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ORIGINAL ARTICLE

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Anticardiolipin antibody in recurrent spontaneous aborting and fertile women

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Objective: To determine the association between the presence of anticardiolipin antibody and a history of recurrent spontaneous abortion. **Study design:** clinical controlled study **Location:** Department of Gynecology and Obstetrics - University of Campinas (UNICAMP) **Subjects:** 52 individuals with recurrent spontaneous abortion were included in Group 1 and 104 individuals with at least one live born child in Group 2. Elapsed time from last delivery to blood sampling varied from six months to two years. **Method**: Between November 1993 and November 1994, patients' blood samples were screened for anticardiolipin antibody by ELISA, as described by Triplett, Barna and Unger (1993). **Analysis:** Chi-square and Fisher's Exact tests were used for statistical analysis. Student's "t" test was used to compare the means. **Results:** There was no statistical difference in the presence of the anticardiolipin antibody between Group I (zero and 2.9%) and Group 2 (7.7 and 5.8%). **Conclusion:** There was no association between the presence of anticardiolipin antibody and recurrent spontaneous abortion.

Uniterms: Anticardiolipin antibody. Recurrent spontaneous abortion. Immunology. Antiphospholipid syndrome.

INTRODUCTION

The definition of recurrent spontaneous abortion is a reproductive history of three or more spontaneous and successive abortions. It occurs at a frequency of 0.5% to 1% of the population. There are reports on the association of recurrent spontaneous abortion with genetic, hormonal and uterine factors.

Antiphospholipid antibodies have been identified as possible factors in recurrent spontaneous abortion. The most frequently reported antiphospholipid antibody is the anticardiolipin antibody. The association between antiphospholipid antibodies and recurrent spontaneous abortion is greatly recognized.[1-5] Women positive to antiphospholipid antibodies and recurrent spontaneous abortion have better gestational results when these antibodies are suppressed.[4,6] It has been suggested that women with recurrent spontaneous abortion or fetal death can benefit from having circulating antiphospholipid antibody tests performed.[7] The presence of antiphospholipid antibodies with clinical manifestations such as venous and/or arterial thrombosis, thrombocytopenia, recurrent abortion and neurological complications is called "primary antiphospholipid syndrome".[8] Retrospective and prospective series of pregnant women positive to antiphospholipid antibodies show a higher frequency of fetal death,[1] especially with the IgG sub-type of anticardiolipin antibody.[9]

The literature strongly suggests an association between antiphospholipid antibodies and recurrent spontaneous abortion but the predictive value of these antibodies for pregnancy loss is still under debate. Antiphospholipid antibodies may be present in normal, successful pregnancies and may persist even beyond the breast-feeding phase.[1]

Anticardiolipin antibody titration may vary during pregnancy and an increase in its level may be related to bad gestational prognosis.[5] Persistent elevated levels have

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been reported,[9] and such patients have a greater risk of complications.[3]

Differences in established cut-off values can determine the variability of the results. In 1990, Love & Santoro[26] reviewed 29 published series and observed that with different cut-offs (among about 1000 systemic lupus erythematosus patients), the prevalence of lupus anticoagulant and anticardiolipin antibody was 34% and 44%, respectively. Vianna et al.[27] found an anticardiolipin antibody prevalence of 14% in 95 systemic lupus erythematosus patients using a cut-off value of 5 standard deviations,

Kwal et al.[5] described women who aborted despite immune therapy and had a dramatic increase in antiphospholipid antibody titration when compared with women who had live-born children. In this group titrations remained stable or decreased at the beginning of pregnancy.

There is only one study reported in Brazil on antiphospholipid antibodies and recurrent spontaneous abortion.[10] This study was performed to determine the association between anticardiolipin antibody and recurrent spontaneous abortion in a group of Brazilian patients.

