Effects of Hormone Replacement Therapy on Plasma and Tissue Fibrinolytic Activity in a Rat Model of Surgically Induced Menopause

Abstract

Purpose: The purpose of this study was to analyze the effects of estrogen deficiency and hormone replacement therapy (HRT) on fibrinolytic activity in a rat model of surgically-induced menopause.

Methods: Twelve-week-old, sexually mature female Sprague-Dawley rats, each weighing 200–250 g, were randomly divided into four groups: (1) sham-operated group, (2) ovariectomy group, (3) ovariectomy group followed by oral administration of daily 17β-estradiol (0.02 mg/kg/day) (E2) + norethisterone acetate (0.01 mg/kg/day), and (4) ovariectomy group followed by oral administration of daily 17β-estradiol (0.01 mg/kg/day) + drospirenone (0.02 mg/kg/day). Tissue plasminogen activator (tPA) antigen, plasminogen activator inhibitor-1 (PAI-1) antigen, and PAI-1/tPA levels were measured as markers of fibrinolysis in plasma and liver and brain tissue.

Results: Compared with sham-operated rats, ovariectomized rats showed higher levels of fibrinolytic activity; however, the increased fibrinolytic activity in plasma and liver tissue was significantly reduced by HRT regimens. No change was observed in the levels of fibrinolytic activity in brain tissue.

Conclusions: HRT showed beneficial effects by decreasing fibrinolytic activity related to surgically-induced menopause. Short-term HRT treatment was associated with a shift in the procoagulant-anticoagulant balance toward a procoagulant state.
Due to the increase age of the global population, the proportion of women who spend up to one-third of their life in the postmenopausal period will increase, and they will be increasingly affected by the consequences of menopausal hormonal changes [1]. Reports on the beneficial effects of various combinations of postmenopausal estrogen/progesterone therapies on cardiovascular risk parameters such as the lipid profile, low-density lipoprotein (LDL) oxidation, vascular function and the fibrinolysis/coagulation system are variable and contradictory [2]. The beneficial effects of hormone replacement therapy (HRT) are evident in the primary target tissues of menopausal therapy, such as bone, cardiovascular system, vagina and brain [3]. Fibrinolysis and coagulation during menopause, and the effects of HRT on the delicate balance between them, are particularly important for risks of acute cardiovascular conditions, such as stroke, myocardial ischemia and infarction, and deep vein thrombosis, during the postmenopausal period.

Endogenous fibrinolysis is regulated predominantly by tissue plasminogen activator (tPA) through enzymatic conversion of plasminogen to plasmin. Plasma levels of active tPA are determined by the combined effects of its synthesis from vascular endothelium [4], binding to plasminogen activator inhibitor-1 (PAI-1) and other inhibitors [5], and by hepatic clearance [6]. Elevated tPA antigen levels, which reflect both active free tPA and inert tPA bound in activator/inhibitor complexes, may indicate coagulation activation and enhanced fibrinolytic function. The level of the PAI-1/tPA complex, a novel fibrinolytic marker, increases during pregnancy-associated hypercoagulable states, vascular spasms and atherosclerotic states [7]; therefore, identification of the PAI-1/tPA complex may provide valuable prognostic information with respect to patients with breast cancer [8] and myocardial infarction with manifested coronary heart disease [9]. Oral HRT, but not transdermal HRT, is associated with PAI activity and t-PA antigen levels that are lower than those found in postmenopausal HRT non-users. These effects of oral HRT may reflect a “first-pass” effect on hepatic PAI-1 synthesis [10].

To date, the effects of two commonly used postmenopausal hormone replacement therapy regimens, 17β-estradiol plus norethisterone acetate and 17β-estradiol plus drospirenone, on fibrinolytic activity remain unclear. The components of the fibrinolytic system may be influenced by many individual factors, such as age, stress, hypertension, glucose intolerance, diabetes and surgical or natural menopause in humans. Therefore, we used a rat model of surgical menopause to neutralize or eliminate the effect of these confounding factors and investigate only the effect of HRT.

In the present study, the effects of ovariectomy (surgical menopause) and the aforementioned two HRT regimens on fibrinolytic activity were investigated in a surgical menopause rat model.

**Materials and Methods**

The experimental procedure was approved by Experimental Animal-Research and Breeding Laboratory, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey. Parameters and data regarding oxidative stress and fibrinolysis in the present study were derived from a previous study by our group [11].

