Heparanase procoagulant activity is elevated in women using oral contraceptives

Moshe Matan1, Elena Axelman2, Benjamin Brenner2, and Yona Nadir2,*

1Faculty of Medicine, The Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel 2Thrombosis and Hemostasis Unit, Department of Hematology, Rambam Health Care Campus, Haifa, Israel

*Correspondence address. Tel: +972-4-8453520; Fax: +972-4-8543886; E-mail: ynadir@netvision.net.il

Submitted on September 12, 2012; resubmitted on March 31, 2013; accepted on May 23, 2013

STUDY QUESTION: What is the effect of estrogen on heparanase procoagulant activity?

SUMMARY ANSWER: Estrogen increases heparanase procoagulant activity.

WHAT IS KNOWN ALREADY: Estrogen therapy increases the risk of thrombosis and was previously found to up-regulate heparanase expression. Heparanase is involved in angiogenesis and metastasis, and has been shown to form a complex with tissue factor (TF) and also shown to enhance the generation of factor Xa.

STUDY DESIGN, SIZE, DURATION: A case–control study. Thirty-four healthy women using oral contraceptives (OC) and 41 women not using hormonal therapy and not pregnant per history were enrolled, over a 5-month period, at the Rambam Medical Center, Haifa, Israel. In vitro, estrogen receptor-positive (MCF-7) and -negative (MDA-231) cell lines were incubated with estrogen, tamoxifen and ICI-182.780 a pure estrogen receptor antagonist. The cell medium was evaluated for TF/heparanase complex activity, TF activity and heparanase procoagulant activity by chromogenic substrate.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Exclusion criteria included age < 18 years, post-menopausal women, concomitant medications other than supplement minerals and vitamins, acute or chronic illness.

MAIN RESULTS AND THE ROLE OF CHANCE: The study demonstrates increased risk of high heparanase procoagulant activity in OC users. When a cutoff level of 0.25 (absorbance 405–490 nm) was set, the odds ratio was 131 (P < 0.0001). When all results were studied by quartiles, in quartiles 3 and 4 the results were almost exclusively of the OC users (P < 0.0001). In cell cultures, estrogen and tamoxifen increased heparanase procoagulant activity in the medium of estrogen receptor-positive (MCF-7) cells.

LIMITATIONS, REASONS FOR CAUTION: The main limitation of the current study is that the two estrogens given to the women and cell cultures, ethinyl estradiol (EE) and 17-β-estradiol (E2), respectively, may have different effects on the coagulation system, although an increase in heparanase procoagulant activity was demonstrated in both of them. Although the sample size of the study group was limited, significant differences in the activation of the extrinsic coagulation pathway were demonstrated.

WIDER IMPLICATIONS OF THE FINDINGS: The clinical relevance of the heparanase procoagulant activity assay as a screening tool in thrombophilia work-up should further be elucidated.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was sought for this study. Authors Nadir and Brenner are named in a US Provisional Patent Application No. 29509/WO/12 filed on 18.01.2012. The other authors have no conflict of interest to declare.

TRIAL REGISTRATION NUMBER: N/A.

Key words: heparanase / coagulation / oral contraceptives / estrogen / tamoxifen

Introduction

The use of oral contraceptives (OC) is a well-established risk factor for venous thrombosis. Evidence on hormonal contraceptive is derived almost exclusively from observational studies and points to a 2–6-fold increased relative risk of venous thromboembolism (Deitcher and Gomes, 2004). Acquired protein C resistance resulting from reduced levels of protein C, protein S and elevated factor VIII is the main
mechanism known today to explain the increased risk of venous thromboembolism among users of OC (Rosing et al., 1999).

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate (HS) side chains at a low pH (5.8–6.2), yielding HS fragments of still appreciable size (~5–7 kDa) (Freeman and Parish, 1998; Pikas et al., 1998). Heparanase activity is implicated in tumor growth, neovascularization and inflammation (Dempsey et al., 2000; Parish et al., 2001; Vladovskv and Friedmann, 2001). Applying active site-mutated heparanase devoid of enzymatic activity, it was noted that heparanase also exerts non-enzymatic activities, at physiological pH, associated with tissue remodeling, angiogenesis and cell invasion (Goldsmith et al., 2003; Gingis-Velitski et al., 2004; Zetser et al., 2006). Recently, we have shown that heparanase is directly involved in the activation of the coagulation system. Heparanase was demonstrated to interact with tissue factor (TF) and enhance the generation of factor Xa (Nadir et al., 2011a). In addition, we lately developed an assay to evaluate heparanase procoagulant activity showing significant increased heparanase procoagulant activity in women at the end of pregnancy and in patients following orthopedic surgery (Nadir et al., 2011b; Peled et al., 2012). Elkin et al. identified four putative estrogen response elements in the heparanase promoter region and found that heparanase promoter genes were significantly up-regulated in estrogen receptor-positive MCF-7 human breast carcinoma cells after estrogen treatment. In vivo, exposure to estrogen augmented levels of heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with increased plug vascularization (Elkin et al., 2003).

