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Effects of conjugated estrogen and bazedoxifene on hemostasis and thrombosis in mice

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Abstract

Estrogen–progestin therapy was previously considered as the standard of care for managing bothersome symptoms associated with menopause, but it increases risks of breast cancer and of thromboembolism. The combination of conjugated estrogen (CE) with bazedoxifene (BZA) named tissue-selective estrogen complex (TSEC) was designed to minimize or even abrogate the undesirable effects on breast, while maintaining the beneficial effects such as prevention of osteoporosis and suppression of climacteric symptoms. The risk on thromboembolism associated with TSEC is unknown, although the clinical available data are reassuring. The aim of this study was to define the impact of a chronic administration of CE, BZA or CE + BZA on hemostasis and thrombosis in ovariectomized mice. As expected, CE, but not BZA neither CE + BZA, induced uterine and vagina hypertrophy. As previously demonstrated for 17β-estradiol (E2), we found that CE (i) increased tail-bleeding time, (ii) prevented occlusive thrombus formation in injured carotid artery and (iii) protected against collagen/epinephrine-induced thromboembolism. Thus, whereas BZA antagonized CE action on reproductive tissues, it had no impact on the effect of CE on hemostasis, thromboembolism and arterial thrombosis in mice. CE + BZA shared the anti-thrombotic actions of CE in these mouse models. If a similar process is at work in women, CE combined with BZA could contribute to minimize the risk of thrombosis associated with hormone replacement therapy.

Introduction

Menopause is defined as the permanent cessation of menstruation and ovulation due to ovarian failure. The decline in estrogen and progesterone levels in women can lead to a number of bothersome symptoms. The most common include bone loss, vasomotor symptoms (hot flushes and sweats) and genito-urinary disturbances, affecting sexual function, relationships and quality of life. These symptoms are usually relieved by the administration of an estrogen. However, hormone replacement therapy (HRT) has been associated with adverse effects such as an increased incidence of breast cancer and thromboembolic events (1, 2, 3). Venous thromboembolism events (VTEs), which encompasses deep venous thrombosis (DVT) and pulmonary embolism (PE), accounts for about one-third of all potentially fatal cardiovascular events in postmenopausal women on HRT (4). Determinants of the risk of VTE among HRT users have been identified; they include the route of estrogen administration and the type of progestogen associated with it (5, 6, 7, 8).

Concerns about the range of potential risks of standard HRT have led to an active search for innovative therapies. A new generation of molecules called tissue selective estrogen complex (TSEC) (9) combines a selective estrogen receptor modulator (SERM) with one or more estrogens. In this context, Pfizer has developed the first menopausal HRT without a progestogen for nonhysterectomized women: Duavee® pairs bazedoxifene (BZA), the first of the third-generation of SERMs, with conjugated equine estrogen (CE) (Premarin®) (10). It must be emphasized that BZA is not only a SERM, but it also induces a rapid degradation of the estrogen receptor α (ERα) in some tissues, such as the uterus and the breast (11).

The efficacy and safety of CE+BZA in postmenopausal women and the impact of this drug combination on quality of life was evaluated through a series of five pivotal phase 3 randomized, double-blinded, multicentered, active and/or placebo-controlled studies, called the Selective estrogens, Menopause And Response to Therapy (SMART) trials (9, 12, 13, 14, 15, 16). From these trials, CE+BZA were found to be associated with significant benefits such as a reduction in the frequency and severity of vasomotor symptoms, the prevention of bone loss, improved sleep and better menopause-specific quality of life. Moreover, CE+BZA did not adversely affect lipid metabolism, provide an acceptable level of protection against endometrial hyperplasia and did not increase mammographic breast density (13, 17, 18, 19, 20, 21, 22, 23). In clinical trials up to 2 years, CE+BZA seemed to have a safe profile and the rates of venous thromboembolism were similar to placebo, but these trials were not powerful enough to fully answer this question.

We recently attempted to investigate the complexity of sex hormones actions on hemostasis and thrombosis in mice (24, 25, 26). We reported that chronic E2 (estradiol-17β) treatment administered subcutaneously increased tail-bleeding times and protected animals against collagen/epinephrine-induced thromboembolism and carotid artery thrombosis in comparison to ovariectomized or sham-operated mice (24). Here, our goal was to explore *in vivo* the effects of a chronic administration of BZA combined with CE on hemostasis and thrombosis in ovariectomized mice.

