Can anti-ovarian antibody testing be useful in an IVF-ET clinic?

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Abstract

Objective To establish importance of anti-ovarian antibodies (AOA) testing in infertile women.

Design A clinical reproductive outcome comparative study between two groups of women undergoing IVF-ET. Group 1 consists of women tested positive for AOA, put on corticosteroid therapy, reverted to AOA negative and then taken up for IVF-ET. Group 2 were seronegative for AOA.

Setting Major urban infertility reference centre and National research institute.

Patient(s) Five hundred seventy infertile women enrolled for IVF-ET.

Intervention(s) AOA testing, corticosteroid therapy and IVF-ET/ICSI.

Capsule Infertile women seeking assisted reproduction should have their serum tested for anti-ovarian antibodies prior to initiation of hormonal stimulation protocols. This would not only ensure efficacy of the IVF-ET but also save on the huge time and money invested on this protocol.

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Main outcome measure(s) Comparable clinical outcome and significance of AOA testing established.

Results AOA positive serum samples were sent periodically to re-investigate presence of AOA after corticosteroid therapy and women turned AOA negative were taken up for IVF-ET. Of the 70/138 women in group 1 who were treated with corticosteroids and turned seronegative for AOA, 22/70 were poor responders and needed donor oocyte-recipient cycles. Results demonstrated that fertilization and clinical pregnancy rates between both groups are comparable. Nevertheless, it is also observed that there is poor response to stimulation protocol, smaller number of oocytes retrieved and more spontaneous abortions in group 1 women. Hence not all outcomes following the treatment are comparable between the two groups. Usefulness of the test was established in two case studies.

Conclusions AOA testing could be included in the battery of tests investigating and treating infertility.

Keywords AOA, Infertility in women · IVF-ET success · IVF-ET treatment · Ovarian autoimmunity

Introduction

The hypothesis of an underlying autoimmune mechanism has been reported in some cases of in vitro fertilization (IVF) failure and in premature ovarian failure (POF) (also known as primary ovarian insufficiency-POI) where the presence of anti-ovarian antibodies (AOA) has been described [1–6]. These AOA could affect egg and embryo development and could be responsible for implantation failures. It has been demonstrated in some cases that AOA appear after follicular aspiration while in other cases, pre-existing AOA levels have been shown to increase with the

number of IVF attempts [4]. These AOA produced by the B-cells of the immune system are known to be influenced by a class of steroids called as corticosteroids.

Corticosteroids mediate a variety of immunological actions and are commonly utilized in the treatment of a wide range of diseases. They have a profound effect on many regulatory systems of the body, including the reproductive system. Their use has been extended in the in vitro fertilization embryo transfers (IVF-ET) programs [7]. Some programs use daily oral methyl prednisolone while others prescribe oral dexamethazone commencing about 10 days prior to initiating ovarian stimulation with gonadotropins, and continuing until the diagnosis of pregnancy. It was Kemeter and Feichtinger in 1986 who first reported a positive effect of corticosteroids on the pregnancy rate in a prospective randomized trial enrolling 146 IVF patients [8]. On the other hand some studies failed to demonstrate the efficacy of immunosuppressive treatments on pregnancy rates [9, 10].

The only randomized, placebo-controlled trial using corticosteroids and human menopausal gonadotropin (hMG) in 36 idiopathic POF/POI patients for 2 weeks did not show any positive effect as none of these patients became pregnant or even ovulated while under the treatment [11]. In fact, the presence of specific AOA was not assessed in these patients. It has been suggested that contradictory results may be a consequence of the very heterogeneous inclusion criteria and methodology used in the above mentioned studies, which suggests that not all infertile women are likely to benefit from such therapeutic adjuncts [7]. This further illustrates the need for accurate diagnostic tools in order to analyse the effect of corticosteroids in a selected population where women demonstrate ovarian autoimmunity. Hence, it is essential to assess the presence of serum AOA when a woman first presents herself with infertility to the clinician. Specificity of AOA testing has been questioned and the available tests reported in literature have been challenged [12] due to high rates of false positives seen using sera from normal fertile controls. This problem of non-specificity has been overcome in a specific, simple and non-invasive diagnostic test developed by us [13]. Using this test we could show new and true molecular and histochemical targets in the ovary in case of ovarian autoimmunity [5, 14]. The next step would be to suppress the activity of these autoantibodies before commencing the ovarian stimulation protocol with a standardized dose of corticosteroids. In the present study, we have established the importance of the AOA test, firstly for screening women for AOA prior to and during corticosteroid treatment and secondly to establish comparable clinical reproductive outcome following IVF-ET in corticosteroid treated women who turned seronegative with those who were initially seronegative.

