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ASSISTED REPRODUCTION TECHNOLOGIES

Reduced protein C Global assay level in infertile women prior to IVF-ET treatment

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Abstract
Purpose In the last few years more robust evidence is emerging to point out at an increased rate of prematurity and low birth weight in singleton pregnancies following ART. Whether this increased rate is related to ART practice or to infertility per se, is still an open question. Our aim in this study was to explore this question by evaluating Protein C (ProC) Global assay in infertile women before ART treatment.
Methods A cohort of 95 unselected and consecutive infertile women, eligible for ART, was prospectively recruited for the study. The control group included 77 matched healthy fertile women with a history of spontaneous conceptions. Pro C Global assay was evaluated in both groups. A full thrombophilic work-up was performed in the study group.
Results ProC Global assay level was found to be significantly lower in the study as compared to the control group, corresponding to 0.78±0.16 and 0.88±0.16, respectively ($P<0.01$). As well, abnormal ProC Global assay level of ≤0.8 was significantly higher in the study as compared to control group corresponding to 53 % and 29 %, respectively. ProC Global assay level was significantly lower in women within the study group found to have APCR, factor V Leiden and high factor VIII level, any thrombophilia or combined thrombophilia when compared to women without these thrombophilic risk factors.
Conclusions Reduced ProC Global assay level is encountered in infertile women prior to ART treatment. This finding may suggest a unique anticoagulation Protein C pathway in infertile as compared to fertile women. Further studies are encouraged to explore this finding.

Capsule Protein C Global assay results are significantly lower in infertile patients, before ART treatment as compared to fertile healthy controls. This may support the notion that the increased perinatal risks in singleton pregnancies following ART treatment, is related to infertility per se and not to the technology.

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Introduction

In the last three decades pregnancies achieved following assisted reproductive technologies (ART) treatment has been implicated with several concerns. These included increased risk for prematurity, low birth weight, birth defect and delayed neurological development, genetic and epigenetic abnormalities [1]. However, in most of these cases the evidence so far has been controversial and remote from being conclusive. Furthermore, these risks were mainly attributed to higher multiple pregnancy rate [2, 3] and advanced maternal age [4] in pregnancies achieved following ART as compared to those naturally conceived.

Nevertheless, in the last few years more robust evidence is emerging to point out at an increased rate of prematurity and low birth weight in singleton pregnancies following ART. This has been estimated to have an approximate 2-fold

increased risk of pre-term birth and low birth weight in singletons controlled for the maternal age [5–10]. Whether this increased rate of adverse pregnancy outcome in singleton pregnancies is related to ART practice; such as employment of medications, manipulation of gametes and use of culture media or to infertility per se, is still an open question. The reason for such behavior is still an enigma and the literature has been far from giving indicative clues to answer these questions.

In a previous prospective controlled study examining Protein C (ProC) Global assay in ART singleton pregnancies compared to naturally conceived pregnancies, our group has shown that ProC Global decrease gradually during pregnancy in both groups [11]. These findings were corroborated later by another group showing decreasing ProC Global assay levels in healthy women during pregnancy [12]. In our study, the decrease in ProC Global was found to be significantly more pronounced in the ART group as compared to the healthy controls. Interestingly, when both groups were compared 6 weeks or more following delivery, ProC Global was found to be significantly lower in the ART group as compared to controls [11], suggesting that infertile women before ART could be a priori distinct from fertile healthy women conceiving spontaneously.

ProC Global assay is an activated partial thromboplastin time (APTT)-based plasma assay that has been developed, to test the global function of the protein C anticoagulation pathway. The assay has been shown suitable to detect hereditary or acquired defects in the protein C system, including protein C deficiency, protein S deficiency, activated protein C resistance and factor V Leiden mutation [13–16]. Moreover, ProC Global assay has also been suggested to potentially reveal yet undetermined abnormalities in the protein C pathway [17].

