Effects of oral combined hormone replacement therapy on platelet aggregation in postmenopausal women

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ABSTRACT

The effects of combined oestrogen/progestin hormone replacement therapy (HRT) on platelet aggregation were studied using women on HRT or placebo. The study involved 32 postmenopausal women (aged 50–75 years) who were enrolled in a double-blind randomized controlled trial, and who received either oral continuous combined HRT (Kliogest®; 2 mg of oestradiol † 1 mg of norethisterone) or placebo for a minimum of 6 months. Platelet aggregation was measured by whole-blood impedance aggregometry in response to the agonists collagen, arachidonic acid and ADP. To determine whether the effects of oestrogen on platelets were influenced by platelet-derived nitric oxide, exposure to collagen was repeated in the presence of the nitric oxide synthase inhibitor N\textsubscript{G}-monomethyl-L-arginine (L-NMMA). Mean platelet volume was similar in the two groups. Compared with the placebo group, the women on HRT had similar rates and maximum values of platelet aggregation in response to collagen, arachidonic acid and ADP. Addition of L-NMMA did not alter the aggregation response to collagen in either the HRT or the placebo group. In conclusion, postmenopausal women on oral combined continuous HRT comprising oestradiol and norethisterone had similar whole-blood platelet aggregation rates and maximum platelet aggregation responses to higher doses of platelet agonists when compared with those on placebo. The endogenous platelet nitric oxide system did not appear to affect aggregation in either group.

INTRODUCTION

Epidemiological studies suggest that hormone replacement therapy (HRT) reduces the risk of acute myocardial infarction in postmenopausal women [1,2]. Underlying this observation may be favourable alterations in lipid profiles [3] combined with direct effects on the vessel wall, such as improved vasodilatory responses [4], reduced neo-intimal smooth muscle proliferation in response to injury [5] and reduced atherosclerotic plaque formation [6]. However, oestrogen contained in both oral contraceptives and lower-dose HRT has adverse, procoagulant effects. This activation of coagulation leads to an increased risk of thrombotic events, despite concomitant oestrogen-stimulated fibrinolysis [7–9]. Indeed, this is supported by clinical data showing an increase in both venous thrombosis and arterial vascular events in women with pre-existing atherosclerotic disease during the first year of HRT use [10].

While this apparent pro-thrombotic effect of oestrogen may be due to changes in coagulation, it is possible that changes in platelet aggregation may also contribute. However, there is a paucity of data on this subject, with conflicting results with regard to the influence of HRT on platelet function [11,12]. It is plausible that HRT may impact on platelet function, as oestrogen receptors have

Key words: collagen, nitric oxide, oestrogen, progesterone.

Abbreviations: HRT, hormone replacement therapy; l-NMMA, N\textsuperscript{G}-monomethyl-L-arginine; WBIA, whole-blood impedance aggregation.

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been identified on megakaryocytes, which are platelet precursors [13]. Furthermore, endogenous oestrogen appears to affect platelet function, with increased aggregation in pregnancy when oestrogen levels are high. Also, platelet aggregation fluctuates during the menstrual cycle in line with changes in oestrogen levels [14].

In vivo, platelets are involved in the first phases of haemostasis, with a platelet plug being formed in response to vascular injury. The process is regulated by a complex dynamic balance between factors released from both the endothelium and the platelet; these include nitric oxide (NO) and prostacyclin (which inhibit platelet aggregation) and thromboxane (which potentiates aggregation) [15]. On the other hand, aggregation studies in vitro may indicate changes in the platelet aggregation system at the level of the platelet, rather than in the modulatory effects of endothelial factors. Platelet aggregation can be studied using whole-blood impedance aggregometry (WBIA) [16], a technique which examines aggregation in a physiological setting, albeit independently of endothelial effects. Results obtained using this impedance technique correlate with the optical method using platelets in plasma or artificial medium [16–18], with the sensitivity of WBIA being comparable with, if not better than, that of the optical method [16].

The present study has focused on the still controversial effects of combined HRT on platelet function, and was undertaken in 32 postmenopausal women to assess the effects of a minimum of 6 months of combined continuous oral HRT (oestradiol 2 mg + norethisterone acetate 1 mg) on the functional aggregation of platelets in whole blood in response to three commonly used agonists: collagen, arachidonic acid and ADP.

**MATERIALS AND METHODS**

**Study design**

This study involved a consecutively recruited subgroup of 32 postmenopausal women, 50–75 years of age, who were recruited by advertisement from the local community to participate in an ongoing long-term study on the role of HRT in cardio-protection; the wider study involved 59 women over 2 years. Participants were healthy postmenopausal women who had been amenorrhic for at least 12 months and who had a follicle-stimulating hormone level of > 20 units. Exclusion criteria included medications known to influence platelet function [19] (specifically aspirin or non-steroidal anti-inflammatory drugs); smoking; diabetes; alcohol abuse; uncontrolled hypertension, history of venous thrombosis, breast or endometrial cancer; abnormal uterine bleeding; abnormal papsmear or mammogram results. The women were randomly allocated to one of two groups: one group received oral oestradiol (2 mg) combined with continuous norethisterone acetate (1 mg) (Kliogest®; Novo-Nordisk, Copenhagen, Denmark), and the other received a placebo of identical appearance. The randomization schedule was determined by an experienced researcher not otherwise involved in the study, using computer-generated random numbers. All other researchers were blinded to the schedule. Medical review was ongoing throughout the study, with medication compliance checked by counting returned tablets and by questionnaire. Ethical approval was obtained from the Monash Medical Centre Human Research and Ethics Committee, and all participants gave informed consent. All patients studied were compliant with medications (> 85% of tablets taken, based on returned tablet count and questionnaire). Several women had withdrawn from the main study before the platelet studies because of side effects of HRT, including vaginal bleeding and breast tenderness.

