—Original—

Inhibitory effect of ethinylestradiol on coagulation factors in rats

Yanira FRANCO-MURILLO and Ruth JAIMEZ

Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, Z.P. 04510, México

Abstract: Epidemiological and experimental data have indicated the beneficial and adverse effects of estrogenic replacement therapy. In the present study, we explored the effect of ethinylestradiol (EE) and 17β-estradiol (E_2) on screening tests, prothrombin time (PT) and activated partial thromboplastin time (APTT), as well as the activity of coagulation factors (FVII, FX, FXI, and FXII) in male Wistar rats. Animals were injected subcutaneously during three consecutive days with EE or E_2 (1, 3, 10, and 30 mg/kg) and propylene glycol (0.3 ml; vehicle, V). EE produced significant increments ($P<0.05$) on PT (8, 13, 15, and 10%) and APTT (32, 35, and 28%), whereas E_2 did not show any effect. EE diminished the activity of factors VII (−10, −13, and −10%) and X (−10, −9, −15, and −14%; $P<0.05$), and E_2 (1 mg/kg) produced a modest increment (8%; $P<0.05$) on FX only. E_2 (10 mg/kg) showed a diminution of 9% ($P<0.05$), while EE did not produce any response on factor XII. EE diminished (−15, −14, −19, and −17%) but E_2 augmented (10, 14, 24, and 24%) factor XI activity ($P<0.05$). Our findings suggest that EE and E_2 produce different effects on coagulation and that EE seems to act across an inhibitory mechanism of coagulation factor activity in the present experimental model.
Key words: coagulation, estrogens, male rat

Introduction

Epidemiological and experimental data have reported the effects of estrogens on humans as well as on animals. The female sex hormone estrogen regulates functions and development of its classical target organs, such as the breast and uterus, and has effects on other organs and tissues too [16]. Estrogens have been used in contraception and hormone replacement therapy. Despite the beneficial effects of estrogens on bone and the cardiovascular system, there are several reports indicating that estrogens produce changes in the coagulation system, resulting in an imbalance between pro- and anticoagulant states [5]. Moreover, there is evidence of a dual effect of estrogens on blood clotting time in rodents [15]. In past reports, we published differential results on blood coagulation time, between estradiol and ethinylestradiol in male Wistar rats and on procoagulant effects of estradiol in ovariectomized mice [9, 13].

In order to explore the effect of these estrogens on specific coagulation tests, we evaluated and compared the effects of estrogen, 17β-estradiol (E_2), and the most widely used compound in contraception, 17α-ethinylestradiol (EE), on male rats.

In this study, we found that EE elicits an inhibitory effect on extrinsic, intrinsic, and common pathways, unlike E_2.

Materials and Methods

Materials

EE (17α-ethinyl-1,3,5 (10)-estratrien-3,17β-diol), E_2 (1,3,5 (10)-estratrien-3,17β-diol), tert-amyl alcohol, 2,2,2-tribromoethanol, and propylene glycol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thromboplastin C Plus, Actin FS, coagulation factor-deficient plasmas (FVII, FX, FXI, FXII), standard human plasma, and imidazole buffer solution were purchased from Dade Behring (Marburg, Germany). Vacutainer® blood collection tubes with buffer sodium citrate and Safety-Lok™ collection sets were purchased from Becton, Dickinson and Co. (Flanklin Lakes, NJ, USA).

Animals

Domestic adult male Wistar rats (*Rattus Norvegicus*; 250 ± 10 g) from animal facilities of the Faculty of Medicine, Universidad Nacional Autónoma de México (UNAM), were used. Animals were housed under 12-h light/12-h darkness conditions with free access to food and water. All experiments were conducted in accordance with the Norma Oficial Mexicana (NOM-062-Z00-1999), and the technical specifications for the production, care, and use of lab animals of the Faculty of Medicine. The protocol was approved by the Ethics Committee in Research of the Faculty of Medicine, UNAM.

Experimental design

Animals were distributed in groups according to body weight. Each group of five received subcutaneously EE (1, 3, 10, or 30 mg/kg), E_2 (1, 3, 10, or 30 mg/kg), or propylene glycol (0.3 ml) during three consecutive days.

Blood and plasma collection. A day after the last injection, animals were anesthetized with Avertin (ip, 250 mg/kg, tert-amyl alcohol/2,2,2-tribromoethanol). Blood was collected from iliac artery using a Vacutainer® system with citrated (0.105 M) tubes (Dickinson and Co., Franklin Lakes, NJ, USA). Samples were centrifuged at room temperature for 10 min at 3,000 rpm (800 g), and then plasma was separated and stored at −20°C until assays were performed.

