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Abstract

Pulmonary thromboembolism (PTE) is one of the leading causes of maternal mortality. We previously reported that possible contamination of amniotic fluid (AF) into maternal circulation accelerated thrombin production and activated platelet function in maternal blood through the extrinsic pathway, which may be associated with the high incidence of PTE in early puerperium. However, it remains unclear whether the maternal anticoagulation system, e.g., the activated protein C (APC) pathway, contributes to the hypercoagulable condition induced by AF. Our previous study using an endogenous thrombin potential (ETP)-based assay revealed that sensitivity to APC was reduced during the postpartum first day, i.e., immediately after delivery, when parturients were supposed to be exposed to AF. Our aim is to investigate the susceptibility of maternal plasma to APC when mixed with AF. We collected plasma from 51 pregnant females and mixed with AF as well as APC. APC-sensitivity ratio (APC-sr) was calculated using the ETP-based assay. Addition of AF to maternal plasma showed a significant increase of ETP in the presence of APC. APC-sr was significantly increased, indicating decreased sensitivity to APC, after AF mixture to maternal plasma. The present APC-sr difference with AF contamination was smaller than that we reported previously in venous thromboembolism cases. The inhibitory

effects of AF on the APC anticoagulation pathway may contribute, at least partly, to further promotion of thrombin production induced by AF. Combined with other classical thrombophilic risk factors, the present findings support possible involvements of AF exposure in the high incidence of PTE in early puerperium.

Key points

1. Amniotic fluid accelerated thrombin production and activated platelet function in maternal whole blood through the activated tissue factor pathway based on our previous *in vitro* report using rotational thromboelastometry.

2. Our previous study showed that the sensitivity to activated protein C was reduced in late pregnancy and continued to decrease during the postpartum first day, immediately after delivery, when parturients were supposed to be exposed to amniotic fluid.

3. In the present study, amniotic fluid slightly but significantly decreased the anticoagulation function of activated protein C pathway in maternal plasma in an endogenous thrombin potential-based assay.

4. The inhibitory effects of amniotic fluid on the activated protein C pathway may be one of the factors contributing to the development of venous thromboembolism during the

peripartum period.

5. Further study is needed to identify the potential factors contributing to reduction in sensitivity to APC in amniotic fluid.

Keywords: Activated protein C; Amniotic fluid; Coagulation; Endogenous thrombin potential; Pregnancy; Pulmonary thromboembolism.

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Introduction

Pulmonary thromboembolism (PTE) is one of the leading causes of maternal deaths worldwide [1]. Its incidence was reported to be the highest during the postpartum first day and its risk was 22-fold higher in cesarean delivery than in vaginal delivery [2]. Amniotic fluid (AF) typically contacts with maternal blood at membrane rupture and/or during cesarean section. Its components were present locally [3] and systemically [4] during the peripartum period, indicating that AF directly entered the maternal circulation. Previous study with the coagulation-related molecular markers showed that thrombin production was activated immediately after delivery and continued during early puerperium [5]. By focusing on significant changes in blood coagulation function immediately after delivery, we previously showed using rotational thromboelastometry that the *in vitro* treatment of AF promoted thrombin production and activated platelet function, thereby accelerating blood coagulation in maternal whole blood through the extrinsic pathway [6]. This finding prompted us to speculate that AF contamination in the maternal circulation may be responsible for the high incidence of PTE in early puerperium after exposure to AF, particularly during cesarean delivery [6]. However, it currently remains unclear whether the maternal anticoagulation system, i.e., the physiological functions

essential for the regulation of blood coagulation and prevention of thrombus formation, contributes to the hypercoagulable condition induced by AF.

Therefore, we herein specifically focused on the activated protein C (APC) pathway. APC is an anticoagulant serine protease enzyme that inhibits the procoagulant functions of activated Factor V (FVa) and VIII (FVIIIa) with protein S (PS) as a co-factor [7]. We previously demonstrated using an endogenous thrombin potential (ETP)-based assay that sensitivity to APC was reduced in late pregnancy and continued to decrease during the postpartum first day [8], suggesting that parturients diminished susceptibility to APC throughout delivery, when they were supposed to be exposed to AF [3]. The present study was planned as an *in vitro* study to investigate whether a treatment with AF affects the maternal APC anticoagulation system based on alterations in thrombin production and sensitivity to APC with an ETP-based assay.