Material and methods

Patient recruitment

The present study was approved by the Institutional review board (IRR75-23/08/2002) and informed consent was obtained from the patients who participated in this study. A total of 570 women (mean age 33 years) presented themselves for infertility treatment at the Department of Assisted Reproduction and Genetics at the Jaslok Hospital and research centre. Inclusion and exclusion criteria for patients enrolled in this on-going study have been described earlier in detail [5]. In brief, women less than 40 years of age at the time of enrollment in the study presenting with infertility and for whom IVF-ET had been determined to be the next treatment for conception. These women had a normal baseline pelvic ultrasound examination and a normal uterine cavity. Patients with the following condition were excluded from the study: those who had pelvic surgery within the past 8 weeks, including appendicitis or uterine or ovarian surgery, excluding diagnostic laparoscopy; those who had clinical evidence of pelvic inflammatory disease within the past 3 months and who were treated for any viral infection; endometriosis; women who had submucous or intramural fibroids of any size and women who had hydrosalpinx and had polycystic ovary syndrome. None of the patients' had been treated for any form of autoimmune diseases such as systemic lupus erythematosus, thyroid or adrenal autoimmunity and diabetes mellitus. None of these women had received any corticosteroid treatment previously. As part of the investigative work-up, serum samples of these patients were screened for the presence of AOA at the Gamete Immuno Biology (GIB) laboratory. Of the 570 women who were enrolled in the study 138 were found to have serum AOA and these women (group 1, age 33.4±4.3 years) were taken up for the study. As a study control, 121 of the 432 women who were declared AOA negative by our test consented to participate in the present study (group 2, age 32 ±3.1 years) and were enrolled simultaneously.

SDS-PAGE Western blot analysis

Protein extracts were prepared and processed for electrophoresis as well as for transfer as discussed earlier [14]. Briefly, the transferred ovarian proteins were visualized using Ponceau S (Sigma, USA). The individual lanes were appropriately marked, isolated and blocked for 1 h at room temperature with 5 g% non-fat dry milk in phosphate buffered saline pH 7.4 (NFDm-PBS). Sera from patients collected 1, 2 and 3 months post corticosteroid therapy were added to the strip and incubated at 4°C overnight.

Following day the membrane strips were washed with 0.1% Tween 20 in PBS six times and then incubated with suitably diluted (1: 100,000) goat anti human secondary antibody HRP labeled (SIGMA, USA) in 1 g% NFDM in PBS for 1 h at room temperature. Strips were then washed as described above. Detection was done using enhanced chemiluminescence plus (ECL) kit (GE Healthcare, USA) as per standard protocol with Luminol serving as substrate.

Corticosteroid therapy

Serum samples of patients recruited for the study were screened for presence of AOA. Corticosteroid therapy was initiated in women who tested positive for AOA by Western blot analysis for a period of at least 1, 2 and 3 months. The dose of the corticosteroid used per patient was 0.5 mg oral dexamethasone twice a day. Post corticosteroid therapy for at least 3 months serum samples were re-screened for the presence of AOA by Western blotting. Patients' sera who tested AOA positive turned negative by Western blotting post corticosteroid treatment were reconfirmed AOA negative using immunohistochemistry on ovarian sections prior to taking them up for IVF-ET.

