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PAPER

Ovarian reserve in women with primary antiphospholipid syndrome

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Objective: The objective of this paper is to evaluate ovarian reserve in primary antiphospholipid syndrome (PAPS) women and the association between ovarian reserve tests and clinical and laboratorial parameters, and anti-corpus luteum antibody (anti-CoL). **Methods:** We screened 85 female patients between 18 to 40 years old with APS. Of these, 67 patients were excluded because of association with other autoimmune diseases ($n=42$), contraindication or unwillingness to stop hormonal contraceptive ($n=21$), current pregnancy or breastfeeding ($n=3$) and previous ovarian surgery ($n=1$). Therefore, a cross-sectional study was conducted in 18 PAPS patients and 24 healthy women. They were evaluated at early follicular phase with measurement of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and anti-Müllerian hormone (AMH) and sonographic antral follicle count (AFC). Serum measurement of anti-CoL was determined by immunoblot analysis. All analyses were performed after at least six months from the last intake of hormonal contraceptive and resumption of menstruation. **Results:** The mean age was comparable in PAPS and controls (33.0 ± 5.0 vs. 30.4 ± 7.0 years; $p=0.19$). Regarding ovarian reserve tests, the frequencies of low AFC (≤ 10) (56% vs. 22%, $p=0.04$) and very low AFC (≤ 5) (37% vs. 9%, $p=0.04$) were significantly higher in PAPS patients than controls. Trends of higher frequencies of reduced (<1.0 ng/ml), low (<0.5 ng/ml) and negligible (<0.2 ng/ml) AMH levels were found in PAPS patients ($p=0.08$, $p=0.07$ and $p=0.07$, respectively). FSH, LH and estradiol were similar in patients and controls. There was no association between low ovarian reserve and specific types of antiphospholipid antibodies. Anti-CoL was solely observed in PAPS patients (11% vs. 0%; $p=0.177$) and was not related to ovarian reserve tests. **Conclusion:** Women suffering from PAPS possessed reduced ovarian reserve, with prevalence greater than 50%. *Lupus* (2014) **23,** 862–867.

Key words: Antiphospholipid syndrome; autoimmune diseases; anti-Müllerian hormone; antral follicle count; ovarian reserve test

Introduction

Antiphospholipid syndrome (APS) is an autoimmune thrombophilic condition that occurs either as an isolated condition known as primary APS (PAPS) or in association with other autoimmune diseases, particularly systemic lupus erythematosus (SLE).[1] Females afflicted by PAPS have an increased risk of complications during pregnancy, and studies have also implied that they might have additional burdens on their reproductive health.[1,2]

Likewise, it is known that aging, smoking and ovarian surgery decrease the quantity and quality of primordial follicles in the ovaries and, ultimately, the ovarian reserve (OR).[3,4] Conditions such as autoimmune oophoritis,[5,6] ovarian ischemia[7,8] and corpus luteum hemorrhage[9] may lead to diminished OR. Females suffering from PAPS are susceptible to the above-mentioned complications, yet the medical literature lacks information on how this relevant autoimmune disease affects the OR of these women.

Accordingly, we assessed the OR profile of women suffering from PAPS by performing hormonal evaluations and state-of-the-art pelvic ultrasound scanning, and we compared these results with the OR profile of healthy women.

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Materials and methods

Patients and controls

Eighty-five patients aged 18 to 40 years old with APS were followed at the Antiphospholipid Outpatient Clinic of the Rheumatology Division of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and were invited to participate in this study. These patients were screened for PAPS during the period from April 2010 to March 2013. All patients had a previous history of thrombosis, and none were newly diagnosed. None had thrombosis at entry and all were on long-term use of warfarin. The median time of warfarin use was 5.05 years (0.7–12.9). Participants were excluded from the study if they were pregnant, breastfeeding, unwilling to participate in the study, had medical contraindications, possessed an increased risk of corpus luteum bleeding after stopping use of progestogen-only contraceptives,[9] underwent previous ovarian surgery, required systemic chemotherapy, underwent pelvic radiotherapy, were currently using a long-acting gonadotropin release hormone agonist (GnRH-a), presented with an additional autoimmune disease, or had an end-stage renal disease. None of them were under estrogen contraceptive during the study or anytime during their treatment.