Screening tests: prothrombin time (PT) and activated partial thromboplastin time (APTT). PT was determined using Dade® Thromboplastin C Plus according with Quick's method [20] in a semiautomatic instrument (Fibrintimer BFT II®). Each sample (50 μl) was incubated 60 s at 37°C, and Dade® Thromboplastin C Plus reagent (100 μl) was added to start the reaction until clot formation.

APTT was evaluated with Dade® Actin FS reagent in a semiautomatic Fibrintimer BFT II® [19]. Each sample (50 μl) was mixed with Actin FS® (50 μl) and incubated 120 s at 37°C. A reaction was activated with $CaCl_2$ (50 μl) to induce clot formation. Results obtained were compared with values from the control group (vehicle, 100%), and represented as the percentage of response.

Standardization of factors VII and X (extrinsic and common pathways). Different dilutions were prepared with imidazole buffer and plasma as follows: 1:20 (100%), 1:40 (50%), 1:200 (10%), and 1:2,000 (1%). Samples (50 μl) were mixed with coagulation factor-deficient plasma VII or X (50 μl), incubated 60 s at 37°C, and activated with Thromboplastin C Plus (100 μl, Dade Behring®). The standard curve was constructed with the seconds vs. percentage.

Standardization of factors XII and XI (intrinsic pathway). Dilutions of plasma with imidazole buffer were prepared as follows: 1:5 (100%), 1:10 (50%), 1:50 (10%), and 1:500 (1%). Each sample (50 μl) was mixed with coagulation factor-deficient plasma (50 μl) and Dade® Actin FS (50 μl), incubated 120 s at 37°C, and activated with $CaCl_2$ (100 μl). The standard curve was constructed with the seconds *vs* percentage.

All techniques used for standardization were in accordance with García-Manzano *et al.* [9].

Determination of the effects of ethinylestradiol and estradiol on coagulation factors VII, X, XII, and XI. Plasma samples obtained from the EE, E_2, or vehicle (control group) groups were diluted. To determine the activity of factors VII and X, each sample was diluted 1:20 with imidazole buffer whereas factors XII and XI were diluted 1:5 in imidazole buffer.

Factors VII and X were quantified in accordance with the PT protocol: samples (50 μl) were mixed with coagulation factor-deficient plasma VII or X (50 μl), incubated 60 s at 37°C, and activated with Thromboplastin C Plus (100 μl, Dade Behring).

Factors XII and XI were processed in accordance with the APTT protocol: each sample (50 μl) was mixed with coagulation factor-deficient plasma (50 μl), and Dade Actin FS® (50 μl) incubated 120 s at 37°C, and activated with $CaCl_2$ (100 μl).

The time of reaction for each factor (FVII, FX, FXII, and FXI) was extrapolated in standard curves from rat plasma, and a percentage of activity was obtained. All
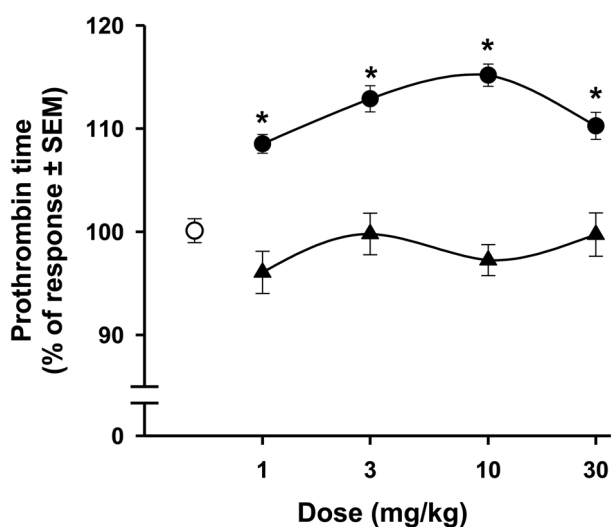
Fig. 1. Effect of ethinylestradiol (●) and estradiol (▲) on prothrombin time (PT). Each point represents the mean ± SEM of 36 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.
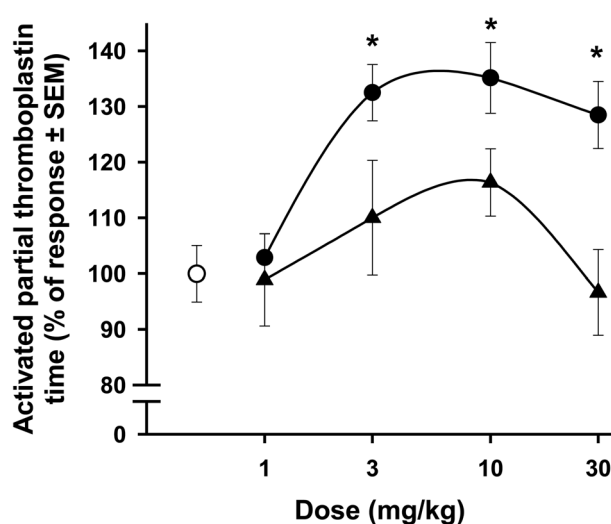

