Antenatal supplementation with micronutrients and biochemical indicators of status and subclinical infection in rural Nepal¹⁻³

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ABSTRACT

Background: Previously we showed that women in rural Nepal experience multiple micronutrient deficiencies in early pregnancy. Objective: This study examined the effects of daily antenatal micronutrient supplementation on changes in the biochemical status of several micronutrients during pregnancy.

Design: In Nepal, we conducted a randomized controlled trial in which 4 combinations of micronutrients (folic acid, folic acid + iron, folic acid + iron + zinc, and a multiple micronutrient supplement containing folic acid, iron, zinc, and 11 other nutrients) plus vitamin A, or vitamin A alone as a control, were given daily during pregnancy. In a subsample of subjects ($n = 740$), blood was collected both before supplementation and at $\approx 32$ wk of gestation.

Results: In the control group, serum concentrations of zinc, riboflavin, and vitamins B-12 and B-6 decreased, whereas those of copper and α-tocopherol increased, from the first to the third trimester. Concentrations of serum folate, 25-hydroxyvitamin D, and undercarboxylated prothrombin remained unchanged. Supplementation with folic acid alone or folic acid + iron decreased folate deficiency. However, the addition of zinc failed to increase serum folate, which suggests a negative inhibition; multiple micronutrient supplementation increased serum folate. Folic acid + iron + zinc failed to improve zinc status but reduced subclinical infection. Multiple micronutrient supplementation decreased the prevalence of serum riboflavin, vitamin B-6, vitamin B-12, folate, and vitamin D deficiencies but had no effect on infection.

Conclusions: In rural Nepal, antenatal supplementation with multiple micronutrients can ameliorate, to some extent, the burden of deficiency. The implications of such biochemical improvements in the absence of functional and health benefits remain unclear. Am J Clin Nutr 2006;83:788–94.

KEY WORDS Micronutrients, pregnancy, Nepal, infection, supplementation

INTRODUCTION

Although it is generally accepted that multiple micronutrient deficiencies during pregnancy are common in developing countries, few studies have examined the changes in serum concentrations of micronutrients over the course of a pregnancy or the effect of antenatal micronutrient supplementation on these concentrations. Pregnancy is a period of numerous physiologic and metabolic changes. Because micronutrients are involved in regulating these processes, it is plausible that micronutrient deficiencies could alter pregnancy outcomes (1–3).

There is growing interest in elucidating the effect of daily antenatal supplementation with multiple micronutrients on pregnancy and newborn outcomes in areas of the world where micronutrient deficiencies are common. We conducted a cluster-randomized, double-masked, controlled trial in rural Nepal in which we observed that an antenatal multiple micronutrient supplement failed to provide any additional benefit above that seen with folic acid and iron for an outcome such as birth weight (4). Furthermore, it failed to show an apparent reduction in infant mortality of $\approx 20\%$, which was observed with folic acid and iron (5), providing little evidence for the use of such an intervention for enhancing pregnancy and infant outcomes in Nepal. Also, iron and hematologic status in our trial (6) and another one in Mexico (7) did not improve with multiple micronutrient supplementation beyond the improvement observed with iron and folic acid alone.

Previously, we also published data from our trial in Nepal, which showed that micronutrient deficiencies in early pregnancy are common and coexist in rural Nepal (8). The deficiencies are exacerbated due to increased metabolic demands as the pregnancy advances. Plasma concentrations of micronutrients can also be modified by the extent of plasma volume expansion, which complicates the interpretation of serum biochemical indicators in pregnancy. Some studies do show that supplementation with micronutrients enhance their circulating concentrations in pregnancy (9, 10).

In this article we compare changes in maternal micronutrient status from the first to the third trimester of pregnancy in the control group, relative to that observed with daily supplementation with 4 combinations of micronutrients that included a multiple micronutrient supplement. Serum indicators of B complex vitamins, copper, zinc, and vitamins D, E, and K are examined.

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Furthermore, we also present data on the effect of micronutrient supplementation on acute phase markers of subclinical infection during pregnancy. These data provide information regarding the success or failure of such a supplementation strategy for enhancing nutritional status and correcting micronutrient deficiencies during a critical life stage—one of the objectives for considering its implementation in the developing world. It also allows the exploration of nutrient-nutrient interactions that provide plausible pathways for understanding the mechanisms responsible for the lack of beneficial effects and even perhaps adverse effects previously noted (4, 5).

