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Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial

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Objective: To study the effects of folic acid and zinc sulfate treatment on semen variables in fertile and subfertile men.

Design: Double-blind, placebo-controlled interventional study.

Setting: Two outpatient fertility clinics and nine midwifery practices in The Netherlands.

Participant(s): One hundred eight fertile and 103 subfertile men.

Intervention(s): Both groups were randomly assigned to receive one of four treatments for 26 weeks: folic acid and placebo, zinc sulfate and placebo, zinc sulfate and folic acid, and two placebos. Folic acid was given at a daily dose of 5 mg, and zinc sulfate was given at a daily dose of 66 mg.

Main Outcome Measure(s): Before and after treatment, standardized semen and blood samples were obtained for determinations of sperm concentration, motility, and morphology according to World Health Organization guidelines; semen morphology according to strict criteria; and blood folate and zinc concentrations. Effects of the four interventions were evaluated separately in subfertile and fertile men.

Result(s): Subfertile men demonstrated a significant 74% increase in total normal sperm count and a minor increase of 4% abnormal spermatozoa. A similar trend was observed in fertile men. Preintervention concentrations of folate and zinc in blood and seminal plasma did not significantly differ between fertile and subfertile men.

Conclusion(s): Total normal sperm count increases after combined zinc sulfate and folic acid treatment in both subfertile and fertile men. Although the beneficial effect on fertility remains to be established, this finding opens avenues of future fertility research and treatment and may affect public health. (Fertil Steril® 2002;77: 491–8. ©2002 by American Society for Reproductive Medicine.)

Key Words: Intervention, folate, zinc sulfate, semen parameters, male fertility

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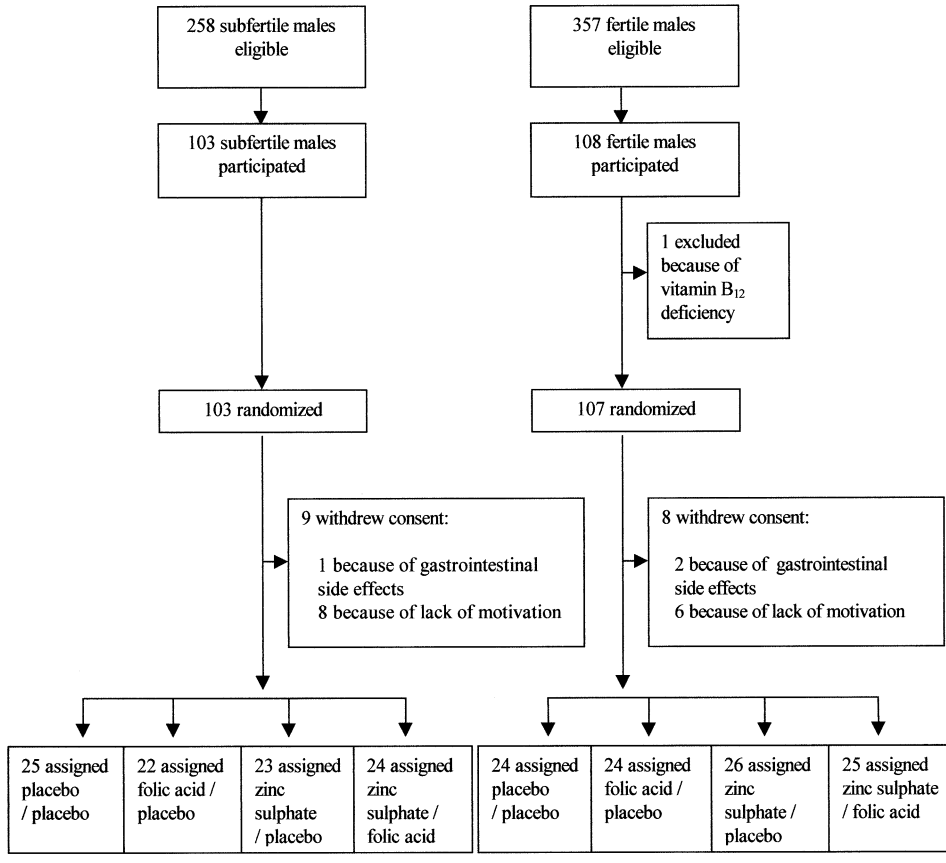
The reported decrease in semen quality may result from interactions between genetic and environmental factors that can easily be manipulated (1–4). Animal research data suggest that nutrition affects spermatogenesis (5, 6). However, data on the influence of specific nutrients on spermatogenesis in humans is scarce.