Protein C pathway abnormalities are the most frequent biological risk factors for venous thromboembolism (VTE) and they can be demonstrated in up to 30 % of white patients with thrombophilia [18–20]. Moreover, the majority of thrombophilias associated with placental vascular complications during pregnancy have been related to the protein C pathway [21, 22].

Taken together, growing evidence is accumulating to suggest that singleton pregnancies achieved following ART are at an increased risk of prematurity and low birth weight. Whether these increased risks are the result of the technology (ART) itself or infertility per se is still an open question. Recent preliminary evidence coming from the ProC Global assay may suggest that these perinatal risks may be associated with infertility per se and not only the technology.

Since our previous study, was performed in pregnant women and in the postpartum period [11], our intention in this study was to gain insight into the relation between ProC Global assay an infertile women eligible for ART treatment before starting therapy and prior to pregnancy achievement. By this way any hormonal treatment or pregnancy related affect on ProC Global assay would be eliminated. Therefore, our aim in this study was to explore this question by evaluating Protein C (ProC) Global assay in infertile women before ART treatment. In addition, our goal was to investigate the association between ProC Global assay and different inherited and acquired thrombophilias.

Methods

The study was prospectively performed at the Poriya and Rambam Medical Centers. A cohort of healthy infertile women eligible for in-vitro fertilization and embryo transfer (IVF-ET) was recruited for the study. The control group included matched healthy fertile women that their blood samples were taken for the measurement of the reference range in the Thrombosis and Hemostasis laboratory at the same time period the blood was taken for the study group. Women in both groups did not have a history of venous thrombo-embolism, known acquired or congenital thrombophilia, a history of repeat pregnancy loss or previous vascular gestational complication and did not receive heparin or low molecular weight heparin upon recruitment or during a previous pregnancy. The exact information about pregnancy history of the control group is not available.

The study group included 95 unselected and consecutive infertile women between 18 and 44 years of age and body mass index of 18–34 kg/m^2. All women were spontaneously menstruating with two intact ovaries and no previous ovarian surgery. Women in the study were determined to have normal uterine cavities by hysterosalpingography and/or hysteroscopy. As well, patients with uncontrolled thyroid disease, diabetes mellitus or hyperprolactinemia were excluded from the study. The matched control group included 77 healthy women with the same age and ethnicity evaluated at the same time period as the study group.

The research project was approved by the Institutional Review Board, and an informed consent form was signed by each woman participating in the study.

In vitro fertilization and embryo transfer treatment, including patient screening and recruitment, ovarian stimulation and endometrial preparation, handling of oocytes, sperm, zygotes and embryos, as well as the embryo transfer technique were similarly performed in all women as routinely carried out in our centers [23, 24].

Venous blood was drawn for ProC Global assay in the study group before ART treatment. Blood samples were collected in both study and control groups by venipuncture into 3.2 % sodium citrate tubes and centrifuged at 2,000g for

15 min. Plasma samples were frozen after second centrifugation at 2,000*g* for 15 min. Supernatant plasma aliquots were frozen at –70±5 °C for further evaluation of ProC Global. Before testing, plasma aliquots were thawed in 37±0.5 °C water bath for 15 min.

ProC Global is based on activation of endogenous protein C by Protac (an extract from Agkistrodon contortrix venom). The assay was performed on the STA-R evolution analyzer (Diagnostica Stago, Gennevilliers, France). Protein C activation time (PCAT) was measured either with Protac® or buffer (to determine PCAT-0). Results were expressed as PCAT normalized ratio (PCAT-NR) calculated by dividing the PCAT ratio (PCAT/PCAT-0) of sample's plasma by a PCAT ratio of lyophilized standard human plasma (ORKL; Siemens Healthcare Diagnostics Product, Marburg, Germany) and multiplying by a lot-specific sensitivity value (SV) defined by the manufacturer for each batch of standard plasma. The intra-assay coefficient of variation was determined for the PCAT-NR from the mean of two separate runs of 10 replicates each and was found to be 5.0 %. The inter-assay coefficient of variation for the PCAT-NR was calculated from the mean of seven separate runs and was found to be 3.2 % [25]. The cutoff level of 0.8 was found to have a sensitivity of 100 % for factor V Leiden mutation [14, 15]. Therefore, PCAT-NR less than 0.8 (≤ 0.8) was considered abnormal.

