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The Role of Mycoplasmal Infection and Anticardiolipin Antibodies as Autoimmune Parameters in Pregnancy Loss

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We aimed to find a relationship between repeated abortions of unknown etiology and autoimmune disease either caused by *Mycoplasmas* infection and/or Anticardiolipin antibodies (IgG and IgM). Twenty three women (21-41 years) with history of recurrent abortion, intrauterine fetal death and/ or neonatal death (after exclusion of other factors as cause abortion) and ten women with normal pregnancy outcome with the same age were chosen as controls. *Mycoplasma hominis* and *Ureaplasma urealyticum* were detected in blood by PCR. Anticardiolipin antibodies (IgG and IgM) were detected in blood using ELISA technique. *Mycoplasma hominis* could be detected by PCR in 7/23(30.4%) in women with pregnancy losses, but was not detected in control group. *Ureaplasma urealyticum* could not be detected in the two groups. No relation was observed between infection with *Mycoplasma* and number of pregnancy losses. High levels of Anticardiolipin antibodies (IgM and IgG) were observed in 10/23 (43.5%) and 4/23 (17.4%) of cases, respectively. Six cases (26.1%) showed high levels of both IgG and IgM. No significant differences in their mean values was observed compared to the control group, however, significant difference was observed in patients with four abortions or patients with adverse outcome compared to controls. The role of autoimmune abnormalities induced by *Mycoplasma* infection in the etiology of pregnancy losses was proposed; therefore we recommend that all women with poor pregnancy outcome, before planning a subsequent pregnancy, should test for the presence of antiphospholipid antibodies and/or bacterial infections.

Key words: Repeated pregnancy loss, anticardiolipine, *Mycoplasma*, autoimmune

INTRODUCTION

Recurrent pregnancy loss is an exceedingly frustrating and discouraging problem for patients and physicians alike. The etiologies of recurrent miscarriage are diverse and may be divided into genetic defects, such as chromosomal anomalies; maternal reproductive anatomic disease, both developmental and acquired, such as septet uterus or cervical incompetence and systemic maternal disease such as diabetes and infective factors (Kartz and Kuller, 1994). A possible relationship between recurrent abortion and autoimmune abnormalities was reported by Festin (1997).

Infection with *Mycoplasma hominis* (Mh) or *Ureaplasma urealyticum* (Uu) two members of the class Mollicutes) are considered as the possible etiological agents in the family causing pregnancy loss since they frequently produce asymptomatic infection and are not identified by routine microbiological techniques (Munday *et al.*, 1984).

Kotb (1995) proposed that the mode of interaction between a group of microbial proteins known as super antigens and the immune system has opened a wide area of investigation into the possible role of these molecules in human diseases. Super-antigens produced by *Mycoplasma* species, are either secreted or membrane-bound proteins. A unique feature of these proteins is that they can interact simultaneously with distinct receptors on different types of cells, resulting in enhanced cell-cell interaction and triggering a series of biochemical reactions that can lead to excessive cell proliferation and the release of inflammatory cytokines.

Mycoplasmas may be responsible for triggering autoimmune responses, first, during their intracellular replication and release from host cells. Mycoplasmas can capture antigens from the host cell surface and incorporate them into their cell membranes. This can lead to immune responses against these antigens and possibly autoimmune reactions. Second Mycoplasmal antigens can mimic host antigens and trigger immune responses against these antigens with resulting cross reactivity against host antigens. Third, they can cause apoptosis of host cells with subsequent release of normal host antigens (Nasralla *et al.*, 1999). Niclson (2000) stated that *Mycoplasmas* have been found at significantly higher incidence in blood and tissue specimens obtained from patients with various chronic illnesses compared to healthy controls as well as, he had obtained these microorganisms from patients with various chronic illness compared to healthy controls.

Festin (1997) and Marai *et al.* (2004) determined that the antiphospholipid antibody syndrome is considered as

autoimmune disease which causes recurrent pregnancy loss characterized by elevated titres of antiphospholipid antibodies: Lupus anticoagulant and anticardiolipin antibody (aCl). These antibodies are believed to cause thrombosis in the maternal circulation, leading to the events that lead to fetal losses.