Methods

We recruited healthy Japanese females in the third trimester of pregnancy for the present study. All patients were scheduled for elective cesarean section, labor induction, and delivered at the Hamamatsu University Hospital between May 2018 and February 2020.

Patients had no complications involving platelets or blood coagulation as well as no history of antiplatelet and anticoagulant medication. We collected 5 mL of blood using our reported technique [6]. Whole blood was centrifuged at 3,000 rpm at 4°C for 10 minutes; thereafter, the supernatant (citrated plasma) was aliquoted and immediately stored at -30°C until analyzed. Normal pooled plasma from healthy male volunteers was used as the control because we considered that non-pregnant female plasma was not appropriate for ETP-based assays as the control due to the following reasons. 1) We found significant increase of ETP levels during the luteal phase compared with those during the follicular phase among non-pregnant female volunteers as described in **Table S1** and **Figure S1**. 2) The same trend of ETP increase during the luteal phase has been reported in the previous study [9]. 3) Therefore, it is difficult to obtain reliable results in ETP-based assays with high reproducibility when we use female plasma as the control. 4) On the other hand, we did not find specific trends in the change of ETP levels in male volunteers on the different 2 days with two weeks interval (**Table S1**). The antigen levels of PS, FV, and FVIII in maternal plasma were assessed with the enzyme-linked immunosorbent assay kits of AssayMax Human Protein S, AssayMax Human Factor V (Assaypro, MO, USA), and VisuLize Factor VIII (Affinity Biologicals, ON, Canada), respectively. We used the same

AF samples collected in our previous study [6]. The mixture of crude AF was stored at -80°C and thawed at room temperature just before the assay.

The ETP-based assay required the defined amount of 80 μL of sample or control plasma for each well in a 96-well microtiter plate (Corning, NY, USA). AF was mixed with plasma at a volume of 0, 1, 4, and 8 μL with the replacement of an identical amount of sample plasma (80, 79, 76, and 72 μL of plasma, respectively), thereby producing a total amount of 80 μL in each well. The volume of AF mixed into maternal plasma was selected based on our previous study [6]. A thrombin calibrator, tissue factor/PPP-Reagent, fluorogenic substrate, and buffer (Thrombinoscope, Maastricht, the Netherlands) as well as purified APC (Teijin Pharma, Tokyo, Japan) derived from human were used in the assay. The ETP-based APC sensitivity assay was performed and analyzed as we reported [8]. The suppression rate of ETP was calculated as ETP with APC divided by that without APC. The APC-sensitivity ratio (APC-sr) was defined as the suppression rate of sample ETP divided by that of the control male pooled plasma. Reduced sensitivity to APC was indicated by an elevated APC-sr value. Data were shown as numbers or means with standard deviations. ETP values between each pair with and without APC at the same amount of AF, among samples in the presence and absence of APC, and APC-sr were

examined by a one-way repeated measures analysis of variance. A two-sided P value < 0.05 was defined as being significant. Each P value was adjusted to account for multiple comparisons. Prism version 7 (GraphPad Software, CA, USA) was used for statistical calculations. The present study was approved by the Institutional Review Board of Hamamatsu University School of Medicine (No. 16-165) and written informed consent was obtained from patients.

Results

We measured ETP with four different amounts of AF in all 51 subjects included in the present study, followed by the calculation of APC-sr. The baseline characteristics of patients are outlined in **Table 1**. **Figure 1a** shows ETP changes with the mixing of a specific volume of AF in the presence and absence of APC through the ETP-based assay. In comparisons between each pair with the same amount of AF, all ETP levels were significantly reduced after the addition of APC. Without APC, ETP levels were significantly lower when 8 μL (one-tenth volume of maternal plasma) of AF was mixed than when 1 μL was mixed, which may have been due to the dilution effect by AF. On the other hand, under the anticoagulation effect by APC, ETP levels were significantly higher

with mixed AF volumes of 1, 4, and 8 μL than without the contamination of AF. APC-sr of maternal plasma significantly increased in an AF volume-dependent manner (**Fig. 1b**).

