Activated Protein C Resistance and Antiphospholipid Antibodies in Recurrent Fetal Loss: Experience of a Single Referral Center in Northern Iraq

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Abstract The current study was initiated to determine the prevalence of activated protein C (APC) resistance, factor V Leiden and antiphospholipid antibodies (APA) in Iraqi women with recurrent fetal loss (RFL), and evaluate the outcome of intervention in those with such states. For this purpose a total of 103 Iraqi women referred to a major teaching hospital in Northern Iraq with two or more consecutive fetal losses, as well as 100 age matched women with no history of fetal loss and at least one live birth were enrolled. After appropriate clinical evaluation, the enrolled subjects were tested for APA as well as APC resistance. Subjects who were APC resistant were further tested for factor V Leiden mutation using a polymerase chain reaction and reverse hybridization. Patients with documented APA and/or with APC resistance, were put on low dose aspirin with or without low molecular weight heparin during pregnancy, and followed for a minimum of 5 years. The results revealed that among patients' group, APA were detected in 19.4 % compared to 1.0 % of the controls (OR 23.9, $p = 0.00005$). On the other hand, APC resistance was documented in 9.7 % compared to 1.0 % of the controls

(OR 10.6, $p = 0.01$). Factor V Leiden was detected in 3.9 % of patients and 1 % of the controls ($p = 0.38$). Among 17 patients with APA available for follow up, there were 24 pregnancies, 18 of which ended with live births (75 %). While among the ten patients who had factor V Leiden or were APC resistant non-carriers, there were 13 pregnancies, 12 ended with live births (92.3 %). In conclusion, this study has demonstrated that among the enrolled Iraqi women, APA and APCR and not factor V Leiden were significantly associated with RFL, and that treatment with aspirin (with or without low molecular weight heparin) had lead to live births in 80.6 % of pregnancies.

Keywords APC resistance · Antiphospholipid antibodies · Recurrent fetal loss · Iraq

Introduction

Recurrent fetal loss (RFL), defined as the loss of two or more consecutive pregnancies, affects up to 5 % of all pregnant women and is a heterogeneous condition with many possible causes and contributory factors [1]. Various genetic, anatomical, endocrine and infective factors as well as thrombophilic states have been implicated [2]. Antiphospholipid antibodies (APA) are the most frequent of thrombophilic disorders documented. The latter comprise a heterogeneous group of autoantibodies directed against negatively charged phospholipids and are detected by immunoassays [anticardiolipin antibodies (aCL)] and functional coagulation tests [lupus anticoagulant (LAC)]. These antibodies are frequently associated with arterial and venous thrombosis as well as fetal loss [3]. Another thrombophilic state is activated protein C resistance

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(APCR), and this maybe inherited due mainly to a mutation involving the factor V gene, rendering the resultant factor V resistant to the action of APC (factor V Leiden) or it may be acquired [4].

The current study aims to evaluate the association of two thrombophilic states namely: APA and APCR in the pathogenesis of RFL among women referred to a single center in Northern Iraq, and to evaluate the outcomes of intervention in those with such states.

Patients and Methods

A total of 103 unselected non-pregnant Iraqi women (aged 19–47, median 33 years) presenting with a history of two or more consecutive fetal losses and referred to a major teaching hospital in northern Iraq were enrolled. The criteria for inclusion necessitated that at least 3 months have elapsed since the last miscarriage, and that the patient is on no medications. Those who had documented anatomical cause or hormonal disturbance were excluded. The study also included a control group of 100 age matched (range 21–47, median 31.5 years) non-pregnant healthy women with no history of fetal loss and at least one live birth and were on no medications at the time of sampling.

After a detailed obstetrical history, the patients and controls had 10 mL of blood withdrawn by clean venipuncture and divided between three tubes, one with sodium citrate for coagulation studies, another with EDTA for DNA studies as well as a plain tube to obtain serum for aCL antibodies. Platelet poor plasma was obtained by double centrifugation of the citrated blood, and was used to perform activated partial thromboplastin time (APTT) (Diagnostica Stago-France), Kaolin clotting time (KCT) and first generation activated protein C (APC) resistance test. The KCT was performed by a method modified from Exner et al 1978 [5], and the KCT-index was calculated by dividing the KCT of a mixture of 80 % normal and 20 % patient's plasma by the KCT of normal plasma [6]. For patients with prolonged APTT, a repeat APTT of 50/50 mix of patient's and normal pooled plasma was performed. Failure of correction of prolonged APTT by the latter procedure and/or a KCT index ≥ 1.2 , was followed by performing a hexagonal phospholipids neutralization test using StacLOT-LA kit (Diagnostica Stago-France) to confirm the presence of a LAC [7].