In addition, all women in the study group underwent a full thrombophilia panel testing. All coagulation assays were performed on thawed frozen plasma samples on the STA-R evolution analyzer (Diagnostica Stago, Gennevilliers, France). APC-R was performed by Coatest APC resistance (Chromogenix - Instrumentation Laboratory SpA Milan, Italy) kit. Protein C and anti-thrombin III activity were measured by STA-STACHROM® Protein C and anti-thrombin kits, respectively, (Diagnostica STAGO, France). Levels of free protein S antigen were determined by immunoturbidimetric assay by STA-Liatest® Free Protein S kit (Diagnostica STAGO, France). Levels of coagulation factor VIII activity were measured by one-stage assay using factor VIII deficiency plasma (Stago Diagnostica).

Lupus anticoagulant was evaluated by the DRVVT assay (Screen and Confirm, Gradipore, North Ryde, Australia) and APTT assay (Diagnostica STAGO, France). Positive results were further confirmed by diluted PT assay. Anti-Cardiolipin antibodies (Ig G and Ig M) were evaluated from plasma by the enzyme-linked immuno-sorbent assay (ELISA), (AESKU. Diagnostica, Mikro-forum Ring-2, Wendelsheim, Germany).

The genetic thrombophilic risk factors; i.e. Factor V Leiden, Pro-thrombin (PT) G20210A and MTHFR C677T mutations were evaluated by Real-Time PCR Using Fluorescent Resonance Energy Transfer (FRET) Method on the Rotor Gene 3000 (Corbett Research, Sydney, Australia).

Statistical analysis

We analyzed all data using the Software Package for Social Sciences (SPSS) for windows version 15.0 (SPSS Inc. 2006, Chicago, IL, USA). Descriptive procedure was used to evaluate patients' characteristics and each variable is presented as mean±SD. Normal distribution was analyzed prior to statistical tests using Wilk-Shapiro Test. *T*-test for two independent samples were performed to compare (a) the ProC Global assay levels in the study and control groups in relation to number of previous IVF cycles (≤ 3 or ≥4 cycles) and (b) the ProC Global assay level in accordance with presence or absence of thrombophilia. Furthermore, a multivariate linear logistic regression analysis was performed to detect which factor may have affected ProC Global assay level or which factor may have been associated with a normal (> 0.8) or abnormal ProC (< 0.8) Global assay level. Spearman and Pearson correlation tests were performed to examine the relationships between ProC Global assay level and the existence of thrombophilia. P value of <0.05 was considered as statistically significant.

Results

Table 1 summarizes the patients' characteristics of the 95 infertile women enrolled for the study eligible for IVF-ET treatment (study group). The control group comprised a matched 77 healthy fertile women evaluated for ProC

Table 1 Patients characteristics' in the study cohort. Values are presented as mean±SD or as numbers (and incidence)

	Study group (*n* = 95)	Range
Age	34.4±5.7	21–44
BMI (Kg/m2)	27.5±6.5	18.4–37.7
Infertility duration (y)	6.4±4.3	1–19
Infertility order		
Primary infertility	40 (42.1 %)	
Secondary infertility	31 (32.6 %)	
Primary and secondary	24 (25.3 %)	
Infertility etiology		
Male factor	44 (46.3 %)	
Mechanical factor	13 (13.7 %)	
Unexplained	24 (25.3 %)	
Others	14 (14.7 %)	
Number of past IVF cycles	3.6±3.9	0–15
Chronic Smoking	20 (21.0 %)	
Gravidity	1.0±1.2	0–5
Parity	0.5±0.70	0–3
Number of children	0.6±0.70	0–3
Number of abortions	0.4±0.8	0–3

Global assay during the same time period of the study group. The mean age of both groups did not differ significantly, corresponding to 34.4±5.7 and 35.6±6.2 years, in the study and control groups, respectively.