Discussion

The present study was the first to demonstrate that a treatment with AF significantly reduced sensitivity to APC in maternal plasma in the third trimester. The attenuation of APC anticoagulation functions by a mixture of AF may contribute partly to enhancements in maternal blood coagulation as one of the procoagulant systems, in addition to the increased production of thrombin and activated platelet function, which we previously reported [6]. We previously demonstrated that sensitivity to APC was reduced during pregnancy and continued to decrease immediately after delivery [8]. In the present study, the results of the *in vitro* experiment showed that AF reduced sensitivity to APC in maternal plasma, indicating that exposure to AF during delivery is associated, at least partly, with this marked alteration and further with the high incidence of PTE in early puerperium. More detailed studies are needed to prove this speculation.

ETP levels were significantly reduced without APC in a mixture of 8 μL of AF, which suggested dilutional effects on maternal coagulation factors by AF; however, this

decrease in ETP was cancelled in the presence of APC **(Fig. 1a)**. We speculated that the treatment with AF may predominantly inhibit anticoagulation by APC rather than its dilutional effect under the specific condition of suppressed thrombin production. Furthermore, the most prominent difference observed in APC-sr in the present study, i.e. by approximately 1 between the mean of APC-sr without AF and that with 8 μL of AF **(Fig. 1b)**, was similar to the change reported between the late gestational period and the first day of puerperium [8]. While we previously demonstrated that decreased responses to APC contributed to venous thromboembolism (VTE) [10], the present APC-sr difference between groups was markedly smaller than that reported in VTE cases (by approximately 3) in our previous study [8]. Therefore, the inhibitory effects of AF on the APC anticoagulation pathway may be one of the factors contributing to the development of VTE during the peripartum period. Based on the potential role of AF exposure in the development of VTE, the inhibition of the APC anticoagulation pathway may partly contribute to the promotion of thrombin production, which may further accelerate blood coagulation via the extrinsic pathway in addition to the other procoagulant mechanisms we reported [6]. Combined with other classical risk factors, such as immobilization, thrombophilia, obesity, and surgery, the present findings support a possible involvement of

AF exposure in the high incidence of PTE, particularly immediately after delivery. Some serine protease inhibitors, such as alpha 1-antitrypsin [11] and protein C inhibitor [12], which are present in AF, are potential factors contributing to reductions in sensitivity to APC with the exposure of maternal blood to AF. The limitations of the present study were as follows: 1) this study was an *in vitro* investigation using maternal plasma; therefore, it wasn't possible to assess platelet function, and 2) plasma obtained during labor and/or in the immediate postpartum period wasn't included because the Institutional Review Board didn't permit plasma collection under these conditions for research purposes.

Conclusion

AF reduced susceptibility to APC and attenuated the maternal APC anticoagulation system, which may have, at least partly, contributed to enhanced maternal blood coagulation and further possibly to the incidence of PTE under the specific situation in early puerperium.

Declarations

Funding

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Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material and Code availability

We made sure that all data and materials as well as software application supported our claims.

Authors' contributions

Divyanu Jain participated in data acquisition and analysis and preparing the manuscript. Tomoaki Oda contributed to the main concept and design of the study and performed the research, including sample collection, data analysis and interpretation, and writing of the manuscript. Kenta Kawai, Yoshimasa Horikoshi, Masako Matsumoto, Megumi Narumi, Yukiko Kohmura-Kobayashi, Naomi Furuta-Isomura, Chizuko Yaguchi, Toshiyuki Uchida, and Kazunao Suzuki contributed to sample collection and data acquisition. Naohiro Kanayama and Hiroaki Itoh supervised the research and contributed to its concept and design. Naoaki Tamura contributed to the interpretation of data, the writing and revision of the intellectual content of the manuscript, and supervised the research. All authors have approved the final version.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. The present study was approved by the Institutional Review Board of Hamamatsu University School of Medicine (No. 16-165).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figure Legends

Figure 1 (a-b). Effects of amniotic fluid on the maternal activated protein C anticoagulation system.