Materials and methods

Mice

Female C57BL/6J mice were purchased from Charles River Laboratories. All procedures were performed in accordance with the principles established by the National Institute of Medical Research and were approved by the local animal ethics committee (Toulouse, France) (protocol number 15/1048/9/67). Mice were anesthetized by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg) and ovariectomized at 4 weeks of age. To study the chronic effects of CE, BZA and CE+BZA, 2 weeks after ovariectomy, mice were implanted with subcutaneous osmotic minipumps (Alzet; Alza, Palo Alto, CA, USA) that released BZA (10 mg/kg/day), CE (3 mg/kg/day) or CE+BZA for a 3-week period. The powders were solubilized in a solution of hydroxypropyl-β-cyclodextrin in HEPES buffer. The treatment doses of CE and BZA were chosen according to results from previous *in vivo* studies (27). Blood was collected from the inferior vena cava of these mice to explore coagulation factor defects (Diagnostica Stago).

Tail bleeding time

After anesthesia by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg), tail bleeding time was measured using a 3-mm tail-tip transection. Blood drops were removed every 15 s with the use of a paper filter. If bleeding did not reoccur within 30 s of cessation, it was considered stopped. Experiments were terminated after 30 min if blood flow had not ceased.

Thromboembolism

Acute systemic vascular thromboembolism was induced by injecting a mixture of collagen (0.4 mg/kg) and epinephrine (60 µg/kg) into the right jugular vein of anesthetized mice. Mice were killed 10 min after injection of the mixture for histological analysis.

Carotid artery thrombosis

The Vevo2100 high-frequency ultrasound system (HFUS) (Visualsonics, Toronto, Canada) was used to monitor thrombus formation in the right carotid artery of mice as previously described (26). This micro imaging system consists of a single element probe of 18-38 MHz frequency. Animal heart rates were monitored. Body temperature was monitored using a rectal probe and was regulated with a heating pad. The carotid artery was dissected free from surrounding tissues and $FeCl_3$ was used to induce vascular injury. A 1×4 mm strip of paper saturated with 7% $FeCl_3$ solution was applied

to the adventitial surface of the left carotid for 3 min and then removed. Warm ultrasound transmission gel was applied to enable visualization and optimize image quality.

Histological analysis of vagina

Paraffin-embedded transverse sections (4 μm) from formalin-fixed vaginal specimens were stained as previously described (28) with anti-Ki-67 (RM-9106; ThermoScientific) and anti-ERα antibodies (MC-20, Santa Cruz Biotechnology). Sections were examined after numerization using a NanoZoomer Digital Pathology®. To examine the proliferative effects of each treatment, the ratio of Ki-67-positive epithelial cell/total cell number from two microscopic fields of measurement at ×20 magnification for each vaginal section was evaluated.

Statistical analysis

Results are expressed as means±s.d., as indicated. Statistical analyses were performed using Graph Pad (one-way ANOVA and Fisher's exact test) *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

Body and uterine weights – vagina weight and structure

After a 3-week administration of BZA (10 mg/kg/day), CE (3 mg/kg/day) or CE+BZA to ovariectomized mice, the vaginas and uterus were excised and weighed. As no difference in body weights between ovariectomized untreated mice (mean = 22.4 g, s.d. ±2.1), BZA- (21.8 g ± 1.6), CE- (23.8 g ± 0.5) and CE+BZA- (21.4 g ± 1.2) treated mice were observed, we compared directly uteri weights.