METHODS

A clinical controlled study was carried out from November 1993 to November 1994 with two groups of patients. Group 1 included 52 patients from the Recurrent Abortion Outpatient Clinic of the Department of Gynecology and Obstetrics, University of Campinas (UNICAMP). These patients had a history of three or more successive spontaneous abortions. Group 2 included 104 patients from the Family Planning Outpatient Clinic of the Department of Gynecology and Obstetrics, University of Campinas (UNICAMP), during the same period. Patients in the control group had had at least one successful pregnancy and fulfilled the following exclusion criteria: no history of recurrent spontaneous abortion or unexplained fetal death; venous or arterial thromboembolic disease; gestational hypertension or low birth weight. Elapsed time from last delivery to blood sampling varied from six months to two years. Patients were informed about objectives and methodology, and signed an informed consent form.

Anticardiolipin antibodies assay

Anticardiolipin antibody was detected by enzyme-linked immunosorbent assay (ELISA), as previously described.[11] Blood was drawn from patients without anticoagulants and centrifuged for 10 minutes at 1600 rpm. Serum was stored at minus 80^0C and thawed at room temperature immediately before performing the assay. The antigen (bovine heart cardiolipin) was diluted in ethanol (45µg/ml). 30µg of this solution was applied to the first four rows of ELISA plate wells (96 wells, in 8 rows and 12 columns). Antigen was not added to the first column, which remained "blank". Another four rows were coated with ethanol without the antigen to establish non-specific bounding values. These values were subtracted from the anticardiolipin coated plates to give specificity to the method. The plates were allowed to dry overnight. 100µl of phosphate buffered saline was added to the wells on the next day, except for the "blank" column. After one minute, the phosphate-buffered saline was poured out and the plate was beaten out over a paper towel until dry. 200µl of phosphate buffered saline with 10% fetal calf serum was added to all wells as a blocking buffer and incubated for 1.5 hours. The blocking buffer reagent was then poured out from the plates, which were once again beaten out over a paper towel until dry. This last step was repeated once more with a one minute incubation of phosphate-buffered saline. 50µl of the patients' serum was added to all wells, except for the "blank" one and dried at room temperature for two hours. The plates were washed three times with 200µl of phosphate buffered saline. The plates were dried and 50µl of indirect antibody conjugated reagent was added to the wells, except for the blank column. Rows one to four received conjugated IgM and rows five to eight received conjugated IgG. After one hour of incubation at room temperature, the plates were washed with 200µl of phosphate-buffered saline and beaten out to dry. 50µl of diethanolamine substrate was added to all wells and incubated at 37ºC in the dark. The reaction was stopped by the addition of 50µl of NaOH 3M. The optical density was determined at 405nm in a Tibertek densitometer.

Quality control

Positive controls were taken from highly positive samples: Dr. EN Harris's cardiolipin standards with known concentrations of IgG and IgM.[12] A normal donor was used as the negative control. Patients' serum samples were run

Table I
Results and percentage (%) distribution of anticardiolipin antibody presence by study group

ACA	Group 1	Group 2	p
Present	4 (7.7)	9 (8.7)	N.S.*
Absent	48 (92.3)	95 (91.3)	
Total (%)	52 (100)	104 (100)	

* N.S. - Not Significant

Table II
Results and percentage (%) distribution of anticardiolipin antibody by study group

ACA	Group 1	Group 2	p
Positive	2 (50.0)	2 (22.2)	N.S.*
Intermediate	2 (50.0)	7 (78.8)	
Total (%)	4 (100)	9 (100)	

* N.S. - Not Significant

Table III
Results and percentage (%) distribution of IgM anticardiolipin antibody presence by study group

IgM ACA	Group 1	Group 2	p
Present	-	3 (2.9)	N.S.*
Absent	52 (100)	101 (97.1)	
(n)	52 (100)	104 (100)	

* N.S. - Not Significant

in duplicates. The mean result for each patient was used as final result. The inter-assay coefficient of variation was 6.8% and the intra-assay one was 7.2%.