Each plot with a bar represents the mean and standard deviation of data collected from 51 pregnant subjects.

(a) Changes in the endogenous thrombin potential (ETP) with and without activated protein C (APC) following contamination with amniotic fluid.

All ETPs decreased after the addition of APC in each pair of the same mixed volume of amniotic fluid. In the presence of APC, ETP levels were significantly higher with the mixing of amniotic fluid at amounts of 1, 4, and 8 μL than in the absence (0 μL) of amniotic fluid contamination (filled circles).

(b) The activated protein C-sensitivity ratio (APC-sr) with the mixture of amniotic fluid. APC-sr was significantly increased by the contamination of amniotic fluid in a

volume-dependent manner, indicating that increasing volumes of amniotic fluid resulted in further reductions in maternal sensitivity to APC.

Table S1. Endogenous thrombin potential (ETP) levels in non-pregnant females during the different menstrual periods and those in males on the different 2 days with two weeks interval.

Non-pregnant female subject No.	Age (year)	Follicular phase			Luteal phase		
		E_2 (pg/mL)	P_4 (ng/mL)	ETP (nM×min)	E_2 (pg/mL)	P_4 (ng/mL)	ETP (nM×min)
1	34	39.5	0.23	1126	60.1	4.77	1665
2	26	< 20	0.30	1267	99.5	11.2	1274
3	43	< 20	0.23	1126	81.2	9.84	1227
4	33	40.3	< 0.2	1275	128	16.86	1476
5	31	26.5	0.58	1330	140	17.98	1570
6	28	53.8	0.37	1164	43.7	3.09	1178
7	31	< 20	0.15	1106	149	10.60	1206
Male subject No.	Age (year)	Initial blood collection			Two weeks after initial blood collection		
		E_2 (pg/mL)	P_4 (ng/mL)	ETP (nM×min)	E_2 (pg/mL)	P_4 (ng/mL)	ETP (nM×min)
1	33	< 20	0.43	1044	< 20	0.34	1139
2	35	< 20	0.32	1306	< 20	0.36	1149
3	27	< 20	0.53	1181	< 20	0.70	1049

We measured estradiol (E_2) and progesterone (P_4) levels among seven non-pregnant female as well as three male volunteers by competitive assays with AIA-900 (TOHSOH, Tokyo, Japan). The lower thresholds of E_2 and P_4 are 20 pg/mL and 0.2 ng/mL, respectively. ETP levels were evaluated with the same method in the present study.

Abbreviations: E_2, estradiol; P_4, progesterone; ETP, endogenous thrombin potential.

