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Haemostatic and metabolic abnormalities in women with unexplained recurrent abortion

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The objective of this study was to establish whether or not patients with unexplained recurrent abortion have an increased incidence of haemostatic or metabolic abnormalities. Fifty-two patients with a history of unexplained habitual abortion (two or more spontaneous abortions before 16 weeks' gestation) were tested for protein S, protein C and antithrombin (AT) III deficiency, activated protein C (aPC) resistance, hyperhomocysteinaemia and anticardiolipin antibodies (ACA). The control group consisted of 67 healthy women with a history of only uncomplicated pregnancies. Blood samples were taken for measuring protein S, protein C, AT III, ACA and activated protein C resistance and a methionine loading test was performed. Of the 46 patients tested for protein S deficiency, 8 (17.4%) were positive. Of the 43 patients tested, two (4.7%) were protein C deficient and none was AT III deficient. Of the 42 patients tested for ACA, eight (19.1%) had detectable antibodies. Of the 44 patients tested for aPC resistance, two (4.6%) were positive. Finally, 35 patients were tested for hyperhomocysteinaemia and six (17.1%) were positive. It was concluded that parous women with a history of unexplained recurrent abortion have an increased incidence of hyperhomocysteinaemia and a trend of increased incidence of ACA can be found.

Key words: anticardiolipin antibodies/hyperhomocysteinaemia/protein C and S deficiency/recurrent abortion

Introduction

Recurrent abortion affects 2–5% of the couples attempting to reproduce (Coulam *et al.*, 1997). The aetiology remains unresolved in 24–60% of all cases of recurrent abortion (Wouters *et al.*, 1993).

Although it seems that certain disorders, for example chromosome abnormalities, corpus luteum insufficiency, congenital malformations of the uterus, systemic hypertension, diabetes mellitus and hyper- or hypothyroidism may explain recurrent pregnancy loss (Helio de Lima *et al.*, 1993), these conditions are rather infrequent. Recently, the relationship between coagulation abnormalities (Preston *et al.*, 1996) and metabolic abnormalities (de Vries *et al.*, 1997) has been described. This leads to a pro-thrombotic state and an increased risk of fetal loss after 16 weeks' gestation. Wouters *et al.* (1993) found evidence for a relation between metabolic abnormalities and fetal loss before 16 weeks' gestation. In this study we tried to determine whether or not both underlying haemostatic and metabolic abnormalities are associated with recurrent abortion.

Materials and methods

In the period between January 1995 and January 1996, 60 consecutive patients with a history of recurrent abortion, defined as fetal loss before 16 weeks' gestational age, were screened by the following tests: endometrium biopsy, IgG and IgM cytomegalovirus-antibodies, karyotyping of the patient and partner, endocrine evaluations for luteal insufficiency, thyroid dysfunction, diabetes mellitus and a hysterosalpingography. In these 60 patients, we found three patients with a uterus bicornis, one woman had a uterus subseptus, one woman was exposed during intrauterine life to diethylstilboestrol (DES), another woman had systemic lupus erythematosus and there were two couples with a chromosome translocation.

In the remaining 52 women, no abnormalities were discovered in these tests. These 52 patients with a history of unexplained recurrent abortion were tested for the presence of protein S, protein C and antithrombin III deficiency, activated protein C resistance, hyperhomocysteinaemia (HHC) and immunoglobulin (Ig)G and IgM anticardiolipin antibodies (ACA).

Of the 52 women tested, 15 had experienced two spontaneous abortions, 18 three, 11 four, four five, two six, one eight and one patient 18 miscarriages. The total number of pregnancies was 186 (average 3.6). Not one of the abortions included in this study was of the blighted ovum type. Embryo presence had been documented by either ultrasound or inspection of the abortion material.

Of the 52 women included in the study, 18 had had a successful pregnancy. Six of these 18 women had consecutive abortions.

As a control group for protein S deficiency, aPC resistance, HHC and ACA, we used data from our recent study on underlying disorders in pre-eclampsia (van Pampus *et al.*, 1997). This control group consisted of 67 healthy women with a normal menstrual cycle and an obstetric history of only uncomplicated pregnancies.