Interpretation of the results

Results were considered negative, intermediate, positive and highly positive, according to the established cut-off value. The cut-off optical density value for IgG and IgM anticardiolipin antibody was determined for 40 normal donors. Optical density value was obtained for each donor and means and standard deviation values were calculated for the group. Patients' optical density values were compared to the cut-off. Values between 2 and 3 standard deviations were considered intermediate; positive above 3 standard deviations and highly positive above 7 standard deviations. Results were negative when the optical density value was less than 2 standard deviations. The optical density cut-off value for IgM anticardiolipin antibody was 0.17nm and for IgG anticardiolipin antibody it was 0.20nm. Chi-square and Fisher's Exact tests were used for statistical analysis. Student's "t" test was used for comparing the means. The significance level was set at $p < 0.05$.

RESULTS

The mean number of abortions was 3.75 in Group 1 (range: 3 to 8) and 78.9% were primary abortions. In Group 2, 81.7% of the patients had not had previous abortions, while 15.4% had one and 2.9% had two abortions.

Results for anticardiolipin antibody are shown in Table I. There was no statistical difference in anticardiolipin antibody presence between the groups. Group 2 had more patients with intermediate results than Group 1, but this difference was not significant (Table II). IgM anticardiolipin antibody was present in three patients from Group 2 and in none from Group 1 (Table III). There was no statistical difference in IgM anticardiolipin antibody between groups. There was also no statistical difference in IgG anticardiolipin antibody between Groups 1 and 2 (Table IV). Studying sub-types of anticardiolipin antibody (Table V), Group 1 had IgG sub-type exclusively, while Group 2 had 66.7% of this class. This difference was not statistically significant.

DISCUSSION

We studied the association between the presence of anticardiolipin antibody in women's blood and the history of recurrent spontaneous abortion. The literature shows that women who are recurrent spontaneous aborters have a higher incidence of antiphospholipid syndrome than fertile women. 7.7% of recurrent spontaneous abortion patients had positive anticardiolipin antibody results. These results are similar to others reported in the literature.4 8.7% of the fertile patients had positive anticardiolipin antibody results, which is not in accordance with previous data.13,14

The difference in these results may be due to the immune characteristics of this population compared to those analyzed by other authors. Racial miscegenation is widespread in this country and could bring about genetic features not seen in the Caucasians who form the majority of populations studied around the world.7,16,17 Polyclonal activation of B lymphocytes can also occur from intestinal flora, external aggression and the effects of drugs.18 It is possible that this population has not been exposed to the same immune stimuli as in other countries.

There were no statistical significant differences in anticardiolipin antibody levels comparing women with recurrent spontaneous abortions (Group 1) and women who had at least one successful pregnancy (Group 2). It must be emphasized that 78.9% of Group 1 patients were primary aborters. Previous data shows that women who have the anticardiolipin antibody are mainly secondary aborters.15 In Group 1, four women had anticardiolipin

Table IV
Results and percentage (%) distribution of IgG anticardiolipin antibody presence by study group

IgG ACA	Group 1	Group 2	p
Present	4 (7.7)	6 (5.8)	N.S.*
Absent	48 (92.3)	98 (94.2)	
Total (%)	52 (100)	104 (100)	

* N.S. - Not Significant

Table V
Results and percentage (%) distribution of anticardiolipin antibody class by study group

ACA class	Group 1	Group 2	p
IgM	-	3 (33.3)	N.S.*
IgG	4 (100)	6 (66.7)	
Total (%)	4 (100)	9 (100)	

* N.S. - Not Significant

antibody, but only two of them were secondary aborters. The low incidence of anticardiolipin antibody in the group of aborters may relate to the great proportion of primary aborters. This is not acceptable because the proportion of primary and secondary aborters is the same as previously reported.

