Introduction

Spontaneous abortions occur in approximately 40% of pregnancies (Abir et al., 1994). A large proportion of miscarriages result from chromosomal abnormalities or other defined medical factors. Recurrent early pregnancy loss is a multifactorial disease, and the causes for some of these abortions are still unknown. Carmi et al. (1994) found a significantly increased rate of spontaneous abortions (48%) in pregnancies preceding those of fetuses with neural tube defects (NTD). Eskes et al. (1996) demonstrated that a derangement of methionine-homocysteine metabolism was found in approximately 20% of cases with NTD and recurrent spontaneous abortion (RSA), which could be the underlying mechanism of pathogenesis. The authors hypothesized that both forms of reproductive failure could have one factor in common: hyperhomocysteinaemia, which interferes with embryonic development as well as with vascular function. As vitamin B12 and folic acid catalyse the remethylation of homocysteine to methionine, hyperhomocysteinaemia may be caused by a deficiency of folate metabolites or cobalamin. Therefore, the presence of appreciable amounts of folate and vitamin B12 was claimed to be essential for normal embryogenesis and embryonic growth. This is in agreement with several authors (Martin et al., 1965; Mukherjee et al., 1984) who postulated that low concentrations of folic acid are related to miscarriages, and might be the embryotoxic factor in the sera of women who abort.

This study was undertaken to evaluate the prevalence of folate or cobalamin deficiency in a high risk collective of non-pregnant habitual aborters with no known risk factors for such events in comparison to nulligravidae controls.

Materials and methods

Subjects

Studies were carried out on 29 women with RSA following conception from the same partner. They had been referred to the Department of Obstetrics and Gynecology, University of Würzburg, Germany. The patients participating in the study were recruited consecutively. RSA was defined as three or more consecutive miscarriages within 18 weeks gestation with confirmation by biochemical pregnancy test (β human chorionic gonadotrophin (HCG) ≥ 100 IU/l), sonography, or histopathological examination. Ectopic pregnancies or elective terminations of gestations were excluded. The mean number of abortions was 3.8 ± 0.9 (range 3–6). In total, all study patients experienced 110 abortions. Eight of the 29 women delivered a healthy infant prior to their successive miscarriages (secondary aborters), whereas the remainder had no living children (primary aborters). The mean age of the patients was 31.1 ± 5.1 years (range 18–43). They were selected on the basis of routine clinical investigation, including karyotype of both partners from peripheral blood, which failed to reveal balanced parental chromosome abnormalities as cause for the abortions. Severe uterine abnormalities were ruled out by either hysterosalpingogram, hysteroscopy, or a high level ultrasound scan. Immunological disorders were excluded by tests for anticardiolipin antibodies and lupus anticoagulant. Venepuncture was performed 6 months after completion of the most recent miscarriage.

The control population consisted of 29 healthy nulligravidae women of similar [P(U test) = 0.14] child-bearing age (mean ± SD, 29.5 ± 4.9 years, range 18–44). None of the subjects of either the study or the control group had a known endocrine dysfunction or suffered from gastrointestinal, hepatobiliary, renal or vascular disease. Patients with neurological disorders such as epilepsy were excluded. Vegetarians and women receiving oral contraceptives, vitamin supplementation, or any other medication possibly interfering with folic or cobalamin metabolism during the last 6 months before the venepuncture were also not included in the study. Before admittance, informed consent was obtained from all subjects.

Laboratory evaluation

Dry and heparinized 10 ml vacutainer tubes were used for fasting venous blood collection in the preovulatory phase of the menstrual cycle. Concentrations of folate and vitamin B12 were determined by
Folate and cobalamin in recurrent abortion

Figure 1. Distribution of folate and cobalamin values in recurrent spontaneous abortion (RSA) (study) and control group with mean and normal ranges.

automated fluorometric enzyme-linked assays (Stratus Folate and Stratus Vitamin B12, Baxter Diagnostics Inc., Deerfield, USA) according to the manufacturer’s instructions. The normal range of concentrations for folic acid was 3–17.5 ng/ml and for vitamin B12 271–966 pg/ml. The intra-assay coefficient of variation was 13.3 for the folate assay and 7.3 for vitamin B12. The interassay coefficient of variation was 4.1 and 8.3, respectively.

