Seminal Plasma Cobalamin Significantly Correlates With Sperm Concentration in Men Undergoing IVF or ICSI Procedures

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ABSTRACT: Mild hyperhomocysteinemia is caused by B vitamin deficiencies. We hypothesized that these biochemical derangements detrimentally affect spermato genesis. Therefore, the aim of this study was to investigate the folate, cobalamin, pyridoxine, and homocysteine concentrations in blood and seminal plasma and the associations between these biomarkers and semen parameters in men participating in an in vitro fertilization or intracytoplasmic sperm injection program. From 73 men (median age [range]: 37 years [28-53]), blood and semen samples were obtained for the determination of serum and red blood cell (RBC) folate, serum total cobalamin, whole-blood pyridoxal-5'-phosphate, plasma total homocysteine (tHoC), and serum total testosterone. Semen analysis included sperm concentration, motility, and morphology according to World Health Organization criteria. The B vitamins and tHoC concentrations were significantly correlated in blood but not in seminal plasma. The serum and RBC folate concentrations were significantly correlated also with the total folate concentration in seminal plasma ($r = .44$, $P < .001$ and $r = .38$, $P < .001$, respectively). Likewise, the total cobalamin concentration in serum and seminal plasma was significantly correlated ($r = .55$, $P = .001$). Of interest is that the total cobalamin concentration in seminal plasma was significantly correlated with the sperm concentration ($r = .42$, $P < .001$). This is in contrast to the absence of significant associations between the other vitamins and tHoC in blood and seminal plasma and any of the semen parameters. These findings suggest that folate and cobalamin are transferred from the blood to the male reproductive organs and emphasize the role of cobalamin in spermogenesis in man.

Key words: Assisted reproduction, folate, homocysteine, semen analysis.

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Spermogenesis is influenced by a combination of endocrine, genetic, and environmental factors, including nutrition and lifestyle (Kuroki et al, 1999; Wong et al, 2000). Evidence is increasing that nutritional factors are important in reproduction and thus in spermatogenesis as well. Of main interest are the B vitamins folate, cobalamin, and pyridoxine, which are involved in homocysteine metabolism.

Folate is essential as a substrate in the synthesis of DNA and RNA precursor and in the remethylation of homocysteine into methionine. In folate-dependent homocysteine remethylation, vitamin B12 (cobalamin) is a cofactor for methionine synthase. Vitamin B6 (pyridoxal-5'-phosphate [PLP], however, is a cofactor for cystathionine-β-synthase, which is necessary for the transulfuration of homocysteine into cystathionine and cysteine. A deficiency of these vitamins causes a mild hyperhomocysteinemia in blood plasma, which is associated with several health problems, including cardiovascular and cerebrovascular diseases (Refsum et al, 2004). Furthermore, hyperhomocysteinemia has been associated with reproductive disorders, such as recurrent pregnancy loss, abruptio placentae, and congenital malformations (Steegers-Theunissen et al, 1991, 1992). Folic acid treatment significantly reduces plasma total homocysteine (tHoC) concentrations (Refsum et al, 2004). The beneficial effects of folic acid supplementation in the reduction of congenital malformations can partly be explained by the correction of hyperhomocysteinemia (Refsum et al, 2004). We recently showed a significant inverse association between embryo quality following in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment and the tHoC concentration in seminal plasma and ovarian follicular fluid (Eibisch et al, 2006).
Total seminal plasma folate concentrations are significantly correlated with blood plasma folate concentrations (Wallock et al, 2001). Moreover, folate derivatives other than 5-methyltetrahydrofolate in seminal plasma appear to be correlated with sperm count (Wallock et al, 2001). Further evidence for the role of synthetic folic acid in spermatogenesis is derived from our randomized controlled trial demonstrating a 74% increase in sperm count after folic acid and zinc sulphate intervention for 26 weeks (Wong et al, 2002).

The effect of cobalamin in reproduction is less defined, although there is some evidence that this B vitamin affects sperm parameters (Watson, 1962; Tomaszewski et al, 1963). During the 1960s and 1970s several studies described the successful treatment of subfertility in men and women following treatment of a cobalamin deficiency (Sharpe and Witt, 1962; Jackson et al, 1967; Varadi, 1967; Blair et al, 1968; Mahmood, 1971). There is no information about the effects of pyridoxine on human reproduction. From animal studies it is known that high doses of pyridoxine impair sperm motility and sperm count and cause histopathologic changes, including degeneration of germinal epithelial cells (Plassmann and Urwyler, 2001).

