Thrombosis Research 132 (2013) 621–626

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Enhanced thrombin generation in women with a history of oral contraception-related venous thrombosis

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ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form 2 September 2013
Accepted 9 September 2013
Available online 13 September 2013

Keywords:
endogenous thrombin potential
oral contraception
overall haemostasis potential
thrombophilia
venous thrombosis

ABSTRACT

Introduction: In women who suffer venous thrombosis (VT) during oral contraceptive (OC) use, a transient risk factor (OC) is removed during the acute event, while most co-existing forms of thrombophilia persist and presumably continue to maintain hypercoagulability. The aim of this study was to establish if hypercoagulability persists long after OC-related VT and if it could be attributed to thrombophilia.
Materials and Methods: 60 women (age 33.0 ± 8.5 years) were investigated 5 – 64 (median 33) months after OC-related VT (patients) and compared to 63 apparently healthy women (controls). All women were tested for thrombophilia, activated partial thromboplastin time (APTT), fibrinogen, D-dimer, P-selectin and C-reactive protein. Thrombin generation was measured by Technothrombin® TGA assay. Overall haemostasis potential (OHP) assay with overall coagulation potential (OCP) and overall fibrinolytic potential (OFP) as supplementary parameters were measured by repeated fibrin formation and degradation registration.
Results: In patients increased endogenous thrombin potential (4205 ± 440 nM x min vs 4015 ± 421 nM x min, $p = 0.017$), increased OCP (22.6 ± 4.6 Abs-sum vs 20.8 ± 4.1 Abs-sum, $p = 0.025$), shorter APTT (30.9 ± 3.8 s vs 33.4 ± 3.6 s, $p < 0.001$) and lower antithrombin activity (99, 93-105% vs 104, 100-109%, $p < 0.05$) were observed. Thrombophilia was observed in 22/60 (36%) patients and in 5/63 (7.9%, $p < 0.001$) controls. The only significant difference between thrombophilic and non-thrombophilic patients was higher soluble P-selectin in the former subgroup (22, 20-33 μg/L vs 17, 12-22 μg/L, $p = 0.012$).
Conclusions: In women with a history of OC-related VT persistent hypercoagulability was observed, which, however was not augmented by the presence of thrombophilia.

Introduction

Oral contraceptives (OC) are used by more than 100 million women worldwide and their use is associated with the majority of venous thrombosis (VT) cases in premenopausal women [1,2]. OC users have a 2-6-fold increased risk of VT compared with nonusers [2,3]. However, VT- a potentially life-threatening disease is a rare event in this age group and is frequently a consequence of a combination of OC with an additional (haemostatic) risk factor. It is well known that OC users who are heterozygous carriers of either factor V Leiden or prothrombin G20210A mutation have a 16-30-fold increased risk of VT [1].

An individual's risk of VT cannot usually be detected by routinely used coagulation tests such as prothrombin time or activated partial thromboplastin time (APTT) since they use clot formation as their endpoint, which occurs when only around 5% of all physiologically relevant thrombin is formed [4]. On the other hand, estimation of an individual's potential to generate thrombin may correlate more closely with hypercoagulable states [5]. Thrombin generation, a more than 50-year old assay made available for clinical use by Hemker et al. [6], indicates the potential of plasma to generate thrombin following in vitro activation of coagulation with tissue factor or another trigger. The resulting thrombin generation curve reflects all pro- and anticoagulant reactions that regulate the formation and inhibition of thrombin. It has been demonstrated that increased thrombin generation indicated by increased endogenous thrombin potential (ETP) was predictive of the first VT. [7] Others have shown that peak thrombin concentration [8] or ETP in combination with D-dimer [9] is also predictive of VT recurrence after the first spontaneous VT.