Immunohistochemistry

Ovarian tissues were fixed and processed for immunohistochemistry (IHC) as per the protocol described earlier [13] which involved a novel blocking recipe. Briefly, Bouin's fixed paraffin embedded ovarian sections was melted at 56°C, deparaffinized in xylene, quenched endogenous peroxidase activity and rehydrated. Sections were then blocked with novel blocking solution which consists of NFDM-PBS containing 20% rabbit polyclonal anti albumin antibodies (AAA), for 2 h at room temperature. Sera from patients detected AOA positive and post corticosteroid therapy turned AOA negative (by our Western blot analysis data) were used undiluted or diluted to 1:5 with 2.5 g% NFDM-PBS, incubated at 4°C overnight in a humid chamber. Section serving as 'negative control' was incubated with 2.5 g% NFDM in PBS. The following day slides were washed with PBS thrice and suitably diluted (1: 1000) goat anti human secondary antibody (SIGMA, USA) in 1 g % NFDM in PBS was added and incubated for 2 h at room temperature in a humid chamber. After washing, the immunoperoxidase color reaction was developed by using 3,3'-diaminobenzidine (DAB) substrate chromogen solution (SIGMA, USA), then counterstained for 30 s using Delafield's haematoxylin (Qualigens, India), dehydrated through series of alcohol grades, cleared in xylene and mounted in DPX mounting medium (SRL, India). Slides were examined on a Zeiss Axioscope microscope (Carl Zeiss Inc., USA).

Intra cytoplasmic sperm injection (ICSI) and cumulus aided embryo transfer protocol

A classical standardized ovarian stimulation protocol [15] was applied in all reference cycles and corticosteroid-treated cycles by our clinical collaborators at Jaslok Hospital. The human chorionic gonadotropin (hCG) trigger was given at follicular maturity, followed by transvaginal ultrasound-guided oocyte retrieval. Oocytes were prepared for ICSI by standard protocols and were denuded by exposure to 80 IU of hyaluronidase. The dissociated cumulus cells were washed twice with Quinn's advantage medium with HEPES. The pellet was then diluted with Quinn's advantage fertilization human tubal fluid medium and then transferred to the center-well culture plate and incubated at 37°C in 5% CO₂. After culture for 24 h the medium was replaced with Quinn's advantage cleavage medium. After equilibration of the medium, the pronuclear stage embryos were added to the autologous cumulus coculture. At 36 h, the old medium was discarded and the coculture along with the cleavage embryos was exposed to fresh cleavage medium. Cumulus-aided embryos transfer was performed with day 3 embryos where the embryos were transferred with the autologous cumulus cells. The number of embryos transferred depended on the advancing age of the woman, the quality of embryos for transfer and the decision of the couple. While selecting embryos for transfer, those having equal blastomeres with minimal fragmentation were transferred in both the study groups. As part of a clinical research program, all patients undergoing IVF are routinely tested for serum AOA before hormonal stimulation and prior to initiation of ART treatment. In case results were positive a repeat sample is sent for AOA testing post corticosteroid therapy for a minimum of at least 3 months. Corticosteroid therapy was initiated at a daily oral dose of 0.5 mg dexamethasone twice a day. Only if the patient's serum AOA levels turned negative were they taken up for IVF-ET. Serum hCG was determined 12 days after embryo transfer. In patients who became pregnant, dexamethasone was continued at the same dose for the whole first trimester of pregnancy and then progressively decreased. In all cycles (with and without corticosteroids), the total dose of FSH used to induce a proper ovarian stimulation, the duration of ovarian stimulation, the number of oocytes obtained and transferred, and the occurrence of a clinical pregnancy were carefully monitored. The number of cancelled cycles and those with embryo abortions was noted. The fertilization, pregnancy and live birth rates were also evaluated and compared between the two groups. The statistical analysis wherever required was performed using Student's t-tests on paired series and chi-square Fischer's exact tests with Graph Pad Prism 4 (Graph Pad software, San Diego, CA).

Results

Frequency of AOA positives in the study groups

AOA positive patients' demonstrated immunoreactivity to antigens spanning regions 30–120 kDa in Western blotting as described in details earlier [5]. Of the 570 patients enrolled for this study, 138 were tested AOA positive (24%) by the Western blotting (Fig. 1). The remainder of the women ($n=432$) tested seronegative for AOA (76%) as seen in the pie-chart. The AOA positive women were consented for corticosteroid treatment prior to enrolling them for the IVF-ET protocol.

Not all patients turned AOA negative after 3 months of corticosteroid therapy

Of the 138 women tested seropositive for AOA taken up for corticosteroid therapy, 70 women turned AOA negative post treatment. Of the remaining 432 initially AOA negative subjects, 121 consented to participate in the study and were parallelly taken up for IVF-ET. The Western blot data which is a representative figure as seen in Fig. 2 evidently shows with an example using serum from a patient tested positive for AOA towards the 90 kDa protein, still persists even after 3 months of corticosteroid therapy (Fig. 2 panel a). M 1, 2 and 3 denote sera collected and processed independently at those time points (in months) post treatment with corticosteroids. However, in the same figure (panel 2b), a patient serum tested positive for AOA towards a 45 kDa protein has reverted to AOA negative status after 1 month of corticosteroid therapy. This indicates that not all women respond uniformly to the treatment. The 66 kDa reactivity is the reaction of naturally occurring abundant auto-antibodies to albumin was commonly seen in all patients and controls (lane C) and as described earlier [13]. A 'no primary antibody control' showed no immunoreactivity (lane NC).

