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Changes in haemostatic parameters during the menstrual cycle and subsequent use of drospirenone-containing oral contraceptives

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ABSTRACT

Introduction: Oral contraceptives (OC) increase the risk of venous thromboembolism that depends on the OC formulation and could at least partially be explained by impaired function of the protein C-system (APC resistance) and the tissue factor pathway inhibitor (TFPI)-system. There is limited information available on the effects of OC, containing a newer progestogen- drospirenone (DRSP-OC) on these two major anticoagulant pathways, thrombin generation, reflecting the overall state of coagulation, and other coagulation parameters.
Methods: In a study population consisting of 14 healthy women (age 21–33 years) we investigated the effect of the menstrual cycle and subsequent use of DRSP-OC on APC resistance, the function of the TFPI-system, thrombin generation and on their major determinants, *i.e.* prothrombin, antithrombin, FV, FX, FVIII, protein C, protein $S_{(total\ and\ free)}$ and $TFPI_{(full\text{-}length\ and\ free)}$.
Results: All studied parameters remained unchanged during the menstrual cycle. During DRSP-OC use we observed a significant increase in APC resistance (~2.4-fold), thrombin generation measured at low (~2.2-fold) and high tissue factor concentrations (~1.4-fold), plasma concentrations of prothrombin (19%), FX (31%), FVIII (17%) and protein C (43%). DRSP-OC use impaired the function of the TFPI-system and decreased plasma levels of antithrombin (−6%), FV (−22%), protein S_{total} (−21%), protein S_{free} (−20%), $TFPI_{full\text{-}length}$ (−36%) and $TFPI_{free}$ (−46%).
Conclusions: DRSP-OC caused procoagulant changes in all studied haemostatic parameters. The impairment of the protein C- and TFPI-systems was more pronounced than the impairment of the coagulation pathways and can at least partially account for the increased risk of venous thromboembolism in users of DRSP-OC.
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Introduction

The increased risk of venous thrombosis during the use of combined hormonal contraceptives is not only dependent on the dose of estrogen, but also on the type (generation) and possibly the dose of the progestogen compound [1]. The risk of venous thrombosis is 1.5- to 3-times higher for users of third generation oral contraceptives as compared to second generation OC [2,3]. OC containing the recently introduced anti-mineralocorticoid drospirenone [4] that is sometimes referred as fourth generation progestogen, were also found to be about 2 times more thrombogenic as compared to second generation OC [3,5]. The risk of venous thrombosis introduced by drospirenone-containing OC (DRSP-OC) is considered to be similar to that caused by third generation OC. Moreover, based on a review of recent epidemiologic studies, the Food and Drug Administration concluded that DRSP-OC may be associated with even a higher risk for thrombosis than other progestogen-containing pills, which resulted in "a warning about drospirenone in oral contraceptives" [6].

Combined OC cause changes in the plasma levels of various proteins involved in haemostasis [7]. Although the levels of coagulation factors and anticoagulant proteins mostly stay within the normal range, OC appear to have a general prothrombotic effect that is more pronounced in users of third generation OC than in second generation OC users [8,9]. The hormone-induced changes are thought to result in acquired resistance to an important physiological inhibitor of the coagulation system-activated protein C (APC). APC resistance is a risk factor for venous thrombosis, both in individuals with and without the factor V Leiden mutation [10,11]. Women using OC containing the later progestogens have higher APC resistance as compared to those who use levonorgestrel-

Abbreviations: OC, Oral contraceptives; DRSP-OC, drospirenone-containing oral contraceptives; APC, activated protein C; TFPI, tissue factor pathway inhibitor; SHBG, sex-hormone-binding globulin; ETP_{-APC} and ETP_{+APC} respectively, the endogenous thrombin potential in the absence and in the presence of APC; APCsr, the APC sensitivity ratio; 95% CI, 95% confidence interval.

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containing OC [12], providing thereby a plausible explanation for the different risk of venous thrombosis in these women. Among several available APC resistance tests, thrombin generation-based APC resistance assays appear to be particularly sensitive to the hormone-induced changes in coagulation and can also provide information on the changes in thrombin generation independent of APC.

Although the mechanisms of hormone-induced APC resistance are not completely understood, protein S and tissue factor pathway inhibitor (TFPI) have been proposed to play a major role in the impairment of the protein C system [13]. DRSP-OC decrease the protein S levels and activity in plasma [14–17]. Similarly to users of third generation OC, women using DRSP-OC have lower TFPI levels than those, who use second generation OC [18]. However, interpretation of the physiological meaning of these findings remains difficult, partially due to the heterogeneity of the plasma pool of TFPI (full-length and truncated, free, and lipoprotein-bound TFPI) and differences in the anticoagulant activity of the various TFPI forms. Additionally, protein S can modulate the anticoagulant activity of TFPI [19]. Therefore, evaluation of the functional activity of the TFPI system in plasma is the method of choice to estimate the global effect of the changes in the levels of TFPI and protein S on haemostasis.

The primary objective of our study was to investigate effects of DRSP-OC on the major anticoagulant pathways, *i.e.* protein C and TFPI-protein S systems. To complement the knowledge on the DRSP-induced changes in coagulation we also characterized thrombin generation at different experimental conditions and measured a number of its determinants [20] in plasma. Additionally, we evaluated in the same women the changes in the above-mentioned haemostatic parameters during menstrual cycle preceding OC use.

