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Lack of anti-paternal cytotoxic antibody production in the presence of spermatozoa in recurrent spontaneous abortion couples in contrast to fertile couples: a pilot study

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Background: Anti-paternal cytotoxic antibody (APCA) is a pregnancy alloantibody and is directed to paternal HLA molecules. A recent study showed that this antibody is produced in the presence of the husband's spermatozoa. Several studies have shown the absence or reduction of this antibody in the serum of recurrent spontaneous abortion (RSA) women. Note that the production of APCA has not been determined in the presence of spermatozoa in RSA women. A reason for the reduction or absence of APCA in the serum of RSA women may be the inability of the husband's spermatozoa to induce APCA production by lymphocytes. Therefore, this study aims to investigate APCA production by the wife's peripheral blood mononuclear cells (PBMCs) in the presence of the husband's spermatozoa in RSA couples. Methods: Ten RSA couples and ten fertile couples were included in this study. The wife's PBMCs (peripheral blood mononuclear cells) were cocultured with her husband's spermatozoa and the supernatant assessed for the presence of IgG by the ELISA method and APCA by complement-dependent cytotoxicity (CDC) assay. Results: Results showed that the production of IgG (median = 740 ng/mL in the fertile and 64 ng/mL in the RSA group) and APCA (median = 78% in the fertile and 48.5% in the RSA group) was significantly lower in the RSA group as compared to the fertile group. Conclusions: We concluded that a possible cause for APCA reduction in RSA women may be the inability of the husband's spermatozoa in induction of antibody production by the wife's PBMC. Nevertheless, large studies are needed to confirm the results of the present study.

Keywords

Recurrent spontaneous abortion; APCA; Spermatozoa

1. Introduction

Recurrent spontaneous abortion (RSA) is defined as the occurrence of three or more clinically detectable pregnancy losses [1]. There are a variety of causes of RSA, including; uterine anatomical defects, uterine infections, chromosomal aberrations, hormonal disorders, thrombophilic disorders and immunological abnormalities [1]. Immunological abnormality can result from a defect in the maternal immune response or a defect in the husband's semen or spermatozoa in stimulation of the female reproductive tract (FRT) immune response [2]. Findings reveal that semen and spermatozoa immunogenicity result in the development of regulatory and effector T and B lymphocyte directed to paternal antigens [3, 4] such as human leukocyte antigen (HLA) molecules. A result of this stimulated immunity may be the production of alloantibody against paternal HLA molecules. Therefore, abnormality in semen or spermatozoa can lead to impaired immune response and consequently pregnancy disorder such as preeclampsia [5] and RSA [6].

Our recent studies offer new evidence for the relation between abnormality in the expression of spermatozoa surface molecules and RSA. We show decreased expression of HLA class I & II [7] and toll-like receptor4 (TLR4) on the husband's spermatozoa from couples suffering from RSA [8]. We show that the husband's spermatozoa could induce maternal peripheral blood mononuclear cells (PBMCs) for the production of anti-paternal cytotoxic antibody (APCA) (https://celljournal.org/journal/article/abstract/7157).

APCA (a class of IgG antibody) is a pregnancy alloantibody and is directed to paternal HLA molecules [9]. There are very few studies on the mechanism of the production, activity and function of APCA. The reduction or absence of APCA is associated with recurrent spontaneous abortion (RSA). Successful lymphocyte therapy is accompanied with APCA development and improvement in live birth rates in RSA women [10–12]. This is evidence that indicates that APCA may be produced upon contact with semen and spermatozoa and, based on this evidence, we demonstrated the induction of APCA production by spermatozoa in a recent study. We think that the first step in determining the cause of the lack of APCA in RSA women is to investigate the ability of the husband's spermatozoa in inducing the wife's PBMC for production of APCA. Note that the production of APCA in the presence of spermatozoa in RSA women has not been determined.

Regarding APCA being directed to the husband's HLA and the decreased expression of HLA on the husband's spermatozoa in RSA couples, one reason for the lack of APCA production in RSA women may be the decreased expression of HLA molecules on spermatozoa. To prove our hypothesis, we aim to investigate APCA production by maternal PBMC in the presence of the husband's spermatozoa in RSA couples.

2. Methods and materials

2.1 Subjects

A control group of 10 fertile couples aged 24–45 years with at least one child and a case group of 10 unexplained recurrent spontaneous abortion (URSA) couples aged 20–38 years with no live births were included in the present study. RSA couples with non-immunological causes such as abnormalities of the uterus or cervix, chromosomal abnormality, infection, endocrine and metabolic diseases, congenital thrombophilia, autoimmune diseases and others were excluded from study. The ASA test was negative in the under-study women. The participant women had no history of blood transfusion or organ transplantation. In both groups, the husband of each woman had normal semen status according to criteria from the World Health Organization released in 2010 (WHO). Couples with male partners who had any history of genital tract disorder such as a history of infection, undescended testis, inguinoscrotal surgery such as varicocelectomy, genital trauma or testicular torsion were excluded from the study.

