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Coagulation and Fibrinolysis During the Normal Menstrual Cycle

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ABSTRACT

Thirteen healthy women (age 24-44 yrs) were studied during their menstrual cycle. Samples were taken 2-3 times weekly for six consecutive weeks. Plasma concentrations of oestradiol and progesterone were determined in each blood sample and only women found to be ovulatory were included. From the hormon data the cycles were divided in five phases (early follicular phase, late follicular phase, early luteal phase, late luteal phase and menstrual period). The samples for the coagulation and fibrinolysis assays as well as the venous occlusion (20 min) tests were drawn or performed between 8-9 a m after fasting for 8 hrs. The fibrinogen F VIII:C and AT III were assayed and did not show any variations through the study period. Neither were any differences found in platelet counts, platelet mean volumes or platelet function measured as platelet adhesion and plasma β-thromboglobulin. The fibrinolytic activity after venous occlusion decreased slightly during the late luteal phase (phase 4) as compared with the other phases. Large individual differences were, however, seen and no statistically significant differences between the five different phases were found. The plasminogen activator inhibitor (PAI-1) varied during the cycle but in most individuals within the normal range. In 7/13 women substantial fluctuations of the fibrinolytic activities during the cycle were seen. Four women had a significant fall of the fibrinolytic activity after venous occlusion during the late luteal phase (phase 4) and

3 others during the menstrual phase (phase 5). No co-variation between the fibrinolytic activities and PAI-1 was found. Multiple regression analysis showed a co-variation between fibrinolytic activities and progesterone.
Key-words: Coagulation - Fibrinolysis - Menstrual Cycle.

INTRODUCTION

The hemostatic mechanisms are influenced by steroid hormones. Decreased antithrombin III and impairment of the fibrinolytic system have been reported during pregnancy and during the use of oral contraceptives containing oestrogen (1). However few longitudinal studies deal with variations in blood-coagulation and fibrinolysis during the normal menstrual cycle and the previous reports have yielded conflicting results (2,7). Recent progress in research concerning the fibrinolytic system has focused on the newly discovered rapid inhibitor of the tissue plasminogen activator (PAI-1) and its role in thromboembolic disease (4,20). The aim of this investigation was therefore to study possible physiological fluctuations in blood-coagulation and fibrinolysis, including PAI-1 measurements, during normal ovarian cycle in healthy women.

MATERIALS AND METHODS

13 healthy volunteers were studied. Ages ranging from 24 to 44 years. All had a history of regular menstrual cycles (range 28-33 days). None had a family history of thromboembolism. None was taking any drugs.

Assessment of the time in the menstrual cycle.

In order to allow correlation between coagulation and fibrinolytic parameters to the time in the menstrual cycle, blood samples were obtained from all the volunteers 2-3 times weekly for six consecutive weeks, beginning before and ending after the actual study period. The blood was centrifuged, the plasma withdrawn and stored at -20° C. Plasma concentra-

tions of oestradiol and progesterone were determined by radioimmunoassays (12,19). For this particular study, a cycle was considered to be ovulatory if at least one progesterone meaurement was above 20 nmol/l or at least three were above 10 nmol/l. Only volunteers who met these criteria were included. The day of ovulation was arbitrarily chosen as the day after the oestradiol peak followed by a progesterone elevation. Using the hormonal findings in each individual, the cycles were subdivided into five different phases. The first phase comprised in the early rise of oestradiol (early follicular phase). The second phase included the oestradiol peak until the occurrence of progesterone in plasma (late follicular phase; preovulatory phase). The third phase included the days of increasing progesterone levels up to the progesterone peak (early luteal phase). The fourth phase constituted in the decreasing progesterone levels until the onset of a bleeding (late luteal phase). The fifth phase was the first 3-4 days following the onset of a menstrual bleeding (menstrual period). Depending on the individual differences in cycle length, the duration of these phases varied from one individual to another.

Sampling for measurements of coagulation and fibrinolysis and venous occlusion.