As a result of estrogen treatment, vaginal and uterine weights from CE-treated mice were higher than those of vehicle-treated controls BZA- and CE+BZA-treated mice (Fig. 1A). We then evaluated the changes of the structure of the vagina by histological analysis. Vaginal epithelial proliferation was evaluated by immunohistochemical detection of nuclear expression of Ki-67 antigen and was significantly decreased in CE+BZA-treated mice compared to CE-treated mice (Fig. 1B and C). After chronic exposure, the changes of the uterine and vaginal cellular content induced by CE treatment did not allow a comparison of protein expression by Western blotting. Thus, ERα protein levels were evaluated by immunohistochemistry, which is essentially qualitative rather than quantitative. As previously observed for the
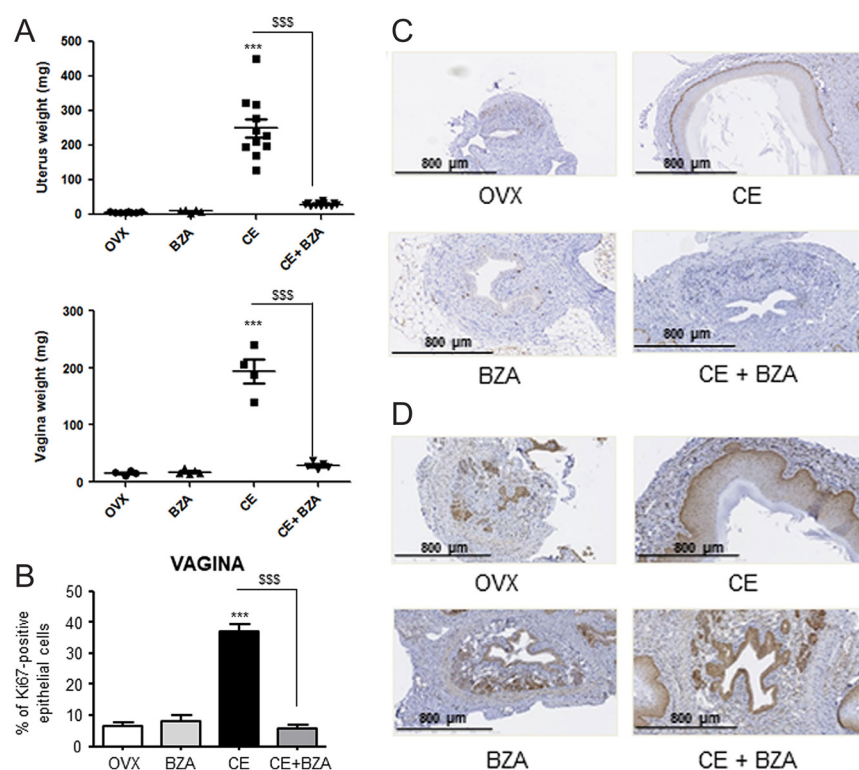

Figure 1

Effect of chronic treatment of CE, BZA and CE + BZA on the vagina in mice. (A) Uterus and vagina weights (mg) after chronic treatment with bazedoxifene (BZA, 10 mg/kg/day), conjugated equine estrogen (CE, 3 mg/kg/day) alone or in combination (BZA + CE). Each point represents 1 individual. (n = 8–10 mice/group). (B) Vaginal epithelial proliferation, as evaluated by quantification of Ki-67-positive cells in epithelium. Results are expressed as means ± s.e.m. To test the respective roles of each treatment, one-way ANOVA and a Bonferroni's multiple comparison test were performed (treatment *versus* placebo: ***$P < 0.001$; CE *versus* BAZ + CE treatment $^{\$\$\$}P < 0.001$; n = 8–10 mice/group). Representative immunodetection of proliferation (anti Ki67 antibodies) (C) and ERα (MC20 antibodies) (D) in vaginal sections.

uteri (27), BZA treatment alone or in combination with CE did not affect the level of vaginal tissue ERα when evaluated by immunohistochemistry (Fig. 1D). A previous publication (27) and the present report demonstrate that BZA antagonizes the action of CE in two sex target tissues: the uterus and the vagina.

Hematologic parameters

We evaluated the effects of a chronic administration of CE+BZA on hematologic parameters. We found that CE-treated mice had lower platelet counts compared to ovariectomized mice, whereas BZA treatment did not induce significant changes to the platelet count (Table 1). Standard coagulation tests, fibrinogen and coagulation factor levels were then determined, and showed that chronic CE, BZA and CE+BZA had no or only very modest impact on these parameters (Table 1).

Effect of chronic CE, BZA and CE + BZA treatments on hemostasis

We previously reported estrogens impact on primary hemostasis in mice by showing that chronic E2 administration decreases platelet responsiveness and increases tail-bleeding times (24). Thus, we first investigated the effect of CE, BAZ and CE+BAZ on tail-bleeding times *in vivo*. As expected, CE treatment prolonged the tail-bleeding time (>10 min) in the majority of the mice (9 of 16). Conversely, tail bleeding times of BZA-treated mice were similar to untreated ovariectomized mice (7.1±4.4 min, $n=10$, and 5.6±2.6 min, $n=12$, respectively) (Fig. 2). A combination of CE and BZA increased the tail-bleeding time, with 7 out of 16 mice having prolonged (>10 min) bleeding times, demonstrating that BZA did

not alter the action of CE on this hemostatic parameter (Fig. 2). Statistical analysis for CE and CE+BAZ seems not relevant because of the prolonged bleeding time which is superior (and not egal) to 30 min.