Materials and methods

Materials

Ecarin, Protac, thrombin, activated factor X (Xa) and activated protein C (APC) were purchased from Kordia Life Sciences, Leiden, the Netherlands. Prothrombin, [21] factor Va [22] and the factor X activator from Russell's viper venom [23] were prepared as previously described. Recombinant human tissue factor was supplied by Dade Innovin®, Behring, Germany. The chromogenic substrates for thrombin D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide (S2238) and for activated protein C pyroGlu-Pro-Arg-pNA · HCl (S2366) were from Chromogenix, Milano, Italy and the fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC was from Bachem, Bubendorf, Switzerland. 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) were from Avanti Polar Lipids, Alabaster, AL, USA. DOPS/DOPE/DOPC, 20/20/60, M/M/M and PS/PC, 10/90, M/M vesicles were prepared as described before [21]. PPP-Reagent Low was from Thrombinoscope BV, Maastricht, the Netherlands.

Collection of plasma samples

Since DRSP-OC are thought to cause similar alterations in the coagulation system as third generation OC, the sample size was estimated on the basis of the data of a pilot-study, in which the functional activity of the TFPI-system was evaluated in users of second and third generations OC [24]. We calculated the number of subjects required to document a significant change in the TFPI activity during OC use. Assuming that the standard deviation of the TFPI activity ratio in women is 0.11 [24], we calculated that 11 women would be required to document a significant change in the TFPI activity ratio of 0.2 during DRSP-OC use ($p = 0.95$ and $\alpha = 0.05$). A sample size calculation based on the differences in the APCsr between users of DRSP-OC and women not using hormonal contraceptives resulted in an even smaller number of participants ($n = 7$) needed to detect statistically significant differences.

Fourteen healthy female volunteers without contraindications to combined OC, who had not been using hormonal contraceptives for at least two month prior to start the study, were recruited. Exclusion criteria were pregnancy, lactation, a personal or family history of venous thromboembolism, coagulation disorders or use of medication that might interfere with blood coagulation. Blood samples were obtained in the follicular (days 3–5) and the luteal (days 22–25) phases of the menstrual cycle. Thereafter, all volunteers were assigned to use DRSP-OC containing 3 mg drospirenone and 30 μg ethinylestradiol for two consecutive cycles and another blood sample was collected between days 18 and 21 of the second OC-cycle. Blood collection and plasma preparation were performed as described elsewhere [25]. The study was approved by the Medical Ethical committee of the Karolinska Institutet, Stockholm, Sweden. Clinical registration number: DRKS00005291. All volunteers gave written informed consent. The participants of this study were partly presented earlier [26].

Pooled normal plasma was prepared by pooling plasma of 23 healthy donors free of medication (17 male and 6 female, mean age 33.3 years) as previously described [25].

Laboratory methods

Prothrombin concentrations were determined after complete activation with 0.5 U/ml Ecarin in a plasma sample diluted 500-fold in buffer A (25 mM Hepes 175 mM NaCl, 5 g/L BSA, pH 7.7) in the presence of 5 mM $CaCl_2$. The reaction was performed at 37 °C and stopped after 15 min by addition of ice-cold buffer B (25 mM Hepes pH 7.9, 175 mM NaCl, 60 mM ETDA and 0.5 g/L ovalbumin). The amidolytic activity of thrombin was subsequently determined at 37 °C using the chromogenic substrate S2238.

Plasma factor V was determined in 1:1250 diluted plasma in buffer A after activation for 10 minutes at 37 °C with 2.84 nM thrombin in the presence of 5 mM $CaCl_2$ and the factor Va was subsequently quantified via a prothrombinase-based assay at limiting amounts of factor Va (<25 pM), 0.3 nM factor Xa, 1 μM prothrombin, 40 μM DOPS/DOPC (10/90 M/M) and 2.8 mM $CaCl_2$ (final concentrations). All participants were screened for factor V_{Leiden} by determining the sensitivity of plasma factor Va for APC [27].

Factor X levels were determined in 1:750 diluted plasma in buffer A after activation for 10 minutes at 37 °C with 0.2 μg/mL factor X activator from Russell's viper venom in the presence of 11 mM $CaCl_2$ and the factor Xa was quantified in a prothrombinase-based assay at limiting amounts of factor Xa (<25 pM), 5 nM factor Va, 40 μM DOPS/DOPC (10/90 M/M), 1 μM prothrombin, and 3 mM $CaCl_2$ (final concentrations).

$TFPI_{full\text{-}length}$ and protein S_{total} were measured by in-house enzyme-linked immunosorbent assays using either 2 μg/ml monoclonal antibody against the C-terminus of TFPI (Sanquin, Amsterdam, the Netherlands) or 4.1 μg/ml polyclonal antibody against protein S (DAKO, Glostrup, Denmark) in Na_2CO_3, pH 9.0 for overnight-coating of microtiter plates at 4 °C, followed by three washing steps with 25 mM Hepes 175 mM NaCl, 0.02% Tween-20, pH 7.7 and a 2 hours-blocking step with buffer A. Thereafter plasma dilutions (1:5 for $TFPI_{full\text{-}length}$ or 1:2000 for protein S_{total}) were incubated in the wells for 1 hour at room temperature, the plates were washed again and antigen was detected with 1:1000 dilutions of either monoclonal anti-TFPI antibody against Kunitz-2 (Sanquin, Amsterdam, the Netherlands, conjugated with peroxidase with the antibody labeling kit from Pierce (Rockford IL, USA) or peroxidase-conjugated polyclonal anti-human protein S antibody (DAKO) in the blocking buffer (1 hour at room temperature) and TMB enzymatic kit (Pierce, Rockford IL, USA). Protein S_{free} was measured similarly to protein S_{total}, except that 3 μg/ml C4b-binding protein in 0.1 M H_2CO_3 pH 9.0 was used for the coating of the plates and all steps starting from the incubation of the samples were performed in the presence of 5 mM $CaCl_2$. [13].

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