Recent food consumption surveys show that intakes of the B vitamin folate and the trace element zinc are marginal (7, 8). Folate, which is mainly present in green leafy vegetables, is essential for DNA, transfer RNA, and protein synthesis. Because DNA synthesis is a main part of spermatogenesis, folate is probably important to this process. Zinc is essential in spermatogenesis as a cofactor of metalloenzymes involved in DNA transcription, expression of steroid receptors, and protein synthesis (9–12). To date, it is unknown whether functional deficiencies in folate and zinc are a risk factor for male factor subfertility.

We conducted a double-blind, randomized, controlled trial in fertile and subfertile men to determine whether administration of folic acid, with or without the addition of zinc sulfate, increases semen quality. Fertile men were included because the effects of the interventions on normal semen quality are unknown.

FIGURE 1

Trial profile.



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MATERIALS AND METHODS

Protocol

From July 1997 to August 1998, fertile and subfertile men were invited to participate in our study, according to a protocol described elsewhere (13). Fertile men were recruited from nine midwifery practices in the surrounding area of Nijmegen, The Netherlands; this group consisted of healthy men with no history of fertility problems whose partners conceived spontaneously within 1 year of regular, unprotected intercourse. Subfertile men were recruited from the fertility clinics of the University Medical Centre Nijmegen and the Canisius Wilhelmina Hospital, Nijmegen. Subfertility was defined as failure of the female partner to conceive after 1 year of regular, unprotected intercourse and a sperm concentration of 5 and 20 million cells/mL on the first routine semen analysis after referral to the fertility clinic. Sperm concentration and results of physical examination and complete endocrine screening were part of the selection procedure after recruitment. After men with known causes of male factor subfertility, such as chromosomal disorder related to the fertility disorder (Y chromosome deletions), cryptorchidism and vasectomy, were excluded, idiopathic subfertility was diagnosed and the men were asked to participate in the trial.

Sperm concentration is highly correlated with motility and morphology. However, to make the study feasible and because sperm concentration is highly predictive of the fertility rate, we chose sperm concentration as a main selection criteria (14, 15).

Exclusion criteria overall were use of folic acid or preparations containing zinc in the 3 months before recruitment. We chose a 3-month wash-out period because spermatogenesis takes 74 days. Because treatment with folic acid masks hematologic symptoms of vitamin B_{12} deficiency, men with vitamin B_{12} deficiency ($<$100 pmol/L) were excluded.

The institutional review board and the medical ethical committee of the University Medical Centre Nijmegen ap-

TABLE 1

Baseline characteristics.