Of the 85 women, 67 were excluded because of an associated autoimmune condition ($n = 42$), contraindications or unwillingness to stop use of hormonal contraceptives ($n = 21$), currently being pregnant or breastfeeding ($n = 3$), or because of previous ovarian surgery ($n = 1$). Therefore, the cross-sectional study was conducted on 18 women with PAPS. The control group included 24 healthy, age-matched control volunteers recruited from the primary care clinic nearby our tertiary hospital. The control volunteers were subject to the same inclusion and exclusion criteria. All PAPS participants voluntarily agreed to discontinue the use of hormonal contraceptives during a period of at least six months and had at least two consecutive menstruations to reveal the onset of normal endocrinological hypothalamic-pituitary-ovarian axis activity. None of our PAPS patients and controls had hypergonadotrophic amenorrhea, which would suggest primary ovarian insufficiency (high levels of FSH >40 IU/l and sustained amenorrhea).[9] The local ethics committee of our university hospital approved this study, and informed consent was obtained from all participants.

Demographic data, PAPS manifestations, and treatment

Each patient's medical records were carefully reviewed concerning clinical, immunological and treatment findings. PAPS manifestations were defined as vascular thrombosis (one or more clinical episodes of arterial, venous, or small-vessel thrombosis in any tissue or organ) or pregnancy morbidity (fetal death; premature birth before the 34th week of gestation because of eclampsia, severe pre-eclampsia, or placental insufficiency; or three or more consecutive spontaneous abortions).[1] All patients were on long-term warfarin treatment. Brazilian socio-economic stratification was carried out according to the Associação Brasileira dos Institutos de Pesquisa de Mercados criteria.[10] Body mass index (BMI) was defined as the weight in kilograms/height in meters^2 (kg/m^2).

Gynecologic evaluation

Complete history and physical examination including history of the age at menarche and detailed obstetric data were recorded.

OR tests

Blood samples were drawn by venipuncture in the early follicular phase (menstrual cycle day 2 through 4). All sera were processed in a centrifuge and stored at -70°C until analysis. Follicle-stimulating hormone (FSH) (reference level: 3.5–12.5 IU/l), luteinizing hormone (LH) (reference level: 2.4–12.6 IU/l) and estradiol (E2) (reference level: $\leq$166 pg/ml) were measured by radioimmunoassay using a commercial kit (Cobas®, Roche, Mannheim, Germany). Intra- and inter-assay coefficients of variation were recommended by the manufacturer and were limited to 5.7 and 3.6%, respectively. FSH concentrations were considered elevated when the levels were equal to or greater than 10 IU/l.[4,11,12]

Anti-Müllerian hormone (AMH) was measured by enzyme-linked immunosorbent assay (ELISA) in duplicated samples (AMH Gen II ELISA, Beckman Coulter Inc, Brea, CA, USA). Intra- and interassay coefficients of variation were limited to 5.4% and 5.6%, respectively. AMH levels were classified by cutoff values: lower than 1.0 ng/ml (reduced), lower than 0.5 ng/ml (low) and lower than 0.2 ng/ml (negligible).[13,14]

Transvaginal ultrasound was carried out to determine the antral follicle count (AFC) and the mean ovarian volume on the same day that

the blood was drawn. Transvaginal ultrasound was not performed in women not engaged in sexual activity. All ultrasound measurements were performed by an experienced, trained, reproductive specialist (LYSY) using a 6.5 MHz endovaginal transducer (HD3, Philips Ultrasound, Bothell, WA, USA) who was blinded to the volunteers' diagnoses and blood test results. Ovaries were scanned in axial and longitudinal planes, and at least two measurements of length (L), width (W) and thickness (T) were obtained and used to calculate mean ovarian volume using the formula for an ellipsoid ($L \times W \times T \times 0.523$). The AFC was calculated by measuring follicles with sizes from 2 to 10 mm in both ovaries.[4,15] AFC measurements were classified clinically as: normal when there were $\geq$10 follicles, low when there were 10 but >5 follicles, and very low when there were $\leq$5 follicles.[14]

Detection of anti-corpus luteum (anti-CoL) and other autoantibodies

The presence of autoantibodies directed toward a 67 kDa protein in the corpus luteum was determined in the patient and control samples using immunoblotting, as described previously.[16] Briefly, crude tissue and cell extracts obtained from bovine corpus luteum (100 mg/well) were submitted to polyacrylamide gel electrophoresis under denaturing and reducing conditions (with sodium dodecyl sulfate and β2-mercaptoethanol). Proteins were then electrophoretically transferred to a nitrocellulose membrane. The membrane strips were further incubated with blocking buffer (5% skimmed milk in phosphate-buffered saline (PBS)) and immunoprobed by incubation with serum samples diluted 1:10. Reactivity was tagged with an anti-human immunoglobulin (Ig)G alkaline phosphatase conjugate and visualized using appropriate chromogenic substrates.