**Chemicals and equipment**

Chemicals and solvents used in the experiments were of highest purity and analytical grade. Deionized water was used in analytical procedures. All reagents were stored at +4°C. The reagents were maintained at equilibrium at room temperature for 30 min before use. All centrifugation procedures were performed using a Jouan G 412 centrifuge.

**Experimental procedure**

All efforts were made to minimize the number of animals used in the study. A total of 32 12 week old, sexually mature female Sprague-Dawley rats, each weighing 200–250 g, were used. All animals were kept in conventional wire-mesh cages with four rats per cage in a room with the temperature regulated at 21 ± 1°C, a humidity of 45–50%, and 12 h light/dark cycles with ad libitum access to food and water throughout the course of the experiment.

Animals were anesthetized by the administration of ketamine (50 mg/kg) and xylazine (10 mg/kg) into the marginal tail veins. The rats underwent either a bilateral ovariectomy or a sham surgical procedure via midline laparotomy incision under sterile conditions followed by appropriate closure of the incisions, as described elsewhere [11].

Rats were randomly divided into four groups: (1) the sham-surgery group (n=8), (2) ovariectomy group (n=8), (3) ovariectomy group followed by oral lavage administration of daily 17β-estradiol (0.02 mg/kg/day) + norethisterone acetate (0.01 mg/kg/day) (Kliogest®; Novo Nordisk, Bagsvaerd, Denmark) (n=8), and (4) ovariectomy group followed by oral lavage administration of daily 17β-estradiol (0.01 mg/kg/day) + drospirenone (0.02 mg/kg/day) (Angeliq®; Schering AG, Berlin, Germany) (n=8).
Blood-sample collection and processing

Blood samples were drawn in the fasting state and processed within 1 h of collection. Samples were collected in tubes containing lithium heparin, EDTA, or no additive depending on the nature of the analysis. For the determination of fibrinolytic parameters, plasma samples containing lithium heparin were stored at −80°C until required. All other routine clinical-chemistry parameters were determined on the day of collection using the Hitachi 704 auto-analyzer (Boehringer Mannheim, Tokyo, Japan).

Preparation of tissue samples

Brain tissue samples (except those from the cerebellum, pons and medulla oblongata) and liver tissues from the rats were immediately removed, washed in cooled 0.15 M NaCl, and placed on an ice-cold plate. Tissue samples were then immediately frozen in liquid N2 until experimentation. Tissue samples (200 mg) were homogenized manually in 2 ml of homogenizing buffer (100 mM KH2PO4-K2HPO4, pH 7.4, plus 0.1% (w/v) digitonin) in a glass homogenizer to avoid disruption of nuclear membranes. In this way, contamination by nucleic acids was minimized [12]. Homogenates obtained from the rats were centrifuged at 5000 × g for 10 min, and the various analytes were assayed using the supernatant fraction.

Assay of tPA, PAI-1 and tPA/PAI-1 levels

tPA, PAI-1, and tPA/PAI-1 levels in plasma and tissues (brain and liver) were measured using Assaypro ELISA kits according to the manufacturer’s instructions (AssayMax ELISA kit; Assaypro, Winfield, MO, USA). Estradiol in serum was measured using the Chemiluminescent Microparticle Immunoassay (CMIA) and the Architect-i 2000 System (Abbott, Abbott Park, IL, USA). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels in serum were determined using the appropriate kits from Sigma Aldrich (St. Louis, MO, USA).

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) for eight animals per group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for Windows (SPSS Inc., Chicago, IL, USA). Post hoc testing was performed for inter-group comparisons using the least-significance-difference (LSD) test. Post hoc tests were conducted using the Bonferroni-Dunn test. A probability value of less than 0.05 was deemed to indicate statistical significance for all comparisons.

Results

Routine clinical and biochemical parameters of the groups subjected to ovariectomy alone and ovariectomy plus both HRT regimens, as well as their respective controls (sham operated), are summarized in Table 1. No allergic or gastrointestinal complications - such as hepatotoxicity - were observed during the study period. No mortality occurred. The 17β-estradiol + drospirenone group lost more weight than the 17β-estradiol + norhisterone acetate group, likely due to the natriuretic properties of drospirenone (Table 1). The information in Table 1 is identical to that included in a previous study by our group [11].

Five weeks after surgery, the serum E2 levels of sham-operated rats had not changed significantly, whereas the serum E2 levels decreased significantly in the ovariectomy group, confirming successful induction of surgical menopause (p < 0.001) (Table 1). Following 5 weeks’ administration of 17β-estradiol + drospirenone (Angeliq®) and 17β-estradiol + norhisterone acetate (Kliogest®), the serum E2 levels were 220.13 ± 77.56 pg/ml and 162.38 ± 34.99 pg/ml, respectively (Table 1). These results confirmed that E2 replacement therapy was effective in ovariectomized rats. In sham-operated rats, no change was noted in any of the parameters compared with the initial values (Table 1).