SUBJECTS AND METHODS

Study design and population

This study was carried out in the Southeastern plains District of Sarlahi in Nepal during 1999–2001. Details of the trial are described elsewhere (4, 5). Briefly, the study area consisted of 30 village development communities in the district that were divided into 426 small units, called sectors. Married women of reproductive age who were not already pregnant, menopausal, sterilized, or widowed were enumerated. They were visited every 5 wk, and the amenstrual women were administered a urine-based human chorionic gonadotropin test to identify new pregnancies. Consenting pregnant women were enrolled into a trial of antenatal and postnatal supplementation with alternative combinations of micronutrients to examine the effect on birth weight and infant survival and health. The pregnant women were randomly assigned to receive daily 400 mg folic acid, 60 mg folic acid + iron, 30 mg folic acid + iron + zinc, a multiple micronutrient supplement containing the foregoing nutrients plus 11 other micronutrients (10 μg vitamin D, 10 mg vitamin E, 1.6 mg thiamine, 1.8 mg riboflavin, 20 mg niacin, 2.2 mg vitamin B-6, 2.6 μg vitamin B-12, 100 mg vitamin C, 65 μg vitamin K, 2.0 mg Cu, and 100 mg Mg), or 1000 μg folic acid, 60 mg folic acid + iron, 30 mg folic acid + iron + zinc, a multiple micronutrient supplement containing the foregoing nutrients plus 11 other micronutrients (10 μg vitamin D, 10 mg vitamin E, 1.6 mg thiamine, 1.8 mg riboflavin, 20 mg niacin, 2.2 mg vitamin B-6, 2.6 μg vitamin B-12, 100 mg vitamin C, 65 μg vitamin K, 2.0 mg Cu, and 100 mg Mg), or 1000 μg RE vitamin A and vitamin A alone as the control group. At baseline, an assessment of variables reflecting household socioeconomic level, literacy, occupation, previous pregnancy history, morbidity, diet, substance use, and strenuous work activities in the previous 7 d was made. Sector workers delivered supplements twice a week to enrolled pregnant women in their homes. Pregnancy outcome was monitored, and a day-of-birth assessment of the newborn and mother was carried out by trained teams of anthropometrists and interviewers.

In 25% of the sectors, a substudy involving blood collection was carried out to assess the effect of supplementation on the women’s micronutrient status. Venous blood was drawn at home by trained phlebotomists at baseline (before supplementation) and again in the third trimester (scheduled at 32 wk of gestation). Detailed methods of this substudy were described previously (6, 8). Blood was collected into 7-mL trace-metal–free vacuum test tubes (Vacutainer; Becton Dickinson Company, Franklin Lakes, NJ), kept on ice, and brought to the project laboratory for centrifugation at 750 x g for 20 min to separate the serum. Aliquots of serum were stored in liquid nitrogen tanks in trace element–free cryotubes (Nalgene Company, Sybron International, New York, NY) and shipped to the Johns Hopkins Bloomberg School of Public Health in Baltimore, MD, where they were stored at −80 °C until analyzed.

Laboratory analyses

Serum was analyzed over the course of 2–2.5 y for 11 different biochemical indicators of micronutrient and infection status. Data on the effect of supplementation on iron-status indicators, including hemoglobin, serum ferritin, iron, and transferrin receptors, were published previously (6) and are not presented here.

