Vitamin C supplementation to prevent premature rupture of the chorioamniotic membranes: a randomized trial\textsuperscript{1–3}

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ABSTRACT
Background: Vitamin C is involved in the synthesis and degradation of collagen and is important for maintenance of the chorioamniotic membranes. Inadequate availability of ascorbic acid during pregnancy has been proposed as a risk factor for premature rupture of the chorioamniotic membranes (PROM).

Objective: The objective of the study was to evaluate the effectiveness of 100 mg vitamin C/d in preventing PROM.

Design: A controlled double-blind trial was performed. Pregnant women ($n = 126$) in their 20th wk of gestation were invited; 120 accepted and were randomly assigned to 2 groups (100 mg vitamin C/d or placebo). Every 4 wk, plasma and leukocyte vitamin C concentrations were measured, and each subject was evaluated for cervicovaginal infection. The incidence of PROM was recorded for each group as an indicator of the protective effect of vitamin C supplementation.

Results: One hundred nine patients finished the study. Mean plasma vitamin C concentrations decreased significantly throughout the pregnancy in both groups ($P = 0.001$), and there were no significant differences between groups. Between weeks 20 and 36, mean leukocyte vitamin C concentrations decreased from 17.5 to 15.23 mg/10\textsuperscript{8} cells in the placebo group and increased from 17.26 to 22.17 mg/10\textsuperscript{8} cells in the supplemented group (within- and between-group differences: $P = 0.001$). The incidence of PROM was 14 per 57 pregnancies (24.5%) in the placebo group and 4 per 52 pregnancies (7.69%) in the supplemented group (within- and between-group differences: $P = 0.001$). The incidence of PROM was 0.26 (95% CI: 0.078, 0.837).

Conclusion: Daily supplementation with 100 mg vitamin C after 20 wk of gestation effectively lessens the incidence of PROM. \textit{Am J Clin Nutr} 2005;81:859–63.

KEY WORDS Vitamin C, ascorbic acid, pregnancy, premature rupture of the chorioamniotic membranes, preterm labor, dietary reference intakes, DRIs, Mexico

INTRODUCTION
Premature rupture of the chorioamniotic membranes (PROM) is a complication affecting 10–20% of all pregnancies. PROM is the main known cause of preterm delivery and is associated worldwide with increased rates of neonatal and maternal morbidity and mortality (1). Thus, it is important to develop public health strategies to prevent PROM. It has been proposed that maintaining adequate vitamin C status, ascertained on the basis of the vitamin C concentrations in leukocytes, could help reduce the incidence of PROM (2, 3). In a previous report, we (3) showed that PROM could be used as a functional test of vitamin C status throughout pregnancy. Our findings derived from the role of vitamin C in collagen metabolism and that of collagen in maintaining the mechanical strength of the chorioamniotic membranes throughout gestation (4, 5). Vitamin C is recognized as a cofactor for collagen posttranscriptional modifications (6) and as a down-regulator of the gene transcription of the 72-kDa type IV collagenase, matrix metalloproteinase (MMP-2), in amnion cells (7); it also controls the catabolism of collagen.

The current recommended dietary allowance for vitamin C does not adequately take into account the metabolic adjustments that occur during pregnancy. Altered patterns of collagen synthesis and diminished concentrations of vitamin C at week 28 of gestation have been associated with subsequent occurrence of PROM (3, 4). It has been postulated that low concentrations of plasma and leukocyte ascorbic acid may result from a dilution effect caused by hemodynamic adjustments of blood volume during the third trimester of pregnancy (3). Leukocytes may act as a store of vitamin C and can provide a more reliable indicator of vitamin C nutritional status (8) than can the ascorbic acid concentration in plasma or serum, which more specifically reflects the recent consumption of vitamin C. We recently conducted a clinical trial measuring the saturation dose of vitamin C in pregnant Mexican women (9) and found that 100 mg vitamin C/d added to the usual diet is sufficient to maintain a leukocyte ascorbate concentration > 18 mg (102 nmol)/10\textsuperscript{8} cells, and this concentration protects against PROM (3).

Other conditions commonly present during pregnancy, such as cervicovaginal or intrauterine infections (which are themselves risk factors for PROM), may also affect the nutritional status of vitamin C (10). Induction of the inflammatory response, which involves the respiratory burst in leukocytes, causes the consumption of antioxidants such as vitamin C (11). The incidence of

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PROM may also reflect the potentiation of chronic or iterative infections or their synergistic effects during pregnancy and the presence of low stores of vitamin C (12). The aim of this study was to evaluate the effectiveness of 100 mg vitamin C/d in preventing PROM.