In sham-operated rats, no change was observed in TC (mg/dl) (70.68 ± 10.23; 74.50 ± 8.02), HDL-C (mg/dl) (20.69 ± 1.97; 21.50 ± 1.05) and TG (mg/dl) (81.33 ± 8.64; 78.17 ± 11.39) levels compared with the initial preoperative values. Additionally, no change was noted in the plasma tPA, PAI-1, and PAI-1/tPA levels in sham-operated rats pre- and postoperatively. The plasma tPA, PAI, and tPA/PAI levels increased significantly (P < 0.001) in ovariectomized rats; however, the plasma tPA, PAI, and tPA/PAI levels were significantly decreased in both groups that underwent HRT regimens compared with those in the ovariectomy group (Tables 2 and 3).

The liver tPA, PAI, and tPA/PAI levels were significantly higher in the ovariectomized rats than in the sham-operated rats (Table 4); however, these parameters were significantly decreased in both groups that received HRT compared with those in the ovariectomized group, but were higher than those in the sham group. Despite the significant changes in parameters in the liver tissues, no change was found in any of the parameters in brain tissues.
TABLE 1. Body weights and plasma 17β-estradiol (E₂) levels.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>E₂ (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td><strong>Sham-operated group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preop. (n=8)</td>
<td>201.00 ± 17.23</td>
<td>298.67 ± 106.60</td>
</tr>
<tr>
<td>Sham (n=8)</td>
<td>204.50 ± 16.60</td>
<td>299.50 ± 104.89</td>
</tr>
<tr>
<td><strong>Ovariectomy group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative (n=8)</td>
<td>198.50 ± 25</td>
<td>256.00 ± 108.16</td>
</tr>
<tr>
<td>Ovariectomy (n=8)</td>
<td>208.17 ± 21.24 (a*)</td>
<td>13.67 ± 2.73 (a***)</td>
</tr>
<tr>
<td>17β-Estradiol+DRSP group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative (n=8)</td>
<td>201.25 ± 17.00</td>
<td>257.88 ± 102.43</td>
</tr>
<tr>
<td>Ovariectomy (n=8)</td>
<td>216.25 ± 19.91 (a**)</td>
<td>14.75 ± 3.85 (a***)</td>
</tr>
<tr>
<td>Ovariectomy+17β-Estradiol+DRSP (n=8)</td>
<td>184.38 ± 16.73 (a***,b**)</td>
<td>220.13 ± 77.56 (a<em>b</em>**)</td>
</tr>
<tr>
<td><strong>17β-Estradiol+ NETA group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative (n=8)</td>
<td>205.50 ± 23.75</td>
<td>251.50 ± 111.24</td>
</tr>
<tr>
<td>Ovariectomy (n=8)</td>
<td>211.50 ± 24.19 (a**)</td>
<td>16.38 ± 3.66 (a***)</td>
</tr>
<tr>
<td>Ovariectomy+17β-Estradiol+NETA(n=8)</td>
<td>201.88 ± 16.93 (b***)</td>
<td>162.38 ± 34.99 (a<em><strong>b</strong></em>)</td>
</tr>
</tbody>
</table>

Abbreviations: DRSP: drospirenone; NETA: norethisterone acetate.
Values represent the means ± SEM and the statistical significance of the analyzed parameters in the study groups. *p<0.05, **p<0.01, ***p<0.001. a Difference with the preoperative group, b difference with the ovariectomy group.
Reproduced from reference 11.

TABLE 2. Changes in plasma levels in preoperative, ovariectomized and ovariectomized rats that received 17β-estradiol + norethisterone acetate (NETA)

<table>
<thead>
<tr>
<th></th>
<th>Preoperative (n=8)</th>
<th>Ovariectomy (n=8)</th>
<th>Ovariectomy + 17β-Estradiol + NETA (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>68.25 ± 10.55</td>
<td>77.88 ± 9.66**(a,c)**</td>
<td>71.75 ± 5.85</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>21.25 ± 2.31</td>
<td>20.25 ± 3.06</td>
<td>22.13 ± 3.14**(b)</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>82.38 ± 15.25</td>
<td>86.00 ± 17.57</td>
<td>82.25 ± 14.46</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>0.75 ± 0.19</td>
<td>1.32 ± 0.27***(a,c)**</td>
<td>0.88 ± 0.27</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>0.73 ± 0.20</td>
<td>1.19 ± 0.38***(a,c)**</td>
<td>0.77 ± 0.65</td>
</tr>
<tr>
<td>PAI-1/tPA (ng/ml)</td>
<td>2.37 ± 0.36</td>
<td>3.08 ± 0.58***(a,c)**</td>
<td>2.53 ± 0.49</td>
</tr>
</tbody>
</table>