The women were fasting at least 8 hours before sampling and were asked not to smoke on the morning of investigation. All samples were drawn between 9-10 a.m. The women were resting in supine position about 10 minutes before and during the test. Blood samples were first drawn from an antecubital vein without a torniquet (for all coagulation and fibrinolysis determinations). Venous occlusion was produced by applying a sphygomanometer cuff to the upper arm and leaving it inflated for 20 minutes to a level half-way between systolic and diastolic blood pressure. Blood samples for fibrinolytic activities were drawn from the antecubital vein immediately before deflating the cuff.

All blood samples were collected in plastic tubes containing 0.13 M-tri-sodium-citrate with siliconized 16G scalp vein needles using free flow technique. Within 30 minutes of collection the blood was centrifuged and plasma was separated.

Preparation of euglobulin precipitate.

In an ice bath, 9.5 ml of 0.014 % acetic acid (final pH 5.4) was added to 0.5 ml of the citrated plasma. This mixture was kept at 8^{o} C for 30 minutes and was then centrifuged at 3000 rpm for 5 minutes in order to bring down the euglobulin fractions. The supernatant was discarded.

Assays.

A pooled human plasma (from thirty healthy blood donors) was used as control in all assays. Reference interval are mean $\pm$ 2 SD from the plasma values from 30 apparently healthy volunteers.

Fibrinogen was assayed by an immunological turbidimetric method using a centrifugal analyzer (Multi-Stat, Instrumentation Laboratory). Reference values 2.4-4.3 g/l. F VIII:C (biologic activity) was assayed by means of titration with plasma using a one-stage clotting assay with F VIII:C-deficient plasma as test base (15). Reference values: 60-160 %. Antithrombin III (AT III) was enzymatically assayed using a chromogenic substrate (S-2238) for thrombin as described in the manual for Anti-thrombin-COATEST (Kabi-Vitrum, Sweden). The reference values was 86-120%.

Platelet count and platelet mean volume were calculated with a Coulter S+. The reference values for platelet count was 150-350 x 10^{9}/l and for platelet mean volume 6.5-10.2 fl. Platelet adhesion to glass beads was determined

by drawing blood through an Adeplat-T column at a constant flow using an Adeplat pump (Adeplat system, Semmelweiss, Milano, Italy).
Reference values were < 27%. β-Thromboglobulin was assayed by means of β-Tg RIA Kit (Amersham, Buckinghamshire, England). Reference values: < 50 µg/l.

The fibrinolytic activity in plasma and euglobulin precipitate was measured by means of the digestion of radiolabelled fibrin coated into plastic tubes as described by Moroz and Gilmore (13). The method was briefly performed as follows: Human fibrinogen was purchased from Kabi, Sweden. The preparation was made plasminogen-free by absorption of plasminogen to lysine-sepharose (Pharmacia, Uppsala, Sweden) as described by the manufacturer. Fibrinogen was labelled with ^{125}Iodine by means of the chloramine-T method. Iodidelabelled fibrinogen was diluted to a working solution of about 200000 cpm/ml in 0.015 M Na-phosphate buffer, pH 8.1. One hundred µl of the fibrinogen working solution were added to 2.5 ml plastic tubes and allowed to rotate at a 45^{o} angle for 2.5 hours after which 0.5 ml bovine albumin (10 g/l) was added and incubated for 20 minutes. This incubation was followed by four washes of the tubes by means of 0.015 M TRIS-NaCl, pH 7.4 : Two hundred µl thrombin (10 U/ml) were added and incubated at 37^{o}C for 10 minutes to transform fibrinogen into fibrin. Finally the tubes were washed twice in the TRIS-NaCl buffer. The fibrin coated tubes were counted in a gamma counter and tubes which deviated more than 10 % in activity from the mean activity of the particular batch were discarded. The tubes were stored until use (maximally three weeks) in the refrigerator. The assay was run as follows: The material to be assayed (100 µl) and 100 µl TRIS-NaCl buffer were added to the tubes and incubated in triplicates at 37^{o}C for 30 minutes. After this 1 ml of buffer was added and the fluid transferred to another tube and the activity in the fluid counted in a gamma counter. The results are ex-

pressed in per cent of the activity of 25 µg trypsin after correction for background activity i e the activity in tubes incubated with buffer alone.