Fig. 1 This pie-chart depicts the percent frequency of anti-ovarian antibodies (AOA) positivity in the present study as demonstrated by our Western blotting analysis. Of the 570 infertile women enrolled for this study, 138 women tested seropositive for AOA accounting to 24% of the infertile population. The remainder of women seronegative for AOA totaled up to 432 in all

Novel AAA blocking in IHC analysis endorse the Western blotting data

The employment of our novel blocking in histochemical analysis of the sera gave us a true picture of AOA status in the study group. Figure 3 is a representative image indicating the status of AOA prior and after to response of corticosteroid treatment in group 1. Prior to the treatment the sera immunostains the oocyte (panel a1) however; no immunostaining to any of the ovarian cell types can be observed after responding to the treatment (panel a2). A persistent of immunoreactivity to the ovarian compartments was observed in patients who did not respond to the corticosteroid treatment (panel b1 and b2 before and after corticosteroid treatment respectively). A 'secondary alone control' showed no immunoreactivity to any of the ovarian cell types (data not shown).

Selection of women in group 1 for IVF-ET and AOA positive patients' characteristics

Of the 138 AOA positive women who were put on corticosteroid therapy, 70 women turned AOA negative (50%) in 3 months as detected by Western blotting. The past obstetric history of the 138 AOA positive patients is as follows: 93/138 (67%) of these women presented themselves with primary infertility while 45/138 (33%) had secondary infertility. Of the 45 women with secondary infertility 21/45 (46%) had single miscarriages and 14/45 (31%) had multiple miscarriages. The 68 women (138-70) still positive were continued on the corticosteroid treatment and would be taken up for IVF-ET after tested seronegative for AOA only. For the present study, the 70 women who turned AOA negative were now taken up for IVF-ET and they underwent 90 IVF cycles. Of these 70 women, 22 women were segregated as extremely poor responders and they underwent donor oocyte-recipient cycles independently. Therefore, 48 women ($70-22=48$) had undergone 66 ovarian stimulation cycles with their own eggs. Out of these, eight cycles were dropped due to inadequate response. Therefore, 48 women now underwent 58 IVF cycles. This is our study group 1. Of the 432 patients who were initially tested AOA negative, 121 women consented to be a part of this study and were taken up for IVF-ET. They served as our study controls and formed group 2 and underwent 121 cycles.

A good and comparable reproductive outcome post corticosteroid treatment

No adverse effects resulting from corticosteroid therapy was observed or reported by the patients. The data compares the clinical reproductive outcome between the

Fig. 2 This is a representative image of a Western blot analysis showing the status of AOA in the study group 1 who were placed on corticosteroid therapy. As seen in the figure (panel a), is an example depicting the persistence of immunoreactivity to a 90 kDa protein in spite of the corticosteroid therapy. In the same figure, (panel b) it can be clearly seen that a patient initially showing immunoreactivity to a 45 kDa protein, after receiving the corticosteroid regime reverts to

AOA negative status. M1, 2 and 3 represent immunoreactivity of serum sample sent 1, 2 and 3 months after corticosteroid therapy. Panel c shows immunoreactivity of control sera (lane C) to the 66 kDa albumin protein (as discussed in Pires et al. 2006 [13]). A 'no primary antibody control' shows no background reactivity to any ovarian proteins (lane NC)