Details of the analytic methods are provided elsewhere (8). Briefly, serum zinc and copper concentrations were analyzed by atomic absorption spectrometry (AAnalyst 600; Perkin-Elmer, Wellesley, MA). Serum folate was measured with a microbiological assay with the use of a chloramphenicol-resistant strain of *Lactobacillus rhamnosus* (NCIMB 10463) (11). Homocysteine was analyzed with a microtiter plate assay (Calbiotech Inc, Spring Valley, CA), which is similar to an enzyme immunoassay and uses a genetically engineered homocysteine binding protein as the capturing agent. Serum vitamin B-12 was determined with a microbiological assay that uses a colistin sulfate–resistant strain of *Lactobacillus lactis* (NCIMB 12519) (12, 13). Serum 25-hydroxyvitamin D [25(OH)D] was determined by immunoassay (Nichols Institute, San Juan Capistrano, CA). Serum retinol and α-tocopherol were determined simultaneously by reversed-phase HPLC (Beckman, System Gold, Columbia, MD) attached with an autosampler (717 Plus AS; Waters Corp, Milford, MA) by using a procedure described by Yamini et al (4) with modifications. Serum riboflavin concentrations were measured as a surrogate for vitamin B-2 with the use of reversed-phase HPLC (model 1100; Agilent Technologies, Foster City, CA) with a fluorescence detector (model FP-1520; Jasco Corp, Easton, MD). The serum concentration of pyridoxal 5'-phosphate, the active form of vitamin B-6, was measured by using HPLC. Serum (100 μL) was deproteinized by the addition of perchloric acid. Precolumn derivatization was performed with potassium cyanide. The fluorescent cyanide derivatives were detected by fluorometry. Undercarboxylated prothrombin (proteins induced by vitamin K absence; PIUKA-II) were measured with a commercial enzyme-linked immunoassay (ELISA) kit (Asserachrom PIUKA II; Diagnostica Stago, Parsippany, NJ).

Markers of inflammation that were examined included α1-acid glycoprotein (AGP) and C-reactive protein (CRP). CRP was measured by ELISA with a commercial kit from ADI (San Antonio, TX), and serum AGP was measured with a radial immunodiffusion assay with commercially available kits (Kent Laboratories, Bellingham, WA).

Statistical analysis

Statistical analyses were based on an intention-to-treat basis. The baseline biochemical status of pregnant women was compared across treatment groups. The mean relative difference (defined as the difference in the change in serum concentrations of various analytes from baseline to follow-up for each supplementation group compared with that in the control group) and 95% confidence limits (CLs) were estimated by using generalized estimating equations linear regression models with an identity link and exchangeable correlation to account for randomization of sectors rather than individuals to treatment groups (15). Each model was adjusted for the baseline concentration of the analyte of interest. We used published cutoff values for defining deficient concentrations of micronutrients. Prevalence ratios (and 95% CLs) for micronutrient deficiencies and subclinical infection, on the basis of a serum AGP concentration >1 g/L and a CRP concentration >5 g/L in the third trimester, were estimated by using a generalized estimating equations binomial regression model with a log link and exchangeable correlation, with the control (vitamin A alone) group as the reference category (15).
Data were analyzed by using SAS version 8.1 (SAS Institute Inc, Cary, NC).

Informed consent was obtained from all participants before enrollment in the study. The study received ethical approval by the Committee on Human Research of the Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, and the Nepal Health Research Council, Kathmandu, Nepal.

RESULTS

Of 1361 pregnant women in the substudy area, 1165 (85.6%) agreed to have their blood drawn at baseline and 779 (59.2%) agreed to having their blood drawn during the third trimester (Figure 1). A total of 740 women contributed both a baseline and a follow-up blood sample. A smaller number of women had both baseline and follow-up blood samples collected because women were eligible to contribute blood at the follow-up visit even if they had not at baseline. The mean (±SD) gestational age at baseline and at follow-up was 10.2 ± 4.1 and 32.6 ± 3.9 wk, respectively. The high nonresponse at the third trimester was due to 1) women having gone to their parental home for delivery, 2) early pregnancy loss, 3) migration, and 4) refusal; the nonresponse rates did not differ significantly by treatment group (Figure 1).

The baseline mean (±SD) concentrations of micronutrients and CRP and AGP did not differ by treatment group (Table 1). In control subjects, serum concentrations of water-soluble vitamins (including riboflavin and vitamins B-12 and B-6) decreased significantly by 32–48% from early to late gestation (data not shown).

Serum concentrations of fat-soluble vitamin E (α-tocopherol) increased by 69%, but vitamins D and K (indicated by PIVKA-II) remained unchanged. Trace mineral concentrations did not exhibit a consistent pattern of change, serum zinc concentrations decreased, and serum copper concentrations increased.