The present study aimed at finding the relationship between fetal losses of unknown etiology and presence of *Mycoplasmas* infection (as a cause of autoimmune disease) and Anticardiolipin antibodies (IgG and IgM).

MATERIALS AND METHODS

Subjects: Two groups of women, ages ranged between (21-41 years) were included in this study. The first group was 23 women with a history of pregnancy loss such as repeated abortion, intrauterine fetal death and/or neonatal death, were referred to Prenatal Clinic in National Research Center. Medical examination and family history, complete physical examination, laboratory investigations were done to exclude other causes of abortion. Complete records were sought of prior pregnancies, gynecologic surgery, non-steroidal estrogen exposure in utero and findings of pathological examinations). The second group (control) composed of ten women who had at least one live birth without pregnancy wastage.

Methods: Blood samples were collected using EDTA as anticoagulant to detect *Mycoplasma hominis* and *Ureaplasma urealyticum* using PCR technique. Serum samples were collected for Immunological tests to detect anticardiolipin antibodies.

DNA extraction: Blood samples were centrifuged at 3500 rpm for 20 min, cell pellets were treated with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 5% SDS with 100 U g mL⁻¹ proteinase k. DNA was extracted using phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated with 0.1 volume of 2 M potassium chloride and double volumes of absolute ethanol, air dried (Ghadersohi *et al.*, 1997). The DNA pellets were suspended in TE buffer and incubated at 37°C for 30 min.

PCR amplification: Amplification of gene sequences was performed in a total volume of 50 µL of PCR buffer (10 mM Tris-HCL, 50 mM KCL , pH 9) containing 0.1% Triton ×-100, 200 µm each of dATP, dTTP, dGTP and dCTP, 100 pmol of each primer and 0.5 µg of DNA. Purified mycoplasmal DNA (0.5- 1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C Extension temperature was 72°C:

finally, product extension was performed at 72°C for 10 min. Negative and positive controls were present in each experimental run.

Primers used as Mycoplasmal group specific (generic) PCR were prepared according to Ossewaarde *et al.* (1996) as follows: upstream primer GPO-3, 5'-GGGA GCAA ACAG GATT AGAT TAGA TACC CT-3', downstream primer MGSO, 5'-TGCAC CATC TGTC ACTC TGTT AACCTC-3' (expected size was 295 bp). Primers used in *Ureaplasma urealyticum* were prepared as follow: U.u.F1: 5'-GCTAA TACCG AATAA TAACA TC -3' and U.u.R1: 5'-ATGGT ACAGT CAAAC TAAAA TC 3' (expected size was 311 bp).

Enzyme Linked Immunosorbent Assay (ELISA): Solid phase immunoassay (enzyme linked immunosorbent assay) is performed on cardiolipine coated plates, usually in the presence of bovine serum β2-glycoprotein I. Anticardiolipin antibodies from patients with the antiphospholipid syndrome are β2-glycoprotein dependent; antibodies from patients with infectious diseases are β2glycoproteinI-independent

For quantitative determination of Anticardiolipin Antibodies (IgG and IgM) as instructed in the kit (BL Diagnostika (cat.No.5B1 5L) .

RESULTS

Results of PCR using primers specific for detection of *Mycoplasma* are presented in Table 1, seven out of twenty three with repeated pregnancy losses had *Mycoplasma hominis* in their blood (30.4%), while negative results were obtained in control group. Presence of *Mycoplasma hominis* differ according to the number of

repeated abortions such that *Mycoplasma hominis* was detected in the blood of cases suffering from three or four losses more than those with 2 or less pregnancy losses. Two cases out of five suffered from one abortion in addition to a history of adverse outcome (neonatal death and/ or intrauterine fetal death) were positive for *Mycoplasma hominis* (40%). None of these women showed presence of *Ureaplasma urealyticum*. In addition none of control women had any of these microorganisms in their blood.