Despite its ability to measure the whole amount of thrombin formed, the thrombin generation assay does not measure the final step of coagulation- fibrin formation. In contrast, the overall haemostasis potential (OHP) assay, described initially by He et al in 1999 and modified in 2001 [10,11], is based on repeated spectrophotometric registraton of fibrin formation in duplicate samples of citrated plasma to which small amounts of thrombin and tissue-type plasminogen activator are added. The overall coagulation potential (OCP) and overall fibrinolytic potential (OFP) are parameters supplementary to OHP, providing details of

Abbreviations: APTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; OC, oral contraceptive(s); OCP, overall coagulation potential; OFP, overall fibrinolytic potential; OHP, overall haemostasis potential; VT, venous thrombosis.

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http://dx.doi.org/10.1016/j.thromres.2013.09.006

underlying changes in coagulation and fibrinolysis. The OHP assay was evaluated in connection with hypercoagulability in normal pregnancy and preeclampsia [10,11], some types of thrombophilia [12,13], coronary heart disease [10], diabetes with microvascular complications [11], and in stroke [14].

In order to elucidate why some women using OC suffer VT whereas the majority do not, we tested the hypothesis that women who suffer VT during OC use have a preexisting prothrombotic tendency, associated with thrombophilic or other defects. While a transient risk factor for VT (OC) is removed during the acute event, most coexisting forms of thrombophilia persist and presumably continue to maintain hypecoagulability. Our aim was to establish if hypercoagulability could be detected long after OC-related VT with global clotting assays (thrombin generation and OHP assay) and if it could be attributed to thrombophilia.

Materials and Methods

Subjects

Consecutive women aged 50 years or less who had suffered VT during OC use between the years 2005 and 2011 were included (patients). VT was confirmed by Doppler ultrasound and patients were treated with low-molecular-weight heparin followed by oral anticoagulants. According to the guidelines [15] OC use was discontinued in all patients. The exclusion criteria were malignant disease, pregnancy, puerperium, antiphospholipid syndrome or other conditions requiring prolonged anticoagulant treatment. Sixty-six patients matched the inclusion criteria. Six women refused to participate or contact was not possible, so 60 patients were finally included in the study. At the time of enrolment (5-64 months, median 33 months after acute VT) all patients had already completed anticoagulant treatment. 63 healthy women of comparable age, who were not OC users or had discontinued OC at least 2 months before inclusion in the study, served as controls.

All participating women were without clinical signs of acute disease at the time of blood sampling. In all women age, body weight, body height and smoking habits were recorded. Patients were asked about the type and duration of the OC used and possible concomitant risk factors for VT (trauma, surgery, immobilization) at the time of the disease. Body mass index (BMI) was calculated as body weight (kg)/body height^2 (m^2). Patients and controls were not related to each other. They gave their full informed written consent to participate in the study, which was approved by the Slovene Ethics Committee.

Blood Sampling, Plasma and DNA Preparation

Blood was obtained on a single occasion in the morning from the antecubital vein with minimal venous stasis after a 20-min rest. For haemostasis assays 4.5 mL of blood were collected in each of two vacuum tubes containing 0.11 M sodium citrate (Becton Dickinson, Vacutaineer System Europe, Heidelberg, Germany), thoroughly mixed with the anticoagulant, placed immidiately in ice water and centrifuged within 4 h of venepuncture at 4 °C and 2000 ×g for 30 min to obtrain platelet-poor plasma. Before freezing plasma for detection of lupus anticoagulants was filtered through a 0.2 μm pore filter (Minisart, Sartorius Stedim Biotech GmbH, Göttingen, Germany). Aliquots of platelet-poor plasma were then frozen in liquid nitrogen and stored at -70 °C until analysed. For preparation of microparticle-free plasma, platelet-poor plasma was filtered through a microfilter unit (Ceveron MFU 500, Technoclone, Vienna, Austria). DNA was isolated from whole blood or buffy coat using a commercially available kit (High Pure PCR Template Preparation Kit, Roche Diagnostica, Indianapolis, USA).

