Thrombophilia and unexplained pregnancy loss in Indian patients

SONAL VORA, SHRIMATI SHETTY, VINITA SALVI, PURNIMA SATOSKAR, KANJAKSHA GHOSH

ABSTRACT

Background. *The role of acquired and congenital thrombophilias in the aetiology of unexplained pregnancy loss in the Indian population has not been studied in detail. We studied the association of acquired and inherited markers of thrombophilia in a large group of patients with unexplained pregnancy loss.*

Methods. *A total of 602 women with pregnancy loss were referred to us for evaluation of thrombophilia between April 2000 and June 2005. After investigations to rule out cytogenetic, hormonal, anatomical and microbiological causes, no cause was ascertained in 430 women for the pregnancy loss. Of these, 49 women, who had a history of only one pregnancy loss, were excluded. The remaining 381 women comprised the study group. These patients and 100 age-matched women who did not have any obstetric complication and had at least one normal healthy child (controls) underwent detailed investigations for the presence of thrombophilia markers. These included screening coagulations tests, tests for lupus anticoagulant (LA), IgG and IgM antibodies to anticardiolipin antibodies (ACA), β2 glycoprotein 1 (β2GP1) and annexin V. The genetic markers studied included protein C (PC), protein S (PS), antithrombin III (AT III), factor V Leiden (FVL), PT gene G20210A, MTHFR C677T, EPCR 23 bp insertion and PAI 4G/5G polymorphisms.*

Results. *Of the 381 women with pregnancy loss, 183 had 2 and 198 had ≥3 pregnancy losses. Early pregnancy loss occurred in 136 patients, late pregnancy loss in 119, and both early and late pregnancy losses in 126. The strongest association was observed with ACA (OR 32.5, 95% CI: 8.6–21.8, p<0.001) followed by annexin V (OR 17.1, 95% CI: 2.9–99.4, p<0.001), LA (OR 8.2, 95% CI: 1.4–47.7, p=0.01) and anti-β2GP1 (OR 5.8, 95% CI: 1.6–22.1, p=0.007). No association of antiphospholipid antibodies with the time of pregnancy loss was found except LA which was significantly associated with early pregnancy loss compared with late pregnancy loss (p<0.05). The risk of pregnancy loss with PS deficiency (OR 17.8, 95% CI: 3.1–102.9, p<0.001) was the highest observed for any heritable thrombophilia followed by PC deficiency (OR 5.8, 95% CI: 1–34, p=0.06). There were no statistically significant differences in the frequency of any of the genetic thrombophilias studied between women with early and late pregnancy loss. A combination of ≥2 genetic factors was observed in 41 (10.8%) while that of genetic and acquired risk factors were observed in 79 (20.7%) patients. No more than one risk factor was observed in any of the controls. In all, 176 (46.2%) patients had at least one acquired thrombophilia while 143 (37.5%) had at least one genetic thrombophilia marker. Overall, 288 patients (75.6%) had either an acquired, genetic or both markers of thrombophilia.*

Conclusion. *Thrombophilia is an important factor in both early and late pregnancy losses. Women with unexplained pregnancy loss should be screened for the presence of thrombophilias.*

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INTRODUCTION

Adverse outcomes of pregnancy, especially pregnancy loss, are a major problem related to women's health—9%–13% of women in the reproductive age group have 1 clinically recognized pregnancy loss, 5% experience ≥2 pregnancy losses while 1%–2% experience ≥3 pregnancy losses.[1–3] In up to 50% of these women, after standard investigations including gynaecological, hormonal and karyotype analyses, no cause is found. Thrombophilia, both acquired and hereditary, has been implicated in the increased susceptibility to adverse pregnancy outcomes such as foetal loss, recurrent spontaneous abortions (RSA), abruptio placentae, intrauterine growth restriction (IUGR) and pre-eclampsia.[4,5] Thrombosis in the decidual vessels of the placenta is believed to be the main cause leading to IUGR, foetal death and possibly recurrent foetal loss.

There are no data from India on the prevalence of acquired and hereditary risk factors in women with unexplained pregnancy loss. We studied a number of acquired and hereditary causes of thrombophilia in a large group of women with unexplained pregnancy loss after ruling out some common aetiological factors.