SUBJECTS AND METHODS

This double-blind study was performed from September 2002 to August 2003 at the Instituto Nacional de Perinatología (INPer) in Mexico City. One hundred twenty-six women attending the prenatal clinic were identified as eligible participants. Inclusion criteria were defined as follows: pregnant women with no acute or chronic diseases, <20 wk of gestation, singleton pregnancy, no consumption of vitamin supplements, and provision of written informed consent. Exclusion criteria were the need for uterine cerclage or the presence of an obstetric indication for cesarean delivery.

Women were allocated to 1 of 2 groups by a random-number table; one group received 100 mg vitamin C/d, and the other group received a placebo of the same size and shape (both provided by Roche Pharmaceuticals, Mexico City). The principal investigator generated the random table, prepared all tablets, and ensured that staff members were blinded as to the grouping codes. Vitamin C or placebo was shipped to each participant directly from the research pharmacy, which concealed the treatment assignment from the investigators. The women were instructed to ingest one tablet with water before breakfast each day.

The sample size was calculated from the overall incidence of PROM at the INPer (18.5%), regardless of gestational age, and on the basis of our hypothesis that intervention with vitamin C would reduce the incidence of PROM by 20%. This would ensure an overall type I error of 5% and an 80% power-to-test reduction in the incidence of PROM. According to these estimations and in the anticipation of losses of 10% during the study, we needed to study 60 cases per group.

Written informed consent was obtained from all subjects. If the pregnant woman was < 18 y old, the written informed consent of a parent or legal guardian was obtained. The protocol was reviewed and approved by the Research and Ethics Board of the institution (protocol registration no. 212250--49311).

Study protocol

During the study, women were evaluated according to standardized procedures by trained nutritionists and faculty members of the Obstetrics and Gynecology Department. At the first evaluation, each woman’s general data, gynecologic and obstetric history, and anthropometric data, including self-reported prepregnancy weight, were recorded. Information on socioeconomic status according to the scale of the Asociación Mexicana de Agencias de Estudios de Mercado (Mexican Association of Marketing and Opinion Research) was also included; this scale measures purchasing power and contains 6 categories ranging from the highest to the lowest purchasing power (13). Each subject completed a food-frequency questionnaire, which considered the habitual intake of the previous year and which was previously validated in Mexican women (14). To obtain information on the habitual intake of vitamin C, intakes were corrected for cooking losses by a factor of 50% for boiled or fried food and by a factor of 25% for steamed food (15).

From weeks 20 to 36, women were evaluated every 4 wk. At each evaluation, a vaginal exploratory examination was performed, and a vaginal swab was taken for Gram staining and microbiologic study. The microorganisms of interest included Gardnerella vaginalis, Candida albicans, Group B streptococcus, Escherichia coli, Neisseria gonorrhoeae, and Ureaplasma urealyticum. Cervicovaginal infection was diagnosed by clinical manifestations and positive results of the Gram stain and culture. If infection was diagnosed, the woman received treatment with antibiotics in accordance with established clinical protocols. Asymptomatic subjects with a positive culture did not receive treatment (16). To evaluate the adherence to treatment, the women were instructed to maintain a personal record to register the daily consumption of tablets; the remaining tablets were counted by research staff at each visit.

At the end of pregnancy, the incidence of PROM was registered as the primary outcome. PROM was defined in accordance with the American College of Obstetricians and Gynecologists as the leakage of amniotic fluid through ruptured chorionamniotic membranes that occurs ≥2 h before the onset of labor and after week 20 of pregnancy (17). The diagnosis of leakage of amniotic fluid was established clinically by physical examination that identified the leakage of amniotic fluid through the cervix and confirmed by crystalization and the nitrazine test. A clinical evaluation was performed at delivery by using the method of Capurro et al (18) to verify the gestational age as estimated from the last menstrual period reported by the subject. A difference of ≤1 wk between the 2 evaluations was accepted; when the difference was > 1 wk, the case was excluded from the study. Preterm labor was established when the delivery occurred before 37 wk of gestation.

Laboratory tests

During the evaluation visits, a 15-mL sample of blood was collected into EDTA-coated tubes to measure plasma and leukocyte vitamin C concentrations. On the day of blood sampling, the women had fasted for 10–12 h and had not consumed the supplement or placebo tablet. Within 30 min after obtaining the blood sample, the plasma was separated by centrifugation at 1800 × g for 10 min, and leukocytes were isolated by using Dextran T500 (Sigma, St Louis, MO) (19). Leukocytes were counted with an automatic cell counter (Coulter Counter T890, version 3D; Beckman Coulter, Miami, FL). After the addition of metaphosphoric acid (Sigma) to prevent vitamin C oxidation, the plasma and leukocyte pellets were frozen at −70 °C. All samples were assayed in duplicate by using HPLC with an electrochemical detector (Perkin Elmer Life and Analytic Sciences, Shelton, CT; 20). Interassay and intraassay CVs were < 5% for all procedures. Laboratory personnel were blind as to patient group allocation.