Intervention	Fertile men (n = 107)				Subfertile men (n = 103)			
	Placebo	Folic acid	Zinc	Zinc and folic acid	Placebo	Folic acid	Zinc	Zinc and folic acid
Age (y)	34.4 ± 4.7	34.2 ± 3.1	35.3 ± 4.4	32.9 ± 4.6	34.5 ± 4.1	34.2 ± 3.3	34.1 ± 4.1	34.1 ± 4.2
FSH level (IU/L)	4.6 ± 2.5	4.1 ± 2.2	4.3 ± 2.6	4.8 ± 2.2	6.6 ± 3.5	6.2 ± 3.1	5.2 ± 2.6	6.1 ± 3.7
Testosterone level (nmol/L)	22.0 ± 7.4	20.1 ± 9.4	19.1 ± 9.4	20.0 ± 7.7	19.0 ± 6.4	19.6 ± 4.2	20.0 ± 5.2	21.5 ± 9.2
Abstinence period (d)	3.8 ± 1.9	4.0 ± 1.5	3.7 ± 1.7	4.0 ± 2.6	3.7 ± 2.4	3.5 ± 1.5	3.4 ± 1.3	3.3 ± 1.1
Sperm concentration[a] ($\times 10^6$ cells/ml)	90.0 (6–190)	70.0 (25–150)	75.5 (20–160)	65.0 (18–160)	7.5 (0.9–110)	10 (2.5–85)	11.5 (2.8–60)	7.5 (1.0–90)
Motility (%)[a]	60 (25–75)	60 (30–75)	58 (30–80)	50 (25–70)	30 (3–70)	30 (4–60)	30 (10–70)	30 (5–70)
Morphology (%)								
World Health Organization (abnormal)[a]	58 (41–80)	64 (35–87)	54 (37–75)	64 (43–83)	80 (63–91)	79 (58–94)	83 (60–92)	80 (56–94)
Strict (normal)[b]	5 (1–12)	5 (1–15)	7 (1–15)	5 (1–19)	2 (1–9)	2 (1–12)	2 (1–8)	2 (1–12)

Note: Data are the mean (±SD) or median (5th–95th percentile).

[a] Determination according to World Health Organization criteria (1992). Normal reference values are sperm concentration of 20×10^6/mL, motility >50%, and morphology <70% abnormal.

[b] Determination according to the strict criteria described by Menkveld et al. (16): percentage of sperm with normal morphology.

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proved the study protocol, and all participants gave written informed consent before participation.

Before and after 26 weeks of treatment, participants provided standardized semen samples, which they obtained at home by masturbation into polypropylene containers after 3 to 5 days of abstinence. These samples were delivered to the fertility laboratory within 1 hour after production.

After liquefaction, an aliquot of semen was centrifuged at 1,400 $\times g$ for 10 minutes. The supernatant seminal plasma was stored at −20°C until assay for folate and zinc. Semen analysis was performed mainly according to 1992 World Health Organization guidelines for volume, pH, sperm concentration, motility, and morphology. Motility was expressed as the percentage of motile spermatozoa and mean velocity (scale of 1 to 6; 1 = immotile, 6 = progressive motility [>100 μm/s]). Morphology was determined according to World Health Organization criteria (15) and according to strict Tygerberg criteria, as described by Menkveld et al. (16).

At the time of semen sampling, overnight fasting venous blood samples were drawn for measurement of folate, zinc, vitamin B_{12}, FSH, and testosterone. Radioassays were used to measure folate and vitamin B_{12} concentrations in blood and seminal plasma specimens (Dualcount Solid Phase Boil radioassay; Diagnostic Products Corp., Los Angeles, CA). Zinc was measured by using flame atomic absorption spectrophotometry. Follicle-stimulating hormone was quantitatively determined in serum (AxSYM; Abbott Laboratories, Abbott Park, IL). Serum testosterone was measured as described elsewhere (17).

After 4, 12, and 20 weeks of intervention, all participants were contacted by telephone to maximize compliance and to obtain information on possible side effects.

To create similar intervention groups, eligible fertile and subfertile men were randomly assigned according to a simple computer-generated randomization schedule in four blocks to receive folic acid and placebo, zinc sulfate and placebo, zinc sulfate and folic acid, or placebo and placebo, which resulted in eight subgroups.

Capsules were coded by the hospital pharmacy according to the randomization list. The research fellow received capsules coded from 1 to 105 for subfertile men and from 501 to 605 for fertile men. Each participant was given the randomized code in order of intake and received one bottle containing folic acid (5 mg) or placebo capsules and a second bottle containing zinc sulfate (66 mg) or placebo capsules. Participants were instructed to take one capsule from each bottle daily after dinner for 26 weeks; to maintain their regular diet; and to keep a diary in which they reported the intake of capsules, side effects, and the eventual use of medicines.