Laboratory Methods

APTT (Pathromtin SL), fibrinogen (Multifibren U), antithrombin activity (Berichrom Antithrombin), protein C activity (Berichrom Protein C, all from Dade/Behring, Marburg, Germany), lupus anticoagulants (LA-screen and LA-confirm, Gradipore, Sydney, Australia), activated protein C (APC) resistance (Coatest APC Resistance, V-S, Chromogenix, Mölndal, Sweden) and D-dimer (TriniLIA Auto-Dimer, Trinity Biotech, Bray, Ireland) were determined on an automated coagulation analyser (Behring Coagulation Timer, Dade Behring, Marburg, Germany). Free protein S was determined by the immunoturbidimetric latex-agglutination test (STA-Liatest Free protein S, Diagnostica Stago, Asnières, France) and soluble P-selectin by enzyme-linked immunosorbent assay (Human sP-selectin/CD62P Immunoassay, R&D Systems, Minneapolis, USA). C-reactive protein (CRP) was determined using the VITROS hs-CRP test (Ortho-Clinical Diagnostics, Rochester, USA). Factor V Leiden and prothrombin G20210A polymorphism were determined by real-time polymerase chain reaction on ABI Prism 7000 SDS (Applied Biosystems, Life Technologies Corp., Carlsbad, USA) using appropriate primers and probes.

Thrombophilia was considered as described previously [14]: if antithrombin or protein C activity were below 0.75 or 0.70 relative to normal plasma, respectively, if the concentration of free protein S was below 0.55 relative to normal plasma, if the ratio between the screening and the confirmatory test for lupus anticoagulants was equal to or above 1.20, if factor V Leiden or prothrombin G20210A polymorphism was present or if the ratio of APTT with APC versus APTT without APC was below 1.93 (APC resistance).

Thrombin generation was determined using a commercial kit (Technothrombin® TGA,Technoclone, Vienna, Austria), which is based on monitoring the fluorescence generated by thrombin cleavage of a fluorigenic substrate over time on activation of the coagulation cascade by tissue factor. The concentration of tissue factor was 5 pmol/L. The following parameters were registered: lag phase, time to peak thrombin concentration, peak thrombin concentration and area under the curve – endogenous thrombin potential (ETP). Amount of microparticle-induced thrombin generation was also determined by measuring thrombin generation in microparticle-free (filtered using 0.2 μm vacuum filtratrion device Ceveron® MFU-500, Technoclone, Austria) plasma versus thrombin generation in non-filtered plasma. The amount of thrombin (peak thrombin concentration) induced by microparticles was calculated (in per cent). The source and procoagulant properties of the filtered microparticles have been reported elsewhere [16].

Parameters of the overall haemostasis potential assay (OHP, OCP and OFP) were determined as described by He et al. [11]. Fibrin-formation time curves were generated in microtitre plate wells and plasma samples were tested in duplicate.

For OHP measurement microtitre wells contained 60 μL plasma and OHP buffer (66 mmol/L Tris, 130 mmol/L NaCl, 17.0 mmol/L $CaCl_2$, pH 7.5) with 0.04 IU/mL thrombin and 348 ng/mL recombinant tissue-type plasminogen activator. OHP curves were generated from automated absorption measurements at 405 nm taken every minute for 40 min. OCP curves were obtained in an identical way, except that the added buffer did not contain recombinant tissue-type plasminogen activator. Values for OCP and OHP were given by the areas under the relevant fibrin-formation time curves calculated by summation of absorption values (Abs-sum). The OFP values in % were calculated as [(OCP-OHP)/OCP] x 100.

Statistical Analysis

Statistical analysis was performed by the Statistica 8.0 (StatSoft Inc., Tulsa, USA) statistical package. Distribution of variables was tested by the Kolmogorov-Smirnov test. Normally distributed data were expressed as means with standard deviations, while skewed data were presented as medians with interquartile ranges. Differences between the two