Effect of chronic CE, BZA and CE + BZA treatments on thrombosis

We also previously showed that the E2-induced changes in primary hemostasis correlate with an impressive resistance to collagen-epinephrine-induced thromboembolism (24). Here, we tested the effects of CE, BZA and CE+BZA on this. All untreated ovariectomized mice and most BZA-treated mice died from occlusive thromboembolism within 5 min following the injection of a collagen/epinephrine mixture into the jugular vein, whereas all CE-treated (7/7) and most CE+BZA-treated (5/7) mice were still alive 10 min after administration of the thrombogenic mixture (Fig. 3A). Histological examination of lung tissue showed marked protection from occlusive thrombi in vessels of CE- and surviving CE+BZA-treated mice compared to ovariectomized and BZA-treated mice. The occlusive pulmonary thrombi in surviving mice were mostly observed in small vessels, and rarely in large vessels (Fig. 3B).

The protective effect of CE+BZA on occlusive thrombus formation was then tested in another *in vivo* model of thrombosis, the $FeCl_3$-induced carotid injury model that leads to artery occlusion. A complete and stable artery occlusion was observed within 20 min in 60% of ovariectomized and BZA-treated mice (Fig. 4). In contrast, CE-treated mice showed protection against occlusive thrombus formation (50% of mice exhibited a complete resistance to occlusion, with unstable occlusions observed in the others) and all of the CE+BZA-treated

Table 1 Hematological parameters.

	OVX	BZA	CE	CE + BZA
Platelet count	554 ± 115	493 ± 40.5	385 ± 145*	512 ± 91
PT	94 ± 3	89 ± 3	110 ± 12	101 ± 7
APTT	81 ± 10	77 ± 9	57 ± 8	90 ± 13
Fibrinogen	2.4 ± 0.5	2.9 ± 0.7	2.6 ± 0.1	4.3 ± 0.6
Factor II	43 ± 6	38 ± 3	40 ± 5	46 ± 4
Factor V	428 ± 28	446 ± 36	462 ± 56	513 ± 106
Factor VII	416 ± 49	605 ± 48	503 ± 43	675 ± 4*
Factor X	103 ± 9	87 ± 7	107 ± 7	103 ± 5
Factor III	154 ± 11.5	132 ± 13	165 ± 20	194 ± 14
Factor IX	16 ± 1	16 ± 1	21 ± 3	23 ± 2
Factor XI	28 ± 2	31 ± 1	39 ± 4*	35 ± 3

Platelet count (G/L), coagulation tests (prothrombin time (PT) and activated partial thromboplastin time (APPT)), fibrinogen and coagulation factors levels in ovariectomized (OVX) and treated mice with BZA (10 mg/kg) or CE (3 mg/kg) alone or in combination. Means ± s.d. One-way ANOVA was performed. *$P < 0.05$ indicates significantly different from OVX.
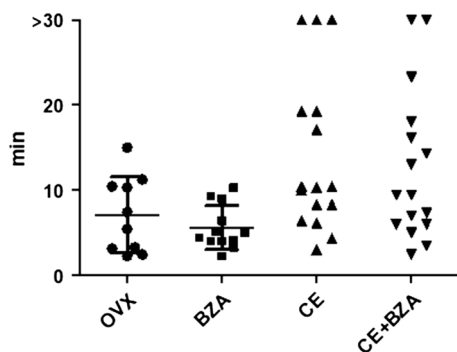
Figure 2
Increased bleeding times in CE and CE + BZA-treated mice. Tail-bleeding times of mice after chronic treatment with BZA (10 mg/kg/day), CE (3 mg/kg/day) alone or in combination (BZA + CE). Each point represents 1 individual.

mice exhibited a complete resistance to occlusion. Taken together, these data suggest that CE+BZA treatment significantly protects mice from occlusive thrombus formation in these two *in vivo* models of thrombosis.