2.2 Purification of spermatozoa

Semen samples were collected by masturbation after 2–3 days of sexual abstinence. To prevent false results due to microbial antigens, sampling was performed under sterile conditions. After assessment of semen quality according to WHO standard guidelines (WHO, 2010), the husbands with normal semen quality were selected for the experiment. Two mL of AllGrad Wash (LifeGlobal® Group, Guelph, ON, Canada) were added to the liquefied semen sample and centrifuged at 350 g for 10 minutes. The pellet was re-suspended in 1 mL of AllGrad Wash®. In a tube, 1 mL of AllGrad Wash® 90% gradient, followed by 1 mL of AllGrad 45% gradient and then 1 mL of the spermatozoa suspension were carefully layered. The tubes were centrifuged at 400 g for 18 minutes. After washing with AllGrad Wash®, the spermatozoa pellet was re-suspended in complete RPMI 1640 medium

2.3 Isolation of peripheral blood mononuclear cells and performing co-culture

Five mL of heparinized venous blood were collected. PBMCs were separated by centrifugation on a Ficoll-Hypaque (Lymphoprep, Sigma, Ronkonkoma, NY, USA) density gradient. Cells at the interface were harvested, washed twice and suspended in complete RPMI 1640 medium supplemented with HEPES, L-glutamine, penicillin (100 U/mL), streptomycin (10 mg/mL), 2-mercaptoethanol (2×10^{-5} M) and 20% autologous serum. 2×10^6 PBMCs were cultured in the presence of 5×10^6 spermatozoa in 24-well plates (final volume: 2 mL). Cells were then incubated at 37 °C in a humidified 5% CO2 atmosphere. After 4 days, cells were washed 3 times and the pellet was re-suspended in 2 mL complete RPMI 1640 medium in which autologous serum was replaced by 20% fetal calf serum (FCS). After 8 days incubation at 37 °C in a humidified 5% CO2 atmosphere, supernatants were harvested, aliquoted and kept at -80 °C for future use.

2.4 ELISA analysis

The concentration of IgG in the supernatant was determined using an enzyme-linked immunosorbent assay (ELISA) kit (IgG (Total) Human Uncoated ELISA Kit, Invitrogen, MA, USA). The test was performed in accordance with the manufacturer's protocol.

2.5 Determining APCA titer in supernatants

APCA percentage was evaluated by cross-matching between supernatants (1/64 dilution) and freshly prepared paternal PBMCs. The test was performed in triplicate in Terasaki plates covered with light paraffin oil. One μL of paternal PBMCs suspension (at a density of 2×10^3 cells/mL) was mixed with 1 μL of supernatant. After 1 hour at room temperature, 5 μL rabbit complement (inno-train, Germany) was added to the plates. One μL Eosin dye was added to wells after 1 hour incubation at room temperature, followed by 5 μL formalin (37%). The test plates were left overnight to allow the cells to settle. Plates were read using a phase contrast microscope (Olympus, Japan). The number of dead cells among 1000 PBMCs was determined and reported as a percentage of APCA.

3. Statistical analyses

Descriptive analysis of IgG concentration and the percentage of APCA in the supernatant included a median and a IQR. A Mann-Whitney U test was used to compare difference in the dependent variable (APCA and IgG) for two independent groups (RSA and fertile groups).

4. Results

4.1 Demographic data

Demographic data of fertile and RSA women are shown in Table 1.

4.2 Semen analysis results

Semen analysis results of every men in both groups were shown in Table 2.

4.3 IgG concentration in the supernatant

Supernatant obtained from coculture of spermatozoa and PBMCs was assessed for IgG by the ELISA method. The Mann-Whitney test for IgG (U = 0, P = 0.005, r = –0.85) showed that the level of IgG (median = 64, IQR = 63) was much less than the control group (median = 740, IQR = 85.5). See Fig. 1.

Table 1. Demographic data of fertile and RSA women.

Parameters	Fertile group										RSA group										U	P value
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10		
Age	32	27	45	26	29	24	34	35	25	30	27	38	33	20	36	37	29	33	36	26	60.5	0.436
Weight	63	54	52	61	48	56	54	52	70	64	64	72	45	57	55	53	62	66	58	54	58.5	0.52
Number of children	2	1	1	3	4	1	2	3	2	2	0	0	0	0	0	0	0	0	0	0	-	-
Number of Abortions	0	0	0	0	0	0	0	0	0	0	3	3	3	5	4	33	5	4	4	3	-	-
Married life span	10	8	5	4	7	3	12	9	6	7	12	5	4	10	11	7	5	12	5	12	59	0.529
Abnormality in reproduction	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	-

F, fertile man; M $\pm$ SD, median $\pm$ standard deviation; N, normal; R, RSA man; the results are significant when *P* value is less than 0.05.