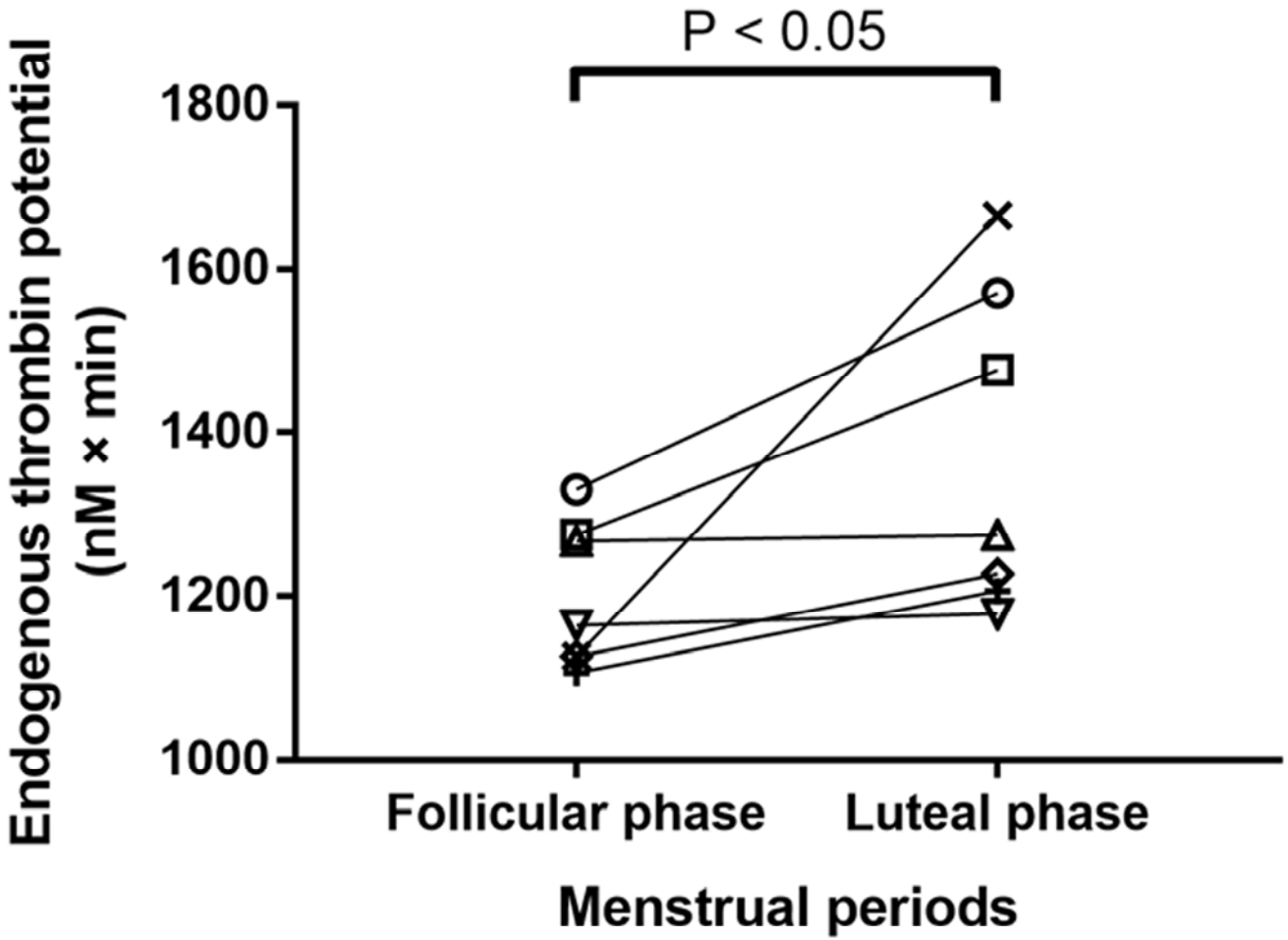


Figure S1. The significant increase of endogenous thrombin potential (ETP) levels during the luteal phase compared with those in the follicular phase in non-pregnant females.