Discussion

CE conjugated with BZA is approved for the treatment of osteoporosis and vasomotor symptoms in postmenopausal non-hysterectomized women (23). This combination of estrogen and SERM presents a tissue-specific action, which has been investigated by five randomized clinical trials. CE+BZA demonstrated agonist effect on bone (prevention of bone loss, reduction of bone turnover) and on vasomotor symptoms (reduction of hot flush frequency and severity) (29). However, since CE prevents breast cancer in the WHI trial (1) and SERMs such as tamoxifen also prevent breast cancer (30), one could expect that the

combination of CE with BZA will be safe in this respect. Although no increased risk of breast cancer was shown in the SMART trials, no conclusion can yet be drawn as this study was not designed to this end and was not powerful enough to answer this question.

According to the phase 3 studies, CE+BZA induced minor effect on coagulation profile (16, 20) and VTE was a rare event, affecting less than 1 person per 1000 patients (31). Indeed, although the risk of VTE is increased by CE and BZA individually, it is not incremented by their combination as TSEC. Indeed, among the 1585 women included in the SMART studies and treated with CE 0.45 mg/BZA 20 mg, 3 women (0.2%) developed deep vein thrombosis and one (0.1%) in placebo (31, 32). Nevertheless, further studies are needed to confirm this low incidence. Again, lack of statistical power of these trials combined with the fact that VTE are rare events, especially in relatively young women (mean 54 years), indicate the need for further experimental preclinical studies. In addition, the impact of SERMs such as tamoxifen in hemostasis and thrombosis is controversial in *ex vivo* model (33, 34). In addition, raloxifene inhibited thrombin generation at high concentrations (35). In this context, we studied here the impact of CE+BZA treatment on hemostasis and thrombosis in mice.

Concerning primary hemostasis, BZA treatment had no effect on this parameter compared to ovariectomized mice. CE administered subcutaneously over 3 weeks induced an increase in tail bleeding time and, when paired with BZA, the association did not antagonize the prolonged tail bleeding. We also explored the impact of this TSEC on induced thrombosis. We showed here for the first time that when paired with CE, BZA did not alter the protective effect of CE against collagen/epinephrine-
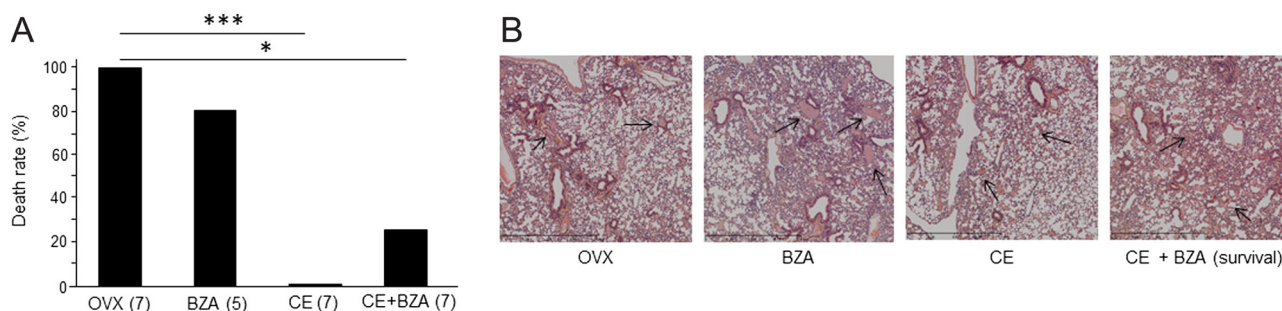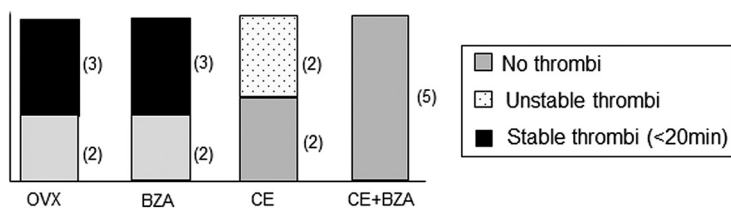

Figure 3
CE + BZA treatment protects mice against thromboembolism. (A) Thromboembolism was induced by injection of a collagen (0.4 mg/kg) and epinephrine (60 µg/kg) mixture into the jugular vein. All ovariectomized mice died within 5 minutes. CE- and CE + BZA-treated mice were protected from thromboembolism. Exact Fisher test was performed. *$P < 0.05$, ***$P < 0.001$. (B) Representative sections of hematoxylin-eosin–stained lungs from ovariectomized mice and BZA-treated mice mouse that died during the assay and from CE- and CE + BZA-treated mouse that survived and was killed 10 min after injection of collagen/epinephrine mixture. Original magnification ×400. The arrows indicate the position of thrombi.