Fig. 2. Effect of ethinylestradiol (●) and estradiol (▲) on activated partial thromboplastin time (APTT). Each point represents the mean ± SEM of 36 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.

values in each experiment were normalized by considering the control group as 100% (vehicle). Experiments were performed at least three times. All samples were processed in a semiautomatic Fibrintimer BFT II®.

Statistical analysis. Statistical significance between treated groups and vehicle (control) was assessed by ANOVA and Dunnett's tests. $P<0.05$ was considered the limit of significant differences. Results were expressed as the percentage (%) of response relative to the control group ± the standard error of the mean (SEM). All experiments were performed in triplicate (SigmaPlot 10, Systat Software, San Jose, CA, USA).

Results

Effects of ethinylestradiol and estradiol on PT and APTT screening tests

Figures 1 and 2 show the effects of EE and E_2 on the PT and APTT screening tests, respectively. The administration of EE (1, 3, 10, and 30 mg/kg) increased PT significantly (9, 13, 15, and 10, respectively; $P<0.05$), whereas E_2 did not show any effect (Fig. 1). Figure 2 shows that EE produced a significant increment ($P<0.05$) in APTT at 3, 10 and 30 mg/kg (32, 35, and 28%, respectively).

Effects of ethinylestradiol and estradiol on extrinsic factors VII and X

Figures 3 and 4 show the effects of EE and E_2 on coagulation factors VII and X, respectively. The administration of EE (3, 10 and 30 mg/kg) significantly decreased the activity of factor VII by −10, −13, and −10% ($P<0.05$), respectively, whereas E_2 showed a significant increment of 8%, but only at 1 mg/kg (Fig. 3). Figure 4 shows that EE produced a significant decrease at all tested doses ($P<0.05$). The values obtained were −10, −9, −15, and −14%, respectively. E_2 did not result in any significant response in this factor.

Effects of ethinylestradiol and estradiol on intrinsic factors XII and XI

Figures 5 and 6 show the effects of EE and E_2 on coagulation factors XII and XI, respectively. The administration of EE (1, 3, and 30 mg/kg) produced a modest but not significant increment in FXII activity (11, 7, and 10%, respectively), whereas E_2 at 10 mg/kg produced a significant decrease (9%, $P<0.05$). Figure 6 shows a significant and dose-dependent increase of FXI produced by EE treatment (10, 14, 24, and 24%; $P<0.05$). This figure also shows that E_2 produced an opposite effect on the same coagulation factor at all doses tested. E_2 reduced FXI by −15, −14, −19, and −17% ($P<0.05$) at 1, 3, 10, and 30 mg/kg, respectively.
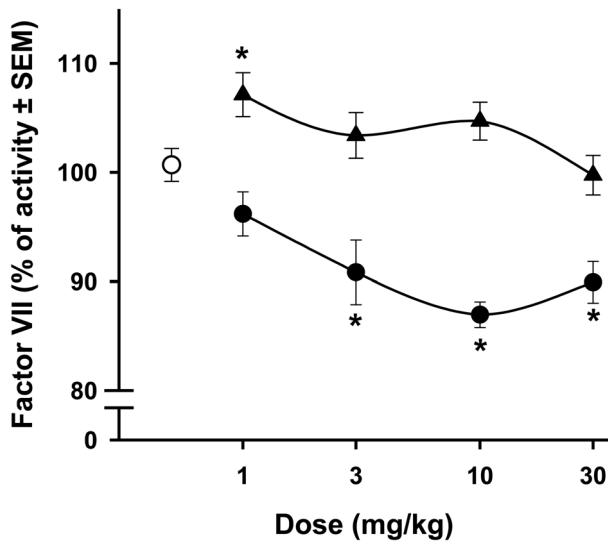
Fig. 3. Effect of ethinylestradiol (●) and estradiol (▲) on activity of coagulation factor VII. Each point represents the mean ± SEM of 24 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.
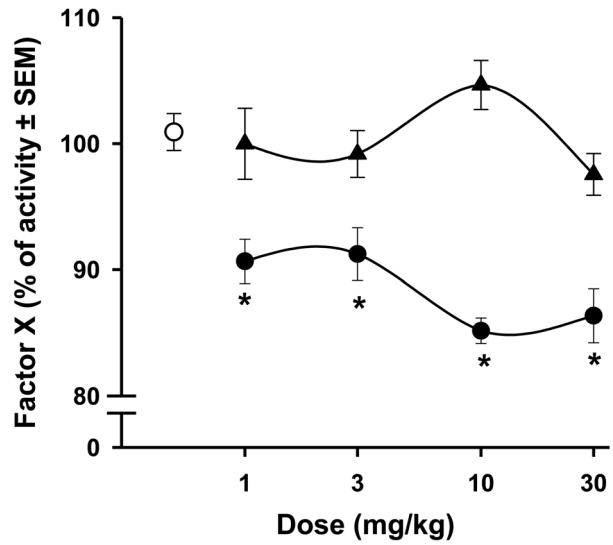

