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Does folic acid and zinc sulphate intervention affect endocrine parameters and sperm characteristics in men?

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**Summary**

We evaluated pre- and post-intervention endocrine and semen parameters in a double-blind, placebo-controlled intervention study to investigate the underlying mechanism of increased sperm concentration after folic acid and zinc sulphate intervention. A total of 47 fertile and 40 subfertile males participated in a 26-week intervention study consisting of a daily treatment with folic acid (5 mg/day) and zinc sulphate (66 mg/day), or placebo. Pre- and post-intervention semen parameters, serum folate, zinc, follicle-stimulating hormone (FSH), testosterone and inhibin B concentrations were measured. The results indicated that intervention treatment significantly increased sperm concentration in subfertile males. Other semen and endocrine parameters were not affected by intervention treatment. At baseline, positive correlations were found between serum zinc and sperm concentration, motility and inhibin B. Serum zinc and FSH were inversely correlated. As (already) well known from previous research, inhibin B positively correlated with sperm concentration, motility and morphology, and was inversely correlated with FSH. The latter was positively correlated with testosterone. In addition, testosterone and inhibin B were inversely correlated. After intervention, the correlations with zinc disappeared. We conclude that the increase in sperm concentration after folic acid and zinc sulphate intervention is not the result of alterations in FSH, testosterone or inhibin B concentrations. Although zinc and folate have several effects on spermatogenesis, the underlying mechanisms involved are not clear.

**Introduction**

Approximately 15% of all couples in the Western world are involuntarily childless, which has a great influence on the quality of life (Wallace, 1995). Male factor subfertility plays a role in about 50% of subfertile couples (World Health Organization, 1987). In up to 40%, the aetiology of male subfertility remains unknown (de Kretser, 1997). Additional knowledge on the cause of male factor subfertility is crucial for better treatments of couples who are involuntary childless.

Unlike genetic causes of male factor subfertility, environmental causes of subfertility are of particular interest, because of the possibility of curative or preventive measures. A significant amendable environmental factor is nutrition. Animal studies have demonstrated the importance of the effects of nutrition on spermatogenesis (Kwiecinski et al., 1989; van Pelt & de Rooij, 1991; Ciereszko & Dabrowski, 1995).

In a first randomized placebo-controlled study, in which fertile and subfertile males were included, we recently demonstrated a 74% increase in normal sperm concentration in the subfertile males after folic acid and zinc sulphate intervention (Wong et al., 2002). The fertile and subfertile males were assigned to four intervention groups, comprising daily doses of folic acid (5 mg) and...
placebo, zinc sulphate (66 mg) and placebo, a combined dose of folic acid and zinc sulphate, or placebo/placebo for 26 weeks. The significant increase of more than 70% in sperm concentration was only observed in subfertile males receiving the combined treatment with folic acid and zinc sulphate. In an extended study, we showed that MTHFR 677CC carriers benefit significantly more from this intervention treatment with regard to the sperm concentration than MTHFR CT/TT carriers (Ebisch et al., 2003).

Natural folate is important for the synthesis of DNA, transfer RNA and proteins, and is therefore suggested to be important in spermatogenesis. Zinc serves as a cofactor for more than 80 metalloenzymes involved in DNA transcription, expression of steroid receptors and protein synthesis. Furthermore, it has been shown that zinc is important in testicular development, spermatogenesis and sperm motility, as reviewed by Wong et al. (2000).

The underlying mechanisms involved in the beneficial effects of synthetic folic acid and zinc sulphate on spermatogenesis are not clarified. Therefore, we hypothesized that folic acid and zinc sulphate may affect endocrine parameters, for example, by stimulating the function of the Sertoli cells. These cells provide the essential microenvironment for normal germ cell production and could therefore be relevant.

Sertoli cells are the main producers of inhibin B in the human body. The serum inhibin B concentration positively correlates with sperm concentration, testicular volume and the state of the spermatogenic epithelium (Pierik et al., 1998; von Eckardstein et al., 1999). Moreover, inhibin B appears to be a marker of advanced stages of spermatogenesis (Anderson & Sharpe, 2000; Andersson, 2000). Thus, the inhibin B concentration reflects the quality of the Sertoli cell function and spermatogenesis and as such can be used as a sensitive marker of spermatogenesis in humans.

The aims of the present study were to assess the effects of folic acid and zinc sulphate intervention on semen parameters and the hormonal levels of inhibin B, follicle-stimulating hormone (FSH) and testosterone, and to determine associations between baseline and post-intervention folic acid and zinc concentrations, and the endocrine and semen parameters.