From this background, we investigated the total folate, total cobalamin, pyridoxine as pyridoxal-5'-phosphate, and 5f hydroxyl concentrations in blood and seminal plasma in men and their associations with semen parameters.

Methods

Study Subjects

As part of an ongoing prospective study focused on the role of nutrition, in particular folate, in fertilisation, implantation, and embryo quality (FOLFO Study), we enrolled couples undergoing an IVF or ICSI procedure at the Erasmus MC, University Medical Center in the Netherlands. Fertile and subfertile men were eligible for enrollment unless frozen or surgically retrieved sperm was to be used for the assistive reproductive treatment. Subfertility is defined by a sperm concentration of less than 20 x 10^9 cells/mL. Of the eligible IVF/ICSI population, 66% of the men participated in the FOLFO Study, resulting in the analysis of the data of 73 participants. The study protocol was approved by the Central Committee for Human Research in The Hague, The Netherlands and the Medical Ethical and Institutional Review Board of Erasmus MC, University Medical Center in Rotterdam, The Netherlands. All participants gave their written informed consent.

Study Protocol

At the intake visit the IVF treatment couples were asked to participate in the study. Between 2 weeks before and 2 weeks after oocyte retrieval men visited the andrology outpatient clinic for fertility evaluation comprising semen analysis, blood withdrawal, and seroval ultrasound. One semen sample and one blood sample were collected from each man. All laboratory analyses were performed without knowledge of the clinical diagnosis of the participants. The ultrasonic volume of each testis was calculated from 3 perpendicular measurements in the equation V (mL) = π × length × width × depth (all in cm)/6. The mean testicular volume was calculated for each participant.

Semen samples were produced via masturbation into polypropylene containers. After liquefication, semen analysis according to the World Health Organization (WHO) guidelines were performed (World Health Organization, 1999). Subsequently an aliquot of semen was centrifuged at 2500 × g for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at −20°C until assayed.

Venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2000 × g, the blood serum was collected before being assayed for the concentrations of total folate, total cobalamin, and testosterone. For the determination of red blood cell (RBC) folate and plasma fHcy, venous blood samples were drawn into ethylenediamine tetraacetate (EDTA)-containing vacutainer tubes. The EDTA-blood samples were kept on ice, and plasma was separated by centrifugation within 1 hour for determination of fHcy. For the determination of pyridoxine, blood was drawn into lithium-heparin-containing vacutainers.

Blood serum and seminal plasma samples from each patient were analyzed during routine laboratory procedures for total folate and total cobalamin using an immunoelectrochemoluminescence assay (E170, Roche Diagnostics GmbH, Mannheim, Germany). Directly after blood sampling, 0.1 mL of blood out of an EDTA tube was hemolyzed with 0.9 mL of freshly prepared 1.0% acetic acid solution. Subsequently the hematocrit of the EDTA-blood was determined on an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen, Germany). The hemolysate was centrifuged for 5 minutes at 1000 × g shortly before the folate measurement. The folate concentration in the hemolysate was recalculated in RBC folate using the following formula: (nM hemolysate folate × 10 hematocrit) − (nM serum folate × [1 − hematocrit/hematocrit]) = nM RBC folate.

Pyridoxine levels in whole blood and seminal plasma and fHcy levels in EDTA plasma and seminal plasma
were determined during routine laboratory procedures using high-performance liquid chromatography with reversed-phase separation and fluorescence detection (Schrijver et al., 1981, Pfieffer et al., 1999). We determined total pyridoxine with PLP as the most common form. Testosterone was measured using the Osteo-Covit radioimmunoassay (Diagnostic Products Corp., Los Angeles, Calif.).

