Use of the etonogestrel-releasing implant is associated with hypoactivation of the coagulation cascade

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BACKGROUND: The role of progestogens in haemostasis is controversial. Our objective is to evaluate the haemostatic effects of an etonogestrel-releasing implant. METHODS: This open-label, self-controlled, longitudinal study involved 20 healthy women receiving subcutaneous etonogestrel-releasing implants. At baseline, 1, 3 and 6 months, we measured the following: activated partial thromboplastin time; prothrombin time; thrombin time; fibrinogen; coagulation factors II, V, VII, VIII, IX, X and XI; von Willebrand factor; activated protein C (APC); antithrombin; free protein S; plasminogen activator inhibitor type 1 (PAI-1); α2-antiplasmin; thrombin–antithrombin (TAT) complex; prothrombin fragment 1 + 2 (F1 + 2); D-dimers; APC resistance. Statistical analyses included the Friedman test and ANOVA. RESULTS: Levels of APC (P < 0.01), factor II (P = 0.02), factor VII (P = 0.006), factor X (P = 0.01) and F1 + 2 (P < 0.001) were reduced, whereas those of PAI-1 (P = 0.01) and factor XI (P = 0.006) were transitory increased. All of these values, however, remained within normal ranges. Surprisingly, TAT concentrations fell below the normal range (P < 0.001). CONCLUSIONS: Our findings suggest that the etonogestrel-releasing implant does not induce a prothrombotic pattern during the first six months of use, and that its use is associated with a reduction in thrombin generation.

Keywords: etonogestrel; progestogens; haemostasis; coagulation; contraception

Introduction

Since the introduction of combined oral contraceptives (OCs), several epidemiological studies have demonstrated a clear association between the use of combined OCs and an increased risk of developing venous thromboembolism (VTE), with the magnitude of the increase having been estimated to be from two to six times greater than that when not using combined OCs (WHO, 1995a; Rosendaal et al., 2003).

This increased risk for VTE was primarily associated with the oestrogenic component of the combined OCs in a dose-dependent manner, causing the levels of ethinylestradiol (EE) in the combined OCs to gradually decrease (from 100 to 15–20 μg). In addition, various progestogens presenting less androgenic activity have been added to the combined OCs with the objective of reducing their metabolic impact. Consequently, the so-called third-generation combined OCs were developed. These OCs incorporate a combination of EE and the new progestogens (desogestrel and gestodene) (Kemmeren et al., 2004).

Since 1995, various independent, publicly funded studies of combined OCs have been conducted. The results have shown that combined OCs containing third-generation progestogens (desogestrel and gestodene) are twice as likely to cause thrombosis as are those containing a second-generation progestogen (levonorgestrel) (WHO, 1995a,b; Kemmeren et al., 2001). Therefore, there was an incentive to discover a plausible biological mechanism that would explain this increased risk, since many women would benefit from progestogens that presented fewer androgenic and mineralocorticoid effects.

It has been shown that combined OCs containing third-generation progestogens cause more pronounced acquired resistance to activated protein C (APC) than do those containing second-generation progestogens (Rosing et al., 1997; Kemmeren et al., 2004), as well as presenting a tendency to induce lower levels of antithrombin, free protein S and higher levels of coagulation factors (Conard, 1999; Tans et al., 2000). These findings could explain the epidemiological observation that the risk for VTE is greater in women using combined OCs that contain third-generation progestogens, since (acquired or inherited) resistance to APC is an important marker for VTE risk (Tans et al., 2003).

Previous studies, evaluating the haemostatic changes resulting from the use of oral progestogen-only preparations, have
demonstrated no procoagulant pattern (Kemmeren et al., 2001, 2004). However, although there are few data on alterations in the coagulation system caused by non-oral progestogen contraceptives, the route of administration of female sex steroid hormones is an important issue concerning the thrombosis risk (Scarabin et al., 2003; Jick et al., 2006; Canonico et al., 2007). There have been no independent studies on the effects that non-oral third-generation progestogens have on haemostasis, especially after four months of use, at which point users of combined OCs have been shown to be at a greater risk of developing thrombosis (WHO, 1995a). Therefore, the objective of this study is to evaluate the effects of a subdermal etonogestrel-releasing implant on the haemostatic system of healthy women during a 6-month period.

Subjects and Methods
An open, self-controlled, longitudinal and prospective study was conducted, in which haemostatic variables were assessed prior to implant placement and after 1, 3 and 6 months of its insertion. The implant was inserted during the early follicular phase of the menstrual cycle.