In the present work, estrogen effect on the procoagulant activity of heparanase was evaluated in breast cancer cell lines and women using OC.

**Materials and Methods**

**Study group**

The study was approved by the institutional Ethic Committee on human research at the Rambam Medical Center. Thirty-four healthy women using OC and 41 women not using hormonal therapy and not pregnant per history were enrolled, over a 5-month period, at the Rambam Medical Center. Exclusion criteria included age < 18 years, post-menopausal women, concomitant medications other than supplement minerals and vitamins, acute or chronic illness. After obtaining informed consent, a total of 6 ml of peripheral blood was collected, with 3.2% sodium citrate as an anticoagulant. Plasma was obtained by centrifugation (1500g for 15 min at 4°C) and all plasma samples were frozen and thawed once.

**Cell culture**

MCF-7 and MDA-MB-231 human breast carcinoma cells were obtained from the American Type Culture Collection. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium (MDA-MB-231 cells) or RPMI 1640 (MCF-7 cells) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), at 37°C. In order to avoid estrogen preinduction or addition, before estrogen treatment, cells were maintained for 96 h in phenol red-free medium supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT, USA). Then, medium was changed to phenol red-free serum-free medium and estrogen with or without tamoxifen (a partial estrogen receptor antagonist) or ICI 182.780 (a pure estrogen receptor antagonist) were added. Estrogen (17β-estradiol, E2) and 4-hydroxy-tamoxifen were obtained from Sigma (St. Louis, MO) and dissolved in absolute ethanol. ICI-182.780 was obtained from Sigma (St. Louis, MO) and dissolved in dimethylsulfoxide (DMSO). Control cultures were treated with the corresponding vehicle (0.1% ethanol or DMSO). Medium was collected after 16 h and refrigerated at ~8°C.

**Reagents and antibodies**

Single-chain GS3 heparanase gene construct, comprising the 8 and 50 kDa heparanase subunits (8 + 50) was purified from the conditioned medium of baculovirus-infected cells. GS3 heparanase was assayed for the presence of bacterial endotoxin by Biological Industries (Beit Haemek, Israel), using the gel–clot technique (Limulus amebocyte lysate–LAL test) and was found to contain <10 pg/ml endotoxin (Nadir et al., 2006). Monoclonal anti-heparanase antibody 1E1 was generated by immunizing Balb/C mice with the entire 65 kDa heparanase protein (Shafat et al., 2006). Polyclonal antibody 1453 was raised in rabbits against the entire 65 kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected HEK-293 cells. The antibody was affinity-purified on immobilized bacterially expressed 50 kDa heparanase GST fusion protein (Zetser et al., 2004). Recombinant human factor VIIa and plasma-derived human factor X were purchased from American Diagnostica (Stanford, CT, USA). Fondaparinux (Arixtra) was purchased from GlaxoSmithKline (London, UK). All coagulation factors were dissolved in double-distilled water.

**Heparanase procoagulant activity assay**

As previously reported (Nadir et al., 2011b), we performed a basic experiment of factor Xa generation in the following manner: the concentrations mentioned are the final concentrations. Twenty-five liters of plasma, recombinant human factor VIII (0.04 μM) and plasma-derived human factor X (1.4 μM) were incubated in a 50 μl assay buffer [0.06 M Tris, 0.04 M NaCl, 2 mM CaCl2, 0.04% w/v bovine serum albumin (BSA), pH 8.4] to a total volume of 125 μl in a 96-well sterile plate. After 15 min at 37°C, chromogenic substrate to factor Xa was added (1 mM). Following 20 min, the reaction was stopped with 50 μl of glacial acetic acid and the level of Xa generation was determined using an ELISA plate reader (Power Wave XS, BIO-TEK, VT, USA). Heparins were shown to abrogate the TF/heparanase complex (Nadir et al., 2011a), so in parallel, the same assay was performed except that fondaparinux (2.5 μg/ml) was added to the assay buffer. The subtraction of the first assay result from the second assay result determined heparanase procoagulant activity. Thus, the assay gives three results: heparanase procoagulant activity, TF activity and TF + heparanase procoagulant activities.