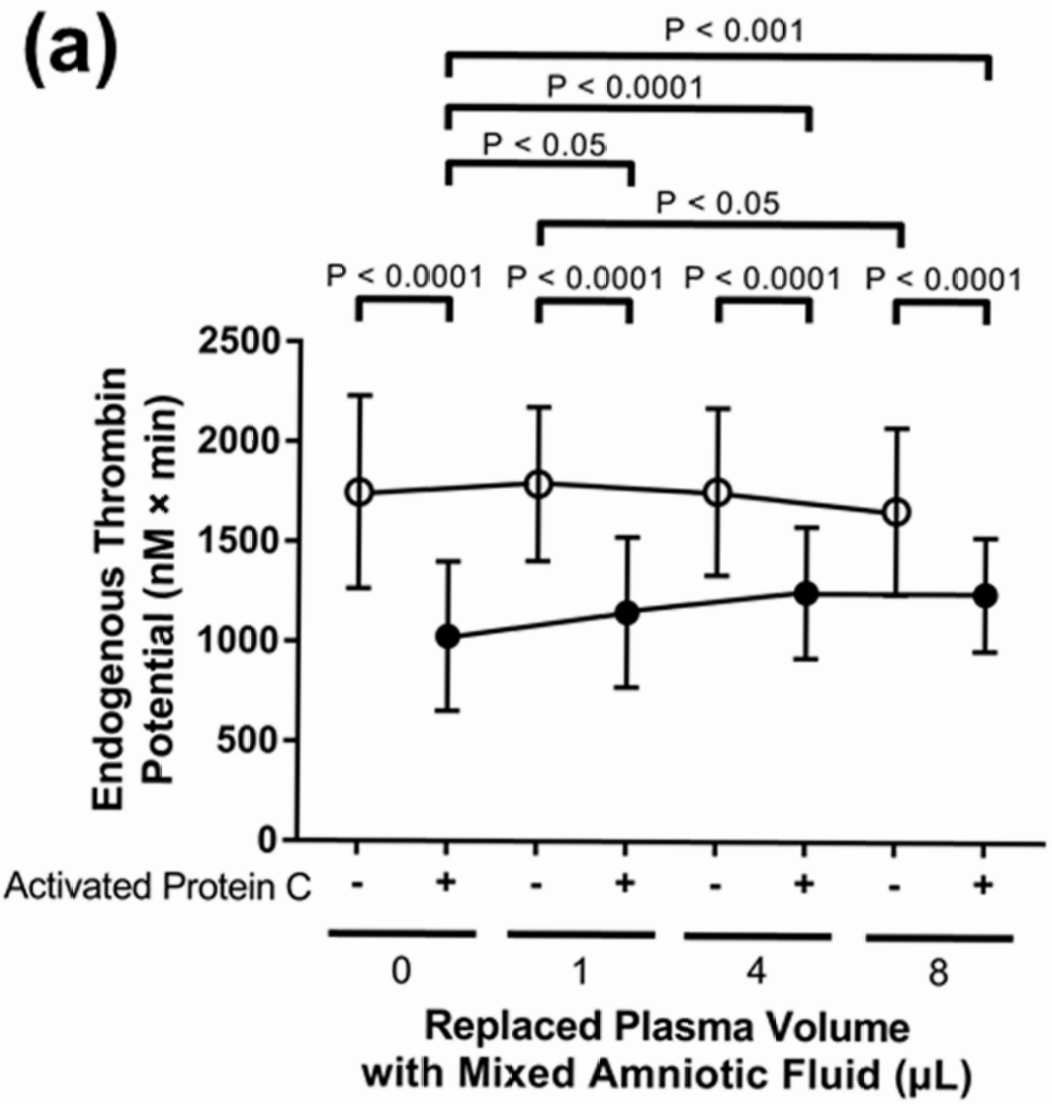
We evaluated ETP levels in seven non-pregnant female volunteers during their follicular and luteal phases with the same method in the present study in the absence of activated protein C. The same symbols indicated the identical subjects. The ETP values increased significantly during the luteal phases analyzed with the Wilcoxon test. The detailed results of estradiol, progesterone, and ETP of the subjects are described in **Table S1**.