Selection of volunteers
Twenty consecutive healthy female volunteers were included in the study and were submitted to a long-term, reversible method of hormonal contraception from March 2002 to September 2003 at the Hospital das Clínicas de Ribeirão Preto (HCRP, Ribeirão Preto Clinical Hospital) of the University of São Paulo at Ribeirão Preto School of Medicine, in Ribeirão Preto, Brazil. Inclusion criteria were being 20–35 years old and having suspended OCs at least 6 weeks prior to participation in the study or 3 months after delivery. Exclusion criteria were as follows: being a smoker, alcoholic or drug addict; having a BMI $\geq 30$ kg/m$^2$; presenting a systemic disease; using any medication that might interfere with blood coagulation or with the assessment of haemostatic variables; having a history (personal or family) of thromboembolic events; presenting alterations in hepatic enzymes (total bilirubin, gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase); being allergic to local anaesthetics (xylocaine).

All volunteers gave written informed consent, and the study was approved by the local ethics in research committee.

The sample size was calculated using the GraphPad StatMate® software program (Graphpad Software, San Diego, CA, USA). Using a two-tailed test with a significance level of 5% and a statistical power of 80%, the estimated minimum sample size required to be able to detect an alteration of 10% or greater in the mean of the resistance to APC, as assessed using the Dahlbäck et al. (1993) test for healthy women, was 16 subjects. However, 20 subjects were included due to the possibility that some subjects would drop out over the course of the study.

Medication used
Subjects received a single implant (Implanon®, Organon, Oss, The Netherlands) containing 68 mg of etonogestrel (3-ketodesogestrel), which is the active metabolite of desogestrel. These implants are programmed to be used for 3 years after insertion. The mean release rate over the 3 years is 40 $\mu$g of etonogestrel/day (Huber, 1998). The implant was inserted subcutaneously on the inside of the upper (nondominant) arm.

Sample collection protocol and laboratory tests
Blood samples were collected, using the atraumatic phlebotomy technique, between 8:00 and 9:00 am (after a fast of at least 8 h) in the HCRP Haemostasis Laboratory.

For each evaluation, 20 ml of whole blood was collected and stored in conical plastic tubes with no vacuum and containing sodium citrate anticoagulant at 3.2% (in a fixed proportion of 9 parts whole blood to 1 part anticoagulant). Certain medications, especially non-steroidal anti-inflammatory drugs and acetylsalicylic acid, can alter the results of the laboratory tests. None of the subjects evaluated used any such medications.

Blood samples were processed within 2 h after being collected. Whole blood was centrifuged at 120 g (700 rpm) in a Sorvall RC 3 centrifuge (Sorvall Kendro Laboratory Products GmbH, Langenselbold, Germany), at room temperature (mean, 22°C; range, 18–24°C) for 15 min. Platelet-rich plasma was collected and transferred to a screw-cap plastic tube in order to maintain the pH. Platelet-poor plasma, which is necessary for the haemostasis tests, was obtained by centrifuging the remainder of the sample (after the collection of the platelet-rich plasma) at 1600 g (2500 rpm), for 30 min, using a Universal 32 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), at 4°C. Polypropylene tubes were used to store the platelet-poor plasma at $-80$°C so that the tests could be performed in a single laboratory assay, with the exception of prothrombin fragment 1 + 2 (F1 + 2) levels, which were evaluated in a separate assay. Each haemostatic variable was measured in duplicate.

The following variables were assessed:

(i) Coagulation times: Thrombin time (TT), activated partial thromboplastin time (APTT) and prothrombin time (PT) were assessed using an automated coagulation analyser (STA compact, Diagnostica Stago, Asnieres-Sur-Seine, France). The reagents used were as follows: for PT, calcium thromboplastin; for APTT, cephalin with silica as a contact activator (STA-PTT automate 5); for TT, thrombin. All reagents were also purchased from Diagnostica Stago.

(ii) Coagulation: Fibrinogen was assessed using the Claus coagulation method, with kits from Diagnostica Stago, in the STA automated coagulation analyser, which detects clots through photometry. The levels of the coagulation factors were determined in an automated manner, using the STA automated coagulation analyser, together with the following kits: for factors II and V (Diagnostica Stago); for factors VII, VIII and IX (Sigma Diagnostics, St Louis, MO, USA); for factors X and XI (Helena Laboratories, Beaumont, TX, USA). Levels of the von Willebrand factor antigen were determined using the enzyme-linked immunosorbent assay (ELISA) method with kits from Diagnostica Stago.

(iii) Activation of the coagulation cascade: Levels of the thrombin–anti-thrombin (TAT) complex and of F1 + 2 were determined using ELISA kits (Dade Behring, Marburg, Germany).