As a reference for the incidence of AT III and protein C deficiency, we used the data from the Leiden Thrombophilia Study (Miletich *et al.*, 1987; Tait *et al.*, 1994).

The patients and controls did not use oral contraceptives or they stopped its use at least 8 weeks before testing. None used vitamins, known to influence homocysteine metabolism (van den Berg *et al.*, 1994), within at least a period of 6 months before performing a methionine loading test. Patients and controls were tested in the second half of a normal menstrual cycle for the presence of protein S, protein C and AT III deficiency, activated protein C resistance,

(HHC) and anticardiolipin antibodies. The 52 patients were tested at least 10 weeks post-abortum.

Laboratory tests

Coagulation tests

Protein C activity was measured with Coamate® (Chromogenix, Mölndal, Sweden) protein C in a microplate system. The test was performed according to the guidelines of the manufacturer. According to the manufacturer the levels of protein C activity measured in 95 healthy individuals, 53 men and 42 women, aged between 19 and 64 years, are in the range of 70–149% (mean 103%, SD 17%) (Vinnazzer and Pangraz, 1987). The normal value of protein C activity in a control group of 30 healthy blood donors in our laboratory is 70–140%.

Total protein S antigen was measured by an enzyme-linked immunosorbent assay with the use of rabbit antihuman anti-protein S from Dakopatts (DAKO). This method has been described in detail by Woodhams (1988). The test was performed according to the guidelines of the manufacturer. The normal range for total protein S antigen is 70–130% according to Woodhams (1988) and DAKO. As an extra control, every batch of patient samples was measured in parallel with a sample of pooled plasma (30 healthy blood donors).

In our laboratory the normal value of total protein S antigen is also 70–130%.

Antithrombin III activity was assayed with chromogenic substrate S-2765 (Chromogenix) in a microplate system (Tollefsen, 1990). The test was performed according to the guidelines of the manufacturer. According to the manufacturer the reference values are 106% ± 19% (2 SD, $n = 50$; male 26, female 24).

In our laboratory the normal value of antithrombin III activity is 75–145%.

Activated protein C resistance was determined by the technique described by Dahlbäck *et al.* (1993) and Koster *et al.* (1993). In short, the resistance to activated protein C was determined by the activated partial thromboplastin time performed in the absence and presence of activated protein C. The results were expressed as the ratio of the two values. The normal value of the activated protein C sensitivity ratio is >2.0.

All patients with an abnormal coagulation test were retested after 6–12 weeks. They were considered to be positive only if the same abnormality was found again at that time.

Anticardiolipin antibodies

In the current study ACA were determined by enzyme-linked immunoassay according to the directives of Harris (1990). Results were evaluated spectrophotometrically (at 492 nm) and expressed as IgG phospholipid units (GPL) and IgM phospholipid units (MPL). Results were considered abnormal if GPL and/or MPL ⩾ 10.

Hyperhomocysteinaemia

Hyperhomocysteinaemia was assessed both in the fasting state and after methionine loading. After an overnight fast, EDTA blood for determination of the fasting homocysteine concentration was obtained at 0800 h. After 10 min of centrifugation at 1800 *g*, plasma was stored at -30^0C until analysis. Subsequently, an oral dose of L-methionine (0.1 g/kg body weight) was administered in orange juice. The patients ate a standardized low-methionine breakfast and lunch (containing 14 mg methionine/g protein) during the test. At 6 h after the methionine load a second blood sample was obtained for determination of the post-load homocysteine concentration. All measurements were performed within 1 week. Total (free plus protein-bound) homocysteine concentrations (disulphide homocysteine plus mixed disulphides) were measured with tri-N-butylphosphine used as a reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4sulphonate used as the fluorochromophore, followed by high-performance liquid chromatography with fluorescence detection (Mudd *et al.*, 1985; Ubbink *et al.*, 1991).

Patients were considered hyperhomocysteinaemic if the homocysteine concentration, measured 6 h after the methionine loading, exceeded the 97.5 percentile in healthy premenopausal volunteers (51 μ mol/l) (van Pampus *et al.*, 1997).