As illustrated in Fig. 1, mean ProC Global assay level was found to be significantly lower in the study as compared to control group, corresponding to 0.78±0.16 and 0.88±0.16, respectively ($P<0.01$). As well, abnormal ProC Global assay level of≤0.8 was significantly higher in the study as compared to control group corresponding to 53 % and 29 %, respectively.

Notably, the mean number of previous IVF cycles in the study group was considerable, 3.6±3.9 cycles (Table 1). In order to examine the impact of this finding on the ProC Global assay level in the study, the study group was divided into two sub-groups; the first with≤3 and the second≥4 previous IVF failures. The two sub-groups had similar ProC Global assay level; in addition, both of these sub-groups had significantly lower ProC Global assay level when compared to the control group. These results negate a possible effect of repeat IVF failure on ProC Global assay findings in our study.

Furthermore, a multivariate linear logistic regression analysis was performed to detect which factor may have affected ProC Global assay level in the study group. Age, infertility duration, BMI, gravidity, parity, number of previous abortions and number of living children as well as number of previous IVF failures were not associated with ProC Global assay level ($P>0.05$).

Similarly, a multivariate linear logistic regression analysis was performed to detect which factor may have been associated with a normal (> 0.8) or abnormal ProC (≤ 0.8) Global assay level in the study group. Age, BMI, infertility duration, order or etiology as well as number of previous IVF failures, smoking, gravidity, parity, number of previous abortions, and number of living children were not associated with a normal or abnormal ProC Global assay level ($P>0.05$).

Table 2 summarizes the association between ProC Global assay results and thrombophilic risk factor levels in the study group. No case of protein S deficiency and only one case each of protein C deficiency, anti-thrombin III deficiency and positive lupus anticoagulants were encountered in the study group; therefore the relation of these thrombophilias to ProC Global assay could not be separately evaluated. ProC Global assay levels were significantly lower in women within the study group found to have APCR, factor V Leiden and high factor VIII level when compared to women without these thrombophilic risk factors. Other thrombophilias tested including anti-cardiolipin anibodies, PT mutation and MTHFR mutation were not related to ProC Global assay results. Furthermore, ProC Global assay was significantly lower in women within the study group with any detected thrombophilia as compared to others with no thrombophilia. Similarly, women with combined thrombophilia had significantly lower ProC Global assay level compared to women with no thrombophilia.

Spearman and Pearson correlation tests were also performed to examine any link between ProC Global assay level and the existence of thrombophilia in the study group. Spearman test found a significant correlation between a low

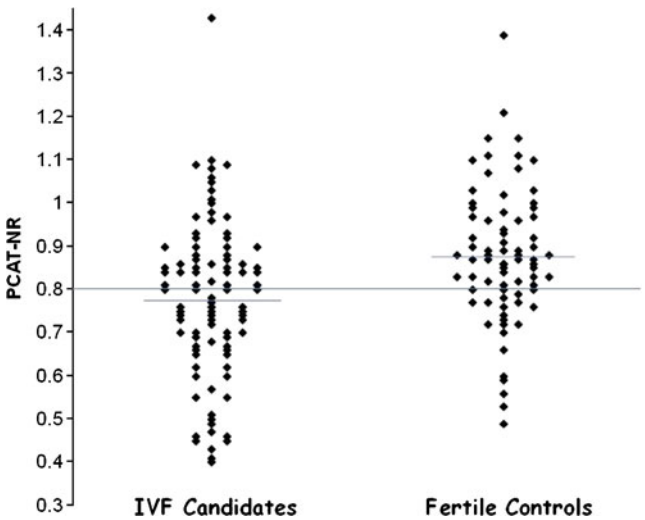

Fig. 1 Protein C Global assay level (PCAT-NR) in infertile women before IVF-ET treatment and fertile controls

Table 2 Comparison of mean ProC Global assay level±SD in accordance with presence or absence of thrombophilia*