A first generation APC resistance test was used to calculate the APC sensitivity ratio (APC-SR). The latter is the ratio of APTT with added purified APC (Diagnostica Stago-France) to that without added APC. Then this ratio was normalized by dividing it with APC-SR of normal pooled plasma measured at the same run to yield a normalized APC-SR (N-APC-SR). Patients were considered

resistant to APC if they had an N-APC-SR lower than 1.96 SD of the mean of N-APC-SR of the normal control group. All those resistant to APC as well as those who had borderline N-APC-SR had their DNA extracted using a phenol–chloroform method. The DNA was thereafter amplified using Primus 25 thermocycler (MWG-Germany) with specific primers (ViennaLab-Austria), and a cycling program consisting of pre-polymerase chain reaction (PCR) at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 s; 58 °C for 30 s; 72 °C for 30 s, and a final extension of 3 min at 72 °C. Factor V Leiden mutation status was determined in the amplified products using reverse hybridization to specific wild and mutant oligonucleotide probes by a colorimetric microwell plate method [8], according to the instructions of the manufacturer (ViennaLab-Austria).

The sera of all patients and controls were used to perform quantitative IgM and IgG ELISA tests for $\beta 2$ glycoprotein I dependent aCL antibodies (in the presence of $\beta 2$ glycoprotein I as a cofactor) according to the manufacturer's instructions (Varlisa, Sweden Diagnostics, Germany). The cut off point for positive IgG and IgM aCL antibodies was set at mean +3 SD (after exclusion of outliers).

Patients who tested APA positive on two occasions and/or were APC resistant (regardless of their factor V Leiden status), were scheduled to receive acetylsalicylic acid (aspirin) 100 mg/day prior pregnancy, while once pregnancy is confirmed and up to 37 week gestation, they were scheduled to receive 100 mg aspirin with or without bemiparin sodium [second generation low molecular weight heparin (LMWH)] at a dose of 2,500 IU/day subcutaneously. The use of bemiparin was based on the patient's compliance as judged by the treating obstetrician. The mode of delivery was chosen based on the patient's obstetrical history and fetal well being during the current pregnancy. The treatment outcomes were checked for all those treated after a minimum of 5 years (median follow up 5.6 years).

The study was approved by the ethical committee at the College of Medicine, University of Duhok, and informed consent was obtained from all enrollees. Statistical analysis utilized the Mann–Whitney *U* test and the χ^2 tests (with Yates correction when appropriate), with $p < 0.05$ considered significant.

Results

The 103 enrolled patients had a median number of fetal losses of four (range 2–11). One hundred patients had at least one mid-trimester abortion, while 12 had early and 18 later fetal loss.

Antiphospholipid Antibodies

Based on the coagulation screening and correction tests and confirmation by hexagonal phospholipids neutralization test a LAC was confirmed in six patients (5.8 %) and in none of the controls. An aCL antibody titre in excess of the set up cut off points was documented in 17 patients (11 IgG, four IgM and two both IgM and IgG), including three of those with LAC. So the total number of patient with APA (aCL and/or LAC) was 20 patients (19.4 %). The control group had only one aCL positive case (IgG) (1.0 %). The above findings would project a highly significant 19.6 fold increased risk of fetal loss in those who were aCL positive [OR 19.6 (95 % CI 2.6–150.1), $p = 0.00028$] and a highly significant 23.9-fold increase in the risk with APA [OR 23.9 (95 % CI 3.1–181.5); $p = 0.00005$]. The presence of LAC, on the other hand, was associated with a lower significant association with fetal loss ($p = 0.042$).