**Heparanase enzyme-linked immunosorbent assay (ELISA)**

Wells of microtiter plates were coated (18 h, 4°C) with 2 μg/ml of anti-heparanase monoclonal antibody in 50 μl coating buffer (0.05 M Na2CO3, 0.05 M NaHCO3, pH 9.6) and were then blocked with 2% BSA in phosphate-buffered saline (PBS) for 1 h at 37°C. Samples (200 μl) were loaded in duplicates and incubated for 2 h at room temperature, followed by the addition of 100 μl antibody 1453 (1 μg/ml) for an additional period of 2 h at room temperature. HRP-conjugated goat anti-rabbit IgG (1:20 000) in blocking buffer was added (1 h, room temperature) and the reaction was observed by the addition of 50 μl chromogenic substrate (TMB; MP Biomedicals, Germany) for 30 min. The reaction was stopped by adding 100 μl H2SO4 and absorbance at 450 nm was measured using an ELISA plate reader (Power Wave XS, BIO-TEK). Plates were washed five times with a washing buffer (PBS, pH 7.4 containing 0.1% (v/v) Tween 20) after each step. As a reference for quantification, a standard curve was established by serial dilutions of recombinant 8 + 50 GS3 heparanase (390 pg/ml-25 ng/ml; Shafat et al., 2006).
Factor Xa chromogenic assay
Chromogenic substrate to factor Xa (I.D. 222, solubility: Tris buffer, pH 8.4) was purchased from American Diagnostica (Stanford). Twenty-five microliters of plasma was added to 50 \( \mu l \) Tris buffer (0.06 M, pH 8.4) and 25 \( \mu l \) of chromogenic substrate to factor Xa (5 mM). After 20 min, the reaction was stopped with glacial acetic and the absorbance at 405 nm–490 nm was measured using an ELISA plate reader (Power Wave XS, BIO-TEK).

Thrombin–antithrombin complex
The complex was evaluated according to the manufacturer recommendations by Enzygnost® thrombin–antithrombin complex (TAT) micro ELISA purchased from Siemens (Marburg, Germany).

D-dimer assay
D-dimer was performed on the STA-R evolution analyzer (Diagnostica Stago, Paris, France) using recombinant STA-LIATEST D-DI kit (Diagnostica Stago).

Statistical analysis
Data were evaluated by SPSS software for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). Statistics on cell culture were calculated by non-parametric Mann–Whitney U-test. Values were reported as median and range. Differences between plasma of OC users and women without hormonal therapy were tested by the t-test for independent variables. Risk of high heparanase procoagulant activity in OC users was studied by log-rank test and odds ratios with corresponding 95% confidence intervals (CIs) were reported. Significance level was set at \( P < 0.05. \)

Results

Heparanase procoagulant activity in MCF-7 estrogen receptor-positive cells
Estrogen (E2) was added to the medium of subconfluent MCF-7 cells. Doses of estrogen were chosen to represent the hormone levels through menarche (0.1–1.0 nM) and typical of pregnancy (10.0 nM) (Calabrese, 2001). The heparanase procoagulant activity assay, developed by our group (Nadir et al., 2011b), gives three results: heparanase procoagulant activity, TF activity and TF + heparanase activity (Fig. 1). Heparanase procoagulant activity increased in a dose–response manner to estrogen addition, reaching a 22-fold increase (Fig. 1A). While TF/heparanase activity (Fig. 1C) increased moderately by 1.4-folds, no significant change occurred in TF activity (Fig. 1B). Although the dramatic change occurred in heparanase activity, since its activity was only \( \approx 10\% \) to that of TF, TF activity was the dominant parameter of the TF/heparanase complex (Fig. 1). When tamoxifen was added in a typical therapeutic level (300 nM) representing the concentration in plasma of women using the drug (Calabrese, 2001), it partially abolished the
procoagulant effect of heparanase (P < 0.005; Fig. 2A). When tamoxifen was added in the absence of estrogen it also increased heparanase procoagulant activity (P < 0.005; Fig. 2A). No significant effect was observed on TF and TF/heparanase activities (Fig. 2B and C). We investigated the effect of the pure estrogen receptor antagonist ICI-182.780 (Wakeling, 1993) on estrogen-mediated increased heparanase procoagulant activity. The presence of ICI-182.780 at a dose of 100 nM, as was previously used (Elkin et al., 2003), completely eliminated the effect of estrogen on heparanase procoagulant activity in MCF-7 cells medium (Fig. 2A). As with tamoxifen, no significant effect was observed on TF and TF/heparanase activities (Fig. 2B and C).