		Mean ProC Global assay level	P***
Anticardiolipin antibodies (IgG+Ig M)	Negative (42)	0.80±0.20	0.09
	Positive (11)	0.70±0.20	
Activated Protein C Resistance (≤ 2.00)	< 2.00 (12)	0.51±0.12	<0.001
	> 2.00 (82)	0.82±0.15	
Factor V Leiden	Normal (80)	0.83±0.14	<0.001
	Homo/hetero (14)	0.48±0.06	
Factor VIII (u/dl)≥180	< 180 (84)	0.80±0.20	<0.05
	≥ 180 (10)	0.67±0.16	
MTHFR mutation	Normal (41)	0.78±0.17	0.82
	Homo/hetero (51)	0.77±0.19	
PT mutation	Normal (89)	0.80±0.18	0.52
	Homo/hetero (3)	0.71±0.34	
**Any thrombophilia	0 (64)	0.84±0.15	< 0.001
	≥ 1 (31)	0.65±0.19	
** Combined thrombophilia	≤ 1 (75)	0.84±0.14	< 0.001
	> 2 (20)	0.56±0.16	

Numbers between brackets are numbers of tests (sample size) performed for each thrombophilia

* Protein S, protein C, anti-thrombin III and lupus anticoagulants calculations were not included in this table since only one single case or no cases of such thrombophilias were found in the study

** MTHFR was not included in the evaluation

*** *T*-test for two independent samples

ProC Global assay level (≤0.8), APCR (r_s=0.359, P<0.001), factor V Leiden (r_s=−0.409, P<0.001), any encountered thrombophilia (r_s=−0.341, P<0.001) and combined thrombophilia (r_s=−0.436, P<0.001). Pearson test found a significant correlation between the absolute ProC Global assay value, APCR (r=0.512, P<0.001), factor V Leiden (r=−0.601, P<0.001), high factor VIII level (−0.205, P<0.05), any encountered thrombophilia (r=−0.453, P<0.001) and combined thrombophilia (r=−0.620, P<0.001).

Discussion

Our prospective cohort matched control study clearly shows that mean ProC Global assay level is significantly lower in infertile women, undergoing ART treatment, as compared to fertile controls. As well, abnormal ProC Global assay level rate is significantly higher in infertile women as compared to fertile controls. These results corroborates with our findings in a previous study examining ProC Global assay 6 weeks or later following delivery [11]. Our present study strengthens the results of our previous publication [11] and totally eliminates any hormonal or pregnancy related explanations for the difference encountered in ProC global assay level results between infertile women and fertile controls.

These results support the idea that infertile women may be unique in their Protein C anticoagulation pathway characteristics. It has been shown that the majority of thrombophilias associated with placental vascular complications during pregnancy have been related to the protein C pathway [21, 22]. It is possible that the lower level we found in ProC Global assay in the infertile women compared to fertile controls could explain their tendency to develop pregnancy complications leading to an increased rate of preterm birth and low birth weight. Whether ProC Global assay could be utilized as a potential bio-marker of perinatal complications before ART treatment in the infertile population is still to be investigated.

ProC Global assay has already been explored in women with recurrent thrombo-emolism [16, 17], thromboemolic phenomena under hormonal therapy [25] repeat pregnancy loss [26] and in women with severe pre-eclampsia and HELLP syndrome [27]. Moreover, it has been recently explored in women throughout pregnancy; following ART [11] or spontaneous conception [11, 12]. To the best of our knowledge, ProC Global assay has not yet been explored in relation to other pregnancy related complications such as placental abruption, intra-uterine growth restriction, pre-term birth and intra-uterine fetal death.

Our study also has found that ProC Global assay results correlated to APCR, factor V Leiden, any encountered thrombophilia and combined thrombophilia. These results corroborate with other studies in the literature exploring the importance of ProC Global assay and strengthen its relation to thrombophilias within the Protein C anticoagulation pathway [13–17]. On the other hand, our study have found that ProC Global assay was not related to age, infertility duration, order or etiology, BMI, gravidity, parity, number of previous abortions and number of living children as well as number of previous IVF failures.