Activated Protein C Resistance

Normalized APC-SR was significantly lower in the patients than it was in the controls ($p = 0.043$). Furthermore, when the cut-off point for APC resistance was used, it was found that 10 (9.7 %) of patients and one (1 %) of the controls were APC resistant, a finding which was statistically significant [OR 10.6 (95 % CI 1.34–84.8), $p = 0.01$]. Molecular studies for factor V Leiden in APC resistant cases as well as those with borderline figures revealed that four patients (3.9 %) (three resistant and one borderline) and one control (1 %) were heterozygotes for FVL, a finding which was not significant ($p = 0.38$). This left seven APC resistant cases, who were non-carriers of the FVL mutation, in the patient's group and none in the control group, a finding which was significant ($p = 0.014$). Among the latter subcategory of patients: two patients had concomitant APA (one aCL, the other LAC).

Treatment Outcome

Twenty nine patients had APA, factor V Leiden, or were APC resistant non-carriers of FVL. Table 1 summarizes their treatments and outcomes. Two patients: one with concomitant APCR and LAC, another with aCL were put on aspirin, but were lost to further follow-up. The four factor V Leiden carriers and the six APCR non-carriers (who had follow-up) had a total of 13 pregnancies, 12 ended with live births (92.3 %). On the other hand, of the 18 patients with APA, one was a diagnosed case of systemic lupus erythematosus and continued to have abortions, leaving 17 patients with primary APS for follow-up. In the latter subgroup, a total of 24 pregnancies were

Table 1 Treatment and outcome, after a minimum of 5-year follow up, in patients with documented thrombophilic states identified by the current study

Thrombophilic state	No.	(No.) treatment	(No.) outcome
Factor V Leiden heterozygous	4	(4) Aspirin	(2) Had one viable baby, one of whom is now 5 m pregnant (1) Had two viable babies (1) Had one viable baby/one abortion (2) Had one viable baby
APCR FVL non-carriers	5	(3) Aspirin (2) Aspirin + LMWH	(1) Had two viable babies (2) Had one viable baby each
APCR FVL non-carriers + APA	2	(2) Aspirin	(1) Had one viable baby, now on contraception (1) Lost to follow-up
APA	18	(6) Aspirin + LMWH (12) Aspirin	(2) Had one viable baby each (1) Had twin viable babies (1) Had two viable babies each (1) Had an abortion followed by one viable baby (1) Had recurrent abortion (SLE) (7) Had one viable baby (2) Had two abortion and then a viable baby each (1) Had two babies (1) Had one abortion (1) Lost to follow up

documented, 18 of which ended with live births (75 %). Overall, all patients with APCR (regardless of FVL status) and 16/17 patients with primary APS had at least one live birth after a minimum of 5 years of follow up.

When the kind of therapy was taken in consideration, it was found that in the subgroup of patients with the APA (excluding the SLE case) put on aspirin and bemiparin (five patients), there were seven pregnancies, six were with successful outcomes (85.7 %). While in those on aspirin only, there were 17 pregnancies, 12 ended with live births (70.6 %). The latter difference in outcome was not significant ($p = 0.63$). On the other hand, both of the two pregnancies in those on aspirin and bemiparin in APC resistant group had successful outcomes, while of the 11 pregnancies in those who had FVL or were APCR non-

carriers and were put on aspirin only, ten ended with live births (90.9 %). Overall, treatment with aspirin (with or without bemiparin) lead to live births in 80.6 % of pregnancies.

Discussion

Antiphospholipid Antibodies

APA are often, although not invariably, associated with adverse obstetric outcomes, including first trimester miscarriage, mid-trimester and later fetal loss [9]. APA-mediated fetal loss has been associated with placental vascular thrombosis, decidual vasculopathy, intervillous fibrin deposition, and placental infarction [10]. However, other mechanisms have also been suggested to contribute, including: APA-mediated inhibition of growth and differentiation of trophoblasts and eventual defective placentation, as well as APA-mediated acute placental inflammation [11].

The frequencies of the two APA associated with RFL namely: LAC and aCL in the current study were 5.8 and 19.4 %, respectively. These figures lie within the ranges documented by many reports from Western countries, where figures of 3–14 and 5–21 % were reported for LAC and aCL respectively [12–17]. Variation in the frequencies of these antibodies in different studies are due to several possible causes including ethnic differences, study designs, timing of the fetal loss, sensitivities and specificities of tests utilized. In LAC, the most important factor is the number and types of the tests employed. In the current study, a combination of two screening (APTT and KCT), correction (50/50 APTT correction and KCT index) and confirmatory tests (hexagonal phospholipid neutralization procedure) was used [7]. In aCL, a key issue is choice of the cut-off point for positive results. The choice of an appropriate cutoff point requires striking a careful balance to ensure adequate sensitivity and specificity of the test, and thus in the current study we employed a compromise mean + 3 SD of the normal control group as a cut-off point, which was also recommended by some previous workers [18].