Reference values for fibrinolytic activities after venous occlusion: Fibrinolytic activity in euglobulin precipitate > 15 %. Fibrinolytic activity in plasma > 8 %. Tissue plasminogen activator inhibitor (PAI-1) in plasma was assayed as described by Chmielewska et al (3) by addition of a standard melanoma t-PA, the remaining t-PA activity being measured in an amidolytic system. Ref values $\leq$ 8 U/ml.

Statistical methods

Statistical differences were estimated by means of Student's t-test. The data for euglobulin precipitate and plasma fibrinolytic activities were further verified by Wilcoxon's signed rank sum test. Multiple regression analysis was performed with Statgraphics statistical computer program.

RESULTS

Distinct individual levels of the different coagulation factors resulted in a wide range of values but there were only small individual cycle variations (Table 1). The average values from the five periods were not statistically different. The fibrinolytic activities after venous occlusion in euglobulin precipitate and in plasma were slightly decreased during the late luteal phase (phase 4) as compared to the other phases (Table 2).

Thus 2/13 individuals had a fibrinolytic activity in euglobulin precipitation below 5 % and 4/13 a plasma fibrinolytic activity after venous occlusion during phase 4 lower than 8 %. Also in the menstrual period (phase 5) some of the women showed a fall of the fibrinolytic activities in the euglobulin precipitate and in plasma after venous occlusion.

However, there were large differences between the individuals and no statistically significant difference between the five phases were seen. Also

Table 1. Some coagulation factors in plasma from 13 women during one menstrual cycle.

Item tested	Reference values	Periods* 1	2	3	4	5
Fibrinogen g/l	2.4- 4.3	3.2± 0.57	3.3± 0.51	3.1± 0.45	3.3± 0.43	3.2± 0.44
Factor VIII:C %	60 - 160	132 ± 38	124 ± 18	129 ± 32	126 ± 44	119 + 44
Antithrombin III %	86 - 120	97 ± 11	96 ± 12	97 ± 12	100 ± 16	101 ± 10
Platelets x 10^9/l	150 - 350	247 ± 47	245 ± 45	240 ± 38	229 ± 47	236 ± 43
Platelet adhesion %	<27	14 ± 10.3	15 ± 6.9	14 ± 6.5	12 ± 10.5	17 ± 10.5
β-thromboglobulin µg/l	<50	37 ± 21	27 ± 17	41 ± 41	35 ± 28	42 ± 32
Platelet mean volume fl	6.5- 10.2	8.8± 0.9	8.9± 1.1	9.0± 0.8	9.1± 1.1	9.3± 0.9

* Results are mean ± SD

Table 2. Fibrinolytic activities after venous occlusion during one menstrual cycle, median values and range (within brackets) are given, and PAI-1 levels in plasma from 13 women.

Item tested	Reference values	Periods 1	2	3	4	5
Fibrinolytic activity	>15 %	37 11-55	38 15-61	42 12-68	32 5-57	35 3-69
Fibrinolytic activity plasma	>8 %	19 4-30	17 9-38	16 7-45	13 2-32	20 4-43
PAI-1 (mean ± SD)	<8 U/ml	3.8±3.1	4.3±2.8	4.3±2.4	5.0±3.3	4.2±3.3

the plasma levels of PAI-1 varied during the cycle although most values stayed within the normal range (Table 2 and 3c).

Concerning the individual results of the different fibrinolytic parameters the 13 investigated women could be divided into two separate groups. Group 1 consisted of 6 women. They all showed distinct levels within the normal range of fibrinolytic activity in euglobulin precipitate, plasma fibrinolytic activity and PAI-1 activity with only small cycle variations. The other group, called group 2, consisted of seven women which showed large individual fluctuations in the fibrinolytic activities during the cycle (Table 3a, b and c).

Table 3a. Fibrinolytic activities in euglobulin precipitate after venous occlusion during one menstrual cycle in group 2.