Serum folate concentrations increased significantly, by ~25 nmol/L, in the groups receiving folic acid or folic acid + iron or the multiple micronutrient supplement (Table 2). The combination of folic acid + iron + zinc failed to increase serum folate as indicated by an increment of ~10 μmol/L with CLs that did not overlap those in the other folic acid groups or in the multiple micronutrient group. Change in serum homocysteine was not significantly different across supplementation groups. Zinc concentrations did not increase in response to supplementation with zinc in combination with folic acid and iron relative to the control, but did so with multiple micronutrient supplementation (0.5; 95% CL: 0.1, 0.99 μmol/L) (Table 2). Serum concentrations of most micronutrients included in the multiple micronutrient supplements increased in that group relative to the control but did not change in the other 3 supplementation groups. The exceptions were α-tocopherol and PIVKA-II, which remained unchanged with supplementation. There was a significant increase in the concentration of 25(OH)D in the women who received folic acid alone, which did not occur in response to folic acid given with zinc or iron (Table 2).

Folate deficiency decreased by 75–86% in the 4 groups who received folic acid (Table 3). This effect was not accompanied by a reduction in the prevalence of high homocysteine concentrations. Zinc deficiency, defined as a serum zinc concentration

FIGURE 1. Study participation and follow-up by supplementation group. R, randomization.
<7.6µmol/L, did not decrease with the folic acid + iron + zinc or the multiple micronutrient supplement, which contained zinc. Deficiencies of vitamins B-12, B-6, riboflavin, and 25(OH)D were 35–77% lower in the multiple micronutrient group than in the control group. Relative to the control group, the prevalence of a PIVKA-II concentration >2.7 ng/mL was lower in the folic acid + iron + zinc group, but not in the multiple micronutrient group, which received a supplement that provided an RDA of vitamin K.

Serum AGP concentrations decreased in the control group (Table 2). Relative to this decrease, serum AGP decreased significantly more in response to folic acid alone or folic acid with zinc with or without iron \((P<0.05)\). Supplementation with multiple micronutrients failed to influence serum AGP concentrations relative to the control supplement. Unlike AGP, CRP increased during pregnancy in all groups, and the decrement relative to the control group was statistically significant only in