Heparanase procoagulant activity in MDA-231 estrogen receptor-negative cells

TF/heparanase activity was three times higher in the MDA-231 cells compared with MCF-7 cells (Figs 2C, and 3C), as in the MCF-7 line, mostly attributed to TF activity (Fig. 3B). Estrogen and tamoxifen did not increase heparanase procoagulant activity (Fig. 3A) supporting an estrogen receptor-dependent mechanism. The experiment described in Fig. 1 was performed in MDA-231 cells using increasing concentrations of estrogen (0.1 – 10 nM) and after 16 h the medium was studied. No significant increase in TF/ heparanase activity, heparanase procoagulant activity or TF activity compared with control, was observed (data not shown).

Activation of the extrinsic coagulation system in the plasma of women using OC

The characteristics of both study groups were similar with respect to age, smoking, days from the commencement of menstrual period. The mean duration of taking OC was ~ 3 years. Most women were taking the third generation pills containing ethinyl estradiol (EE) along with a progestosterone derivative (Table I). TF/heparanase activity, heparanase procoagulant activity, TF activity, factor Xa level (present in plasma without activation) and D-dimer level were significantly higher in the OC group compared with the control group. The most dramatic difference was observed in heparanase procoagulant activity, reaching a 3.3-fold increase (P < 0.0001; Fig. 4). Levels of heparanase and TAT measured by ELISA did not statistically differ among the study groups (Fig. 4).

As the number of results in each formulation used is too small for statistical analysis we compared the two biggest groups: Harmonet (EE 20 mg + gestodene 75 mg) n = 5 + Meliane (EE 20 µg + gestodene 75 µg) n = 6 (together n = 11) versus Yasmin (EE 30 µg + drospirenone 3 mg) n = 8. Using t-test for independent variants, no significant difference was found between the groups in TF/heparanase, heparanase and TF activities (P = 0.8, P = 0.9, P = 0.9, respectively).
**Figure 3** Estrogen effect on heparanase procoagulant activity is via estrogen receptor. Estrogen (E2, $10^{-9}$ nM), Tamoxifen (T, $3 \times 10^{-7}$ nM) and ICI (1 $\times 10^{-7}$ nM) were added to MDA-231 estrogen receptor-negative cells. In the absence of estrogen receptor, estrogen and tamoxifen had no significant effect on heparanase, TF or TF/heparanase activities (A, B, and C, respectively), suggesting an estrogen receptor-dependent mechanism. Results represent median and range of three separate experiments.

**Table 1** Demographic characteristics of the study group.

<table>
<thead>
<tr>
<th></th>
<th>No OC ($n = 41$)</th>
<th>OC users ($n = 34$)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26.4 $\pm$ 0.5 (range 22–37, median 26)$^{a}$</td>
<td>25.9 $\pm$ 0.6 (range 22–32, median 25)</td>
<td>0.56</td>
</tr>
<tr>
<td>Smoking</td>
<td>$n = 3$</td>
<td>$n = 4$</td>
<td>0.2</td>
</tr>
<tr>
<td>Days from commencement of menstrual period</td>
<td>16.4 $\pm$ 1.3</td>
<td>13.3 $\pm$ 1.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Duration of OC use (months)</td>
<td>N/A</td>
<td>39 $\pm$ 7.3</td>
<td></td>
</tr>
<tr>
<td>Type of OC</td>
<td>N/A</td>
<td>Minesse EE 15 µg + gestodene 60 µg</td>
<td>$n = 5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Harmonet EE 20 µg + gestodene 75 µg</td>
<td>$n = 5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Meliane EE 20 µg + gestodene 75 µg</td>
<td>$n = 6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mercilon EE 20 µg + desogestrel 150 µg</td>
<td>$n = 2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feminet EE 20 µg + desogestrel 150 µg</td>
<td>$n = 2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yasmin EE 30 µg + drospirenone 3 mg</td>
<td>$n = 8$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minulet EE 30 µg + gestodene 75 µg</td>
<td>$n = 4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diane EE 35 µg + cyproterone acetate 2 mg</td>
<td>$n = 2$</td>
</tr>
</tbody>
</table>