Subject	Period				
	1	2	3	4	5
1	14	26	27	32	3
2	52	35	22	22	69
6	11	25	52	19	32
9	43	55	37	5	28
10	26	38	15	9	31
12	35	36	58	42	14
13	54	51	55	57	47

Reference values > 15 %.

4/7 showed a similar pattern with a fall of the fibrinolytic activities during the late luteal phase (period 4). Three individuals instead had a fall during the menstrual phase (period 5), two of these (individual 1 and 6) also showed low fibrinolytic activities in phase 1. The menstrual cycle from two of the women in group 2 are presented in figure 1 and 2.

Table 3b. Fibrinolytic activities in plasma after venous occlusion during one menstrual cycle from women in group 2.

Subject	Period				
1	1	2	3	4	5
1	8	14	15	14	4
2	19	15	13	8	22
6	4	11	7	3	17
9	27	33	21	2	20
10	20	17	10	8	19
12	11	12	14	13	6
13	30	27	24	18	8

Reference values > 8 %

Table 3c. PAI-1 levels in plasma during one menstrual cycle from women in group 2.

Subject	Period				
1	1	2	3	4	5
1	0.3	0.3	2.5	0.3	1.3
2	0.5	2.7	4.3	6.0	2.5
6	2.5	4.0	4.0	4.5	0.5
9	8.0	7.5	7.3	8.1	7.5
10	1.0	1.0	0.5	1.0	1.0
12	0.5	3.8	6.5	3.0	1.5
13	5.0	5.0	5.0	5.5	6.5

Reference values < 8 U/ml

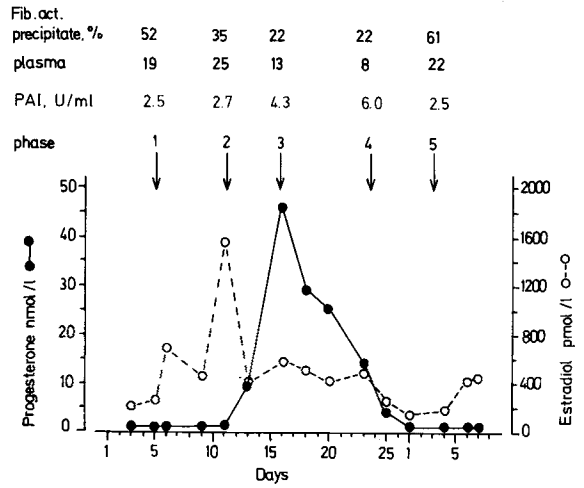

Fig 1. The cyclic variations in fibrinolytic activity after venous occlusion and PAI-I levels in plasma from one woman in group 2.

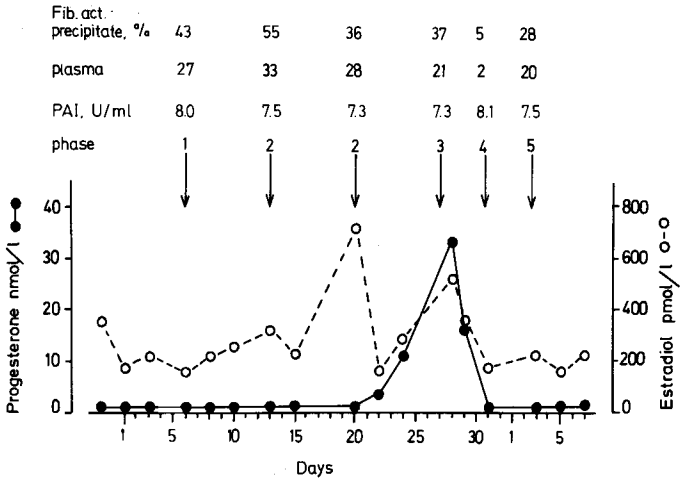

Fig 2. Fall in fibrinolytic activities in euglobulin precipitate and plasma during phase 4 of the menstrual cycle in one woman in group 2. The PAI-I levels did not vary during the cycle.