Materials and methods

Patients
This study is part of our previously described randomized trial on the effects of folic acid and/or zinc sulphate administration on semen parameters of fertile and subfertile males (Wong et al., 2002). Every participant took two capsules per day for 26 weeks after dinner. The randomization schedule was folic acid/placebo, folic acid/zinc sulphate, zinc sulphate/placebo, or placebo/placebo. The dosage of the folic acid capsule was 5 mg per capsule per day, and for zinc sulphate 66 mg per capsule per day. In the current study we evaluated the serum concentrations of folate, zinc, FSH, testosterone and inhibin B, in the available serum specimens, and the semen parameters in the fertile and subfertile males, before and after intervention with folic acid and zinc sulphate or placebo.

For the inhibin B determinations a total of 47 serum samples were available from the original group of 49 fertile males, of which 23 males had received placebo/placebo, and 24 had the combination treatment. From the subfertile males, 40 serum samples were available from the original group of 49 subjects, of which 22 males had received the placebo/placebo, and 18 the combination treatment. The data of the other hormones and folate and zinc concentrations were available from the original data set.

The fertile group comprised healthy males without a history of fertility problems whose partners were pregnant at the start of the recruitment and who conceived spontaneously within 1 year of regular, unprotected intercourse. Subfertility was defined as failure of the female partner to conceive after 1 year of regular, unprotected intercourse and a sperm concentration between 5 and $20 \times 10^6$ spermatozoa/mL at the first routine semen analysis after referral to the fertility clinic. We excluded subfertile males with a sperm concentration of $<5$ million spermatozoa/mL semen, thereby excluding males with a severe, possibly genetically caused, deranged spermatogenesis, who are not very likely to benefit from intervention treatment. The cut-off value of 20 million spermatozoa/mL semen was deducted from the WHO guidelines for fertility. In addition, no further investigations were conducted regarding the fertility of the female partners, as it was not our aim to study the outcome measure, ‘pregnancy’. Recruitment of these males was performed as described previously by Wong et al. (2002).

The study was approved by the Institutional Review Board of the University Medical Centre Nijmegen, and all participants gave written informed consent before participation.

Semen samples
Before and after intervention one semen sample was obtained from every participant for standardized semen analysis according to WHO guidelines (World Health Organization, 1992). The semen samples were produced by the participants via masturbation after an abstinence period of at least 3 days. These samples were delivered at the fertility laboratory within 1 h after production. After liquefaction, the semen parameters such volume, sperm concentration, motility and morphology were determined.
Sperm concentration was determined with a Makler counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel). Motility was expressed as the percentage of motile spermatozoa, and morphology was determined according to WHO criteria (World Health Organization, 1992), after incubation of the sample with trypsin (10 min at room temperature), staining with methylene blue/eosin, feathering and fixation by flame.

Folate and zinc measurements

Serum folate concentrations were measured using radioassays (Duocount Solid Phase Boil radioassay; Diagnostic Products Corp., Los Angeles, CA, USA). Serum zinc was measured using flame atomic absorption spectrophotometry (Perkin Elmer 4100; Perkin Elmer, Norwalk, CT, USA).

Hormone determinations

Follicle-stimulating hormone was quantitatively determined in serum (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Serum testosterone was routinely measured using a specific dextran-coated radioimmunoassay after extraction of serum specimens with diethylether and subsequent isolation of the T fraction by Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column chromatography, using an antiserm raised in rabbits and directed against T-(o-carboxymethyl)oxime–BSA (Dony et al., 1985). Inhibin B was determined using kits purchased from Serotec Limited (Oxford, UK). The intra-assay coefficient of variance (CV) was <9% and the interassay CV was <15%. The lowest detectable inhibin B concentration was 5 ng/L, based on the mean value of the zero-dose standard plus twice the standard deviation. A value of 2.5 ng/L (i.e. half of the value of the undetectable range) was assigned to test results below 5 ng/L.

Statistical analysis

Results are expressed as median (25th–75th percentile) and analysed for statistical significance using nonparametric tests, because of the skewed distribution of the semen parameters and hormone concentrations.

The Mann–Whitney U-test was used to compare age, folate, zinc and hormone concentrations, and the deltas (post-intervention value minus pre-intervention value) between the fertile and subfertile males. To compare endocrine and semen parameters in fertile and subfertile males before and after intervention, the Wilcoxon signed rank test was used. Spearman’s test was used to calculate correlation coefficients between the biochemical and semen parameters.