Another hypothesis to explain the absence of correlation between a history of recurrent spontaneous abortion and anticardiolipin antibody presence is technical. The procedures used in this study were described by Triplett et al.[11] in the XIVth Congress of the International Society for Thrombosis and Hemostasis. Dr. Harris's calibration serum was used for determining normal and standard deviation values.

False-positive results can occur when non-specific binding values are not subtracted. This may happen in patients with hypergammaglobulinemia. To avoid it, the absorbance of non-coated wells must be subtracted.[19] The dilution and blocking buffer used is also discussed in the literature.[20] We used fetal calf serum as blocking buffer, as recommended in the Second International Anti-Cardiolipin Standardization Workshop.[12]

Interpretation of the results was performed as described. Triplett et al.[11] suggested the expression of results in MPL and GPL units. These are units of measurement: GPL for IgG anticardiolipin antibody and MPL for IgM anticardiolipin antibody. One GPL unit is equivalent to one µg/ml of an affinity purified IgG sample; one MPL unit is equivalent to one µg/ml of an affinity purified IgM sample.[12] We calculated means from normal donors' optical density, as described by Triplett et al.[4] Results were expressed using standard deviations based on these means. We chose to adopt qualitative results for establishing a correlation between the presence of anticardiolipin antibody and obstetric history, rather than establishing positive result intervals as is possible when using MPL and GPL units.

The variation of antiphospholipid antibody levels during and after pregnancy is not well established. One isolated sample may not reflect the real immune status of the person. Antibody levels may increase during the abortion process and become normal or almost normal in the inter-gestational period.[5] In this study, we controlled the time interval between last delivery and blood sampling for patients from Group 2, but not the interval between last abortion and blood sampling for patients from Group 1. This may have influenced the results and time interval control between last abortion and blood sampling is therefore suggested in any new series.

We know that higher circulating antibody levels correlate with pregnancy complications such as recurrent spontaneous abortion.[5] We decided to use 3 standard deviations with the risk that we would have a higher sensitivity and lower specificity and even then we did not identify differences between the two groups.

There are other limitations when anticardiolipin antibody results are compared in the literature. The methods used for detection of antiphospholipid antibodies vary in different series. Many studies involve patients who have one or two previous abortions;[22,23] etiologic factors are inclusion criteria in some studies[24] whereas they are exclusion ones in others.[22] "Liberal" definitions of positive results and the lack of standards in tests for anticardiolipin antibody and lupus anticoagulant makes real comparison of the results difficult.[25]

Human hystocompatibility antigens (HLA) are related to antiphospholipid antibody expression.[27] Women who are prone to HLA DQ alpha 0501 develop sub-clinical autoimmune responses in pregnancy and may abort despite therapy.[27] Kwak *et al.*[27] consider that there are combinations of maternal-fetal genotypes that are unacceptable to the maternal organism, particularly those which involve DQ alpha and DQ beta antigens. We hypothesize that if these combinations were not present in the group studied, the frequency of anticardiolipin antibody positive results would be reduced.

There is no evidence for antiphospholipid antibodies having a causal role in abortions during the first three months, although there is data suggesting the

involvement of these antibodies in the pathogenesis of pregnancy losses.[25] No matter which factors are considered, the relationship between anticardiolipin antibodies and pregnancy loss is conflicting.[30] Our results question the established "truths" of this association, especially if patients are tested for anticardiolipin antibodies (and/or lupus anticoagulant) without associating these with other risk factors for the antiphospholipid syndrome. The association between anticardiolipin antibodies and recurrent spontaneous abortion may be influenced by other factors, despite the presence of the anticardiolipin antibody.

CONCLUSIONS

We conclude that there was no association between anticardiolipin antibody presence and the history of recurrent spontaneous abortion in the studied group.

7.7% of recurrent spontaneous abortion patients and 8.7% of successful pregnancy patients were positive to anticardiolipin antibody.

There was no statistical difference in IgM anticardiolipin antibody and IgG anticardiolipin antibody frequencies between the studied groups.