Statistical analysis
Results are given as mean ± standard deviation (SD). In the test of van der Waerden, folic acid was the only variable which showed a Gaussian distribution. Consequently statistical significance was determined using the Mann–Whitney non-parametric U test for differences between means of a variable and the exact chi-square analysis (χ²) of Mehta and Patel for differences between percentages. The Student’s t-test was used for normally distributed variables with similar SD, which was tested by the F-test. The association of two numerical variables was evaluated by Spearman’s rank correlation (coefficient rho). P < 0.05 was considered to be significant.

Results
The distribution of the serum concentrations of folate and cobalamin in both women with RSA and controls is presented in Figure 1. There was no significant difference between the mean vitamin values in the study and the control group (see Table I). Moreover the percentage of reduced or elevated concentrations of folic acid or vitamin B12 was similar in both collectives (see Table II). The majority of patients (75.9% of the study and 72.4% of the control group) had both vitamin concentrations within the normal range, and only five women had low cobalamin values. None of the subjects suffered from a folate deficiency. No correlation was found between age and vitamin concentrations in either the study [P(rho) = 0.60 for folate, 0.60 for cobalamin], the control [P(rho) = 0.11 and 0.12], or the whole population [P(rho) = 0.21 and 0.22]. In the study group the parity had no influence on the concentrations of folate [P(U-test) = 0.31] or cobalamin [P(U-test) = 0.94] (see Table I), whereas the number of previous abortions showed a weak significant negative correlation with folate [P(rho) = 0.036; see Figure 2], but not with vitamin B12 concentrations [P(rho) = 0.62]. Patients with a history of three miscarriages had a significantly higher mean of folate concentration (16.8 ± 5.2) than women who suffered from at least four pregnancy losses [11.1 ± 5.1, P(t-test) = 0.0062], but there was no significant difference [P(t-test) = 0.91] in the folate status of the latter collective in comparison to the control group (11.0 ± 5.3). An association of both substances was observed in

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Collective</th>
<th>Number</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>P(t-U test)</th>
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</thead>
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<td>13.7</td>
<td>5.8</td>
<td>4.5–23.2</td>
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<td>15.3</td>
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<td>6.0–23.2</td>
<td>0.35b</td>
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<td></td>
<td>control group</td>
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<td>11.0</td>
<td>5.3</td>
<td>3.1–23.2</td>
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<td>study group</td>
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<td>218.2</td>
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<td>215.3</td>
<td>193–1190</td>
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</table>

Table II. Percentages of vitamin concentrations within or out of normal range and level of statistical significance of the difference

<table>
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<th>Vitamin</th>
<th>Collective</th>
<th>&lt; Normal</th>
<th>&gt; Normal</th>
<th>P(χ²-test)</th>
</tr>
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<td></td>
<td>control group</td>
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<td>0</td>
<td>86.2</td>
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<tr>
<td>vitamin B12</td>
<td>study group</td>
<td>1</td>
<td>3.5</td>
<td>93.1</td>
</tr>
<tr>
<td></td>
<td>control group</td>
<td>4</td>
<td>13.8</td>
<td>82.8</td>
</tr>
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</table>

Figure 2. Correlation between the number of previous abortions and folate concentrations in the study group (rho = −0.3905, P = 0.036). The regression equation is : y = −1.852879E-5x^2 + 2.068333E1 Answer: The study group the parity had no influence on the concentrations of folate [P(U-test) = 0.31] or cobalamin [P(U-test) = 0.94] (see Table I), whereas the number of previous abortions showed a weak significant negative correlation with folate [P(rho) = 0.036; see Figure 2], but not with vitamin B12 concentrations [P(rho) = 0.62]. Patients with a history of three miscarriages had a significantly higher mean of folate concentration (16.8 ± 5.2) than women who suffered from at least four pregnancy losses [11.1 ± 5.1, P(t-test) = 0.0062], but there was no significant difference [P(t-test) = 0.91] in the folate status of the latter collective in comparison to the control group (11.0 ± 5.3). An association of both substances was observed in
none of the collectives \( P(\rho) = 0.26 \) all subjects, 0.64 study group, 0.41 control group.