Fig. 4. Effect of ethinylestradiol (●) and estradiol (▲) on activity of coagulation factor X. Each point represents the mean ± SEM of 24 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.
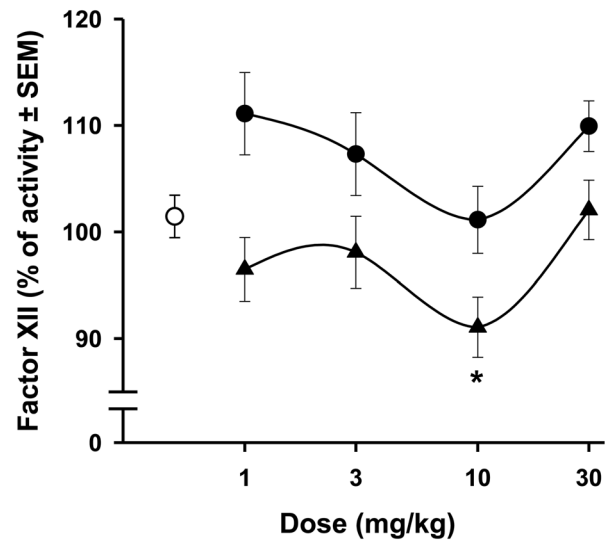

Fig. 5. Effect of ethinylestradiol (●) and estradiol (▲) on activity of coagulation factor XII. Each point represents the mean ± SEM of 24 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.
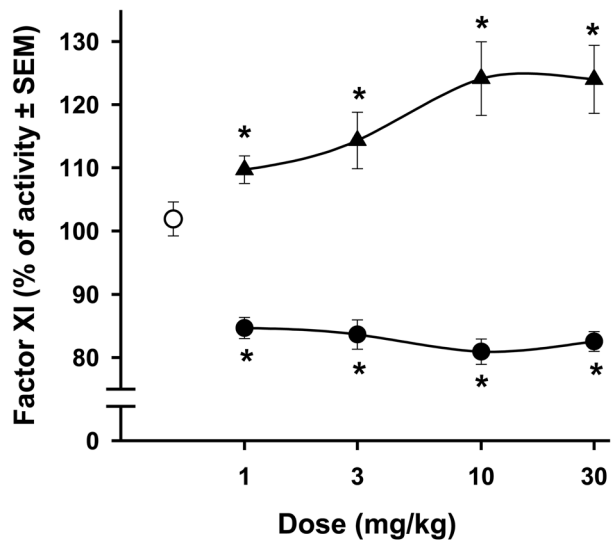

Fig. 6. Effect of ethinylestradiol (●) and estradiol (▲) on activity of coagulation factor XI. Each point represents the mean ± SEM of 24 determinations. *$P<0.05$ vs. vehicle (O) by Dunnett's test.

Discussion

In this study, both PT and APTT were increased after EE treatment but not after E_2 treatment. Our results contrast with previous results where two administrations of E_2 (30 mg/kg) produced increments in PT and APTT [8].

The increment observed in PT (so called factor II, FII)

is in agreement with Bonnar and Sabra [2]. In women, Bonnar and Sabra demonstrated that FII activity increased significantly with three monophasic preparations (ethinylestradiol 30 μg and levonorgestrel 0.15 mg; ethinylestradiol 30 μg and norethindrone acetate 1.5 mg; and mestranol 50 μg and norethindrone 1 mg).