Neither the research fellow and the participants knew whether the participants received folic acid, zinc sulfate, or placebo capsules. Folic acid and placebo capsules were yellow and identical in appearance. Zinc sulfate and placebo capsules were white and identical in appearance.

After 26 weeks, compliance was assessed by counting the remaining capsules and checking the diaries. At the end of the trial, the research fellow received the randomization list that matched the codes from the hospital pharmacy.

Statistical Analysis

Our main goal was to investigate the effects of the interventions on semen variables in fertile and subfertile men. A factorial design was used for statistical analysis. We calculated that 25 patients in each treatment group would be

TABLE 2

Preintervention folate and zinc concentrations in blood and seminal plasma of fertile and subfertile men.

Intervention	Fertile men (n = 107)				Subfertile men (n = 103)			
	Placebo	Folic acid	Zinc	Zinc and folic acid	Placebo	Folic acid	Zinc	Zinc and folic acid
Folate								
Blood serum level (nmol/L)	17 (8.9–30)	15 (10–50)	18 (8.3–34)	18 (9.4–36)	20 (9.3–43)	16 (11–28)	16 (9.1–36)	16 (11–27)
Erythrocyte level (nmol/L)	800 (400–1,500)	780 (420–1,200)	840 (410–1,300)	780 (570–1,400)	870 (480–1,900)	750 (530–1,400)	740 (420–1,100)	780 (500–1,400)
Seminal plasma level (nmol/L)	34 (16–69)	35 (12–53)	38 (17–86)	34 (25–81)	35 (20–80)	30 (11–89)	33 (14–83)	31 (12–100)
Zinc								
Blood plasma level (μmol/L)	19.7 (15.1–27.1)	19.3 (14.3–25.3)	21.4 (15.2–29.1)	20.5 (13.2–27.3)	20.0 (14.8–25.1)	19.9 (15.1–24.1)	19.9 (15.5–28.0)	20.7 (15.3–28.5)
Erythrocyte level (μmol/L)	8.8 (7–11.7)	8.9 (6.3–10.9)	9.2 (7.3–11.7)	8.6 (5.7–10.5)	8.9 (6.9–10.9)	8.6 (5.1–10.9)	8.7 (6.8–11.0)	9.4 (4.5–12.9)
Seminal plasma level (mmol/L)	1.3 (0.1–2.4)	1.3 (0.5–3.1)	1.4 (0.3–3.1)	1.4 (0.4–3.0)	1.5 (0.3–3.0)	1.2 (0.4–2.8)	1.4 (0.4–2.7)	1.4 (0.2–2.7)

Note: Data are the median (range).

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needed to detect an increase of 10×10^6 spermatozoa/mL at a two-sided α of 10% and a power of 80% (assumption of a SD of 20×10^6 spermatozoa/mL).

Nonparametric methods were used for analysis because of the skewed distribution of the variables. The Wilcoxon 1-sample test was used to evaluate the changes before and after intervention in the four intervention groups of fertile and subfertile men. Treatment effects were then adjusted for the placebo effect by computing confidence intervals for the difference between treatment effect and placebo effect. This was done by using the nonparametric method of Conover (18). These data were expressed as means and 95% CIs. Spearman rank correlation coefficients were calculated to determine associations between folate and zinc concentrations and semen variables.

RESULTS

Participant Flow and Follow-up

One hundred eight of 357 fertile men and 103 of 258 subfertile men were enrolled in the trial. One fertile man with vitamin B_{12} deficiency (<100 pmol/L) was excluded after randomization, leaving 107 fertile men (mean [±SD] age, 34.2 + 4.2 years) and 103 subfertile men (mean age, 34.3 + 3.9 years) participated in the study. Two fertile men and 1 subfertile man withdrew because of adverse gastrointestinal side effects, and 6 fertile men and 8 subfertile men withdrew because of lack of motivation (Fig. 1).

Table 1 shows the baseline characteristics of the four groups after randomization. At baseline, fertile and subfertile men differed only in FSH level and semen variables. Preintervention concentrations of folate and zinc in blood and seminal plasma were similar and within the normal ranges in fertile and subfertile men (Table 2).