Furthermore, linear regression models were used to analyse the influence of folic acid and zinc sulphate on inhibin B, FSH, testosterone and sperm concentration. Statistical analysis was performed using SPSS 11.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

In Table 1 the baseline characteristics of the endocrine and semen parameters are depicted in the fertile and subfertile groups. The semen parameters were significantly different between the groups. Although serum folate was not significantly different, serum zinc concentrations were significantly higher in fertile males compared with subfertile males (p < 0.05). The median FSH concentration was higher in subfertile males compared with fertile males (p < 0.05), while the median inhibin B concentration was lower in subfertile males than in fertile males (p < 0.05).

The effects of intervention with folic acid and zinc sulphate treatment vs. placebo treatment on the serum levels of folate and zinc, the hormones of the hypothalamic–pituitary–testis axis and on the sperm concentrations in both fertile and subfertile males are presented in Table 2. After intervention with folic acid and zinc sulphate treatment, the serum folate concentrations increased in both groups (p < 0.001) but the serum zinc concentrations did not significantly increase in fertile and subfertile males (p = 0.06 and p = 0.72 respectively). The sperm concentration increased significantly in subfertile males, but not in fertile males (p = 0.007 and p = 0.45 respectively). After adjusting for the placebo effect by comparing the delta sperm concentration for subfertile males receiving...
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Table 2 Effects of folic acid and zinc sulphate treatment on serum levels of folate and zinc, endocrine parameters and sperm concentration

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Placebo (subfertile n = 22, fertile n = 23)</th>
<th>Zinc and folic acid (subfertile n = 18, fertile n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-intervention</td>
<td>Post-intervention</td>
</tr>
<tr>
<td>Subfertile males (n = 40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>18.0 (15.0–23.0)</td>
<td>17.0 (12.0–24.0)</td>
</tr>
<tr>
<td>Zinc (µmol/L)</td>
<td>18.8 (18.0–20.0)</td>
<td>20.3 (16.9–22.4)</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>5.6 (4.0–9.7)</td>
<td>6.3 (4.9–9.0)</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>18.3 (14.0–23.1)</td>
<td>19.0 (14.0–24.0)</td>
</tr>
<tr>
<td>Inhibin B (ng/L)</td>
<td>28.8 (159.8–300.3)</td>
<td>200.0 (157.8–288.0)</td>
</tr>
<tr>
<td>Sperm concentration (10^6/mL)</td>
<td>7.5 (1.5–25.0)</td>
<td>7.0 (3.8–18.3)</td>
</tr>
<tr>
<td>Fertile males (n = 47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>18.0 (15.0–21.8)</td>
<td>16.0 (13.0–23.0)</td>
</tr>
<tr>
<td>Zinc (µmol/L)</td>
<td>21.3 (18.9–24.1)</td>
<td>19.6 (18.4–21.9)</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>3.7 (2.4–6.5)</td>
<td>3.7 (2.7–6.7)</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>20.5 (17.8–24.7)</td>
<td>21.0 (17.0–25.0)</td>
</tr>
<tr>
<td>Inhibin B (ng/L)</td>
<td>253.0 (163.0–287.0)</td>
<td>246.0 (187.0–271.0)</td>
</tr>
<tr>
<td>Sperm concentration (10^6/mL)</td>
<td>85.0 (45.0–130.0)</td>
<td>80.0 (40.0–110.0)</td>
</tr>
</tbody>
</table>

Data are medians (25th–75th percentile), deltas are calculated as post-intervention value minus pre-intervention value.

*p < 0.01 for comparison between pre- and post-intervention values (Wilcoxon signed rank test); ^p < 0.01 for comparison between ‘delta placebo’ and ‘delta zinc and folic acid’ (Mann–Whitney U-test).

placebo with the delta sperm concentration for subfertile males receiving folic acid and zinc sulphate intervention, sperm concentration in subfertile males having the combination treatment was significantly higher (p = 0.006).

In order to investigate whether this increase in sperm concentration was the result of a lower production of prostate or seminal vesicle fluids, we examined the change in sperm count (ejaculate volume × spermatozoa concentration). The sperm count appeared significantly higher in subfertile males after intervention with folic acid and zinc sulphate compared with the baseline sperm count. The median baseline sperm count in the subfertile males was 28.8 × 10^6 spermatozoa (8.5–46.2), and after folic acid and zinc sulphate intervention it was 44.4 × 10^6 spermatozoa (13.6–151.8) (p = 0.009). From these data we can conclude that the observed increase in sperm concentration after the combined intervention is due to an effect on spermatogenesis, because intervention resulted in a higher production of spermatozoa.