Abbreviations: NETA: norethisterone acetate; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride; tPA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1.
Values represent means ± SEM and the statistical significance of the analyzed parameters in the study groups. *p<0.05, **p<0.01; ***p<0.001. a Difference with the preoperative group, b difference with the ovariectomy group, c difference with the ovariectomy + 17β-estradiol + NETA group.
Table 3. Changes in plasma levels in preoperative, ovariectomized and ovariectomized rats that received 17β-estradiol + drospirenone (DRSP)

<table>
<thead>
<tr>
<th></th>
<th>Preoperative (n=8)</th>
<th>Ovariectomy (n=8)</th>
<th>Ovariectomy + 17β-Estradiol + DRSP (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>65.38 ± 9.29</td>
<td>73.75 ± 10.61*(a)</td>
<td>72.88 ± 10.58.</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>19.25 ± 2.05</td>
<td>18.50 ± 2.33</td>
<td>21.88 ± 2.36*(a,b)</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>80.38 ± 12.64</td>
<td>85.63 ± 12.16</td>
<td>79.50 ± 20.09</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>0.84 ± 0.17</td>
<td>1.36 ± 0.28***(c)</td>
<td>1.09 ± 0.24</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>0.62 ± 0.16</td>
<td>1.01 ± 0.34***(c)</td>
<td>0.64 ± 0.35</td>
</tr>
<tr>
<td>PAI-1/tPA (ng/ml)</td>
<td>2.30 ± 0.25</td>
<td>2.89 ± 0.32****(a)</td>
<td>2.57 ± 0.56</td>
</tr>
</tbody>
</table>

Abbreviations: DRSP: drospirenone; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride; tPA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1.

Values represent means ± SEM and the statistical significance of the analyzed parameters in the study groups. *p<0.05, **p<0.01, ***p<0.001. * Difference with the preoperative group, † difference with the ovariectomy group, ‡ difference with the ovariectomy + 17β-estradiol + DRSP group.
Estrogen administered in postmenopausal hormonal therapy affects directly the biosynthesis and secretion of PAI-1 [30]; however, the progestin component may reduce the beneficial effects of estrogen on the fibrinolytic system [31]. Ruszkowska et al. [32] reported that the route of administration of postmenopausal hormonal therapy had a significant effect on the concentration of PAI-1:Ag in the blood. Higher levels of PAI-1:Ag were found in women who used transdermal postmenopausal hormonal therapy than in those who received oral postmenopausal hormonal therapy. Other studies [30-36] demonstrated that oral postmenopausal hormonal therapy significantly reduced the levels of PAI-1 (by up to 50%); however, no such significant change was associated with transdermal postmenopausal hormonal therapy.

A limited number of studies have reported conflicting results regarding fibrinolytic activity in human and rats [27-36]. Additionally, no report on the influence of norethisterone acetate and drospirenone on the tPA and PAI-1 levels in liver and brain tissue of rats has been published to date. Our study is the first report on the effects of these drugs on fibrinolytic activity: PAI-1 and tPA levels, as well as the PAI-1/tPA ratio, were significantly higher after bilateral oophorectomy, suggesting an increase in fibrinolytic activity, particularly after ovariectomy. The cause may be stimulation of the release of PAI-1 by t-PA. Increased PAI-1 levels in menopause may reveal the risk of thrombotic cardiovascular disease, including coronary heart disease. The liver tPA and PAI levels were significantly higher in ovariectomized rats than in sham-operated rats. After administration of drospirenone and norethisterone acetate in ovariectomized rats, the tPA, PAI, and tPA/PAI levels in liver tissue were decreased significantly. Both HRT regimens antagonized the increase in fibrinolytic activity that occurs after ovariectomy (surgical menopause). HRT caused a decrease in the fibrinolytic activity rates in both plasma and liver tissue of ovariectomized rats. It seems clear that drospirenone decreases the PAI levels of liver tissue more effectively than norethisterone acetate.