All told, a word of caution should be added. Although our results hold up to the idea that the different results concerning ProC Global assay between infertile and fertile controls is related to dissimilar Protein C anticoagulation systems in these patients, other different still uncovered explanation could not be ruled out. It is possible that the lower ProC Global assay in the infertile population is or only a marker/indicator of a different explanation waiting to be revealed. Further studies are encouraged to explore this behavior.

Furthermore, no matter what is the explanation for the lower ProC Global assay in the infertile ART candidates as compared to the fertile controls, our findings support the notion that the increased perinatal risks, encountered in the literature, in singleton pregnancies following ART treatment, is related to infertility per se and not to the technology.

When revising the literature, several maternal factors, closely associated to infertility, may be implicated in the development of preterm birth and low birth weight, following ART therapy, increasing the risk of perinatal mortality and morbidity. These may include uterine anomalies [28], uterine leimyomata [29], hormonal imbalances specifically PCOS [30], the vanishing twin syndrome [31], low ovarian reserve [32] as well as thrombophilia [33]. Most of these factors are encountered in fertile as well as infertile populations making their investigation and total isolation as well as their impact on perinatal risks more complex.

On the other hand several other ART factors, related to the technology itself, have been recently implicated in the increasing perinatal risks in women achieving singleton pregnancy following treatment. High E_2 level above 3,450 pg/mL on the day of hCG administration during COH, was found to be associated with greater odds of developing preeclampsia and delivery of a small for gestational age infant in singleton pregnancies resulting from IVF cycles [34]. In another recent retrospective database analysis, extended culture of embryos from cleavage stage to blastocyst stage has been shown to increase the risk of preterm delivery [35]. As well, there is accumulating data to show that children born after frozen-thawed embryo transfer have higher birth weight and fewer adverse perinatal outcomes than do children born after fresh IVF-ICSI [10, 36].

It could be argued that our ProC Global findings are not representative since a high proportion of repeat IVF failure has been recruited to the study. Current literature has conflicting findings in relation to the role of thrombophilia and its impact

on repeat IVF failure. While some studies have suggested that repeat IVF failure may be associated with an increased incidence of thrombophilia [37–41] others have negated such an association [42–44]. In a recent systemic review and meta-analysis, pooled data from patients in 8 case control studies showed an overall 3-fold increased risk of ART failure in association with factor V Leiden; while in the 3 cohort studies there was no difference in outcome between those with and those without factor V Leiden mutation [45]. Although our study was not targeted to examine the association between thrombophilia and repeat IVF failure, our findings do not support such an association. Furthermore, our results imply that ProC Global cannot be suggested as a bio-marker of such an occurrence. Most important and specifically related to the current study, the significant difference found between ProcC Global assay in the infertile and fertile controls, in our study, is not the result of repeat IVF failure but to other factors that are still to be revealed.

Another drawback of the current study that could be brought up is that the control group did not undergo a full thrombophilic evaluation. It could be argued that the reason for the reduced ProC Global in the infertile women before IVF-ET is a higher incidence of thrombophilia in the study group as related to controls. Although it could be true, it does not alter the finding that infertile women are diverse from healthy ones and this may explain the difference, encountered in the literature, between singleton IVF-ET pregnancies and controls.

In conclusion, in a prospective cohort matched controlled study we have shown that mean ProC Global assay results are significantly lower in infertile patients, candidates for ART treatment as compared to fertile healthy controls. Low ProC Global assay results were correlated with APCR, factor V Leiden and combined thrombophilias implying that infertile women are unique in their Protein C anticoagulation characteristics. While these findings may be or may be not directly or indirectly related to hypercoagulability in infertile patients, they certainly support the notion that the increased perinatal risks, encountered in the literature, in singleton pregnancies following ART treatment, is related to infertility per se and not only to the technology.

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