Lupus anticoagulant (LA) was detected according to the guidelines of the International Society on Thrombosis and Hemostasis.[17] Presence of anticardiolipin (aCL) antibodies and anti-β2-glycoprotein I (anti-β2-GPI) IgG and IgM were analyzed by ELISA.[18] The presence of additional autoantibodies, including anti-double-stranded DNA (anti-dsDNA; indirect immunofluorescence using *Crithidia luciliaea* substrate), anti-ribonucleoprotein (anti-RNP), anti-Sm proteins (hemagglutination assay with rabbit thymus extract), anti-Ro/SS-A and anti-La/SS-B (counterimmunoelectrophoresis using human spleen extract as antigen) were detected.

Statistical analysis

Results are shown as the mean ± standard deviation (SD) or median (range) for continuous variables and as percentages for categorical variables. For continuous variables, data were compared by the *t* test and Mann-Whitney test to evaluate differences between the PAPS group and control group. For categorical variables, differences were assessed by Fisher's exact test. The level of significance was set at 5% ($p < 0.05$).

Results

Histories of arterial thrombosis, venous thrombosis and pregnancy morbidity were detected in 17%, 83%, and 33% of the PAPS patients, respectively. LA, aCL antibodies, and anti-β2-GPI antibodies were detected in 67%, 83% and 11% of the PAPS patients, respectively. All PAPS patients were negative for anti-dsDNA, anti-Sm, anti-RNP, anti-Ro/SS-A and anti-La/SS-B autoantibodies.

The demographic features, gynecologic history, OR test results, and anti-CoL statuses of the PAPS patients and controls are depicted in Table 1. The mean age was similar between the PAPS group and controls (33.0 ± 5.0 vs. 30.4 ± 7.0 years; $p = 0.189$). The BMI, smoking status and the remaining demographic features were similar in both groups ($p > 0.05$). The history of deliveries was superior in PAPS patients when compared to controls (1 (0–6) vs. 0 (0–2), $p = 0.02$), and there were fewer pregnancies per woman in the PAPS group than in the controls (17% vs. 54%, $p = 0.02$).

The frequencies of low and very low AFC were greater in PAPS patients than in controls (low AFC: 56% vs. 22%, $p = 0.042$; very low AFC: 37% vs. 9%, $p = 0.045$). Reduced, low and negligible AMH levels were observed in PAPS patients more often than in controls ($p = 0.08$, $p = 0.07$ and $p = 0.07$, respectively). Serum FSH, LH and estradiol levels were similar in PAPS patients and controls ($p > 0.05$). The occurrence of anti-CoL antibodies was observed in two PAPS patients (11%) and no controls (0%) ($p = 0.177$), and one of these PAPS patients had reduced OR (Table 1).

Discussion

To our knowledge, this is the first study to indicate that more than half of women suffering from PAPS have diminished OR. The main strength of this

Table 1 Demographic features, gynecologic history, ovarian reserve tests, and anti-corpus luteum antibody (anti-CoL) in primary antiphospholipid syndrome (PAPS) patients and controls

Variables	*PAPS* n = *18*	*Controls* n = *24*	p
Demographic features			
Current age, years	33.0 ± 5.0	30.4 ± 7.0	0.19
BMI, kg/m^2	27.0 ± 5.4	24.9 ± 4.5	0.18
Smoking	6	4	1.00
Hypertension	17	4	0.30
Caucasian	67	54	0.53
Socioeconomic class C or D	67	58	0.75
Gynecologic history			
Age at menarche, years	12 (9–15)	12 (11–18)	0.98
Time between menarche and current age, years	20.7 ± 5.0	17.9 ± 6.8	0.14
Parity	1 (0–6)	0 (0–2)	**0.02**
No pregnancy	17	54	**0.02**
Ovarian reserve			
FSH, IU/L	6.1 (3.8–20.1)	5.8 (2.2–14.4)	0.85
FSH ≥10	17	8	0.64
LH, IU/L	5.6 (1.9–9.9)	4.9 (2.1–10.6)	0.56
Estradiol, pg/ml	39 (26–75)	34 (24–128)	0.74
AMH, ng/ml	1.6 (0–4.2)	2.8 (0.1–6.6)	0.16
AMH <1.0	44	16	0.08
AMH <0.5	39	13	0.07
AMH <0.2	28	4	0.07
AFC[a]	12.4 ± 12.3	17.3 ± 8.9	0.16
AFC ≤10 [a]	56	22	**0.04**
AFC ≤5 [a]	37	9	**0.04**
Mean ovarian volume, mm^{3a}	5.1 (2.9–9.3)	5.1 (1.4–17.3)	0.70
Anti-CoL	11	0	0.18