Results represent mean $\pm$ SEM.
EE, ethinyl estradiol; OC, oral contraceptive.
$^{a}$Number of women $>$35 years: 1.
The extrinsic coagulation pathway is activated in women taking oral contraceptive (OC). Plasma of 34 OC users and 41 women without hormonal therapy (C) were tested. Box plots show the changes of parameters. Central horizontal bars, columns and error bars indicate the median, 25th to 75th percentile and 10th to 90th percentile, respectively. TF/heparanase activity, heparanase procoagulant activity, TF activity, factor Xa levels and D-dimer levels were significantly higher in the OC group compared with control. Note the major increase in heparanase procoagulant activity (3.3-fold increase). No difference was found in heparanase and TAT levels by ELISA. *p < 0.05, **p < 0.005, ***p < 0.0001.
Values of the two women using Diana were not extraordinarily high [TF/heparanase activity: 0.48, 0.74 (absorbance 405–490 nm), heparanase procoagulant activity: 0.33, 0.48 (absorbance 405–490 nm), TF activity: 0.15, 0.27(absorbance 405–490 nm)].

T-test for dependent variables was done showing no significant difference between age of OC users and heparanase procoagulant activity or age of non-OC users and heparanase procoagulant activity ($P = 0.7$ and 0.8, respectively).

Range and risk of high heparanase procoagulant activity in the study group

Although the average heparanase activity in the OC group was higher than in controls (Fig. 4), Fig. 5 demonstrates the wide range of heparanase activity in women not using hormones (0.01–0.96) as well as in women using OC (0.03–0.72). *Smoker women and the number of cigarettes per day.

<table>
<thead>
<tr>
<th>Heparanase procoagulant activity (405–490 nm)</th>
<th>C number</th>
<th>OC number</th>
<th>Odds ratio</th>
<th>Confidence interval (95%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.25</td>
<td>31</td>
<td>3</td>
<td>131</td>
<td>(25–695)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;0.25</td>
<td>3</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quartile 1 (0–0.08)</td>
<td>18</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quartile 2 (0.08–0.023)</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>(0.1–17)</td>
<td></td>
</tr>
<tr>
<td>Quartile 3 (0.024–0.035)</td>
<td>4</td>
<td>15</td>
<td>67</td>
<td>(7–670)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Quartile 4 (0.037–0.072)</td>
<td>1</td>
<td>17</td>
<td>306</td>
<td>(18–5289)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

C, control.

Discussion

In estrogen receptor-positive cells, estrogen increased heparanase procoagulant activity while in the absence of the estrogen receptor this effect was not observed, supporting an estrogen receptor-dependent activity (Figs 1–3). These results potentially indicate a new mechanism of hypercoagulability in women using estrogen. In order to gain clinical relevance to this notion we studied the plasma of 34 women using OC and compared them with 41 women without hormonal therapy. The results indicate a significant increase in TF/heparanase activity, mostly attributed to heparanase procoagulant activity, although TF activity also increased (Fig. 4). Heparanase level by ELISA showed no difference between the groups. The ELISA of heparanase dominantly detects the enzymatic active heparanase (50 + 8 kDa) and less sensitive to the pro-heparanase (65 kDa) (Shafat et al., 2006) that is active in the hemostatic system (Nadir et al., 2011a). Therefore, it is possible that the ELISA results do not correlate to the procoagulant activity of heparanase. Previously, we studied the plasma of 35 women at the end of pregnancy compared with the plasma of 20 non-pregnant women (Nadir et al., 2011b). There is a considerable similarity in the results of pregnant women and the OC user group regarding the pattern of changes and level of activities. In pregnant versus non-pregnant women, the TF/heparanase, heparanase, TF and Xa activities were 0.53 versus. 0.17, 0.33 versus 0.05, 0.2 versus 0.12 and 0.34 versus 0.22 (absorbance 405–490 nm), respectively. Accordingly, in the OC users compared with non-users the TF/heparanase, heparanase, TF and Xa activities were 0.6 versus. 0.26, 0.4 versus 0.12, 0.2 versus 0.14 and 0.09 versus 0.05 (absorbance 405–490 nm), respectively. In heparanase ELISA, as in our present data, no significant difference was observed. In the present study results of Xa levels are about four times lower compared with our previous study as the test was done using different methodology (25 μl of plasma with 20 min incubation, and in the previous article, 50 μl of plasma with 30 min incubation). Hence, with the limitation of two separate studies, in the pregnant women group, as in the OC group, TF/heparanase activity was increased and the dominant parameter of the complex was the increase in heparanase procoagulant activity. Downstream the extrinsic coagulation cascade, levels of factor Xa and D-dimer were significantly increased in the OC users, although TAT was comparable between the groups. The fact that no difference was found in the TAT may result from sensitivity limitations of the assay and is in accordance with previous published.
data (Pinto et al., 1997; Wiegratz et al., 2008). Figure 5 demonstrates the wide range of heparanase procoagulant activity in the study group and Table II shows that OC users are at increased risk to have high heparanase procoagulant activity. The clinical significance of the assay as a predictor of thrombosis risk and as a routine test before and/or immediately after starting OC should be addressed in a large clinical trial. Additionally, large-scale study is needed to answer questions regarding the effect of different estrogen dose, progesterone dose and formulation in pills, age and smoking on heparanase procoagulant activity.