Table 2. Semen analysis results.

Parameters	Fertile group										RSA group										U	P value
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10		
Volume of semen (mL)	3	5	2.5	3.5	3.7	4.5	4	4.6	3.8	2.9	3.1	4.5	4.9	2.6	3.5	3.4	3.8	2.8	3	3.4	39	0.434
Liquefaction time (minute)	60	40	35	45	30	40	35	42	35	35	40	40	45	30	60	35	45	40	45	40	62.5	0.353
PH value	7.8	7.3	7.2	7.5	7.5	7.6	7.7	7.5	7.3	7.5	7.5	7.5	7.2	7.6	7.5	7.7	7.2	7.3	7.4	7.8	46.6	0.796
Concentration of spermatozoa $\times 10^6$/mL	70	80	55	50	45	30	120	90	90	80	45	50	70	130	83	100	140	90	85	50	62	0.393
Progressive mobility (%)	65	60	70	55	40	55	45	60	50	50	50	60	65	40	50	60	55	75	70	50	53	0.853
Overall mobility (%)	75	80	90	85	70	75	85	85	75	85	80	80	70	75	90	95	70	85	90	80	53	0.85
Non-progressive mobility (%)	10	20	30	25	30	15	35	40	15	25	30	25	5	25	40	35	15	10	20	30	48	0.912
Immotile morphology of spermatozoa in %	25	20	10	15	30	25	15	15	25	15	20	20	30	25	10	5	30	15	10	20	48	0.853

Leukocyte count in all samples was less than 1 milion/mL. F, fertile woman; M $\pm$ SD, median $\pm$ standard deviation; R, RSA woman; the results are significant when *P* value is less than 0.05.
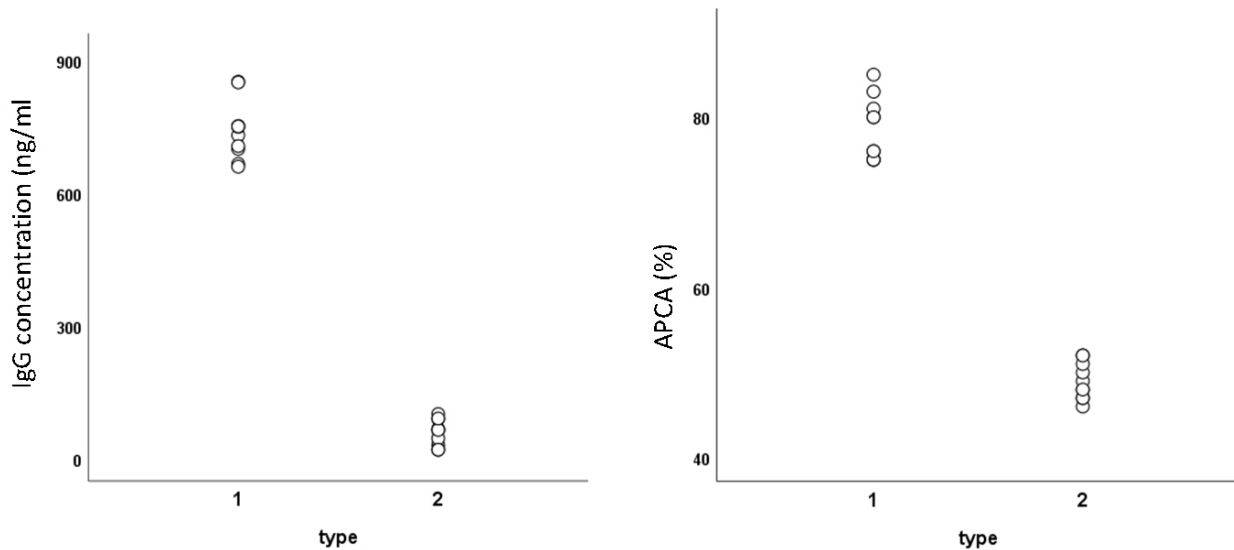

Fig. 1. Dot plot of supernatant concentrations of IgG and percentage of APCA.

4.4 Percentage of APCA in the supernatant

The Cross-match test was used to determine APCA in the supernatants. The Mann-Whitney test for APCA (U = 0, P = 0.005, r = −0.85) indicated a significant reduced percentage of APCA in the RSA group (median = 48, IQR = 4) compared with the control group (median = 78, IQR = 6). See Fig. 1.