(iv) Fibrin turnover: Levels of D-dimers were also determined using ELISA kits (BioMérieux SA, Marcy-l’Étoile, France) in a Mini-Vidas analyser (Vitek Systems®; bioMérieux).

(v) Natural anticoagulants: Protein C and antithrombin levels were determined through the automated chromogenic method using the STA automated coagulation analyser, with protein C kits from Diagnostica Stago and antithrombin kits from Sigma Diagnostics. Free protein S levels were determined using ELISA kits from Helena Laboratories.

(vi) Fibrinolysis inhibitors: The plasminogen activator inhibitor type 1 (PAI-1) and $\alpha_2$-antiplasmin levels were determined.
The age of the volunteers was 26.3 ± 4 years (mean ± standard deviation), and 35% were nulliparous. The mean BMI was 23.3 ± 2.3 kg/m².

There were no differences between the pre-insertion and post-insertion periods in terms of the coagulation times (TT, APTT, or PT). Similarly, levels of the coagulation factors V, VIII and IX, as well as those of fibrinogen and the von Willebrand factor did not change during the 6 months of implant use. However, factor II levels decreased by 8% over the 6-month period ($P = 0.02$). During the first and third months of implant use, factor VII levels decreased by 7% ($P = 0.006$) and 11% ($P = 0.006$), respectively, normalizing by the sixth month. In addition, factor X levels decreased by 7% ($P = 0.01$) after 3 months. Factor XI levels increased by 10% ($P = 0.006$) in the third month but returned to the baseline value by the sixth month. Despite such alterations, the levels of each coagulation factor remained within normal values for the assays performed (Table 1).

Regarding natural anticoagulants, there were no alterations in the levels of antithrombin and free protein S. However, in the first month, there was a 13% reduction in the levels of protein C, which, in comparison with the baseline values, remained low until the sixth month (Table 2) ($P = 0.01$). However, for the method used, the levels of protein C remained within the 70–130% range, which is considered normal for the
3.5 U

with this transitory alteration, the plasma levels of PAI-1 which returned to baseline values by the sixth month. Even subjects developed resistance to APC (Table 2).

During the observation period, none of the women using the etonogestrel-releasing implant (Table 3).

Reduction in TAT complex concentrations in healthy women over a 6-month period

**Table 3:** Effects of the etonogestrel-releasing implant on fibrinolysis inhibitors and D-dimers in healthy women over a 6-month period

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-Antiplasmin (%)</td>
<td>112 (106.5–119)</td>
<td>110 (101.5–119)</td>
<td>109 (100–112)</td>
<td>110 (104–120)</td>
</tr>
<tr>
<td>PAI-1 (U/ml)</td>
<td>2.2 ± 1.0</td>
<td>3.2 ± 1.4*</td>
<td>2.5 ± 1.0</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>D-dimers (µg/ml)</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. the pre-insertion (baseline) value (ANOVA with Tukey’s post-test).

Results are expressed as median and range (first and third quartiles) for α2-antiplasmin levels, whereas PAI-1 levels and D-dimer levels are expressed as means ± SD.

Normal range: PAI-1 = 0.3–3.5 U/ml; α2-antiplasmin = 80–120%; D-dimers < 0.5 µg/ml.

**Figure 1:** Reduction in TAT complex concentrations in healthy women using the etonogestrel-releasing implant

method used. During the observation period, none of the subjects developed resistance to APC (Table 2).

Levels of α2-antiplasmin remained unaltered during the study period. However, in the first month, there was a transitory increase of 43% in the concentrations of PAI-1 (P = 0.01), which returned to baseline values by the sixth month. Even with this transitory alteration, the plasma levels of PAI-1 were within the range considered normal for the test (0.3–3.5 U/ml). The levels of D-dimers did not change during the use of the implant (Table 3).

In the assessment of the in vivo generation of thrombin by the TAT complex, a significant (55%) reduction in the TAT complex concentrations was observed by the sixth month (P < 0.001; mean baseline value: 1.7 ± 0.7 µg/l; mean value at 6 months: 0.8 ± 0.5 µg/l), at which point they were below the normal range (1.4–4.1 µg/l) (Fig. 1). Levels of F1 + 2, another marker of thrombin generation, also presented a significant (15%) decrease by month 6 of implant use (P < 0.001; mean baseline value: 0.55 ± 0.15 nmol/l; mean value at 6 months: 0.47 ± 0.12 nmol/l) but remained within the normal range for this test (0.4–1.1 nmol/l) (Table 1).