Multiple regression analysis

Multiple regression analysis with stepwise forward and backward variable selection with plasma or euglobulin precipitate fibrinolytic activity as dependent variables and all the other coagulation, fibrinolysis and hormon values as independent variables were performed. The analysis showed a significant co-variation between the fibrinolytic activities in plasma and euglobulin precipitates ($p < 0.001$). Platelet mean volume and progesterone levels were the only other variables that significantly ($p<0.01$) added to the explanation of this co-variation.

DISCUSSION

The thirteen women in this study showed very small cycle variations concerning the different coagulation parameters. On the other hand seven women had substantial fluctuations in the fibrinolytic activities during the cycle. This is in agreement with previous investigations (2, 7). However, our results differ from previous investigations in showing two patterns related to the phases of the cycle. 4/7 individuals showed the lowest fibrinolytic activities in luteal (phase 4) and 3/7 women had a fall during the menstrual phase (phase 5). The fibrinolytic pattern in the women studied after venous occlusion was regarded as a measure of the fibrinolytic capacity (5,6,18). The fibrinolytic activity as measured in the euglobulin precipitate is claimed to reflect more of the true fibrinolytic activity, i.e. the plasminogen activator (t-PA) activity released from the endothelial cells on stimuli like venous occlusion. Most of the plasma fibrinolytic inhibitors remain in the supernatant on euglobulin precipitation. The plasma fibrinolytic activity on the other hand should vary with the total fibrinolytic activity thus reflecting both the fibrinolytic activator (release and synthesis) and the inhibitor level. In the present study fibrinolytic activities in euglobulin precipitate and plasma co-variated but there were no discernible co-variation between PAI-1 levels and the

different fibrinolytic activities. This most probably reflects the fact that a more complex system is responsible for the over-all fibrinolytic activity as is measured after venous occlusion. This system represents the synthesis, release, as well as the inhibition of the fibrinolytic activator (s) and fibrinolytic enzymes.

The reason for or mechanism behind the decreased release from the vessel wall and/or low synthesis of fibrinolytic activator in the endothelial cells in some women during part of the menstrual cycle cannot be explained. No simple correlation between the fibrinolytic activities and the hormone levels was found and thus it is difficult to determine wether the changes in the fibrinolytic activities are related to the menstrual cycle or not. Since the material in the present study was small it is therefore difficult to draw firm conclusions for one particular individual compared to menstruating women as a group. However, multiple regression analysis showed that the progesterone levels and the platelet mean volumes added to the explanation of the co-variation between the fibrinolytic activities in plasma and in euglobulin precipitate pointing to a possible role of progesterone in the regulation of fibrinolytic activity. Both t-PA and PAI 1 are synthesized by the endothelial cells. Progesterone may interact with the release of t-PA and/or PAI-1 from the vessel wall, thus d-norgestrel was previously shown to stimulate the t-PA release from human veins in vitro (9). Changes in the platelet mean volume may express different activation and release of PAI-1 from the platelets during the menstrual cycle (11).

In the clinical situation it is well known that the fibrinolytic activity of one individual can vary on different occasions. This may, however, represent only natural cycle variations. As a consequence of this study we emphasize that samples for fibrinolysis tests should be drawn according to a strict standard scheme. Thus, the samples are preferably drawn in the morning.

The reason for this is that PAI-1 levels seem to vary during twenty-four hours with the highest values in the morning (14). Patients should be investigated several times and women at two different phases of the menstrual cycle. It is probably best to sample in the middle of the cycle since this investigation showed the fewest pathological values during phases 2 and 3.

A defective fibrinolytic defense mechanism seems to play an important role in the development of deep vein thrombosis (DVT) (8,10,16,20). It has been claimed that patients developing thrombosis during use of oral contraceptives already have a defective fibrinolytic system and thus have a higher risk of developing thrombosis even without use of the pill (17). It is interesting to speculate whether women with substantial cycle fluctuations within the fibrinolytic system were at risk of thromboembolism if they use oral contraceptives, during pregnancy or after operations, situations known to reduce AT III and the fibrinolytic activity in plasma.

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