The between-run coefficient of variation for serum total cobalamin was 5.1% at 125 pmol/L, and 2.9% at 755 pmol/L; the coefficients of variation for serum total folate were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L; 3.3% at 14.55 pmol/L and 2.3% at 34.23 pmol/L for tHcy; 1.8% at 40 nmol/L and 1.3% at 115 nmol/L for pyridoxine; and less than 7.5% for testosterone. The detection limit for total folate was 1.36 nmol/L; for total cobalamin 22 pmol/L, for pyridoxine 5 nmol/L, for tHcy 4 pmol/L, and for testosterone 0.1 nmol/L. In addition, men filled out a general questionnaire from which the following data were extracted: medical history, education, use of medication, and lifestyle factors, such as smoking and the use of vitamin supplements.

Statistical Analysis

The results were expressed as median (range) or as a percentage and analyzed for statistical significance using nonparametric tests because of a skewed distribution of the semen parameters and B vitamins and tHcy in blood and seminal plasma. Spearman rank correlation coefficients were calculated to determine associations between semen parameters and total folate, total cobalamin, PLP, and tHcy concentrations in blood and seminal plasma. In the figures we present the log transformed variables.

To adjust for possible confounding variables, a multiple linear regression analysis was performed with the logarithm of semen concentration as dependent variable. In this analysis, age, body mass index (BMI), smoking, alcohol, medication, intake of multivitamin supplements, intake of folic acid supplements, serum total testosterone, mean testicular volume measured by ultrasonography, history of urologic surgery, and the presence of a varicocele were considered potential confounding factors.

Results

The population characteristics and semen parameters are presented in Table 1. Due to a low semen volume, concentrations of total folate and total cobalamin could not be determined in 2 samples, and PLP and tHcy concentrations were missing in 7 and 19 semen samples, respectively. The median serum testosterone was 14.5 nmol/L (range: 5.8–31.5). The median of the mean testicular volume was 10.9 mL (range: 5.5–22.0). In the study population, a varicocele was diagnosed in 17 participants (26%) and 5 men (7%) had a history of urologic surgery. Twelve men used medication for the following categories of diseases: cardiovascular (3), endocrinologic (3), psychologic (2), respiratory (2), metabolic (1), and dermatologic (1). None of these drugs are known to affect sperm parameters or the concentrations of B vitamins and tHcy. According to the sperm concentration in the semen samples for this study, 34% of the participants were subfertile (<20 × 10^6 cells/mL).

The total folate and total cobalamin concentrations in serum were significantly lower than in seminal plasma (P < .001 and P < .001, respectively), and RBC folate and pyridoxine concentrations were significantly higher (P < .001 and P < .001, respectively) in blood than in seminal plasma. The tHcy concentrations in serum and seminal plasma were comparable (P = .137) (Table 2). In blood, the total folate, total cobalamin, PLP, and tHcy concentrations were significantly correlated with each other, but no significant correlations between these B vitamins and tHcy in seminal plasma could be determined. In addition, significant correlations were determined among serum, RBC, and seminal plasma.
Table 2. Concentrations of B vitamins and total homocysteine in blood and seminal plasma

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Blood median (range)</th>
<th>Seminal plasma median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate serum (nmol/L)</td>
<td>14.7 (6.8-313.1)</td>
<td>25.7 (13.5-78.6)</td>
</tr>
<tr>
<td>Folate red blood cells (nmol/L)</td>
<td>0.75 (0.58-2.16)</td>
<td></td>
</tr>
<tr>
<td>Total cobalamin (pmol/L)</td>
<td>317 (141-669)</td>
<td>509 (94-1250)</td>
</tr>
<tr>
<td>Presence of a variance (nmol/L)</td>
<td>76 (40-900)</td>
<td>24 (0-500)</td>
</tr>
<tr>
<td>Total homocysteine (umol/L)</td>
<td>11.6 (7.8-20.7)</td>
<td>6.3 (1.7-35.5)</td>
</tr>
</tbody>
</table>

total folate levels as well as between serum and seminal plasma total cobalamin (Figure 1). No significant correlations were observed between the tHcy and PLP concentrations in blood and seminal plasma.