**Discussion**

Multifarious mechanisms are said to play a role in the aetiology of spontaneous and recurrent abortion. Not only immunological and genetic disorders but also endocrine and psychological factors as well as infections or endometriosis may be responsible for embryo loss (Bulletti et al., 1996). There is evidence in the literature that some miscarriages result from embryotoxic factors present in the sera. Previous studies have demonstrated such embryotoxic effects of sera from women with spontaneous abortions in preimplantation mouse embryos and 10.5 day old rat embryos (Abir et al., 1990, 1994). Although folate deficiency is one of the factors that may lead to alterations in DNA synthesis and chromosome structure in rapidly dividing cells (e.g. heritable folate-sensitive fragile site in human autosomal chromosome 1p21.3; Baker and Sutherland, 1991), and the serum concentration is a sensitive indicator of the folate available for replicating cells (Neiger et al., 1993), Abir et al. found the mean serum concentration of folic acid to be similar in so called ‘high risk sera’ from women with at least two abortions and in control sera from women after a normal pregnancy or during a normal second trimester of gestation. Several studies on the relationship of the vitamin B complex, particularly of folate, to spontaneous abortion have been published, but available data with regard to recurrent miscarriage are rare.

Neiger et al. (1993) compared the incidence of spontaneous abortion and adverse perinatal outcome in 151 women with first trimester uterine bleeding and either low serum concentrations of folate \( n = 52 \) and/or vitamin B12 \( n = 7 \) or normal concentrations of these substances. No significant differences \( P = 0.3 \) were found, and it was concluded that low folate concentrations do not appear to be associated with an increased risk of pregnancy loss and adverse outcome. All seven subjects with low vitamin B12 levels miscarried, but the difference did not reach statistical significance \( P = 0.17 \). Chanarin and associates (1968) also found no significant difference in the serum and red cell folate values between patients who were aborting and early pregnant controls. In a randomized controlled trial on the effect of periconceptional multivitamin supplementation on pregnancy outcome, which included 5502 females, Czeizel et al. (1994) were unable to detect a significant difference in the rate of miscarriages between the multivitamin (including 0.8 mg folic acid) group, which was 10.8%, and the trace element groups, with 9.4%. An almost identical rate of miscarriages in folic acid (12.4%, 42/338) and unsupplemented groups (12.7%, 44/346) was also found in the Medical Research Council Vitamin Study (Wald et al., 1991).

Our results indicate that folic acid and vitamin B12 deficiency is not a common metabolic disorder among women with RSA, implying that these two vitamins do not play an important role in the aetiology of habitual abortion. In fact, folate and cobalamin concentrations were similar in the sera of women with this reproductive failure and controls. These results are in agreement with the above mentioned experimental animal and human studies, which found no association between folate or cobalamin deficiency and spontaneous miscarriage. Nevertheless, we found a significant effect of the number of previous abortions on the folate levels in the study group, as reflected by a negative correlation, indicating that, despite the absence of a significant difference in comparison to the control group, there might be an association between the frequency of miscarriages and folate values, even if the issue of cause and effect has not been clarified. Surprisingly, the two patients with six miscarriages had high folate values of 20.7 and 14.1 ng/ml in contrast to a maximum concentration of 18.9 ng/ml in the 11 women with four and 9.0 ng/ml in the three women with five miscarriages. In this context, it has to be taken into account that, although we were able to detect the mentioned significant correlation between the number of abortions and folate concentrations as well as a significant difference of folate values between patients who experienced three and those with at least four such events, these calculations deal with small numbers. We have no other scientific explanation for the high values of the two patients with six abortions than the high variation of values in each subgroup.

Dansky et al. (1992) reported that low blood folate concentrations before and/or early in pregnancy were significantly associated with spontaneous abortion (50% versus 19.2%). He pointed out that experimental studies in a number of animal species, e.g. in the guinea pig (Habibzadeh et al., 1986), had shown folate deficiency to cause intrauterine death. Martin et al. (1965) also observed a significant association between threatened abortion and reduced serum folate concentrations in 43 patients, but the occurrence of a miscarriage in the previous pregnancy of a multigravida was not significantly associated with a lowered serum folate in the current pregnancy. Hibbard (1964) reported a higher prevalence of maternal defective folate metabolism as reflected by a positive formimino glutamic acid (FIGLU) urinary excretion test among 87 patients who aborted at 8–25 weeks gestation without apparent cause (28.7%) in comparison to a control series of 50 normal pregnant women (6%). Among the 29 patients having their second or subsequent consecutive abortion, 41% \( n = 12 \) showed excessive FIGLU excretion. Therefore Hibbard suggested this metabolic defect to be an aetiological factor in a certain number of miscarriages. The findings of these studies were not confirmed in our study.