After 26 weeks of intervention with zinc and folic acid, the median sperm concentration significantly increased from 7.5×10^6 cells/mL to 12.0×10^6 cells/mL in subfertile men ($P<.001$) (Table 3, Fig. 2). The median percentage of abnormal spermatozoa increased from 80% to 84% ($P<.01$) and the median total normal sperm count (sperm concentration $\times$ [100 − percentage abnormal spermatozoa/100] $\times$ volume) increased by 74%, from 5.1×10^6 cells/mL to 8.9×10^6 cells/mL ($P<.05$), after administration of zinc and folic acid. No significant changes in semen variables were observed in the other intervention groups, except for morphology as determined according to strict criteria, which slightly decreased in the groups that received only folic acid or zinc (Table 3).

After adjustment for the effects of the placebo intervention by using the nonparametric method of Conover (18), sperm concentration was still significantly increased ($P<.05$) and total normal sperm count was slightly increased ($P=.07$) after zinc and folic acid treatment in subfertile men. The δ of the mean differences for sperm concentration and total sperm count were

TABLE 3

Semen quality in subfertile (n = 94) and fertile men (n = 99).

	Placebo (n = 25)		Folic acid (n = 22)		Zinc (n = 23)		Zinc and folic acid (n = 24)	
	Preintervention	Postintervention	Preintervention	Postintervention	Preintervention	Postintervention	Preintervention	Postintervention
Subfertile men								
Sperm concentration (10^6/mL)	7.5 (0.7–110)	9 (0.8–80)	10 (1.4–110)	14 (0.9–130)	11.5 (0.8–60)	16 (0.6–80)	7.5 (0.3–130)	12 (0.5–180)[a]
Motility (%)	30 (2–70)	30 (5–80)	30 (2–60)	35 (5–65)	30 (5–80)	35 (10–65)	33 (5–80)	35 (5–70)
Morphology (%)								
World Health Organization criteria (abnormal)	80 (53–98)	79 (61–98)	79 (56–94)	81 (66–93)	83 (55–94)	82 (63–92)	80 (54–100)	84 (60–100)[a]
Strict criteria (normal)	2 (1–9)	2 (1–8)	2 (1–12)	2 (1–5)[a]	2 (1–8)	2 (1–5)[a]	2 (1–12)	2 (1–13)
Total *normal* sperm count ($\times 10^6$ cells/mL)	6.4 (0.3–113)	6.3 (0.1–80)	8 (0.7–61)	9.1 (0.8–32)	9.0 (0.3–70)	12.3 (0.5–108)	5.1 (0–92)	8.9 (0–106)[a]
Mean (±SD) volume (mL)	3.9 ± 1.3	3.2 ± 1.3	3.8 ± 1.7	3.3 ± 1.3	2.9 ± 1.3	2.9 ± 1.4	3.8 ± 2.0	4.1 ± 2.4
Fertile men								
Sperm concentration (10^6/mL)	90 (1.3–200)	85 (9–225)	70 (16–155)	70 (0–155)	74.5 (16–230)	90 (6–220)	65 (12–180)	78 (35–160)
Motility (%)	60 (20–80)	55 (25–80)	60 (30–80)	58 (30–75)	58 (25–90)	50 (10–80)	50 (20–70)	50 (15–80)
Morphology (%)								
World Health Organization criteria (abnormal)	58 (37–91)	62 (38–87)	64 (34–90)	66 (35–86)	54 (31–76)	63 (40–98)[a]	64 (43–90)	65 (42–87)
Strict criteria (normal)	5 (1–12)	5 (1–10)	5 (1–15)	5 (1–17)	7 (1–15)	6 (1–15)	5 (1–19)	5 (1–15)
Total *normal* sperm count ($\times 10^6$ cells/mL)	126 (0.3–414)	76.2 (4.8–547)	68.6 (14.4–410)	89.8 (13.7–167)	118 (11.6–270)	106 (0.5–330)	57.1 (5.1–364)	92.2 (16.4–707)
Mean (±SD) volume (mL)	3.6 ± 1.8	3.4 ± 2.2	3.7 ± 1.9	3.4 ± 1.8	3.2 ± 1.5	3.1 ± 1.6	3.7 ± 1.7	3.6 ± 2.4

Note: Data are the median (range).