### TABLE 2

<table>
<thead>
<tr>
<th>Maximum observations</th>
<th>Control (^1) ((n = 164))</th>
<th>Folic acid (^2) ((n = 126))</th>
<th>Folic acid + iron (^3) ((n = 127))</th>
<th>Folic acid + iron + zinc (^4) ((n = 167))</th>
<th>Multiple micronutrients (^5) ((n = 156))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (nmol/L)</td>
<td>1.8 ± 15.4</td>
<td>25.9 (19.4, 32.5) (^6)</td>
<td>24.9 (19.2, 30.8) (^7)</td>
<td>10.3 (6.9, 13.7) (^8)</td>
<td>27.6 (22.7, 32.5) (^9)</td>
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<tr>
<td>Vitamin B-12 (pmol/L)</td>
<td>−113.6 ± 129(^6)</td>
<td>12.2 (−3.2, 27.6)</td>
<td>7.3 (−10.5, 25.1)</td>
<td>11.0 (−5.3, 27.2)</td>
<td>49.6 (27.8, 71.4) (^4)</td>
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<tr>
<td>Vitamin B-6 (nmol/L)</td>
<td>−11.4 ± 12.4(^6)</td>
<td>0.9 (−0.5, 2.4)</td>
<td>−0.02 (−1.5, 1.5)</td>
<td>−0.3 (−1.7, 1.1)</td>
<td>9.9 (7.0, 11.2) (^4)</td>
</tr>
<tr>
<td>Riboflavin (nmol/L)</td>
<td>−6.2 ± 12.0(^6)</td>
<td>0.9 (−1.0, 2.8)</td>
<td>−1.1 (−3.0, 0.9)</td>
<td>0.05 (−2.1, 2.2)</td>
<td>10.8 (8.3, 13.4) (^5)</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>−1.6 ± 4.5</td>
<td>0.17 (−0.51, 0.84)</td>
<td>0.26 (−0.41, 0.93)</td>
<td>−0.06 (−0.73, 0.61)</td>
<td>0.05 (−0.68, 0.77)</td>
</tr>
<tr>
<td>Retinol (µmol/L)</td>
<td>0.18 ± 0.5</td>
<td>−0.06 (−0.17, 0.05)</td>
<td>0.05 (−0.04, 0.14)</td>
<td>−0.05 (−0.14, 0.03)</td>
<td>0.06 (−0.03, 0.14)</td>
</tr>
<tr>
<td>α-Tocopherol (µmol/L)</td>
<td>7.9 ± 4.6(^6)</td>
<td>−0.3 (−1.4, 0.7)</td>
<td>0.2 (−0.7, 1.2)</td>
<td>−0.6 (−1.5, 0.3)</td>
<td>0.3 (−0.7, 1.3)</td>
</tr>
<tr>
<td>γ-Tocopherol (µmol/L)</td>
<td>0.00 ± 1.3</td>
<td>−0.00 (−0.18, 0.17)</td>
<td>−0.07 (−0.26, 0.12)</td>
<td>−0.12 (−0.28, 0.04)</td>
<td>−0.38 (−0.56, −0.21) (^5)</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>3.2 ± 28.7</td>
<td>15.5 (7.4, 27.5) (^4)</td>
<td>5.1 (−1.9, 12.1)</td>
<td>5.9 (−0.2, 12.0)</td>
<td>17.8 (11.7, 23.8) (^5)</td>
</tr>
<tr>
<td>PIVKA-II (ng/mL)</td>
<td>−0.12 ± 0.9</td>
<td>0.07 (−0.18, 0.34)</td>
<td>−0.03 (−0.21, 0.15)</td>
<td>−0.10 (−0.25, 0.04)</td>
<td>0.00 (−0.19, 0.21)</td>
</tr>
<tr>
<td>Zinc (µmol/L)</td>
<td>−1.6 ± 2.5(^6)</td>
<td>0.1 (−0.5, 0.6)</td>
<td>0.1 (−0.4, 0.5)</td>
<td>0.2 (−0.3, 0.6)</td>
<td>0.5 (0.1, 0.99) (^5)</td>
</tr>
<tr>
<td>Copper (µmol/L)</td>
<td>11.1 ± 9.3(^6)</td>
<td>0.9 (−0.9, 2.8)</td>
<td>0.3 (−1.3, 1.9)</td>
<td>−0.3 (−1.8, 1.2)</td>
<td>−0.5 (2.0, 1.00)</td>
</tr>
<tr>
<td>AGP (g/L)</td>
<td>−0.22 ± 0.29(^6)</td>
<td>−0.04 (−0.08, −0.002) (^5)</td>
<td>−0.03 (−0.06, −0.001) (^5)</td>
<td>−0.05 (−0.08, −0.01) (^5)</td>
<td>−0.01 (−0.05, 0.03)</td>
</tr>
<tr>
<td>CRP (g/L)</td>
<td>0.74 ± 3.46(^6)</td>
<td>−0.41 (−1.04, 0.23)</td>
<td>−0.25 (−0.81, 0.32)</td>
<td>−0.57 (−1.07, 0.56) (^5)</td>
<td>0.00 (−0.74, 0.74)</td>
</tr>
</tbody>
</table>

\(^1\) All values are ± SD. 25(OH)D, 25-hydroxyvitamin D; PIVKA-II, proteins induced by vitamin K absence (undercarboxylated prothrombin); AGP, α1-acid glycoprotein; CRP, C-reactive protein.

\(^2\) Serum values for some analytes missing in 2–5 samples per group.

\(^3\) All values are the mean ± SD change from baseline to follow-up.

\(^4\) All values are mean differences (and 95% confidence limits) in the change from baseline to follow-up relative to the control group, calculated by using a generalized estimating equations linear regression model adjusted for baseline concentration.

\(^5\) \(P<0.05\) (generalized estimating equations linear regression model adjusted for baseline concentrations).