METHODS

Six hundred and two women with unexplained pregnancy loss were referred to our centre from April 2000 to June 2005 for evaluation of thrombophilia. In 430 women, cytogenetic, hormonal (thyroid function test, 17-hydroxy progestorene levels in serum and 24-hour oestriol levels in urine), anatomical and microbiological examination such as TORCH serology and urine examination and culture for evidence of infection revealed no cause and 49 women had had only 1 pregnancy loss. The remaining

National Institute of Immunohaematology (ICMR), K.E.M. Hospital, Parel, Mumbai 400012, Maharashtra, India

SONAL VORA, SHRIMATI SHETTY, KANJAKSHA GHOSH

K.E.M. Hospital, Parel, Mumbai 400012, Maharashtra, India

VINITA SALVI Department of Obstetrics and Gynaecology

Nowrosjee Wadia Maternity Hospital, Parel, Mumbai 400012, Maharashtra, India

PURNIMA SATOSKAR Department of Obstetrics and Gynaecology

Correspondence to KANJAKSHA GHOSH; *kanjakshaghosh@hotmail.com*

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381 women were studied in detail for the presence of acquired and inherited markers of thrombophilia. Among these 381 women, 183 had 2 pregnancy losses and 198 had ≥3 pregnancy losses. Based on the time of loss of pregnancy, 136 women had early pregnancy loss (EPL), 119 had late pregnancy loss (LPL) and 126 had both EPL and LPL. In patients who presented immediately after a pregnancy loss, the samples were collected at least 4 months after the pregnancy loss. If the patient was pregnant at the time of presentation, tests for protein C and S and antithrombin III were repeated 4 months after delivery, and only the post-delivery results were considered.

One hundred normal healthy women matched for age and with at least 1 normal healthy child were included in the study as controls. Those with a previous history of thrombosis or pregnancy loss, those currently pregnant and those who gave a history of taking oral contraceptives were excluded.

Blood collection. Ten ml of blood was collected by venepuncture into 3.18% trisodium citrate (1:9 anticoagulant to blood) and EDTA tubes. Plasma samples were stored at –70 °C until analysed. The cell pellet was preserved at –20 °C for DNA extraction.

The study was approved by the Ethics Committee of all the participating institutes. Each patient and control gave written informed consent before inclusion in the study.

Tests for thrombophilia

Screening tests. These included prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT), and were done using commercial reagents (Organon Teknika, Durham, USA). Any sample with clotting time (APTT) prolonged >5 seconds than the control sample was analysed for the presence of lupus anticoagulant. Fibrinogen was measured by clotting assays using commercial kits (Diagnostica Stago, Asniers, France).

Tests for antiphospholipid antibody (APA) syndrome. Mixing studies were done by mixing the patient's plasma with normal pool plasma (NPP). Kaolin clotting time (KCT) and dilute Russel Viper venom time (DRVVT) were done using commercial reagents (Dade Behring, Germany) as described earlier.[6,7] IgG and IgM antibodies for cardiolipin antibodies, β2 glycoprotein P1 (β2GP1) and annexin V were measured by ELISA using commercial kits (Varelisa, Freburg, Germany). All tests for APA were repeated after a minimum interval of 4 months.

Tests for inherited thrombophilia. Protein C and S were measured by ELISA using commercial kits (Diagnostic Stago, Asniers, France). Antithrombin III (AT III) was measured by chromogenic assays using commercial reagents (Diagnostica Stago, Asniers, France). DNA was extracted from the citrated cell pellet using standard methods.[8]

Factor V Leiden mutation was identified by PCR amplification of a 220 bp fragment followed by digestion of the amplified fragment with Mnl1 restriction enzyme.[9] The PT G20210A polymorphism was identified by Hind III cleavage of the 322 bp PCR amplified product.[10] The C677 T polymorphism of MTHFR was detected using Hinf 1 cleavage of the 175 bp PCR product[11] while EPCR 23 bp insertion was detected by PCR amplification without any further restriction digestion.[12] PAI–1 4G/5G polymorphism was detected by allele-specific PCR amplification using two sets of primers.[13]

Statistical analysis

Univariate odds ratio (OR) and 95% CI were estimated separately for each parameter and for the total number of samples as well as for different groups, i.e. EPL and LPL. Multiple analyses of variants using SPSS12 software were used to calculate the p values. Because of repetitive measurements, Bonferroni correction was also employed.

RESULTS

The median age of the patients (*n*=381) was 24 years (range: 16–41 years) while that of controls (*n*=100) was 24 years (range: 18–30 years). The pregnancy losses in the patients ranged between 2 and 13 with a total of 867 previous pregnancy losses in 818 patients.

Each APA was significantly associated with pregnancy loss as an independent risk factor. ACA was the most common antibody detected followed by lupus anticoagulant (LA), anti-annexin V and β2GP1 (Table I). Overall, 171 of 381 patients (44.9%) were positive for one of the APAs studied compared with 6% of controls. Only LA was significantly associated with EPL ($p<0.05$).