**Discussion**

Since VTE is considered a multicausal and multifactorial disease (Rosendaal et al., 2003), the use of exogenous estrogens, which can cause a hypercoagulable state, is just one of the acquired risk factors. Although the relative risk for VTE is increased with the use of combined OCs, the absolute risk for thrombosis in users of combined OCs is still low (2–3 cases/10 000 women/year) (Jick et al., 2000). However, since approximately 100 x 10^6 women are estimated to be using OCs worldwide (WHO, 1998), finding the least thrombogenic option remains highly relevant.

Progestogen-only contraceptives have been shown to be unassociated with an increased risk of VTE (Vasilakis et al., 1999; Conard et al., 2004) and are therefore appropriate for use in patients at a high risk for VTE. However, desogestrel and its metabolite have not yet been tested.

The most important finding of this study was the statistically significant (55%) decrease (to below the normal range) in the concentration of the TAT complex after 6 months of implant use. This result was confirmed by a reduction in F1 + 2 levels by month 6, albeit a smaller reduction than that observed for the TAT complex. There have been no previous studies assessing the TAT complex and F1 + 2 in users of etonogestrel-releasing implants.

Thrombin is the key effector enzyme of the coagulation system, presenting multiple important biological functions such as platelet activation, proteolytic conversion of fibrinogen into fibrin and feedback amplification of coagulation (activation of coagulation factors V, VIII, XI and XIII) (Dahlbäck, 2000; Brummel et al., 2002). This process involves the generation of thrombin. Thrombin generation is attenuated and finalized by enzymatic inhibitors such as antithrombin and the APC pathway (Brummel et al., 2002). The covalent inhibition of thrombin by antithrombin forms the TAT complex, a specific marker of thrombin generation, together with the release of F1 + 2, which, in the final analysis, represent the activation of the ‘coagulation cascade’ (Boneu et al., 1991; Rivard et al., 2005).

The decreases in the concentrations of TAT and F1 + 2 observed in the present study suggest hypoactivation of the coagulation cascade. This is in contrast with the findings of another study evaluating combined OCs, in which increased levels of F1 + 2 were observed (The Oral Contraceptive and Hemostasis Study Group, 2003). However, the reductions in TAT and F1 + 2 levels seen in the present study remain unexplained. One possible explanation would be a reduction in the hepatic synthesis of prothrombin (factor II). In the present study, there was a gradual decrease in the levels of factor II. This decrease was significant in the sixth month of implant use, although remaining within the normal range.
Etonogestrel has been shown to be a synthetic progestogen that presents affinity not only for progesterone receptors but also for other steroid receptors, such as androgens and glucocorticoids (Schindler et al., 2003). It can play an active role in haemostasis by the effects it has on both of these types of receptors in the endothelium, muscle subendothelial cells, and hepatocytes (Hinchcliffe et al., 1996; Bernauer et al., 2001; Herkert et al., 2001).

Another finding of the present study was that, during six months of etonogestrel-releasing implant use, none of the subjects developed resistance to APC, an important predictor of VTE risk (Tans et al., 2003). This differs from the findings of studies involving the use of combined OCs, which were found to promote an acquired resistance to APC (Kemmeren et al., 2004).

There are two methods of assessing resistance to APC: the APTT-based test (used in the present study) and the endogenous thrombin potential (EPT)-based test (Rosing et al., 1997). The difference between the two tests is that they activate different coagulation pathways: the intrinsic pathway (APTT-based test) and the extrinsic pathway (ETP-based test). Resistance to APC was first associated with an increased risk of VTE using the APTT-based test results (de Visser et al., 1999). Subsequently, Tans et al. (2003) demonstrated that APC resistance is also predictive of VTE when determined through the use of the ETP-based (extrinsic pathway) test. Although APC resistance is predictive of VTE regardless of the test used, the ETP-based test presents greater sensitivity for evaluating acquired APC resistance (contraceptive used, e.g.) than does the APTT-based test (de Visser et al., 2005). Of the studies evaluating progestogen-only oral contraceptives and using the ETP-based test to identify APC resistance, none have identified any such resistance (Alhenc-Gelas et al., 2004; Kemmeren et al., 2004). The APTT-based test and ETP-based test were both used in one study, which was the only study assessing resistance to APC in users of etonogestrel-releasing implants (Lindqvist et al., 2003). The authors found that none of their subjects presented acquired resistance to APC. However, those authors evaluated the subjects after 1 month of implant use, which is prior to the critical period of increased risk for VTE (WHO, 1995a). Differences among the various progestogens in terms of APC resistance were only demonstrated when the individual progestogens were combined with estrogen (Rosing et al., 1997; Kemmeren et al., 2004).