48 AOA positive reverted to AOA negative women who underwent 58 IVF cycles (group 1) versus the 121 initially detected AOA negative who underwent 121 IVF cycles (group 2). Women in group 1 who are tested seropositive for AOA now turned seronegative after corticosteroid treatment are poor responders and have a lower normal response and higher sub-optimal response to ovarian stimulation protocols in comparison with those women in group 2 who were primarily seronegative for AOA (Table 1). The number of cycles dropped by group 1 women (16.6%) is significantly higher than group 2 women (4.9%). The average number of oocytes collected from group 1 women (3.2 ± 1.6) is significantly less in numbers than group 2 women (6.1 ± 2.2). Our findings reveal that there is equivalence in fertilization rates (73.20% in group 1 versus 71.8% in group 2) and clinical pregnancy rates (34.5% in group 1 versus 39.6% in group 2) in women who were AOA positive now turned AOA negative after corticosteroid therapy (Table 1). Nonetheless, it is also observed that the number of spontaneous abortion rates in group 1 (35%) is significantly higher than group 2 (10.4%). An appreciable take home baby rate / live birth rate between group 1 (21%) and group 2 (35.5%) was observed.

Discussion

In spite of a tremendous evolution in assisted reproductive technologies (ART), we still face problems of poor reproductive outcomes. Some of the leading causes for female infertility include polycystic ovarian disease, endo-

metriosis, pelvic inflammatory disease, ovulatory dysfunction and uterine fibroids [29]. It has been well established that anti-ovarian antibodies (AOA) could be a contributing factor towards female infertility. AOA could affect oocyte development, embryo development and also could be responsible for implantation failures [4]. From this study, we observed that women testing positive for AOA are usually poor responders (Table 1). A high correlation between poor responders and the presence of AOA was also seen. It was thus felt that testing for the presence of AOA in women prior to initiation into the IVF-ET program should be recommended as this would help to counsel the patients regarding the reproductive outcome with IVF. Counseling women who test positive for AOA becomes important prior to initiation into an IVF program. Data from our study suggests that [1] women should be tested for serum AOA prior to enrolling them into the ART program, [2] once women are identified to have AOA; they could be recommended low dose oral corticosteroid therapy for at least 1–3 months and [3] each month the serum sample should be sent back for follow-up with the AOA status. Once AOA status turns to negative, they could be taken up for ART program. As seen from the results, the clinical pregnancy rates are almost comparable with the control group. We also observed that the incidence of spontaneous abortion was high in the group 1 women who were taken up for an IVF cycle after reverting to an AOA negative state. Women in this group also demonstrated small numbers of oocyte retrievals as well as poor response to the stimulation protocol. These differences reflects that corticosteroid treated AOA positive turned AOA negative

Fig. 3 This is a representative figure depicting an immunohistochemical localization of a patient serum tested seropositive for AOA before initiation of the corticosteroid treatment as seen in panel (a). Prior to the immunosuppressive treatment, the patient sera showed strong immunoreactivity to the oocyte (pointed by red arrow in a1). The same patient after the treatment now showed no immunoreactivity to the oocyte (pointed by blue arrow in a2). Panel b1 shows serum immunoreactivity to the oocyte by another patient prior to treatment and even after the corticosteroid treatment, immunoreactivity to the oocyte still persist (pointed in red arrows in b1 and b2). Magnification at $\times 200$ times

status still leaves behind some pathological mechanism and hence all outcomes following the offered treatment of their infertility are not comparable between the two groups studied.

Immunosuppressive doses of corticosteroids administered for a short period of time to patients undergoing IVF-ET have been shown to significantly improve the implantation and pregnancy rates [7, 16]. In a pilot study conducted by Forges et al. (2006) highlighted that the indication for corticosteroid treatment was based only on the identification and follow up of organ-specific antibodies directed against ovarian targets in patients with previous IVF failure [7]. In these selected patients they showed that corticosteroids not only reduced the anti-ovarian autoimmune response triggered by follicular aspiration but also increased the live birth rate after IVF. Several mechanisms are proposed to explain the beneficial effect of corticosteroids. The immunosuppressive action of these drugs could inhibit potential adverse effects of AOA on oocyte quality or gamete interaction [17]. Furthermore, corticosteroids could also help with implantation and embryonic development and have therefore been proposed

for the treatment of recurrent pregnancy loss [18]. Corticosteroids are shown to stimulate estradiol and progesterone productions, which are immunoregulating biomolecules, during pregnancy, as it's well known that progesterone is needed to maintain pregnancy [19]. There are numerous anecdotal reports of successful treatment of POF/POI and infertility with low-dose corticosteroid immunosuppression [20]. Women with POF/POI were treated with immunosuppression without screening for ovarian autoimmunity and two pregnancies occurred in 11 women [21]. In another trial without screening for ovarian autoimmunity, danazol was not effective for reversal of POF/POI [21]. However, success with low-dose corticosteroid treatment of POF/POI with autoimmunity has been confirmed by ovarian biopsy [22]. Likewise, infertile women with AOA treated with immunosuppression before IVF stimulation had improved embryo quality and pregnancy rates [23]. Controlled clinical trials to determine dose and side effects have yet not been conducted.