The inhibitory effect of EE on FVII activity is prob-

ably related to the suspected repression of the transcriptional activity of the FVII promoter by estrogenic factors. One possible mechanism to explain the observed suppression of FVII activity in the presence of estrogenic factors could be that estrogen receptor binding to the FVII estrogenic response elements prevents a positively acting transcription factor from binding at this site, which prevents its form displaying any transactivation properties [1]. These findings are consistent with previously reported data in humans showing an increased level of FVII in postmenopausal women compared with that of similar age premenopausal women and also the inverse relationship between FVII and estradiol levels during the menstrual cycle [12, 21]. The increased levels of estrogens during pregnancy and associated increase in FVII levels appear at first paradoxical and opposite to these findings. For instance, oral contraceptive formulations using synthetic estrogenic compounds are generally accepted to results in an increased, not decreased, level of FVII antigen in plasma [2, 17]. Elevated cFVIIa plasma levels thus may represent a pronounced prothrombotic blood capacity, which may be of clinical importance in case of atherosclerotic vascular defects, as tissue factor-producing cells have been identified in atherosclerotic plaques. cFVIIa is a potent hemostatic *stimulus*, as complex formation of FVIIa with tissue factor is the main physiological triggering mechanism of blood coagulation [14, 22].

The common pathway begins with the activation of factor X by the intrinsic system, the extrinsic system, or both. Factor Xa, in the presence of factor V, Ca^{2+}, and phospholipid (PF3), converts prothrombin to its active form, thrombin. Because the common pathway contains factors X, V, II, and I, these factors may be monitored by both the PT and the APTT.

Our data on FX are very similar to those obtained on FVII; EE induced a decrease in FX activity. Unlike FVII, there are no reports about inhibitory effects of estrogens on FX. Our results are the first evidence in a rat model that shows that EE could be involved in inhibitory activity of FX. Once more, our evidence contrasts with several reports in regard to coagulation factor activity being enhanced by estrogens [8].

There are many reports that have pointed out the effect of E_2 on FXII, but there is no evidence about the effect of EE on this factor. In this study, neither EE nor E_2 produced any effect on FXII. This result was unexpected and contrasted with various studies with the evidence supporting that estradiol enhances FXII via regulation of gene transcription is supported. Using livers isolated from rats Gordon *et al*. [10] demonstrated that E_2 and prolactin may raise the plasma titer of factor XII (Hageman factor) by enhancing gene expression at the level of transcription and RNA processing, protein synthesis, or secretion (or a combination of them). Farsetti *et al*. [6] and Citarella *et al*. [3] reported the effect of estrogen on the FXII promoter. They analyzed the hormone induction of FXII gene transcription in transient cotransfection assays in mouse NIH3T3 fibroblasts and human hepatoma cells (HepG2). Additionally, Farsetti *et al*. [7] demonstrated that HNF-4 (hepatocyte nuclear factor 4) usually acts alone or in combinatorial association with other tissue-specific or basal transcription factors, in promotion of the transcription of a wide variety of target genes as well as several coagulation factors. In that study, they demonstrated that HNF-4 is instead capable of antagonizing ERα transactivation properties of the FXII gene promoter.

Finally, we evaluated the effect of EE and E_2 on FXI activity. Factor XI is activated by FXII. In our study, we demonstrated that EE produced an inhibitory effect on FXI activity, while E_2 produced an unexpected and opposite effect in relation to EE. The increment of FXI activity induced by E_2 could be related to a procoagulant activity.

The lack of effect of E_2 on some factors and the opposite effect on FXI with respect to EE could be explained in part by pharmacokinetic differences specific to half-life, because E_2 has a half-life of approximately 70 min (1 h), whereas the half-life for EE is 12 h (8–24 h) [11].

Our results indicate that effects of EE seem to be correlated with a decrease of coagulation factors activity and thus may be implicated in prevention of thrombus. Our findings are in agreement with Mueck *et al*. [18]. He reported a positive influence of EE on the various markers and found improvement of vascular tone, impediment of the development of atherosclerosis and arterial thrombosis, and improvement of water and electrolyte homeostasis. Cleuren *et al*. [4] reported the effect of EE on coagulation factor activity and hepatic transcript levels in ovariectomized mice caused by oral treatment for ten days. They demonstrated a significant reduction in plasma activity levels of combined FII, FVII, and FX combined as well as a reduction in the transcript levels of FII, FVII, FX, and FXII among others.

In this study, we demonstrated for the first time in rat model an inhibitory effect of EE on global screening tests and on some specific coagulation factors, including FVII, FX, and FXI. Moreover, we presented evidence of opposite effects of EE and E_2 on factor XI. Further studies are needed to evaluate the implied mechanisms with inhibitory effects and to understand how different doses can produce diverse effects on distinct *in vivo* and *in vitro* models as well as humans to prevent or diminish adverse effects of natural and synthetic estrogens.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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