A significant correlation was determined between cobalamin in seminal plasma and sperm concentration \( r = .42; P = .001 \) (Figure 2). The other B vitamins and tHcy in blood and seminal plasma were not significantly correlated with any of the semen parameters. We adjusted this significant correlation for possible confounders by multiple linear regression analysis with the logarithm of the semen concentration as dependent variable and the concentration of cobalamin in seminal plasma as independent variable. None of the factors considered as potential confounders (age, BMI, smoking, alcohol, medication, intake of multivitamin supplements, intake of folic acid supplements, serum total testosterone, mean testicular volume measured by ultrasound, a history of urologic surgery, and the presence of a varicocele) reached statistical significance. In this linear regression model, the corresponding standardized regression coefficient was 0.35 \( (P = .012) \). After correction for the previously described variables, the regression coefficient was only marginally different compared with the crude estimate \( r = .34; P = .013 \).

Discussion

In this study, we clearly demonstrated a significant positive correlation between the total cobalamin concentration in seminal plasma and the sperm concentration in men participating in an IVF or ICSI procedure. Moreover, the total cobalamin concentration in serum was significantly correlated with the seminal plasma concentration.

Tomaszewski et al (1963) found lower cobalamin concentrations in men with oligospermia than in men with normal semen concentrations, which is in line with our finding. It was previously suggested that cobalamin influences the maturation of human spermatozoa (Watson, 1962). Our data, however, did not show a significant correlation between sperm morphology and cobalamin concentration. Differences in the distributions of sperm morphology in both studies and the method of determining sperm morphology may explain these differences.

According to the criteria of WHO, all men participating in the present study should be classified as subfertile because of their low sperm morphology scores (World Health Organization, 1999). The threshold for sperm morphology, however, is widely debated and several authors have advocated a lower threshold (Omebe et al., 1997; van der Merwe et al., 2005). For that reason, we defined male subfertility in our study as a sperm concentration of less than 20 x 10^6 sperm/mL.

Of interest is that in the 1960s and 1970s subfertile, cobalamin-deficient men were described who became fertile after treatment with cobalamin (Sharp and Witts 1962; Blair et al., 1968). In our study, however, only 1 male showed a borderline cobalamin concentration (141 pmol/L; normal: >145 pmol/L) with a normal semen analysis (118 x 10^6 sperm/mL, 43% progressive motility, 7% normal sperm morphology). Although the sperm morphology was below the WHO criteria, there is still a lot of discussion about the definition of "normal" sperm morphology (World Health Organization, 1999; Merwe van der et al., 2005).

Another interesting finding is the significant correlation between the total folate concentrations in blood and seminal plasma. The total folate concentrations in serum and seminal plasma were slightly lower and in RBC slightly higher than the preintervention concentrations in fertile and subfertile men reported by us previously (Wong et al., 2002). This may be due to laboratory differences, such as the use of different assays, population differences, and changes in dietary patterns and lifestyles in the last decade in the Netherlands. This is also in line with the slightly higher tHcy concentrations in seminal plasma compared with the concentrations reported by Elbash et al. (2006).

A significant correlation between the total folate concentration in seminal plasma and sperm parameters could not be determined. This is in contrast to the results of Wallock et al. (2001), which demonstrated a significant correlation between nonmethylated folate derivatives, in particular, and sperm count.
difference may be due to the fact that they determined a subgroup of nonmethylated folate derivatives while we measured mainly methyltetrahydrofolate.

The absence of a correlation between tHcy and sperm parameters is consistent with the results from Ebisch et al. (2006). However, in that study we assessed a significant association between the embryo quality and tHcy concentration in both seminal plasma and follicular fluid. This emphasizes the importance of considering both male and female parameters in the investigation of (sub)fertility.

In general, the correlations among total cobalamin, total folate, and PLP in blood are rather small, which is very likely due to the many factors that determine these biomarkers, such as diet, medication, supplement use, metabolism, and genetic variations. The significant correlations between tHcy and total folate and total cobalamin are well known (Refsam et al., 2004). The
In summary, the strong correlation between seminal plasma total cobalamin and sperm concentration supports the role of this vitamin, besides that of folate, in spermato genesis in humans. Because male factor subfertility due to cobalamin deficiency is amenable to curative and/or preventive action by supplementation, studies should focus on the determination of cut-off points of cobalamin concentration in blood and seminal plasma and the efficacy and safety of supplementation on semen parameters and fertility as outcome measures.

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References