Data analysis

We used a repeated-measures analysis of variance with orthogonal polynomial contrasts, adjusted for multiple comparisons, in the general linear models (GLM) program on SPSS software (version 11.0; SPSS Inc, Chicago IL) with Tukey’s correction to evaluate changes in vitamin C concentration in plasma and leukocytes at the return visits. Comparison of the maternal characteristics, infections, and the incidence of PROM in the 2 groups was performed by chi-square test. Relative risks (RRs) with 95% CIs were calculated.
RESULTS

Of 126 participating women, 109 (91%) finished the study: 52 women in the vitamin C–supplemented group and 57 in the placebo group (Figure 1). The groups did not differ in any baseline variable such as age, number of gestations, or nutritional status. None of the women reported smoking cigarettes during pregnancy. According to the socioeconomic status scale, the participants belonged to the lower middle-class, and the 2 groups did not differ significantly in socioeconomic status. A history of PROM was reported by 10.6%. The general and outcome variables of the 2 groups are presented in Table 1.

A cervicovaginal infection at the first evaluation at 20 wk of pregnancy was present in 32 (29%) of the 109 patients; 14 (25%) of the 57 women in the placebo group and 18 (35%) of the 52 women in the vitamin C group ($P = 0.50140$). At week 28, only 10 (18%) of 57 and 7 (14%) of 52 women had a cervicovaginal infection ($P = 0.05$). At week 36, only 10 women had infection (6 in the placebo group and 4 in the vitamin C group; $P = 0.05$).

There were no differences between groups in the incidence of infection, and only one woman had a recurrent infection. The microorganisms most frequently isolated were $C. albicans$ (10.5% of the cases) and $G. vaginalis$ (5.3% of the cases).

According to the food-frequency questionnaire, the mean daily vitamin C intake was 63 and 68 mg in the placebo and vitamin C groups, respectively. In the placebo group, the 25th and 75th percentile intakes were 38 and 135 mg/d, respectively; corresponding values for the vitamin C group were 45 and 118 mg/d. There were no differences in dietary vitamin C intake between groups. Citrus fruit, tomatoes, and chilies (hot peppers) were the most important dietary sources of vitamin C. According to the personal records kept by the subjects, $\approx 80\%$ reported adherence to the supplementation protocol ($\approx 83\%$ in placebo group and $\approx 85\%$ in the vitamin C group).

The overall mean plasma vitamin C concentration at week 20 of gestation was 0.76 ± 0.28 mg/dL (43.15 ± 15.9 μmol/L). Plasma vitamin C concentrations decreased similarly throughout pregnancy in both groups (between-subjects differences: $P < 0.001$), and they followed a significant ($P < 0.001$) linear trend with time (Table 2).

The mean leukocyte vitamin C concentration in the groups diverged as gestation progressed (between-subjects differences: $P < 0.001$). The concentration increased significantly between weeks 20 and 36 in the vitamin C–supplemented group, but it decreased over the same period in the placebo group (between-subjects differences: $P < 0.001$; Table 2).

The incidence of PROM was 4 (8%) of 52 women in the vitamin C–supplemented group and 14 (25%) of 57 women in the

### Table 1

<table>
<thead>
<tr>
<th>Characteristics and outcome variables</th>
<th>Placebo group ($n = 57$)</th>
<th>Vitamin C group ($n = 52$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics of the mothers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>27.4 ± 7.7$^2$</td>
<td>27.5 ± 7.4</td>
</tr>
<tr>
<td>Number of gestations ($n$)</td>
<td>2.3 ± 1.2</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>155.4 ± 6.7</td>
<td>155.0 ± 6.9</td>
</tr>
<tr>
<td>Prepregestational BMI (kg/m²)</td>
<td>23.8 ± 3.86</td>
<td>24.2 ± 4.2</td>
</tr>
<tr>
<td>Schooling (y)</td>
<td>11.0 ± 3.1</td>
<td>10.3 ± 2.7</td>
</tr>
<tr>
<td>Married [$n$ (%)]</td>
<td>35 (61)</td>
<td>33 (63)</td>
</tr>
<tr>
<td>Characteristics of the fetuses</td>
<td></td>
<td></td>
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<tr>
<td>Percentage of weight expected for height and gestational age at week 20 (%)</td>
<td>109 ± 24</td>
<td>104 ± 12</td>
</tr>
<tr>
<td>Gestational age at prenatal care (wk)</td>
<td>20 ± 3</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Outcome results</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3015 ± 629</td>
<td>3015 ± 513</td>
</tr>
<tr>
<td>Gestational age at birth (wk)</td>
<td>38.0 ± 3.1</td>
<td>38.5 ± 2.0</td>
</tr>
<tr>
<td>Premature births [$n$ (%)]</td>
<td>14 (24)</td>
<td>7 (13.4)</td>
</tr>
<tr>
<td>PROM [$n$ (%)]</td>
<td>14 (24)</td>
<td>4 (7.6)$^4$</td>
</tr>
</tbody>
</table>

