538 ($p<0.001^*$)	-0.703 ($p<0.001^*$)	0.297 ($p=0.019^*$)
Cobalamin in seminal plasma (pmol/l)		0.466 ($p<0.001^*$)	-0.283 ($p=0.026^*$)
Folate in seminal plasma (nmol/l)			-0.278 ($p=0.028^*$)

^a statistically significant results are given in bold and denoted by *

and 5th and 95th percentiles; categorical parameters were described using percentages of individual categories. Statistical significance of differences between groups of patients' was tested by means of Mann-Whitney U test for continuous parameters and by means of maximum likelihood chi-square test for categorical parameters. Spearman rank order correlation was used to analyze correlations between biochemical parameters of serum and seminal plasma, sperm/testis characteristics and BMI. Relationships between AZ type and biochemical parameters were analysed by means of Kruskal-Wallis test; for post hoc comparison of differences between individual types of AZ multiple comparison of mean ranks test was applied. The critical limit for the level of significance was set to $\alpha=0.05$ for all applied tests. Statistical analyses were performed using Statistica 8.0 (Statsoft, Inc.) and SPSS 17 (SPSS Inc.).

Results

A total of 72 men with AZ and 62 men with NZ were evaluated in this study. Basic descriptive characteristics and parameters are presented in Table 1. Most frequent type of AZ was non-obstructive (55 men, 76.4%).

Homocysteine and cobalamin concentrations in seminal plasma were significantly lower in men with AZ ($p<0.001$),

but folate was similar in both groups. The Hcy concentrations in seminal plasma of men with AZ were below detection limit ($0.2 \mu\text{mol/l}$) in 11 samples tested. These samples were allocated a value of $0.1 \mu\text{mol/L}$ (half of the lowest detectable value for Hcy in seminal plasma). No significant differences were found in plasma homocysteine and serum folate and cobalamin between the groups of NZ and AZ. As expected, the testis volume, semen volume and testosterone concentration were higher in men with NZ. Body mass index and the use of medication (cardiovascular, respiratory and metabolic) were significantly higher or more prevalent, respectively, in AZ men. Patients' age and the extent of smoking (more than three cigarettes per day) were similar between both groups.

Spearman rank order correlation was used to analyse correlation of biochemical parameters in serum and seminal plasma (Table 2). In the NOA group only folate in seminal plasma correlated with serum folate (0.334 ; $P<0.004$), in OA more correlations of both seminal plasma folate and cobalamin were found. In the NZ group significant correlations of Hcy, folate and cobalamin in seminal plasma and serum were more frequently determined, but significant correlation of these values with sperm concentration and progressive movement was not found. Kruskal-Wallis test was used to analyze relationship of seminal plasma Hcy,

Table 3 Relationship of seminal plasma homocysteine, folate and cobalamin with azoospermia type (median and 5th and 95th percentile, statistically significant results are given in bold and denoted by *)

Azoospermia type	Cobalamin in seminal plasma (pmol/l)	Folate in seminal plasma (nmol/l)	HCY in seminal plasma (μmol/l)
Non-obstructive (<i>N</i> =55)	286.0 (151.0–611.0)	20.7 (11.6–34.3)	1.1 (0.1–3.4)
Obstructive (<i>N</i> =17)	411.0 (176.0–834.0)	31.5 (19.5–41.2)	1.5 (0.4–2.9)
<i>p</i> -value	0.014*	0.001*	0.095

folate and cobalamin with AZ type. Folate concentration was significantly higher in OA than in NOA. Cobalamin concentration was significantly higher in OA than in NOA (Table 3). Significant correlations were found between testis volume and seminal plasma homocysteine (0.260; $P=0.028$), folate (0.361; $P=0.002$) and cobalamin (0.469; $P<0.001$) in AZ men. In the NZ group only seminal plasma cobalamin significantly correlated with testis volume (0.295; $P=0.020$) and smoking ($P<0.001$).

Discussion

In this study we demonstrated significant differences between Hcy and cobalamin levels in seminal plasma of men with AZ and NZ, while blood levels of Hcy, cobalamin and folate in NOA, OA and NZ were similar and within the range given in previous literature [1, 6, 18]. In NZ men seminal plasma levels of cobalamin and folate were higher than those in blood, in NOA men only seminal plasma folate was higher than serum folate. These correlations suggest an active transfer of these substances as the values are higher in seminal plasma than in blood. Similar results and conclusions about active transport from blood to ejaculate have been reported in literature [6, 18, 19].

Seminal fluid is a mixture of combined secretions of the male accessory sex glands. Thus, it is difficult to determine where folate and cobalamin in seminal plasma comes from and how much is secreted from each gland. High affinity folate-binding sites were detected in human testicular tissue [20], as well as 5,10-methylenetetrahydrofolate reductase (MTHFR), which proved to be five times higher in the mouse testis than in other tissue [21], but the cellular origin of this activity has not been determined. In OA the ductal transport system is not patent, but we found significantly higher folate and cobalamin in seminal plasma of OA men than NOA men. This finding implies that their secretion is more related to testicular function than transport through ductal system.

The low levels of Hcy and cobalamin in seminal plasma of men with AZ may occur as a result of severe testicular pathology [9]. The correct composition of ejaculate depends on intratesticular and extratesticular regulatory

processes, the functions of the intertubular microvasculature, the Leydig cells and other cellular components of the male reproductive organs [22]. We found correlations between volume of the testis and seminal plasma Hcy level in AZ. Correlation with serum testosterone level was not found.

Varicocele was insignificantly ($P=0.181$) more frequent in men with AZ (23.6%) than in the NZ group (14.5%). No correlation with Hcy, folate and cobalamin seminal plasma concentrations was found. This is also in line with the study of varicocele and reactive oxygen species [23] and current varicocele management [24].

In the NZ group, the absence of a correlation between Hcy and sperm parameters is consistent with the results from other studies [2, 5]. The other significant correlations (Table 2) of folate and cobalamin in serum and seminal plasma in NZ group have been also described [7, 25].

Homocysteine and cobalamin metabolism is involved in a large number of physiological and pathophysiological processes in the testis. Altered testicular function could result in decreased homocysteine and cobalamine concentrations in seminal plasma of men with AZ and decreased cobalamin and folate in NOA. The exact mechanisms underlying these phenomena still need to be further investigated.

Conclusions

Lower concentrations of homocysteine and cobalamin (but not folate) were found in azoospermic seminal plasma than normozoospermic. Folate and cobalamin were higher in seminal plasma from obstructive azoospermia than in non-obstructive azoospermia patients.

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