It is not likely that folate and zinc affected the endocrine parameters, because FSH, testosterone and inhibin B concentrations were not significantly changed by the intervention treatment in the fertile and subfertile groups.

In Table 3 the correlation coefficients of pre-intervention (italics) and post-intervention (bold) in the pooled group of fertile and subfertile males are shown for serum concentrations of folate, zinc, endocrine parameters and semen parameters. Before intervention, significant correlations were observed between serum zinc and sperm concentration, motility, FSH and inhibin B, which disappeared after intervention. In addition, at baseline, inhibin B was significantly correlated with sperm concentra-

Table 3 Spearman correlation coefficients between serum folate (Fol-ser) and zinc concentrations (Zn-ser), semen and serum endocrine parameters, of the pooled data of 47 fertile and 40 subfertile males

<table>
<thead>
<tr>
<th></th>
<th>Fol-ser</th>
<th>Zn-ser</th>
<th>Sperm conc.</th>
<th>Motility</th>
<th>Morphology</th>
<th>FSH</th>
<th>Inhibin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fol-ser</td>
<td>−0.11</td>
<td>−0.06</td>
<td>−0.03</td>
<td>−0.02</td>
<td>−0.02</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Zn-ser</td>
<td>0.16</td>
<td>0.36</td>
<td>−0.002</td>
<td>−0.27</td>
<td>−0.18</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Sperm conc.</td>
<td>0.008</td>
<td>0.004</td>
<td>0.52</td>
<td>0.52</td>
<td>−0.50</td>
<td>−0.09</td>
<td>0.49</td>
</tr>
<tr>
<td>Motility</td>
<td>−0.02</td>
<td>0.08</td>
<td>0.62</td>
<td>0.48</td>
<td>−0.34</td>
<td>−0.16</td>
<td>0.29</td>
</tr>
<tr>
<td>Morphology</td>
<td>−0.02</td>
<td>−0.05</td>
<td>0.48</td>
<td>0.26</td>
<td>−0.26</td>
<td>−0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>FSH</td>
<td>−0.03</td>
<td>&lt;0.001</td>
<td>−0.22</td>
<td>−0.22</td>
<td>0.25</td>
<td>0.24</td>
<td>−0.65</td>
</tr>
<tr>
<td>Inhibin B</td>
<td>−0.11</td>
<td>0.11</td>
<td>0.08</td>
<td>0.07</td>
<td>0.11</td>
<td>−0.26</td>
<td></td>
</tr>
</tbody>
</table>

The r-values in italics indicate correlations observed before intervention and the r-values in bold indicate correlations observed after intervention.

*p ≤ 0.001; ^p ≤ 0.01; †p ≤ 0.05
In this study we confirm our previous data (Wong et al., 2002) on the beneficial effect of folic acid and zinc (standardized regression coefficient = 0.24; p < 0.001) and inhibitin B concentration (standardized regression coefficient = 0.32; p < 0.001), but the sperm concentration did not influence inhibitin B, FSH, testosterone and morphology. The FSH concentration was predicted by sperm motility (standardized regression coefficient = 0.22; p = 0.03), sperm morphology (standardized regression coefficient = −0.32; p = 0.002) and inhibitin B concentration (standardized regression coefficient = 0.24; p = 0.03). We evaluated age and duration of sexual abstinence as possible confounders with regard to the prediction of sperm concentration, but found that they are, in fact, not so in our study population. Therefore they need not be included in the regression analysis. Folic acid and zinc sulphate concentrations did not influence inhibitin B, FSH, testosterone and sperm concentrations.

After intervention, the results from the linear regression models remained the same for the predicting variables of inhibitin B and FSH concentrations, but the sperm concentration was significantly predicted by sperm motility (standardized regression coefficient = 0.31; p = 0.001), sperm morphology (standardized regression coefficient = −0.42; p < 0.001) and FSH concentration (standardized regression coefficient = −0.30; p = 0.004).

Discussion

In this study we confirm our previous data (Wong et al., 2002) on the beneficial effect of folic acid and zinc sulphate intervention on sperm concentration in subfertile males in a subset of both study groups. We could not demonstrate an effect of this intervention on FSH, testosterone and inhibitin B concentrations, reflecting the endocrine status of subfertile and fertile males. By interpreting these data we have to take into account that only an 18% increase in sperm concentration of subfertile males after intervention was now observed.