\(^6\) \(P<0.05\) (paired \(t\) test).
the folic acid + iron + zinc group (0.57 g/L; P < 0.05), although the trend was evident with the other 2 groups who received folic acid. The frequencies of third-trimester concentrations of AGP and CRP above the thresholds considered to reflect infection (>1 and >5 g/L, respectively) were lower in the folic acid + iron + zinc group than in the control group, but the 95% CL for AGP did not exclude 1.0 (Table 3). Supplementation with multiple micronutrients failed to affect either of these indexes of infection.

**DISCUSSION**

Our community-based study provides data on changes occurring in circulating concentrations of vitamins and minerals and the biological response to daily supplementation with 4 different combinations of micronutrients among rural pregnant Nepali women living with chronic dietary deficits. The trial that generated these data showed no benefit of a multiple micronutrient supplement over folic acid + iron in improving birth weight (4) and perhaps even an adverse effect on infant survival (5). Thus, the biochemical data need to be interpreted in light of these previous findings.

**Change in micronutrient status during pregnancy**

In the present study, concentrations of most water-soluble vitamins decreased by 20–50% from early to late gestation, a finding that has also been recorded in healthy populations (24). Concentration of homocysteine did not decrease, unlike the decreases that have been described due to pregnancy-related endocrinologic changes (25). Fat-soluble vitamins E and K transported by plasma lipoproteins are known to increase during pregnancy (24, 26). An increase in vitamin D, acting as a calcitropic hormone, is crucial for meeting the increased need for calcium during pregnancy (27–29). In our study, unlike vitamin E, concentrations of vitamins D and K did not increase during pregnancy. With regard to the 2 minerals, their change was expectedly in the opposite direction; serum zinc concentrations decreased in contrast with significant increases in copper, as shown before (30).

**Effects of supplementation on status**

Improvements in maternal biochemical status during pregnancy associated with micronutrient supplementation may primarily be due to correction of underlying deficiency. Another mechanism may be related to the effect on subclinical infection known to lower circulating concentrations of micronutrients caused by an acute phase response. A lack of response in a measured indicator may, perhaps, be masked by the plasma volume expansion of pregnancy that may in turn have been influenced by micronutrient status (3), or changes in endocrine regulations of pregnancy may facilitate channeling of nutrients to the fetus without altering maternal status. Other reasons for a nonresponse could be related to an inadequate dose or an inhibitory effect of one or more nutrients when provided simultaneously.

Folic acid singly or in combination with iron resulted in an increase in serum folate concentrations. A significant attenuation of this effect was apparent in combination with zinc, which points to a negative interaction between zinc and folate. Old in vitro and in vivo studies have shown that a mutual inhibition exists at the cellular level (34), which is contrary to our study findings. We found no published evidence that zinc supplementation per se (alone or in combination with folic acid or iron) affects folate metabolism. In the present study, however, multiple micronutrients, which also contained zinc, completely reversed the negative effect of zinc on serum folate, although which one or more micronutrients in this mixture could have alleviated this inhibition remains unclear.

Multiple micronutrients succeeded in enhancing the status of B vitamins as indicated by their circulating concentrations. With the exception of folate, this has not been demonstrated to our
knowledge for other vitamins in pregnancy. Daily supplementation with the Recommended Dietary Allowance (RDA) of these vitamins, however, was insufficient to lower deficiency for some by much. For example, the prevalence of vitamin B-6 and B-12 deficiencies was reduced by only 30–35% in late gestation. Homocysteine did not decrease with folic acid or in combination with vitamins B-6 and B-12 supplementation. Unlike these findings, changes in homocysteine were previously noted with folic acid supplementation and fortification in the United States and other countries (35–37), although deficiencies of both vitamin B-12 and vitamin B-6 may also affect homocysteine concentrations (38, 39). Persisting deficiencies of these vitamins, despite supplementation, may provide an explanation for the lack of effect on homocysteine in our study.

Concentrations of 25(OH)D increased in the group that received an RDA of vitamin D. This increase was also observed with folic acid supplementation alone. We found no previously described evidence linking folate status with either the synthesis of vitamin D in the skin, the synthesis of the vitamin D–binding protein, or the hydroxylation of vitamin D, which suggests that the mechanism remains to be elucidated.