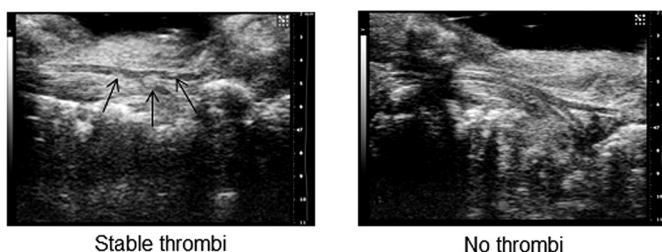|	OVX	BZA	CE	CE+BZA
Time to stable occlusion (minutes)	20, 15, 10	11, 11, 10	Unstable thrombi at 8 and at 20	No thrombi
Mean (SD)	15 (±5)	11 (±0.6)		

Figure 4

CE + BZA treatment protects mice against thrombosis in the carotid artery. Thrombi in the carotid arteries were visualized by high-frequency ultrasound after ferric chloride injury. Longitudinal view of mice with a stable thrombus (left panel) and without thrombus (right panel). Representative images are shown. The arrows indicate the position of the thrombi.

induced thromboembolism. In addition, CE+BZA-treated mice exhibited a strong resistance to occlusive thrombus formation in an *in vivo* model of $FeCl_3$-induced carotid injury. It is noteworthy that these effects of CE+BZA on hemostasis and thrombosis were not correlated with modification of platelet counts and blood coagulation factors. Consistently, we have previously shown that chronic treatment with E2 has a protective effect against experimental occlusive thrombosis, independently of modification of coagulation factors (24, 25). We confirmed here the favorable impact of estrogens on thrombosis in mice and the neutral effect of BZA alone.

The effect of this TSEC on uterine tissues is also reported by the SMART trials. Compared with traditional progestin-containing HRT (CE and acetate of medroxyprogesterone acetate), CE+BZA offers similar protection against endometrial hyperplasia (21, 29). In addition, BZA alone did not increase endometrial thickness in women (13, 36). Here, we confirmed that chronic treatment with BZA alone had no impact on uterus and we extend these conclusions to vagina, another sex target tissue of CE and BZA. Indeed, the increase of weight and epithelial proliferation, qualitatively evaluated by immunohistochemistry, observed with CE treatment, was abrogates by BZA. This is in accordance with the results of the SMART clinical trials which observed a significant increase in vaginal superficial cells, but a decrease in parabasal cells, at 12 weeks in the CE+ BZA group compared with placebo and bazedoxifene alone. Despite the antagonism of uterotrophic effect of CE

by BAZ, BZA+CE treatment in women has been reported in SMART 3 trial to improve vaginal maturation index and ease of lubrication, with a positive impact on quality of life (14, 29).

After a chronic treatment, BZA did not impact the level of ERα in vaginal tissue (Fig. 1D) as on the uterus tissue (27). BZA has been described to modify the conformation of ERα, inducing proteosomal degradation (37). BZA would act like a SERM rather than like a SERD in the female reproductive tract after chronic treatment. Madak-Erdogan *et al.* emphasized that the TSEC exhibited pathway-selective activities distinct from those of CE, E2 or BZA alone (38).

Conclusion

Our results clearly indicate that, after a chronic CE+BZA treatment, mouse models are protected from *in vivo* induced thrombosis without significant functional alterations to coagulation factor levels. Concerning the question of the thrombotic risk of TSEC, only observational studies will contribute to be reassuring, or even better a large clinical trial. However, in the meantime, if a similar effect is at work in women at the level of the haematopoeitic system as now clearly demonstrated in mice, it could help to counterbalance the stimulatory effect of estrogens on liver-derived coagulation factors, and thereby could attenuate the increased risk of VTE during TSEC administration.

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