Activated Protein C Resistance

This study has shown that APC resistance (regardless of FVL mutation) is significantly associated with RFL, while factor V Leiden on its own was not. Such findings are consistent with those of Rai et al. (2001), who reported on APC resistance in 1111 recurrent aborters, and found that 15.5 % of their cases were APC resistant, compared to 11.3 % of the controls, and resistance was due to FVL mutation in 6.7 % of the patients and 8 % of the controls.

APC resistance in the latter study was proposed acquired in 8.8 % of the patients compared to 3.3 % of the controls and similar to the current study, APC resistance (non-FVL related) was more frequently encountered in those with RFL, but FVL mutation was not [16]. The higher overall figures of APC resistance in the latter British study is evidently due to the higher background prevalence of factor V Leiden in the population than its prevalence in our population, in which FVL is much less common, as shown in our control group and in a previous study [19]. Although the frequency of FVL in our patients with recurrent abortion was higher than controls, this was not significant, which is in contrast to several previous studies reporting significantly increased frequency of the factor V Leiden alleles among women with recurrent miscarriage, including studies from countries in Eastern Mediterranean region like Egypt and Iran [20, 21]. Furthermore, two meta-analyses analyzing case-control studies on factor V Leiden and RFL, concluded that FVL carriers have double the risk of experiencing two or more miscarriages, when compared with those without such a mutation [22, 23]. However, failure of documentation of the association between FVL and RFL has also been reported by several other investigators [13, 24–26].

It maybe appropriate to assume that APC resistance in those who are not carriers of the Leiden mutation is mostly acquired, based on the fact that other mutations like factor V Cambridge and the HR2 haplotype, also known to be associated with APC resistance are both rare and no common genetic cause for APC resistance apart from the factor V Leiden mutation has been reported [16]. In contrast, high concentrations of coagulation factor VIII, V and IX, APA, pregnancy and the combined oral contraceptive pill are associated with acquired APC resistance [4, 27, 28]. In this study, no woman was taking the contraceptive pill, and all were tested at least 12 weeks after their last pregnancy. On the other hand, two of our APCR cases had APA concomitantly.

It is worth noting that women in the current study were tested after their miscarriages, thus establishing a temporal relationship between the development of acquired APC resistance and pregnancy loss would not be straight forwards. However, it appears logical to assume that an acquired APC resistance would certainly be amplified by pregnancy, which also induces an APCR state [4]. This may lead to placental thrombosis and infarction and consequent abortion.

Outcome of Therapy

Sixteen out of the 17 ladies with APA, who had follow up, had at least one live birth after more than 5 years of follow up (94.1 %). Several previous studies have found that

aspirin with or without LMWH would produce a high success rate in APA syndrome and that there is no added advantage to LMWH addition [29, 30]. However, a recent study reported that bemiparin (the LMWH used in the current study) was slightly superior on its own, when compared to aspirin on its own [31].

Data on the effectiveness of antithrombotic therapy in patients with APCR or FVL are sketchy, with some studies reporting benefit, others disputing it [32, 33]; however, it is interesting to note that in all APCR patients (regardless of the Leiden mutation), at least one successful outcome was obtained. The intervention is justified since it has been documented that carriers of FVL are more likely to abort, than those who are non-carriers [34].

The value of aspirin as a single agent in APA and APCR and the good outcome associated with it, as shown in the current study, although on a relatively small number of patients, is important, because it is simple to use, cheap (important in a setting of a less developed country) and it has not been linked conclusively to congenital defects if given in early pregnancy [35], and certainly less cumbersome than added injectable LMWH.

In conclusion, this study has demonstrated that APA and APCR, and not factor V Leiden, are significantly associated with RFL in Iraqi women, and that treatment with aspirin (with or without LMWH) would lead to live births in 92.6 % of the patients. Further larger prospective studies on patients with APCR are needed to confirm the extremely favorable results obtained in this category of patients by the current study.

Conflict of interest The authors declare that they have no conflict of interest.

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