Our study clearly proves the usefulness of the test for follow-up post corticosteroid treatment. It gives the clinician an indication as to which patient is likely to benefit

Table 1 Comparable clinical outcome between women anti-ovarian antibody (AOA) positive reverted to AOA negative after corticosteroid treatment versus women seronegative for AOA

Parameters	AOA positive reverted to negative Group 1	Originally AOA negative Group 2	<i>p</i> -value
Number of subjects	48	121	NA
Mean age (years)	33.4±4.3	32±3.1	0.02
Number of IVF cycles	58	121	NA
Number of cycles dropped	8	6	NA
Suboptimal response (%)	65.5	16.5	0
Normal response (%)	34.5	83.5	0
Average number of oocytes collected	3.2±1.6	6.1±2.2	0
Fertilization rate (%)	73.2	71.8	1
Cleavage rate (%)	99.2	98	1
Clinical pregnancy rate (%)	34.5	39.6	0.67
Abortion rate (%)	35	10.4	0
Take home baby rate (%)	21	35.5	0.08

This table illustrates the importance of AOA testing before enrolling and taking up women for ART. The table also shows comparison between the two groups in the study with regards to their clinical reproductive outcome of the IVF-ET. Group 1 tested seropositive for AOA and were placed on corticosteroid therapy and once their sera tested seronegative for AOA they were taken up for the IVF-ET and results were compared to the group 2 women who were primarily seronegative for AOA. From the table, one can appreciate the comparable results between the two groups with regards to their fertilization, cleavage and clinical pregnancy rates. The statistical analysis was performed using Student's *t*-tests on paired series and chi-square Fischer's exact tests (*NA* not applicable)

post corticosteroid treatment in an IVF treatment cycle. Based on our findings, we propose that the AOA test should be a part of the battery of tests included for infertility diagnosis and management. The AOA test allows clinicians to make an early assessment of a possible 'poor responder' before initiating a stimulus protocol particularly when women have responded poorly in past IVF cycles. It also gives a strong clue to the clinician to modulate the corticosteroid treatment and to decide when this treatment should be discontinued. Parallely, this will not only save on time utilized in the success of the ART program employed by the clinical team but it would also assist in reducing the cost factor to be incurred by the patient.

Reviewing literature, it can be seen that most researchers have employed in-house or commercially available ELISA for AOA detection. Very few have confirmed the presence of these using histochemical findings. It has been reported by Luborsky et al. (2002) that the IHC results by several researchers have been tabulated as positive or negative, but specific reaction sites are rarely depicted pictorially [24]. Reactivity of AOA to ovarian cell types has been shown by

indirect immunofluorescence (IIF) [25–28]. However, there was no specificity and it varied according to source of ovarian tissue and maturation state. Novosad et al. (2003) reported poor specificity with a commercially available kit which tested positive in nearly one-third of normally menstruating women [12]. The group thus affirmed that IIF is not a reliable tool to detect AOA. Although various investigators have demonstrated that there is multiplicity of molecular and cellular targets in human ovarian autoimmunity, there is still a need to identify all specific ovarian targets [29, 30]. However, from our earlier findings we have investigated the cause of the non-specificity and were able to demonstrate true molecular and histochemical targets [5, 13]. Once the patient tests positive for AOA by Western blot analysis, the serum is screened by us to identify the cell types which are targeted, using IHC which employs a novel blocking recipe [13]. Our AOA combo test (Western blotting followed by IHC) is very useful as it not only demonstrates whether the patient has AOA but also highlights the cellular targets of these antibodies. IHC is a simple technique revealing a lot of cellular information and had it not been for this IHC data, it would have been very difficult for the treating clinician to decipher the reason for the failed cumulus-aided embryo transfers prior to AOA testing. Described below are two case reports which strongly support the usefulness of our test and was found to be useful by the clinician in her treatment strategy.