**TABLE 4. Changes in brain and liver tissue levels in the sham-operated, ovariectomy and ovariectomy plus hormone replacement therapy groups**

<table>
<thead>
<tr>
<th>Liver tissue</th>
<th>Preoperative (n=8)</th>
<th>Ovariectomy (n=8)</th>
<th>Ovariectomy + 17β-Estradiol + DRSP (n=8)</th>
<th>Ovariectomy + 17β-Estradiol + NETA (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA (ng/g tissue)</td>
<td>2.61 ± 0.51</td>
<td>4.87 ± 1.15*** (a,c)</td>
<td>2.97 ± 0.27*** (b,d)</td>
<td>3.79 ± 0.78*** (a,b,c)</td>
</tr>
<tr>
<td>PAI (ng/g tissue)</td>
<td>2.78 ± 1.04</td>
<td>4.63 ± 1.16*** (a,c,d)</td>
<td>2.88 ± 0.83*** (b,d)</td>
<td>3.25 ± 0.86*** (a,b,c)</td>
</tr>
<tr>
<td>tPA/PAI (ng/g tissue)</td>
<td>2.35 ± 0.81</td>
<td>3.73 ± 0.39*** (a,c,d)</td>
<td>2.98 ± 0.36*** (a,b)</td>
<td>2.84 ± 0.78*** (a,b)</td>
</tr>
<tr>
<td>Brain tissue</td>
<td></td>
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</tr>
<tr>
<td>tPA (ng/g tissue)</td>
<td>1.23 ± 0.62</td>
<td>1.50 ± 0.38</td>
<td>1.27 ± 0.29</td>
<td>1.29 ± 0.47</td>
</tr>
<tr>
<td>PAI (ng/g tissue)</td>
<td>1.92 ± 0.85</td>
<td>2.31 ± 0.62</td>
<td>2.07 ± 0.31</td>
<td>2.03 ± 0.40</td>
</tr>
<tr>
<td>tPA/PAI (ng/g tissue)</td>
<td>0.79 ± 0.29</td>
<td>1.13 ± 0.33</td>
<td>0.80 ± 0.30</td>
<td>0.80 ± 0.15</td>
</tr>
</tbody>
</table>

Abbreviations: DRSP: drospirenone; NETA: norethisterone acetate; tPA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1.

Values represent means ± SEM and the statistical significance of the analyzed parameters in the study groups. *p<0.05, **p<0.01, ***p<0.001.

*p difference with the sham-operated group, *b difference with the ovariectomy group, *c difference with the ovariectomy + 17β-estradiol + DRSP group, *d difference with the ovariectomy + 17β-estradiol + NETA group.
one acetate. Previously, Post et al. [36] showed that oral use of postmenopausal hormonal therapy resulted in stimulation of the synthesis of many proteins in the liver (including t-PA and PAI-1) and increased clearance in hepatocytes. Our results are consistent with those of Post et al. [36]. Coagulation and fibrinolysis may also influence cardiovascular risk; however, the relationship of the lipid profile to these processes in menopause is unclear. The significance of increased PAI-1 remains unclear; several observational studies reported high concentrations of PAI-1 to be a risk factor for cardiovascular disease, but the significance of PAI-1 as a predictor often disappears after adjusting for lipids and BMI [37].

The use of rat as model of surgically-induced menopause and the manner in which the data were presented here do have some limitations. Surgery itself may alter fibrinolytic activity through wound healing and other processes. The effects of sham surgery on fibrinolytic activity in this group were not evaluated this information, which would have served as an additional “baseline” was not available.

In conclusion, our results suggest that oral HRT demonstrates beneficial effects on the menopause-mediated decrease in fibrinolytic activity. Short-term oral HRT seems to be associated with a shift in the procoagulant-anticoagulant balance towards a procoagulant state. The conflicting results from human and animal studies of heart disease prevention by estrogens and progestins suggest the need for further investigation of nuclear hormone receptor function and interaction. Long-term, controlled, and prospective studies are needed to evaluate this hypothesis and confirm our findings. Furthermore, studies including an increased number of rats and using other HRT protocols should be performed to confirm our suggestion that HRT has a protective effect on fibrinolysis in ovariecctomized rats.

Acknowledgments

This paper is dedicated to the memory of Professor Tuncay Altug of the Experimental Animal-Research and Breeding Laboratory, Istanbul University, Cerrahpasa Medical Faculty, Turkey, who kindly provided experimental animals for our current research. We thank also Professor Deniz Topcuoglu for assisting with research coordination.

References


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References