Zinc concentrations were not responsive to supplementation, which has been shown before in pregnancy (40). It is likely that the bioavailability of zinc was compromised by the presence of iron and folic acid (33, 41, 42). Zinc, in combination with other micronutrients, did increase serum zinc concentrations by 0.5 μmol/L.

The combination of folic acid + iron + zinc reduced the risk of PIVKA-II > 2.7 ng/mL, which suggests that zinc promotes vitamin K status. Previously, an in vitro study showed that zinc sulfate caused a dose-dependent prolongation of prothrombin and partial thromboplastin times as well as shortened thrombin clotting time (43). A rat experiment of the effect of vitamin K2 (menaquinone-7) on bone metabolism showed an enhancement with zinc (44). In patients with alcoholic cirrhosis, zinc supplementation increased plasma prothrombin and serum alkaline phosphatase concentrations (45).

Copper supplementation did not change plasma concentrations of copper, which increased significantly during pregnancy. Previously, copper supplementation in pregnant ewes, mares, or cows resulted in increases in liver copper concentrations without altering plasma concentrations (46–48). Even long-term exposure to a high copper index in men showed no changes in plasma concentration of copper, although other indicators, such as urinary copper, ceruloplasmin activity, benzylamine oxidase, and superoxide dismutase, were significantly elevated (49). Our study showed no evidence of copper status being affected by zinc supplementation at 30 mg/d. Neither was there evidence that copper supplementation affects zinc status because serum zinc response was the highest in the multiple micronutrient group, who received 2 mg Cu. Recently, zinc supplementation was found not to affect plasma copper concentrations in infants (50) or extracellular superoxide dismutase activity in healthy pregnant women (51).

**Effect of supplementation on subclinical infection**

Micronutrient status, such as that of vitamin A or zinc, is known to modulate the immune system. We found that folic acid alone or in combination with iron, with or without zinc, decreased mean concentrations of AGP and in combination with iron and zinc decreased CRP during pregnancy. This suggests that folic acid and zinc may ameliorate the inflammatory process in pregnancy, which has implications for reproductive health outcomes. However, the multiple micronutrient supplements, which included the above nutrients, failed to show this reduction, which suggests an inhibitory interaction with the other nutrients present in the supplement.

**Conclusion**

In addition to dietary interventions, supplementation may be a reasonable approach for addressing the problem of micronutrient deficiencies in pregnancy. The data presented in our article support this conclusion, although several considerations are necessary before such an approach is adopted broadly. First, if the goal of such a strategy were just to alleviate deficiency (on the basis of known indicators of status), then the formulation tested in our study achieved this goal for some nutrients (folate, riboflavin, and vitamin D), but had only a modest effect on others (vitamins B-6 and B-12) and even failed to affect it for some (zinc, copper, and vitamin K). Second, both negative and positive nutrient-nutrient interactions may occur. Knowledge of these interactions is critical in creating combinations that will work best together and perhaps even synergize each other. Finally, the usefulness of biochemical indicators for assessing benefits of supplementation is limited. Instead, functional outcomes as true indicators of the effect are needed and should be assessed as endpoints in studies. Methods for the safe delivery of micronutrients to correct the high levels of deficiency that are clearly apparent among women in South Asia are urgently needed. Testing different combinations and doses of micronutrients and alternative delivery mechanisms (food fortification, sprinkles) on both short- and long-term health and functional outcomes in the mothers and their infants should receive priority.

Apart from the authors, several members of the Nepal study team helped in the successful implementation of the study and laboratory analysis, including the field managers, supervisors, and phlebotomy team. Tracey Wagner conducted the laboratory analyses, Joanne Katz was a co-investigator, Kerry Schulze performed the PIVKA-II analysis and provided comments on the paper, Elizabeth K Pradhan and Gwendolyn Clements were responsible for computer programming and data management, and Lee Wu provided statistical support.

PC was the principal investigator and analyzed and wrote the paper. TJ was the laboratory director, oversaw all the biochemical analyses, and provided edits for the manuscript. SKK was country director and implemented the study. SCL participated in the procedure development, study design, and edits to the article. SRS supervised the field work and data collection. KPW assisted in the development of the research idea, study design, protocol, and manuscript preparation. None of the authors had a personal or financial interest to declare.

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