[a] $P<.05$ for comparison between baseline and post-treatment values.

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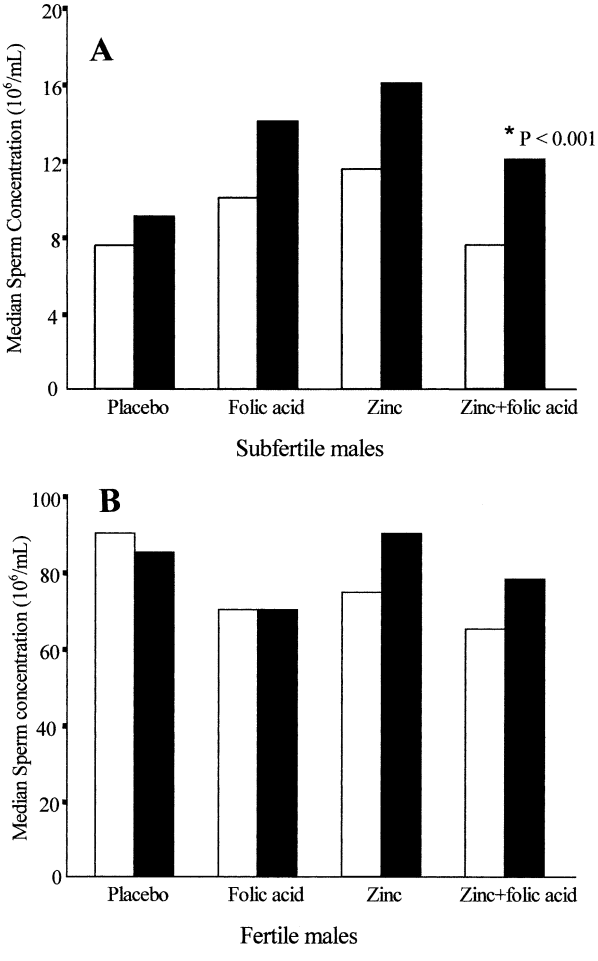
FIGURE 2

Median preintervention and postintervention sperm concentrations in subfertile (**A**) and fertile (**B**) men. *$P<.001$ for comparison between baseline and posttreatment values. ■ pre-intervention; □ postintervention.



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15.2 million cells/mL (95% CI, 0.0–19.6 million cells/mL) and 15.0 million cells/mL (95% CI, −0.5 to 16.5 million cells/mL), respectively.

No significant changes in semen variables except sperm morphology were observed in fertile men (Table 3). After adjustment for the placebo effect, fertile men had no significant increase in sperm concentration ($P=.49$) or total normal sperm count ($P=.27$) after treatment with zinc and folic acid. The δ of the mean differences were 6.3 million cells/mL (95% CI, −18 to 31 million cells/mL) and 32.8 million cells/mL (95% CI, −23.4 to 65.7 million cells/mL), respectively.

Administration of folic acid to subfertile and fertile males significantly increased folate concentrations in serum, erythrocytes, and seminal plasma, whereas administration of zinc administration did not affect zinc concentrations (data not shown). Concentrations of FSH and testosterone did not significantly differ in any group.

DISCUSSION

We found a 74% increase in total normal sperm count after adjustment for the concomitant increase in the percentage of abnormal spermatozoa (4%) in subfertile men who received both zinc sulfate (66 mg) and folic acid (5 mg) daily for 26 weeks.

Although this improvement in sperm count did not always result in sperm concentrations greater than the reference value of 20 million cells/mL, the observed increase suggests a beneficial effect on the quantitative aspect of spermatogenesis. This is supported by nonrandomized controlled studies showing that oral zinc supplementation improves sperm concentration in subfertile males with idiopathic asthenozoospermia or oligozoospermia (19, 20). However, Landau et al. (21) reported that daily supplementation with 10 mg of folic acid for 30 days had no beneficial effect on sperm concentration in 40 normospermic and oligozoospermic men.