$^2$ PROM, premature rupture of the chorioamniotic membrane.

$^2 x \pm SD$ (all such values).

$^4$ Significantly different from placebo group, $P = 0.018$ (chi-square test).
in the vitamin C group but decreased in the placebo group, and leukocyte concentration is considered an indicator of the stored amount of vitamin (13, 23). We measured plasma vitamin C concentration in fasted subjects because its concentration in plasma is coregulated with blood glucose by insulin (24).

Plasma vitamin C concentration decreased progressively during gestation regardless of supplementation. This decrease most likely reflects the effects of hemodilution (3, 25) and of active transport of vitamin C to the fetus, which increases throughout pregnancy (26). Leukocyte vitamin C concentration differed significantly between groups. Because the 2 groups did not differ in their dietary intake of vitamin C, these changes most likely reflect the effect of supplementation, which suggests that the extra vitamin C was taken up by the leukocyte compartment. Thus, leukocyte vitamin C concentration might serve as a direct marker of compliance.

The amount of vitamin C contained in the supplement tablet (100 mg/d) is less than the highest intake limit of 2000 mg/d established by the Institute of Medicine (27); this explains the lack of adverse effects of supplementation in our study. A daily dose of 100 mg ascorbate in combination with the average dietary intake of 65 mg vitamin C appears to be sufficient to maintain leukocyte an ascorbic acid concentration of >18 µg (102 mmol)/10⁹ cells, which has been shown to prevent PROM (3). In our study, the incidence of PROM in the placebo group was 3 times that in the supplemented group, which represents a 76% protective effect of vitamin C supplementation. To our knowledge, this is the first example of a beneficial effect of vitamin C in lowering the incidence of PROM.

Our data are consistent with a previous study of >2000 women that found an inverse relation between vitamin C intake and the incidence of PROM (28), although only diet and clinical cervicovaginal infection were evaluated. Another recent trial of vitamin C supplementation during pregnancy failed to show any beneficial effect on the incidence of preterm labor (29). However, the latter study used preterm labor as the primary outcome and did not evaluate the effect of supplementation on the incidence of PROM. Moreover, the dose of vitamin C was much higher (500 mg/d) than that in our study, and the method of evaluating vitamin C concentration was colorimetric assay.

Large doses of vitamin C can cause adverse reactions, such as those associated with oxidative stress (30). Reactive oxygen species can enhance collagen degradation in chorionicamnion membranes (31), which can interfere with the protective effect on the gene expression of metalloproteinases (eg, MMP-2) and can lead to increased collagen degradation, a condition thought to be an important mechanism in the genesis of PROM (32–34). This potential, undesirable effect must be taken into account when establishing the supplement dose of vitamin C. We propose that vitamin C status could be used as a functional indicator of the risk of PROM and that a leukocyte vitamin C concentration > 18 µg (102 mmol)/10⁹ cells is an indicator of adequate bioavailability of the vitamin (35).

We found no relation between infection and PROM, although 38% of the women had cervicovaginal infections by week 20 of gestation. We treated these infections and found no infection relapses in the women.

In conclusion, our data support the concept that vitamin C at dietary doses (100 mg/d) can prevent PROM. Because PROM may trigger ≥40% of all preterm labor, supplementation could be a valuable tool in sustaining pregnancy to term.
The next challenge is to design educational programs to promote adequate intakes of vitamin C through the consumption of fruit and vegetables and not through the taking of supplements during pregnancy.

We thank the volunteer women for their valuable participation.

EC assisted with the development of the research study protocol, coordinated the study, performed the statistical analyses, interpreted the data, and took the major responsibility for writing the manuscript. CR assisted with the recruitment and study of the subjects and collected data. MC and RMM performed the laboratory experiments and collected data. PV assisted with coordination of the study and with writing the manuscript. FP assisted with the obstetric evaluations of the subjects. FVO assisted in the development of the research protocol, review of the literature, interpretation of the data, and revision of the manuscript. None of the authors had a personal or professional conflict of interest.

REFERENCES