Table I. Underlying disorders in 52 women with a history of unexplained habitual abortion

Test	Tested (no.)	Positive (no.)	Positive (%)	Positive control[a] (%)
Prot S def	46	8	17.4	9[c]
Prot C def	43	2	4.7	0.1–0.3[d]
AT III def	43	0	0.0	0.1[e]
ACA[b]	42	8	19.1	7.5[c]
APC-R	44	2	4.6	1.5[c]
HHC	35	6	17.1	4.5[c]

Prot S def = protein S deficiency; ACA= anticardiolipin antibodies IgG and/orIgM; Prot C def = protein C deficiency; aPC-R = activated protein C resistance; AT III def = antithrombin III deficiency; HHC = hyperhomocysteinaemia.
[a]Control, percentage of abnormal findings in normal populations.
[b] >10 GPL and/or MPL ACA.
[c]Pampus van *et al.* (1997).
[d]Miletich *et al.* (1987).
[e]Tait *et al.* (1994).

Statistical analysis

Data were analysed using Fisher's exact test. A two-sided P value < 0.05 was considered to indicate a statistically significant difference.

Results

The overall results of the analysis of the 52 patients with a history of unexplained recurrent abortion are presented in Table I.

The mean (±SD) level of protein S in the eight patients with a protein S deficiency was 62.5 ± 5.15 %. Although the incidence of protein S deficiency was higher in patients (17.4%) than in the control population (9%), the difference was not statistically significant, which may be due to small sample size.

The difference between the incidence of protein C and AT III deficiency and aPC resistance in the patient population versus the control group was not statistically significant.

Although there appears to be a marked difference in the ACA regarding the patient population (19.1%) and the control population (7.5%) this difference was not statistically significant ($P = 0.12$) which may also be due to the small sample size. (Typical odds ratio 2.9; 95% CI: 0.88–9.6).

The difference concerning HHC in the overall patient group versus the controls just failed to reach statistical significance ($P = 0.059$; odds ratio 4.4 and 95% CI: 1.03–18.8).

Of the 35 women tested for hyperhomocysteinaemia, 14 were parous and 21 were non-parous. In these subgroups, the incidence of HHC was respectively 28.6% and 9.5%. The control group consisted of only parous women, so the difference between the parous patients (28.6%) and the parous controls (4.5%) was statistically significant ($P = 0.015$, odds ratio 8.5; 95% CI: 1.6–43.9).

Of the 52 women, 18 had had one successful pregnancy. Of

Table II. The relationship between the number of abortions and the incidence of underlying abnormalities

Number of abortions	Number of women	Fully examined	Abnormality found (number of patients, %)	Abnormality found and consecutive abortions (number of patients)
2	15	7	2 (28.6)	2
3	18	12	7 (58.3)	7
4	11	8	5 (62.5)	3
5	4	4	3 (75)	1
6	2	2	1	0
8	1	1	1	0
18	1	1	1	0

these 18 women, eight women (44%) had an underlying abnormality. Of the remaining 34 non-parous women, 15 women (44%) had an underlying abnormality, so there was no difference between these subgroups.

In 29 (56%) women no abnormality was detected. However, of these patients, only 15 patients had completed the full protocol of six tests.

There were three women with a combination of underlying abnormalities, one patient had hyperhomocysteinaemia and high-positive ACA, one patient had hyperhomocysteinaemia and protein S deficiency and the third patient had protein S deficiency and activated protein C resistance.

The percentages of patients who were fully examined and the presence of underlying abnormalities are shown in Table II.

Discussion

Recurrent abortion affects 2–5% of the population and because its aetiology often remains unsolved, it is a tragic event for both partners (Coulam *et al.*, 1997).

In The Netherlands, recurrent abortion is traditionally defined as three or more consecutive spontaneous abortions before 16 weeks' gestation. However, in recent articles, a definition of two or more spontaneous abortions is frequently used, because doctors and couples tend to investigate sooner for potential causes. In a previous study, this tendency appeared to be justified by the observation of an equal distribution of possible aetiological factors in couples with a history of two or more miscarriages and those with three or more (Wouters *et al.*, 1993). The results of this study suggest that there is a positive association between the number of failed pregnancies and the chance of revealing haemostatic or metabolic abnormalities.