Presence of Anticardiolipin Antibodies (aCL) (IgG and IgM) were detected in blood using ELISA technique. Table 2 shows comparison between mean (±SD) of aCL IgG and IgM values according to the number of pregnancy losses compared to the control group. It was noticed that the mean values (± SD of aCL IgG and IgM were significantly increased only in cases who had more than four repeated pregnancy losses and Cases suffered from abortion and adverse outcome compared to control group, (p = 0.48) . However, there was no significant difference in aCL (IgG and IgM) values between all groups.

Table 3 demonstrates the relationship between aCL values in women with repeated pregnancy losses and presence of *Mycoplasma hominis* in their blood. It was observed that high titer of IgM only was found in 10/23 women (43.5%); three of them had positive PCR results of *Mycoplasma hominis* (30%). High titer of aCL IgG only was observed in 4/23 women with repeated abortion (17.4%); two of them showed positive PCR results for *Mycoplasma hominis* (50%). Six women showed high titer of both IgG and IgM ; of them 1/6 (16.7%) had positive PCR results. One woman had positive PCR for *M. hominis* and normal titer of both IgG and IgM.

Table 1: Frequency distribution of PCR- positive *Mycoplasma hominis* in relation to No. of pregnancy loses

	No. of pregnancy losses			Cases suffered from abortion and adverse outcome	Total	Control
	No. of repeated abortions					
<i>Mycoplasma hominis</i> PCR +ve	>4	3	2			
	2/8 (25%)	3/5 (60%)	0/6 (0%)	2/5 (40%)	7/23 (30.4%)	0/10

Table 2: Mean±SD and test comparing Anticardiolipin Antibodies (IgG and IgM) values in groups tested

Parameters of aCL	No. of pregnancy losses			Cases suffered from abortion and adverse outcome	Total	Control
	No. of repeated abortions					
	>4	3	2			
IgG (Mean±SD)	8±3.2*	6.4±3.5	9.2±2.16	12.25±6.8	8.6±4.2	5.27±2.4
P	0.05	0.48	0.01	0.012		
IgM (Mean±SD)	12±4.3**	6±2.3	7.8±3.3	7.5*±1.7	9±4.1	3.8±1.8
P	0.001	0.068	0.09	0.04		

*Significant (p<0.05), **Highly Significant (p<0.001)

Table 3: Percentage of aCL (IgG and IgM) in women with repeated pregnancy losses in relation to Infection with *Mycoplasma*

Parameters of aCL	No. and % of women classified according to level of IgG and /or IgM	%PCR-positive for Mh and aCL
High level of IgG	4/23(17.4%)	2/4(50%)
High level of IgM	10/23(43.5%)	3/10(30%)
High levels of IgG and IgM	6/23(26.1%)	1/6(16.7%)
Normal level of IgG and IgM	3/23(13%)	1/3(33.3%)

*High levels were considered when: >10 GPL/mL for IgG and >7 MPL/mL for IgM

DISCUSSION

The relation between Mh and Uu infection and repeated pregnancy loss was observed previously by several authors. In the present study, Mh was detected in blood of 7/23 (30.4%) women with repeated pregnancy losses and not in normal women (control), however, Uu were not detected in any of the tested samples.

Similar studies proposed an explanation to clarify the persistence of these microorganisms for long time despite treatment (Nasr Allah, 1999). Simecka *et al.* (1993) concluded that the interactions between mycoplasmas and pro-inflammatory markers, which consist primarily of the development of specific antibody and non-specific interactions with B lymphocytes or antibody which responses are important in the resistance to mycoplasmal disease in humans. However, the ability of mycoplasmas to survive in their host despite vigorous responses suggests that these play a limited role in the host's recovery from infection accounting for the appearance of systemic mycoplasmal infections. In some cases, antibody responses may contribute to disease pathogenesis through the development of hypersensitivity responses or the deposition of immune complexes leading to autoimmune reactions.