Values expressed in mean ± standard deviation, median (range) or *n* (%). PAPS: primary antiphospholipid syndrome; BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; AMH: anti-Müllerian hormone; AFC: antral follicle count. [a]*n* = 16 PAPS patients and 23 controls.

research was the evaluation of most reliable OR markers, including AFC and serum AMH, which were obtained at the early follicular phase to avoid intramenstrual cycle variations in AMH levels.[19,20] We used strict criteria to define PAPS. Moreover, all patients discontinued use of hormonal contraceptives for at least six months and had resumed two or more consecutive menstrual cycles, suggesting that ovarian function was reestablished. These precautions were taken to obtain consistent OR evaluations because it is known that AMH concentrations decrease during hormonal contraceptive use.[21–23] However, the main limitation of this study is the low sample size, which, in part, is accounted for by the strict definition of PAPS and the low incidence of this illness.

Low OR, as indicated by a significant reduction in AFC, implies that primordial follicle cohort growth may suffer insults within an antiphospholipid milieu that manifest as a reduced follicle cohort. Follicle atresia, apoptosis, the location of primordial follicles near the granulosa cell layer, and ovarian blood supply could make primordial follicle cohorts more susceptible to microvascular ischemia events. Furthermore, diminished serum AMH levels might be brought about by insults to the ovarian microvasculature. A parallel dysfunction could be drawn in male PAPS and SLE-related antiphospholipid patients, in whom morphofunctional penile alterations were observed.[24,25]

Diminished OR was most likely not related to the use of warfarin as a PAPS therapy. Furthermore, unlike patients with other autoimmune diseases, our patients did not use immunosuppressive agents, such as cyclophosphamide[26–29] or glucocorticosteroids,[30] that could result in low OR.

OR evaluation in patients with chronic autoimmune diseases may be influenced by age, thrombosis, autoimmunity, drug therapies, oral contraceptives, genetics and environmental factors such as smoking.[26,27,29,31] Previous use of medroxyprogesterone acetate, an option of contraception for women with positive aCL and/or LA,[32] was not a relevant factor for this analysis since the drug was discontinued for at least six months and menstrual cycle had to be resumed. Additionally, patients and controls had comparable ages and very low tobacco smoking habits, which negates these factors as a causes of low OR. Additionally, none of our patients had clinical evidence of ovarian thrombosis, which is in accordance with the findings described for PAPS patients.[33]

Regarding OR evaluation, basal serum FSH, although widely available, has been demonstrated to have low accuracy in diagnosis of diminished OR, especially at early stages, the most important phase to be identified.[27] Today, AMH and AFC are the best non-dynamic tests to predict ovarian performance in human reproductive treatment.[26,28] AMH is produced by granulosa cells of early-stage follicles and has the advantage of being FSH independent, which makes its level relatively stable during all menstrual cycles, and it can be used in early evaluation.[26] AMH assessment was first performed in SLE patients[31] and it is considered a reliable ovarian marker.

It is unlikely that autoimmunity influenced the low OR observed herein, which contrasts with our female SLE patients who showed an association between anti-CoL antibodies and ovarian dysfunction.[16] Antibodies directed to pre-ovulatory follicle cells and not to corpus luteum-specific antigens may have an impact on OR in PAPS patient. This field of study deserves further research.

The presence of a relatively high parity among PAPS patients is conceivably due to the fear of childlessness in these women and to their high pregnancy morbidities.[1] The elevated number of childless women in the control group may reflect an unanticipated selection of women interested in learning about their OR. The focus of our work was to assess OR markers in a cross-section study. A future study evaluating OR markers for prediction of pregnancy outcomes will be required.

In conclusion, the present report identifies a high prevalence of diminished OR in PAPS patients. Further studies are necessary to uncover the mechanisms by which PAPS causes ovarian impairment.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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