Obviously this is not the first study regarding unexplained recurrent abortion and haemostatic and metabolic abnormalities. However, to our knowledge this is the first report where an extended screening on both thrombotic and metabolic factors was performed.

There are various hypotheses concerning the causes of recurrent spontaneous abortion. There is much controversy concerning the impact of psychological stress upon recurrent abortion. A study by Bergant *et al.* (1997) showed that psychological factors seem to be of subordinate importance as a cause for recurrent spontaneous abortion. The detection of the antiphospholipid syndrome as an aetiological cause for recurrent abortion has substantiated the thrombosis theory of repeated fetal loss (Younis *et al.*, 1997). Although the pathophysiology of the antiphospholipid syndrome associated thrombosis is not well characterized, antithrombotic treatments employing the combination of aspirin and heparin have proved to be effective in these women (Rai *et al.*, 1997). In another study, treatment with low-dose aspirin was evaluated (Tulppala *et al.*, 1997). The administration of aspirin caused a desirable biochemical effect but it did not improve pregnancy outcome in women with recurrent abortion with or without detectable ACA. Other mechanisms must also be considered. The possibility of direct damage to the placenta by these antibodies is supported by histologic observations of placentae from patients who experienced intrauterine fetal death. The most significant difference between placentae from patients with antiphospholipid antibodies and patients without these antibodies was decreased vasculo-syncytial membranes. Although the effect on syncytial membranes was attributed to hypoxia secondary to thrombosis, a counter hypothesis is that thrombosis occurred secondary to syncytiotrophoblast damage (Rote, 1997).

Inherited thrombotic disorders, termed 'familial thrombophilia', are the result of specific defects in the genes for plasma coagulation proteins involved in heparin-antithrombin III and protein C anticoagulant pathways. These are relatively rare familial disorders that are usually inherited as autosomal dominant disorders. Resistance to activated protein C is almost always caused by a single point mutation in the factor V gene. This new mutation has been termed factor V Leiden and is also inherited autosomal dominant. Preston *et al.* (1996) has substantiated the association between familial thrombophilia and increased fetal loss, particularly stillbirth. Although in studies of severe early onset pre-eclampsia the incidence of aPC resistance as an underlying abnormality was found to be increased (Dekker *et al.*, 1995) this was not found in this study on recurrent spontaneous abortion. Also Balasch *et al.* (1997) found that first-trimester repeated abortion is not associated with activated protein C resistance. The hypothesis given by the authors is that the pregnancy-associated development of resistance to activated protein C develops mainly after 14 weeks' gestation and is more evident near term.

Protein S deficiency may also cause thrombo-embolism and thus recurrent abortion. In our study we used precise testing criteria. All patients were tested twice at least 10 weeks post-abortum. They were considered to have protein S deficiency only if the protein S antigen level was below 70% on both occasions. The incidence of protein S deficiency in this

population of patients with unexplained recurrent abortion is about two times higher (not significant) than in the control population.

We found that the incidence of hyperhomocysteinaemia in the patient population was about four times higher (17.1%) than the controls (4.5%). Hyperhomocysteinaemia is more common in parous patients (28.6%) compared to non-parous patients (9.5%). The study of Wouters *et al.* (1993) also showed this discrepancy (33% versus 14%). Besides causing thrombosis, hyperhomocysteinaemia could also cause recurrent abortion because of its embryotoxicity (Unander *et al.*, 1987). A study by Sütterlin *et al.* (1997) evaluated the folate and cobalamin serum levels in women with recurrent spontaneous abortion. The levels showed no significant differences between the patients and the controls. A negative association between folate metabolism and parity has been suggested in the literature (Stone *et al.*, 1967). In this study we did not measure the folate levels.

In three out of 52 women more than one abnormality was found. There was no difference in the incidence of underlying abnormalities in parous or non-parous women.

In summary, patients with a history of unexplained recurrent abortion should be tested for the presence of protein S deficiency, hyperhomocysteinaemia and anticardiolipin antibodies, since these were the most common abnormalities found in this patient group. Abnormalities were found in 44% of these patients.

Finding these abnormalities may have an impact on general health and may also influence management of subsequent pregnancies.

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