In the present study, we also have reported some significant favourable alterations (reductions in the levels of factors VII and X) and some procoagulant changes (increases in the levels of factor XI and PAI-1; reduction in protein C). Such alterations, however, were transitory and within the range of normality, probably having little clinical significance.

In the remaining haemostatic tests, the variables TT, APTT, PT, factor V, factor VIII, factor IX, fibrinogen, the von Willebrand factor, free protein S, antithrombin, α2-antiplasmin and D-dimers were unaltered after the use of the implant for 6 months.

The measurement of D-dimer levels has been proven to be a useful negative predictor of VTE, with greater sensitivity than that of measurement of the TAT complex and F1 + 2 (Boneu et al., 1991). Despite its high sensitivity for predicting VTE (96%), the measurement of D-dimer levels has demonstrated lower specificity (40–50%), and D-dimer levels can be elevated in numerous situations in which there is no thrombin generation (Stein et al., 2004). Since D-dimers are used to indicate activation of the coagulation cascade indirectly (by fibrin dissolusion) rather than directly (by measuring the amount of thrombin produced), it is not known whether the D-dimer test is sensitive enough to detect a reduction in thrombin generation. Therefore, our findings of normal D-dimer concentration, despite the decreased TAT and F1 + 2 levels, could be explained by the fact that the reductions in these markers of thrombin formation would not change D-dimers levels to the same degree. With normal D-dimers levels, we can only conclude that there is no increased fibrin generation. Our results were similar to those reported by Winkler et al. (1998), who found decreased levels of F1 + 2 in users of desogestrel only, despite the normal D-dimer levels.

Previous studies of the effects that the etonogestrel-releasing implant has on haemostasis showed that the variables under study presented either no alterations or discrete alterations, and that the values were always within the normal ranges for the tests performed. In a 6-month study, Egberg et al. (1998) compared women using etonogestrel-releasing implants with those using levonorgestrel-releasing implants. The authors found that the two types of implants had similar effects on the haemostatic system, with no procoagulant tendency, as evidenced by a significant increase in antithrombin levels and a significant decrease in the levels of coagulation factor VII. Lindqvist et al. (2003) assessed haemostatic variables 1 month after the insertion of etonogestrel-releasing implants, comparing them with those obtained during the pre-insertion period. The authors found a discrete increase in the serum levels of natural anticoagulants (protein S and antithrombin), a small decrease in protein C levels and no resistance to APC. Finally, the results of another study carried out in our laboratory showed a transitory decrease in platelet aggregation in healthy female users of the implant (Vieira et al., 2005).

Regarding the effects that oral administration of progestogen-only contraceptives has on haemostasis, our findings are in agreement with those of previous studies in which no procoagulant pattern was reported (Winkler et al., 1998; Kemmeren et al., 2004). In fact, we found that the use of the implant had a more pronounced effect on markers of activation of the coagulation cascade than did oral administration. This might be attributable to the fact that the implant releases etonogestrel in a continuous, steady manner, which does not occur in oral administration (Huber, 1998).

Although we showed that the etonogestrel-releasing implant is not related to hypercoagulability, our study did not have sufficient power to evaluate the risk of thrombosis, which would require a much larger patient sample. Another limitation of this study is the fact that we presented haemostatic changes related to etonogestrel-releasing implant use over a 6-month period. Therefore, we do not know what happens to the coagulation system after this time. In fact, it is well known that the increased risk for VTE is apparent after 4 months of contraceptive use and is unaffected by the duration of use (WHO, 1995a).
Consequently, the best period during which to study haemostasis is that evaluated in the present study. Nevertheless, it would be quite interesting to evaluate the long-term effects of the etonogestrel-releasing implant.

In conclusion, the present study has added relevant information to what is already known regarding the effects that the use of etonogestrel in isolation has on haemostasis. Such effects were found to include decreased levels of markers of thrombin formation (TAT and F1+2) after 6 months of implant use, suggesting hypoactivation of the coagulation cascade and the absence of a prothrombotic pattern. It is tempting to speculate that such findings characterize biological mechanisms implicated in the lower risk for thromboembolic phenomena when this progestogen-only implant is used. However, further studies are necessary in order to assess the long-term risk of VTE associated with etonogestrel-releasing implants.

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Brummel KE, Paradis SG, Butenas S, Mann KG. Thrombin functions during activation to speculate that such findings characterize biological mechanisms implicated in the lower risk for thromboembolic phenomena when this progestogen-only implant is used. However, further studies are necessary in order to assess the long-term risk of VTE associated with etonogestrel-releasing implants.

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