REFERENCES

1. Lockshin MD, Qamar T, Levy RA. Anticardiolipin and related antibodies: thrombosis and fetal death. In: Scott JS, Bird HA, editors. Pregnancy, autoimmunity, and connective tissue disorders. 5th ed. Oxford: Oxford University Press 1990;185-211.
2. Branch DW, Silver RM, Blackwell JL, Reading JC, Scott JR. Outcome of treated pregnancies in women with antiphospholipid syndrome: an update of the Utah experience. Obstet Gynecol 1992;80:614-20.
3. Out HJ, Bruinse HW, Christiaens GCML, Van Vliet M, De-Groot PG, Nieuwenhuis HK, Derksen RHWM. A prospective, controlled multicenter study on the obstetric risks of pregnant women with antiphospholipid antibodies. Am J Obstet Gynecol 1992;167:26-32.
4. Triplett DA. Obstetrical complications associated with antiphospholipid antibodies. In: Coulam CB, Faulk WP, McIntyre JA, editors. Immunological obstetrics. London: Norton Medical Books 1992;377-403.
5. Kwak JYH, Barini R, Gilman-Sachs A, Beaman KD, Beer AE. Down-regulation of maternal antiphospholipid antibodies during early pregnancy and pregnancy outcome. Am J Obstet Gynecol 1994;171:239-46.
6. Rosove MH, Tabsh K, Wasserstrum N, Howard P, Hahn BH, Kalunian KC. Heparin therapy for pregnant women with lupus anticoagulant or anticardiolipin antibodies. Obstet Gynecol 1990;75:630-4.
7. Branch DW, Scott JR, Kochenour NK, Hershgold E. Obstetric complications associated with the lupus anticoagulant. N Engl J Med 1985;313:1322-6.
8. Arthurs M, Morgan OSC, Deceulaer K, Browne B. Primary antiphospholipid syndrome: a case report. West Indian Med J 1994;43:27-9.
9. Lynch A, Marlar R, Murphy J, Davila G, Santos M, Rutledge J, Emlen W. Antiphospholipid antibodies in predicting adverse pregnancy outcome: a prospective study. Ann Intern Med 1994;120:470-5.
10. Costa HLFF, Moura MD, Ferriani RA, Anceschi MIS, Barbosa JE. Prevalence of anti-cardiolipin antibody in habitual aborters. Gynecol Obstet Invest 1993;36:221-5.
11. Triplett DA, Barna L, Unger G. Laboratory identification of lupus anticoagulants. XIVth Congress of the International Society on Thrombosis and Haemostasis. Indiana, 1993. New York: Hilton & Towers 1993;70-9.
12. Harris EN, Phil M. The second international anti-cardiolipin standardization workshop: the Kingston anti-phospholipid antibody study (KAPS) group. Am J Clin Pathol 1990;94:476-84.
13. Branch DW. Immunologic disease and fetal death. Clin Obstet Gynecol 1987;30:295-311.
14. Pattison NS, Chamley LW, McKay EJ. Prevalence of antiphospholipid antibodies in a normal pregnant population. Clin Exp Rheumatol 1988;6:210 [Abstract].
15. Cowchock S, Smith JB, Gocial B. Antibodies to phospholipids and nuclear antigens in patients with repeated abortions. Am J Obstet Gynecol 1986;155:1002-10.
16. Clauvel JP, Tchobroutsky C, Danon F, Sultan Y, Intrator L, Brouet JC. Spontaneous recurrent fetal wastage and autoimmune abnormalities: a study of fourteen cases. Clin Immunol Immunopathol 1986;39:523-30.
17. Bocciolone L, Meroni P, Parazzini F, et al. Antiphospholipid antibodies and risk of intrauterine late fetal death. Acta Obstet Gynecol Scand 1994;73:389-92.
18. El-Roeiy A, Gleicher N. Definition of normal autoantibody levels in an apparently healthy population. Obstet Gynecol 1988;72:596-602.
19. Lenzi R, Rand JH, Spiera H. Anticardiolipin antibodies in pregnant patients with systemic lupus erythematosus. N Engl J Med 1986;314:1392-3.
20. Rupin A, Gruel Y, Watier H, Girard AC, Leroy J, Bardos P. ELISA for the detection of anticardiolipin antibodies: high specificity based on the use of adult bovine serum as buffer and systematic subtraction of non-specific binding. J Immunol Meth 1991;138:225-31.
21. Harris EN, Phil M. A reassessment of the antiphospholipid syndrome. J Rheumatol 1990;17:733-5 [Editorial].