An Enzyme Linked Immunosorbent Assay (ELISA) was used in this study for the detection of anticardiolipin antibody (aCL). The mean values of aCL (IgG and IgM) were significantly higher in women with four or more pregnancy losses and women with adverse outcome compared to control group. High IgG, IgM levels were observed in 6/23 (26.1 %) of our patients, aCL-IgG alone was present in 17.4% while aCL -IgM alone was present in 43.5%. In agreement to our results, Costa *et al.* (1993) analyzed blood of habitual aborters, they detected aCL in 4/20 (20%) of them, while not found among controls (women without pregnancy wastages). Similar results were observed by De Carolis *et al.* (1994) who found that the overall incidence of aCL in their studied group was 20.5% and their findings confirm that aCL are strongly linked to fetal loss. In addition, Yasuda *et al.* (1995), on

analyzing aCL by ELISA found a positive result in 7% of aborted women but they assumed a positive relation between aCL and adverse outcome of pregnancy such as repeated abortion, pre-eclampsia and fetal growth restriction. Daboubi (2001) found that high levels of aCL activity were detected among 19.23% of women complained with habitual abortions. In contrast, Tsapanos *et al.* (1989) and Couto *et al.* (1998) concluded that there was no association between the presence of aCL and recurrent abortions.

However, Sheth and Sheth (2001) found that 11.79% only of women had aCL IgG and 14.04% had aCL IgM, which are less than our results. They concluded that presence of aCL is a major cause of recurrent fetal loss and many pregnancies can be saved if diagnosed and treated adequately. In a previous study in Egypt, Hussein *et al.* (2003) had detected aCL IgG and IgM in women with repeated abortions in 32 and 18%, respectively. Also Gordon (2004) had reported the association between presence of aCL antibodies and repeated fetal loss. We conclude that patients with high aCL level are at risk for recurrent fetal loss.

Catteau *et al.* (1995) reported two cases of *Mycoplasma pneumoniae* infections associated with Stevens-Johnson syndrome and antiphospholipid antibodies and noted that though mainly described in systemic lupus erythematosus and autoimmune diseases, anticardiolipin antibodies and lupus anticoagulant have been found in many infectious disorders. It has been considered by many authors as "non pathogenic" or "non prothrombotic" on epidemiologic and immunologic data. They suggested that antiphospholipid antibodies could possibly play a role in their pathogenesis especially as the mechanisms are not to date clearly understood.

The infectious origin of antiphospholipid syndrome (APS) has been proposed by Celli *et al.* (1999); Blank *et al.* (1999), Blank *et al.* (2002) and Pierangeli *et al.* (2004), they reported that the antiphospholipid syndrome (APS) is characterized by the presence of pathogenic autoantibodies against β 2-glycoprotein-I (β 2GPI). The factors causing production of anti- β 2GPI remain unidentified, but an association with infectious agents has been noticed. Their studies established a mechanism of molecular mimicry in experimental APS, demonstrating that bacterial peptides homologous with beta2GPI induce pathogenic anti-beta2GPI antibodies along with APS manifestations.

In addition, Nowicki and Locksmith (2005) described the relation between the infection and presence of antiphospholipid saying that phospholipids (PL) molecules are ubiquitous in nature and are present in the inner surface of the cell. Therefore, during infectious

disease processes, including different viruses or bacteria like *Mycoplasma* disruption of cellular membranes may occur during cell damage (PLs) release and stimulate PL antibodies. Pandey *et al.* (2005) reported that majority of cases with unexplained cause of abortion are found to be associated with certain autoimmune antibodies that may play a role in the immunologic failure in pregnancy and may lead to abortion.

In conclusion, we propose a role of autoimmune abnormalities induced by *Mycoplasma* infection in the etiology of pregnancy losses. We propose a relation between presence of *Mycoplasma* and aCL antibodies leading to pregnancy loss. We recommend that all women with poor pregnancy outcome, before planning a subsequent pregnancy, should test for the presence of antiphospholipid antibodies and/or bacterial infections.

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