5. Discussion

APCA as a protective antibody is detected in the serum of multiparous women who have had successful pregnancy; whereas this antibody is usually absent in the serum of RSA women [9, 13, 14]. We recently showed that APCA (a class of IgG antibody) is produced in the presence of the husband's spermatozoa when these cells are cocultured with maternal PBMC in fertile couples (https://celljournal.org/journal/article/abstract/7157).

Following the above study, we performed the present study and showed a decrease of APCA and IgG production by the maternal PBMCs in the presence of the husband's spermatozoa in RSA couples. As far as we know, this is the first time that this study had been performed and no similar studies exist.

There are few studies which examine the mechanisms of APCA production by the maternal immune system and the mechanisms of the action of APCA in supporting pregnancy. Therefore, our current study was conducted to obtain more information about APCA. As mentioned in the introduction, insemination results in the induction of inflammation in the FRT that accompanies recruitment of immune cells such as neutrophils, macrophages and lymphocytes to the FRT [3, 4]. This response constitutes a pre-embryo implantation immune response that has a major role in the improvement of embryo implantation and successful pregnancy [4, 15]. We hypothesize that APCA may be produced in the context of this activated immunity and is directed to HLA molecules on spermatozoa. In the recent article (mentioned above), we discussed a possible mechanism of the action of APCA, namely participation of APCA in implantation via antibody dependent cell-mediated cytotoxicity (ADCC) activity of natural killer (NK) cells. ADCC is an immune mechanism through which Fc receptor-bearing effector cells (such as NK cells) can recognize and kill antibody-coated target cells [16]. NK cells and cytotoxic T lymphocytes take part in the implantation process through cytotoxic activity [17, 18].

We hypothesize that APCA passing through the placenta may be absorbed by fetal tissue and contributes to the development of fetal tissue, such as the brain. It may be via the complement system. Recently, studies showed that components of the complement system, such as C5a component, have roles in brain development. These roles include neuron development, formation and refinement [19–21]. But what can activate the complement system in the brain? We suppose that APCA could be the cause of activation of the classic pathway of the complement system and lead to the generation of factors that contribute to brain development. Because of the lack of the expression of final complement components, which assist membrane attack complex (MAC) formation in some parts of the brain [22, 23], cell lysis and death mediated by MAC does not happen.

Some studies have documented that HLA class I molecules are expressed by brain cells and participate in the development and plasticity of the central nervous system (CNS). For instance, it is mentioned that MHC-I molecules, by binding to their receptors on brain cells, inhibit neural development, axonal and dendritic growth and so on [23–25]. Thus, APCA may play a role in the regulation of the mentioned function for HLA molecules by binding to these molecules.

Experiments on serum APCA have shown that APCA is directed to paternal HLA [9, 12]. We recently showed that HLA class I & II molecules are expressed on spermatozoa [26] and we revealed a decreased expression of these molecules on spermatozoa obtained from men whose wives suffer from RSA [7]. This result is accordant to our hypothesis that APCA production is induced by HLA molecules on spermatozoa. In other words, a reason for the absence of APCA in RSA women can be decreased expression of HLA molecules on the husband's spermatozoa.

The present study was limited by small sample size; however, the results go some way towards enhancing our understanding of the mechanisms of producing APCA and their role in embryo implantation and successful pregnancy.

6. Conclusions

This study suggests that APCA production by maternal PBMC may be induced by HLA molecules on spermatozoa and that decreased expression of HLA on spermatozoa may be a cause of the absence of APCA in RSA women. Further work needs to be carried out to determine the cause of decreased expression of HLA on spermatozoa and its effect on the production of APCA. This study could not achieve sufficient study power because of small sample size. Therefore, large studies are needed to confirm the results of the present study.

Key points

- Recently, it was shown that APCA is produced by maternal leukocytes in the presence of the husband's spermatozoa.
- The cause of the lack or reduction of APCA in the serum of RSA women is not known.
- An abnormality of spermatozoa antigenicity may be a cause for the lack of APCA production in RSA women.

Author contributions

NS contributed to conception and design, experimental work, data and statistical analysis, interpretation of data, manuscript writing and editing. RA and MR contributed to all experimental work and interpretation of data. VAP contributed data and statistical analysis. DW contributed to writing and editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of this study was approved by the Ethics Committee of Asadabad School of Medical Science (Asadabad, Iran) (The ethics committee approval letter: IR.ASAUMS.REC.1399.001). Informed consent was obtained from all couples who participated in this study.

Conflict of interest

The authors declare no conflict of interest.

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