22. Barbui T, Radici E, Cortelazzo S, et al. Antiphospholipid antibodies in early repeated abortions: a case-controlled study. Fertil Steril 1988;50:589-92.
23. Sanchez-Guerrero J, Alarcón-Segovia, D. Course of antiphospholipid antibodies in patients with primary antiphospholipid syndrome before, during and after pregnancy treated with low dose aspirin: relationship of antibody levels to outcome in 7 patients. J Rheumatol 1992;19:1083-8.
24. Lubbe WF, Butler WS, Palmer SJ, Liggins GC. Lupus anticoagulant in pregnancy. Br J Obstet Gynaecol 1984;91:357-63.
25. Harris EN. Antiphospholipid antibodies. Br J Haematol 1990;74:1-9 [Annotation].
26. Love PE, Santoro SA. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders: prevalence and clinical significance. Ann Inter Med 1990;112:682-98.
27. Kwak JYH, Ober C, Barini R, Beer AE. Maternal autoimmune abnormalities and fetal HLA-DQ A1 alleles in women with recurrent spontaneous abortion (RSA). In: Proceedings of the thirty-ninth annual meeting of the Society for Gynecologic Investigation. San Antonio, Texas, March 18-21: Society for Gynecologic Investigation 1992;363.
28. Vianna JL, Haga HJ, Tripathi P, Cervera R, Khamashta MA, Hughes GRV. Reassessing the status of antiphospholipid syndrome in systemic lupus erythematosus. Ann Rheum Dis 1992;51:160-1.
29. MacAean MA, Cumming GP, McCall F, Walker ID, Walker JJ. The prevalence of lupus anticoagulant and anticardiolipin antibodies in women with a history of first trimester miscarriages. Br J Obstet Gynaecol 1994;101:103-6.
30. Harris EN, Phil M. Solid-phase anti-cardiolipin test revisited. Am J Med 1988;85:599-601 [Editorial].

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RESUMO

Objetivo: Determinar a associação entre a presença do anticorpo anticardiolipina e o antecedente de aborto espontâneo recorrente. **Tipo de estudo:** Estudo clínico-controlado. **Local:** Departamento de Tocoginecologia da Faculdade de Ciências Médicas da UNICAMP. **Participantes:** No grupo 1 foram incluídas 52 pacientes que apresentavam antecedente de aborto espontâneo recorrente e, no grupo 2, 104 pacientes com antecedente de pelo menos uma gestação bem sucedida. O prazo máximo entre o último parto e a data da coleta de sangue foi de dois anos; o mínimo foi de seis meses. **Intervenção:** Entre novembro de 1993 e novembro de 1994 as pacientes foram submetidas à pesquisa sérica do anticorpo anticardiolipina por ELISA, segundo técnica descrita por TRIPLETT, BARNA, UNGER (1993). **Mensuração:** Os resultados foram analisados através dos testes Qui-Quadrado, Exato de Fisher e t de Student. **Resultados:** Não foi encontrada diferença estatisticamente significativa na freqüência do anticorpo anticardiolipina entre os grupos I e II para IgM (zero e 2,9%) e IgG (7,7 e 5,8%). **Conclusões:** Não foi encontrada associação entre a presença do anticorpo anticardiolipina e o antecedente de aborto recorrente.