Case study

Mammalian cumulus cells play a very important role during oocyte growth and maturation. Cumulus cells are known to nurture the oocyte while it is in the ovarian follicle and at the time of ovulation. It is speculated that it is through the dynamic contacts via transzonal cumulus cell projections and via intercumulus projections that this close association between the cumulus cells and the oocyte is maintained [31]. After oocyte maturation, however, the transzonal cumulus cell projections are disconnected while the intercumulus projections still remain, and thus, the cumulus-cumulus cell communications are preserved. Studies by our collaborators group demonstrated the importance of this co-culture system [15]. They indicated the importance of the intercumulus interactions which not only functions in cell to cell adhesion but also transferring essential nutrients to the embryo and providing an adhesive surface for uterine implantation. Using these cells in such a co-culture set up where the embryo was no longer free floating but appeared to be stuck to the uterine wall.

Two patients coded CP1 and CP2 (CP indicates serum AOA reactivity to cumulus cells and oocytes) presented themselves at the IVF clinic at our collaborative centre at Jaslok Hospital for treatment of infertility. These two

women were of age 32 and 31 years with no children and had POF/POI. Our collaborators had carried out IVF-ET for both these patients in the past and were unsuccessful. The clinician then used donor oocytes but there was no success in spite of several attempts. Unaware of the outcome, the clinician sent the sera from both these patients to our laboratory for AOA testing. This was carried out using our AOA combo tests viz., Western blotting and IHC. Both CP1 and CP2 were identified to be seropositive for AOA by both the tests. Serum from CP1 reacted to two proteins of molecular weight 85 and 97 kDa while CP2 reacted to an 85 kDa protein in Western blot analysis (Fig. 4a). IHC analysis revealed that both sera showed strong immunoreactivity not only to oocyte but the cumulus cells of the follicle were also immunostained (Fig. 4b, red arrows).

Cumulus cells immediately surround the oocyte and bidirectional communication between the oocyte and surrounding cumulus cells is critical to the development and function of both the cell types [32]. These cumulus cells provide substrates that are used by the oocytes for the energy metabolism necessary to promote oocyte meiotic maturation [33]. Cumulus-aided embryo transfers demonstrated an increase in pregnancy and implantation rates [15]. The continuation of secretions of growth factors from the cumulus cells which are known to improve embryo

morphology and play a putative role in the implantation process constitute to a successful IVF-ET. In these two cases, AOA positivity was demonstrated by Western blotting and IHC where anti-oocyte and anti-cumulus antibodies were observed. These antibodies were the probable cause of the IVF-ET failures. These AOA were possibly affecting embryo development and could be responsible for implantation failures. This finding thereby validates the test developed by us [13] and strongly establishes its clinical significance and its importance when carrying out several investigative workups for planning treatment strategies for women with infertility. Therefore the need arises for the use of specific and sensitive diagnostic test to detect true status of AOA. The observation made based on these two case studies clearly indicates the need for inclusion of this diagnostic test in screening of infertile couples. The combo test has exhibited the diagnostic utility in predicting success of pregnancy following IVF-ET protocols.

Conclusions

In summary, our combo test (Western blotting as well as IHC) for AOA detection suggests that it is important to get the AOA test done in women presenting themselves with

Fig. 4 Panel **a** shows Western blotting analysis of two patients labeled as CP1 and CP2 (CP designated for presence of AOA to cumulus-oocyte complex). From the panel it is clearly seen that CP1 and CP2 react to a common 85 kDa protein while CP1 additionally reacts to a 97 kDa protein. The immunoreactivity at the 66 kDa locus is commonly observed in patients and controls as discussed in our earlier communications. From panel **b**, one observes the pattern of immunostaining using sera from CP1 and CP2 which stains not only the oocyte but also the cumulus cells of the follicle as shown by the *red arrows*. The sera from controls (**c**) do not react to any ovarian cell type pointed in *green arrows*. The novel blocking recipe was employed for all IHC reactions. Magnification at $\times 400$ times

infertility followed by appropriate treatment. If tested seropositive for AOA these women should be advised low dose corticosteroid treatment; serum samples should be re-checked to ensure seronegative for AOA and only then recruit the patient for ART program. Knowing the AOA status in the infertile women and planning appropriate immunosuppressive therapy would not only ensure the effectiveness of the IVF-ET but also reduce the time invested by the clinician and the cost to be incurred by the patients and thereby restore fertility.

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