There were no significant differences in the mean levels of protein C, protein S and AT III between the patients and controls or between women with EPL, LPL, or EPL and LPL. The most common defect was protein S deficiency (Table II).

Among the inherited thrombophilic polymorphisms studied, 13 of 381 patients (3.4%) were positive for factor V Leiden mutation compared with 1% of controls. PT gene polymorphism was not found in any of the patients or controls. MTHFR T/T polymorphism and EPCR 23 bp insertion polymorphism were

TABLE I. Frequency of antiphospholipid antibodies in patients and controls

Marker	Controls (*n*=100)	Early pregnancy loss (*n*=136)			Late pregnancy loss (*n*=119)			Total* (*n*=381)		
	n (%)	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value
Lupus anticoagulant	1 (1)	14 (10.3)	11.4 (1.9–68.4)	0.003	4 (3.4)	3.4 (0.5–23.2)	0.4	29 (7.6)	8.2 (1.4–47.7)	0.01
Anticardiolipin antibody	2 (2)	40 (29.4)	20.4 (5.3–78.4)	<0.001	35 (29.4)	20.4 (5.3–78.8)	<0.001	112 (29.4)	32.5 (8.6–21.8)	<0.001
	n=100		*n*=118			*n*=99			*n*=332	
β2 glycoprotein 1	2 (2)	6 (5.1)	2.6 (0.6–11.6)	0.3	9 (9.1)	4.9 (1.2–20.6)	0.03	35 (10.5)	5.8 (1.6–22.1)	0.007
Annexin V	1 (1)	15 (12.7)	14.4 (2.4–86.7)	0.001	16 (13.4)	19 (3.1–114.6)	<0.001	49 (14.8)	17.1 (2.9–99.4)	<0.001

* Includes women who had both early and late pregnancy losses Comparison of the groups of women with early pregnancy loss and women with late pregnancy loss showed the following results: Lupus anticoagulant: OR 3.3, 95% CI: 1.1–9.8, p=0.04; Anticardiolipin antibody: OR 1, 95% CI: 0.6–1.7, p=1.0; β2 glycoprotein 1: OR 0.6, 95% CI: 0.2–1.6, p=0.3; Annexin V: OR 0.8, 95% CI: 0.4–1.7, p=0.6

seen in 10 (2.6%) and 22 (5.8%) women, respectively. Homozygous PAI 4G/4G polymorphism was observed in 82 (21.5%) and 10 (10%) of the patients and controls, respectively (Table III).

Combination of risk factors

A combination of two or more genetic risk factors were observed in 41 (10.8%) patients while genetic and acquired risk factors were observed in 79 (20.7%) patients. No more than one risk factor was observed in any of the controls. In all, 176 (46.2%) patients had any one of the acquired thrombophilias while 143 (37.5%) had any of the heritable thrombophilias studied. Overall, 288 patients (75.6%) had either acquired, genetic or both forms of thrombophilia.

DISCUSSION

The aetiology of pregnancy loss is an important yet unresolved clinical problem. Data from various centres have focused on the role of abnormal procoagulant activity in the pathogenesis of unexplained pregnancy loss and other obstetric complications.

We assessed the presence of both acquired and genetic thrombophilia in a cohort of women with unexplained pregnancy loss and analysed these in patients with EPL and LPL. All the women studied had no previous live births.

The prevalence of APA in unexplained pregnancy loss has been found to range between 7% and 40% in various studies.[14,15] However, most studies did not test for all the antibodies. Thus, the reported prevalence rates are probably not the actual rates of prevalence of APA in the groups studied. Except antiprothrombin antibodies, we included all the antibodies, both IgG and IgM, and about 41% of our patients were positive for any one of the APAs as against 6% of controls. The conventional LA and ACA assays were informative in 108 (28.3%) patients and in only 3% of controls. Pregnancy loss was significantly associated with all the APA studied including LA. However, a significant association with EPL was observed only with LA. This is consistent with the recent systematic review of thrombophilias and pregnancy.[16] Though there are some reports of an association between LA and LPL,[17,18] the risk of EPL has been reported to be high.[19,20] None of the other APA studied (ACA, β2GP1 or anti-annexin V) showed a significant difference between the EPL and LPL groups, though as an independent risk factor each APA antibody was strongly associated with unexplained pregnancy loss compared with controls.