Although this is a true effect, because significance remained after adjusting for the placebo effect compared with the 74% increase described in the original paper by Wong et al. (2002), this increase is much smaller. This may certainly have affected the negative findings on the endocrine parameters. Particularly the data on inhibitin B should be interpreted with caution, because this hormone could only be determined in samples of 47 fertile and 40 subfertile males from the original study.

The baseline serum inhibitin B concentrations were significantly lower in subfertile males compared with fertile males. Only one study also made a direct comparison of inhibitin B concentrations between fertile and subfertile males (Lee et al., 2001), and reported that the mean inhibitin B concentration in subfertile and fertile males were 163 and 146 ng/L, respectively, and were not significantly different. The relatively high values we found may be due to the timing of blood collection. The serum inhibitin B concentration shows a diurnal rhythm in which the levels in the early morning are 30% higher than in the late afternoon (Andersson, 2000).

Another explanation for the different inhibitin B levels is the different selection of subfertile males. In contrast to the group of Lee et al. (2001), we excluded subfertile males with a known cause for infertility and with a sperm concentration of <5 million spermatozoa/mL semen, thereby excluding males with a severe, possibly genetically caused, deranged spermatogenesis and concomitant low inhibitin B levels. Analytical differences of the immunoassay procedures might explain the differences in inhibitin B concentration as well. Finally, the difference in inhibitin B levels could be explained by the wide range of sperm concentration with a 10-fold increase between the fertile and subfertile groups.

Furthermore, it remains to be established as to which specific type of germ cell determines inhibitin B secretion. It has already been suggested that pachytenic spermatocytes or early spermatids are the most important determinants of circulating inhibitin B (Foresta et al., 1999; Andersson, 2000; Pierik et al., 2003). As no significant change in circulating inhibitin B concentration was found after intervention, it is possible that the effects of the intervention on sperm concentration are exerted in later stages of spermatogenesis (e.g., late spermatids, spermiogenesis), thereby not influencing circulating inhibitin B concentrations.
We demonstrated a significantly positive effect on sperm concentration in subfertile males after 26 weeks of folic acid and zinc sulphate treatment. It is well known that natural folate plays an important role in the synthesis of purines and pyrimidines for tRNA and DNA, which are both important in spermatogenesis. A study by Bentivoglio et al. (1993) showed that oral folic acid supplementation improved sperm concentration and motility in infertile males presenting with a high sperm concentration of immature germ cells, e.g. spermatids and spermatocytes. In our study we could not demonstrate any correlations between serum folate concentrations and semen parameters.

Of interest is the significantly positive correlation between serum zinc concentration and sperm concentration. The zinc content in the adult testis is high, and the prostate has an even higher concentration of zinc than any other organ in the human body (Mann, 1964; Zai-chick et al., 1997). Moreover, hypogonadism is a clinical feature of zinc deficiency in humans (Sandstead et al., 1967; Prasad, 1985a,b) and sperm defects have been observed in zinc-deficient rats (Hamdi et al., 1997). It has also been shown that zinc improved spermatogenesis in animals (Hamdi et al., 1997), and increased sperm concentration (Stankovic & Mikac-Devic, 1976; Hartoma et al., 1977), motility (Kynaston et al., 1988) and morphology in subfertile males (Tikkiwal et al., 1987).

In this study we observed a significantly higher serum zinc concentration in fertile males compared with subfertile males; however, both fertile and subfertile males had serum zinc concentrations within the normal ranges. In our previous study by Wong et al. (2002), no difference was observed regarding serum zinc concentrations between these two groups. This could be explained by the smaller sample size in the present study.

Another striking result is the lack of rise in serum zinc concentrations after intervention with folic acid and zinc sulphate, also observed in our previous study (Wong et al., 2002). Possible explanations for this phenomenon are the absence of zinc deficiencies, the relatively low dose of zinc used to avoid gastrointestinal side-effects and lack of compliance. The latter explanation is not very likely considering the marked increase of folate concentration after the combined intervention with folic acid and zinc sulphate.

**Conclusion**

Despite the absence of an effect of folic acid and zinc sulphate on endocrine parameters, we conclude that the correlations between zinc and sperm concentration and the increase in sperm concentration after intervention in subfertile males, should stimulate research on nutrition and environmental factors in the pathogenesis and prevention of fertility disorders.

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