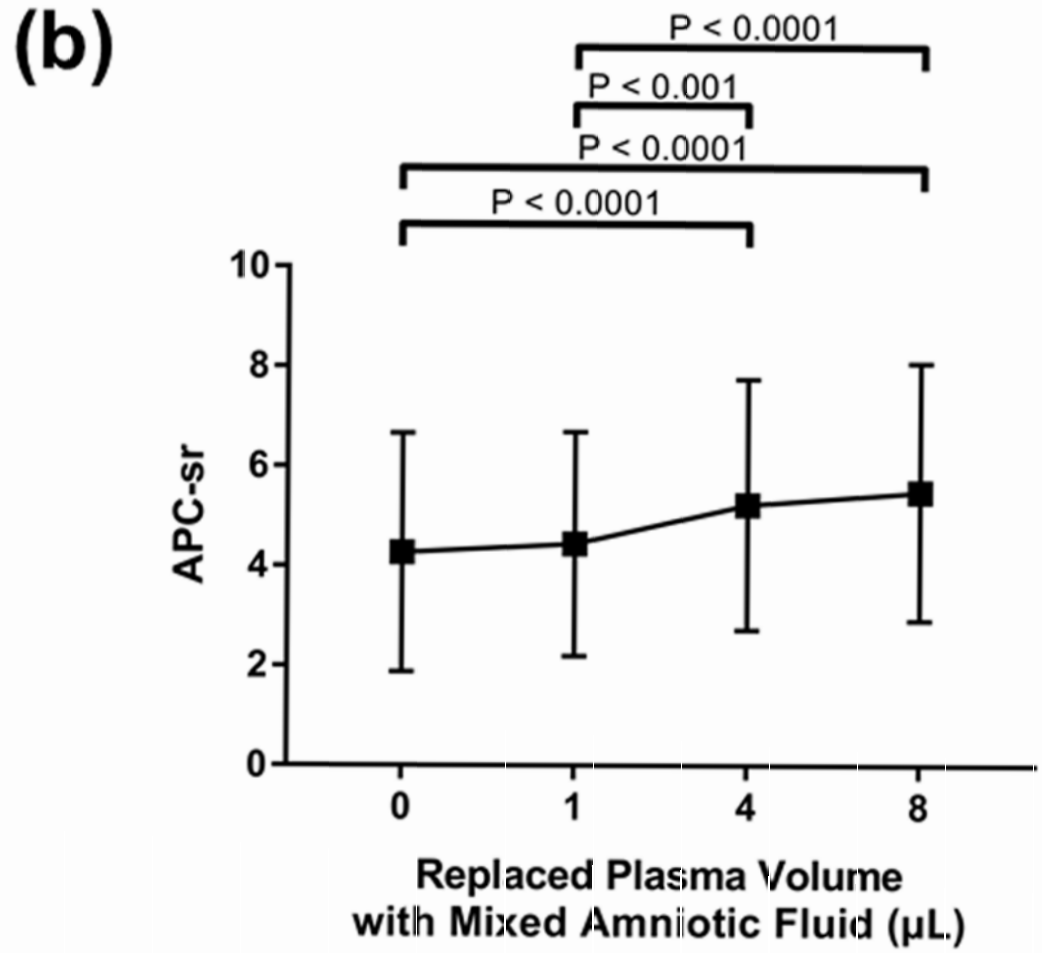
Table 1. Backgrounds of patients as a mean (SD) or number.

	Study group (n = 51)	
Age (year)	33	(4.0)
Parity (case)		
0	15	
1	28	
2 or more	8	
Body mass index at plasma collection (kg/m^2)	26.6	(4.6)
Gestational age at plasma collection (day)	264	(7.2)
Platelet count ($\times 10^9/\mu L$)	249	(68.9)
PT-INR	0.91	(0.05)
Fibrinogen (g/L)	4.41	(0.64)
Factor V antigen (μg/mL)	1.51	(0.75)
Factor VIII antigen (μg/mL)	1.69	(0.80)
Protein S antigen (μg/mL)	10.9	(3.40)

Abbreviations: SD, standard deviation; PT-INR, prothrombin time-international normalized ratio.

Figure 1.


(a)
P < 0.001
P < 0.0001
P < 0.05
P < 0.05
P < 0.0001
P < 0.0001
P < 0.0001
P < 0.0001
Endogenous Thrombin Potential (nM × min)
2500
2000
1500
1000
500
0
Activated Protein C
- + - + - + - +
0
1
4
8
Replaced Plasma Volume with Mixed Amniotic Fluid (μL)


(b)
P < 0.0001
P < 0.001
P < 0.0001
P < 0.0001
APC-sr
10
8
6
4
2
0
0
1
4
8
Replaced Plasma Volume with Mixed Amniotic Fluid (μL)