Tamoxifen, a partial estrogen receptor antagonist most widely used drug in endocrine therapy of breast cancer, is known to increase the risk of thrombosis by 2–3-fold (Hernandez et al., 2009). Cohen et al. (2007) demonstrated that tamoxifen induced heparanase expression in MCF-7 cells. According to our results, tamoxifen partially inhibited the procoagulant effect of heparanase in the presence of estrogen but increased heparanase procoagulant activity in the absence of estrogen (Fig. 2A). Following chemotherapy treatment the menstrual cycle may stop intermittently or permanently, causing a drop in estrogen level in the plasma. Tamoxifen is usually started after chemotherapy for 2–5 years. It is possible that in women with a drop in normal estrogen level, tamoxifen may induce increased heparanase procoagulant activity and a risk of thrombosis. This notion is in accordance with the data of Hernandez et al. (2009) showing that in women at the age of 45–69, older women were at increased risk of thrombosis during tamoxifen treatment.

According to the results, there is a discrepancy in the contribution of heparanase activity to the TF/heparanase complex in the women’s plasma compared with the medium of the cancer cell lines. The contribution is calculated as heparanase activity/TF + heparanase activity. While in the plasma the contribution in women without pills was 45%, and in women using pills was 65% (Fig. 4), in medium of MCF-7 cells without estrogen the heparanase procoagulant activity was 1% and in the presence of estrogen it reached 20% (Fig. 2). The reason for the discrepancy could be secretion of TF bearing apoptotic bodies to the medium over the 16 h of incubation in serum-free medium or different setups of hormonal effect in health versus malignancy.

The main limitation of the current study is that the two estrogens given to the women and cell cultures: EE and E2, respectively, may have different effects on the coagulation system, although an increase in heparanase procoagulant activity was demonstrated in both of them. Different ways of estrogen administration, i.e. orally or transdermally may also influence the procoagulant effect. These important issues should further be investigated.

In summary, the present work suggests a new mechanism for activation of the coagulation system during estrogen treatment. Although the sample size of the study group was limited, significant differences in the activation of the extrinsic coagulation pathway were demonstrated. The clinical relevance of the heparanase procoagulant activity assay as a screening tool in thrombophilia work-up ought to be elucidated in the future.

**Acknowledgements**

We would like to thank Prof. Israel Vlodavsky and Dr Neta Ilan for providing the heparanase ELISA, and to Dr Anat Keren for the assistance with the D-dimer assay. The study was supported by the Israel Science Foundation (ISF), Legacy Heritage Clinical Research (2009–2012).

**Authors’ roles**

M.M., E.A. and B.B. made substantial contributions to acquisition of data and analysis and interpretation of data and in the drafting of the article. Y.N. made substantial contributions to the concept and design, acquisition of data, analysis and interpretation and also contributed in drafting the article and revising it critically for important intellectual content. All authors are responsible for the final approval of the version to be published.

**Funding**

No external funding was sought for this study.

**Conflict of interest**

Y.N. and B.B. are named in a US Provisional Patent Application No. 29509/WO/12 filed on 18.01.2012. Entitled: Methods and kits for assessing heparanase procoagulant activity, compositions comprising heparanase, and methods for the treatment of coagulation-related disorders. The other authors have no conflict of interest to declare.

**References**