The common inherited thrombophilias (protein C, protein S and AT III) were significantly associated with unexplained pregnancy loss and protein S deficiency had the strongest association. This prevalence was much higher than that reported in our earlier study in a large series of patients with deep vein thrombosis.[21] A strong association of protein S deficiency with recurrent pregnancy loss has been reported previously.[22,23] The prevalence of protein C, AT III deficiency, factor V Leiden were almost similar as in our earlier report on patients with deep vein thrombosis[21] and other published reports.[12,24–28]

The PAI 1 4G/4G polymorphism has a key role in the inhibition of fibrinolysis and has been reported to affect binding of nuclear proteins involved in the regulation of *PAI 1* gene transcription.[29,30] Homozygosity for the PAI 1 4G allele has been found to be associated with increased transcription of

TABLE II. Frequency of protein C, protein S and antithrombin III deficiency in patients and controls

Marker	Controls (*n*=100)	Early pregnancy loss (*n*=136)			Late pregnancy loss (*n*=119)			Total* (*n*=381)		
	n (%)	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value
Protein C	1 (1)	12 (8.8)	9.6 (1.6–58.2)	0.009	6 (5)	5.3 (0.8–33.7)	0.1	21 (5.5)	5.8 (1–34)	0.06
Protein S	1 (1)	21 (15.4)	5.3 (0.8–33.7)	0.1	18 (15.1)	17.6 (2.9–105.3)	<0.001	58 (15.2)	17.8 (3.1–102.9)	<0.001
Antithrombin III	1 (1)	3 (2.2)	2.2 (0.3–15.8)	0.6	3 (2.5)	2.6 (0.4–18.1)	0.6	8 (2.1)	2.1 (0.3–13.2)	0.7

* Includes women who had both early and late pregnancy losses Comparison of the groups of women with early pregnancy loss and women with late pregnancy loss showed the following results: Protein C: OR 1.8, 95% CI: 0.7–4.8, p=0.3; Protein S: OR 1, 95% CI: 0.5–2, p=1; Antithrombin III: OR 0.8, 95% CI: 0.2–3.9, p=1

TABLE III. Frequency of genetic markers of thrombophilia in patients and controls

Marker	Controls (*n*=100)	Early pregnancy loss (*n*=136)			Late pregnancy loss (*n*=119)			Total* (*n*=381)		
	n (%)	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value	*n* (%)	OR (95% CI)	p value
Factor V Leiden	1 (1)	7 (5.1)	5.4 (0.8–33.9)	0.1	2 (1.7)	1.7 (0.2–13.1)	0.1	13 (3.4)	3.5 (0.5–21.1)	0.3
EPCR	1 (1)	6 (4.4)	4.6 (0.7–29.3)	0.4	8 (6.7)	7.1 (1.1–44.5)	0.04	22 (5.8)	6.1 (1–35.8)	0.06
PAI 4G/4G	10 (10)	23 (16.9)	1.8 (0.8–4)	0.2	30 (25.2)	3 (1.4–6.5)	0.005	82 (21.5)	2.5 (1.2–4.9)	0.01
MTHFRT677T	1 (1)	5 (3.7)	3.8 (0.6–24.7)	0.2	3 (2.5)	2.6 (0.4–18.1)	0.6	10 (2.6)	2.7 (0.4–16.3)	0.5

* Includes women who had both early and late pregnancy losses Comparison of the groups of women with early pregnancy loss and women with late pregnancy loss showed the following results: Factor V Leiden: OR 3.2, 95% CI: 0.7–13.7, p=0.2; EPCR: OR 0.6, 95% CI: 0.2–1.8, p=0.6; PAI 4G/4G: OR 0.6, 95% CI: 0.3–1.1, p=0.1; MTHFR T677T: OR 3.8, 95% CI: 0.5–24.7, p=0.2

PAI 1 gene. Not many studies are available on the association of this polymorphism with recurrent spontaneous abortions. As against a 2-fold increase in patients with pregnancy loss, in our study on patients with deep vein thrombosis and foetal loss we had found a 4-fold increase.[31] Most reports on the association of PAI 1 4G/5G polymorphism and recurrent spontaneous abortions have shown an increased risk in association with other thrombophilic markers.[13]

It has been documented that the presence of two or more thrombophilia markers increases the risk for pregnancy loss.[32] The presence of multiple thrombophilias in a large number of patients also confirms these findings. Except LA, we did not find any significant differences between the EPL and LPL groups. There are problems in attributing thrombophilia as the cause of pregnancy loss in the EPL group. They can have a higher frequency of genetic and structural anomalies in the early stage of embryonic development and some of the recurrent spontaneous abortions could be due to karyotypic abnormalities in the foetus. The incidence of such abnormalities has been reported to be high,[33] but we do not have the karyotypic data of the foetuses. Lymphokine-activated NK cells have also been found to be associated with recurrent spontaneous abortions.[34]

Thus, to conclude, thrombophilia is likely to be an important cause of both EPL and LPL. Detecting these abnormalities may allow appropriate therapeutic measures to be instituted, where available.

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