According to our knowledge, the only investigations with regard to the incidence of folate and vitamin B12 serum concentrations in women with unexplained recurrent early pregnancy loss were published by Pietrzik et al. (1992) and the group of Steegers-Theunissen (1992), Wouters (1993) and coworkers. Pietrzik described a highly significant association of reduced folate values in 46 habitual aborters in comparison to a healthy reference group with normal pregnancy. In contrast to these data, Wouters et al. demonstrated in their most recently published survey (1993) that the mean fasting concentrations of serum vitamin B12 and serum folate were not significantly different between 102 women with a history of at least two abortions and 41 females who had delivered at least one live child and had not suffered from abortion. The majority of tests (73%) were performed within 6 months after completion of
the last pregnancy. The following problem arises from the design of the aforementioned studies: it is known from the literature (Bruinse et al., 1985) that maternal serum folate values decrease significantly and progressively between the 16th week of gestation and parturition. Within 6 months after pregnancy there is no statistically significant increase, so that the mean value at that time is considerably lower than the mean value of a non-pregnant female reference group. Serum vitamin B12 concentrations in normal pregnancy are also lower than in non-pregnant controls (Karlgård et al., 1964; Benjamin et al., 1966). As folate decreases progressively during pregnancy, patients experiencing an abortion at, for example, 14 weeks gestation might have a folate acid deficiency due to a metabolic disorder, whereas women who delivered at term 3 months ago might show low folate values due to physiological changes during pregnancy. When comparing these groups, no statistically significant difference in folate values would be found, and no effect of folic acid on pregnancy outcome would be apparent. Therefore, in contrast to the study of Wouters et al. (1993) and Pietrzik et al. (1992), we chose a control group which consisted of nulligravidae, being well aware of the uncertainty concerning the most appropriate control population. Blood samples of women with RSA were collected 6 months after the most recent abortion, and we have no data for the period immediately preceding the miscarriage. Moreover, nulligravid controls of similar reproductive age are not an unusual control population in studies among habitual aborters (Nicotra et al., 1994; Bussen and Steck, 1995). Another difference between our study and prior investigations is the number of previous miscarriages. The study groups of other publications consisted of women with a history of two or more consecutive abortions. We selected a high risk collective with at least three previous miscarriages to maximize the probability of a significant difference in comparison to a suitable control series. As the influence of nutritional factors (Janelle and Barr, 1995) as well as the impact of hormones (Amatayakul et al., 1984; Tyer, 1984) on the concentrations of folate and cobalamin is contradictory in the literature, vegetarians and women receiving oral contraceptives were excluded. Hibbard (1964) reported that in 1484 women examined the number of previous pregnancies had a significant impact on the incidence of folic acid deficiency. Age did not have any significant influence. We were unable to detect any significant effect of parity on folate values, but we observed a weak significant correlation between the number of previous miscarriages and folate values. Furthermore our results confirm the findings of Hibbard et al. (1994) with regard to the lack of influence of age on vitamin concentrations.

According to the literature, after iron deficiency, folate is the most common deficiency found in the pregnant woman (Neiger et al., 1993). The incidence of folate deficiency in gestation was approximately 30% in 1970 using non-pregnant standards of folate concentrations, whereas vitamin B12 deficiency was uncommon in pregnancy (Rothmann, 1970). In our study, among a small collective of non-pregnant women, five subjects were diagnosed as having low vitamin B12 serum values, but none suffered from folate deficiency. Therefore while hyperhomocysteinaemia has been claimed to be a risk factor for RSA (Wouters et al., 1993), this disorder does not seem to be associated with folate or cobalamin deficiency. Further studies on the role of homocysteine metabolism in women with RSA should consider other aetiological factors for hyperhomocysteinaemia than decreased concentrations of folic acid and vitamin B12.

In summary, our analysis revealed no significant alterations of folate and vitamin B12 concentrations in non-pregnant women with recurrent spontaneous abortion when compared to nulligravidae controls, and consequently, in the absence of marked anaemia, recommendations for a periconceptional prophylaxis with folic acid or vitamin B12 following spontaneous abortion to prevent further miscarriages seem to be of questionable value. We would like to point out that our data do not allow us to comment on the efficacy of periconceptional folate supplementation with regard to the prevention of neural tube defects (Wald et al., 1991).

References
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