The sperm concentration increased, but not significantly so, in fertile men receiving zinc only (median increase, 15.5×10^6 cells/mL) or zinc plus folic acid (median increase, 13×10^6 cells/mL). The lack of significance can be explained by the large variability in sperm concentration in these men compared with subfertile men.

Animal in vivo and in vitro studies have shown that zinc deficiency alters the absorption and metabolism of dietary folate (22–24). Although folic acid and zinc are essential for transfer RNA and DNA synthesis, the underlying mechanisms by which these micronutrients affect spermatogenesis are not known (25).

Although food consumption surveys conducted in the past decade suggest marginal overall intake of folate and zinc in the general population, we did not find deficient folate or zinc blood levels or significant differences between fertile and subfertile men in preintervention concentrations of these substances (7, 8). In contrast, Chia et al. (26) found that seminal plasma zinc concentrations differed significantly between fertile and subfertile men.

Folate concentrations in blood and seminal plasma markedly increased after folic acid administration and decreased during the wash-out period, as previously reported (27). Zinc administration did not affect zinc concentrations in blood and seminal plasma in either group. This may be explained by the absence of zinc deficiency or by the relatively low dose of zinc used in an effort to avoid gastrointestinal side effects. Zinc concentrations in serum or erythrocytes may not reflect the intracellular semen zinc status. Moreover,

physiologic zinc concentrations in blood may not respond to zinc administration (28). Zinc is mainly excreted by the prostate in high concentrations, and zinc administration may not substantially increase levels of zinc in seminal plasma.

Lack of compliance is not likely, considering the marked increase in folate concentrations after folic acid administration and selective intake of micronutrients. Among subfertile and fertile men, compliance rates of 97% and 95%, respectively, were calculated on the basis of the number of men who did not take fewer than 20 capsules (median range, 4 [0–20] and 5 [0–40] capsules, respectively).

In general, physiologic doses of micronutrients have a better effect on absorption, transport, and metabolic processes, as long as no major disorders exist. Therefore, we hypothesize that an even larger beneficial effect may have been achieved if lower doses of folic acid or zinc sulfate had been administered.

Semen analyses must be carefully interpreted because data may be biased by intraindividual biological fluctuations in semen variables, limitations and inaccuracies of the methods used, and intraobserver and interobserver variability (14, 29). In addition, the use of one semen sample to distinguish fertile from subfertile men may have introduced some nonrandom misclassifications and bias. However, because this confounding would equally affect either group, we expect that the effects may be larger than we could demonstrate in this study.

We further evaluated the above matter by reclassifying the men according to their baseline sperm concentration. The data clearly show stronger effects on sperm concentration (15.8×10^6 cells/mL [$P=.002$]), total normal sperm count (19.8×10^6 cells/mL [$P=.002$]), and percentage of abnormal spermatozoa (3.7% [$P=.01$]) in subfertile men after treatment with folic acid and zinc sulfate. Treatment with zinc significantly increased sperm concentration in subfertile males (18.5×10^6 cells/mL [$P=.02$]).

Our results cannot be explained by regression to the mean, because a placebo group was included among fertile and subfertile men. We would have liked to obtain more semen samples per participant before and after intervention, but this was not feasible, especially among fertile men.

Male factor subfertility is a multifactorial disorder. Our findings emphasize the importance of two micronutrients on spermatogenesis. Unlike genetic factors, nutritional factors can be changed by increasing intake. However, whether the improvement in sperm concentration observed after administration of folic acid and zinc will lead to an increase in pregnancy rates remains to be established. Before wide-scale implementation of combined zinc and folic acid administration, we recommend that a larger randomized, placebo-controlled study on the efficacy and safety of these nutriceuticals be done. Nevertheless, our findings suggest new avenues of future fertility research and treatment.

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