**Blood collection**

Bloods were collected before 11.00 hours, by a single medical practitioner trained in non-traumatic phlebotomy. Venepuncture was performed with a 19-gauge needle, and blood was taken directly into two trisodium citrate (0.129 M) tubes (Vacutainer; Becton Dickinson; final blood/citrate ratio 9:1) for platelet aggregation studies. The samples were collected and stored at 25 ºC until aggregation studies were completed. All studies were completed within 3 h of blood collection. Platelet counts and indices were measured by using a Coulter STKR haematology analyser (Coulter Electronics Inc., Hialeah, FL, U.S.A.).

**Platelet aggregation studies**

Aggregation was measured by a single trained medical scientist experienced with WBIA. Blood samples were diluted with isotonic NaCl (0.154 M) to 1:1 (v/v) and preheated for 15 min to 37 ºC for aggregation studies, which were performed at a stirring speed of 800 rev./min on a dual-channel impedance aggregometer (Chronolog Corp., Philadelphia, PA, U.S.A.) based on the technique described by Challen et al. [20]. Baseline aggregation responses to collagen [2 µg/ml final concentration; from a solution of 1 mg of equine type I collagen dissolved in isotonic glucose (pH 2.7) and stored at 4 ºC], arachidonic acid (1 mM; from a 50 mM stock stored at −70 ºC, prepared by addition of bovine albumin to the vial after centrifugation) and ADP (8 µM; from a 1 mM stock solution prepared from 2.5 mg of ADP reconstituted with 5 ml of 0.9% saline) were studied. Doses of aggregating agents selected were in the lower range of those recommended by the manufacturers, and were chosen on the basis that they induced submaximal platelet aggregation. These doses have been used for many years both in our laboratory and by other groups working in whole-blood platelet aggregometry. Indeed, identical aggregation methodology has been applied in both cross-
sectional and human interventional studies by our group, which demonstrated significant effects of diet on platelet aggregation [21,22]. Aggregation responses to collagen were repeated 15 min after \textit{in vivo} addition of the nitric oxide synthase inhibitor $N^\omega$-monomethyl-L-arginine (L-NMMA).

Upon addition of an aggregating agent (e.g. collagen), platelets aggregated on the electrodes, increasing the impedance, which was then recorded on a dedicated analogue-to-digital computer recording system (sampling rate 4/s), attached to the aggregometer, for 7 min (see Figure 1, upper panel), following which the recording was stopped. The signal was differentiated and the rate of aggregation was generated simultaneously (see Figure 1, lower panel). Impedance results were reported both as ohms per min (i.e. rate) and as ohms at a fixed time (6 min) after the start of the aggregation (i.e. impedance amplitude or maximum aggregation). The electrodes were cleaned thoroughly between each aggregation study with sodium hypochlorite (Ajax Chemicals; 0.5% solution) and NaCl (9 g/l). Data from all participants were included in the analysis, with the exception of one sample, which demonstrated a poor response to all aggregation agents. The subject who donated the sample was found subsequently to have been taking medication containing non-steroidal anti-inflammatory agents.

**Statistics**

Statistical calculations were performed using the SPSS statistical package, version 9 (SPSS Inc., Chicago, IL, U.S.A.). Results are expressed as means $\pm$ S.E.M. Comparisons between the HRT and placebo groups were based on either two-way or one-way ANOVA and Student’s $t$-tests. All data except platelet counts showed homogeneous variances. In the case of platelet counts, the non-parametric Mann–Whitney $U$ test was applied, with results expressed as median plus 25–75% confidence intervals. Values of $P < 0.05$ were considered statistically significant.

**RESULTS**

The two groups were not significantly different in terms of age, height, weight, body mass index, or blood pressure (Table 1). In addition, platelet count and mean platelet volume were not statistically different between the two groups (Table 1).

![Figure 1 Measurement of platelet aggregation](image)

The upper panel shows an aggregation curve (maximum aggregation was determined at 6 min), and the lower panel shows a differentiated slope derived from the aggregation curve (the rate of aggregation was determined from the peak of the slope).

**Table 1 Characteristics of the study groups**

Results are presented as means $\pm$ S.E.M., except the platelet count, which is given as mean (25–75% confidence interval). BMI, body mass index; MPV, mean platelet volume; BP, blood pressure. No characteristics were significantly different between the two groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HRT ($n = 13$)</th>
<th>Placebo ($n = 18$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62 $\pm$ 2</td>
<td>63 $\pm$ 2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.6 $\pm$ 0.01</td>
<td>1.6 $\pm$ 0.01</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67 $\pm$ 4</td>
<td>76 $\pm$ 6</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26 $\pm$ 1</td>
<td>29 $\pm$ 2</td>
</tr>
<tr>
<td>Platelet count ($\times 10^9$/litre)</td>
<td>277 (246–208)</td>
<td>265 (206–295)</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>6.9 $\pm$ 0.2</td>
<td>7.1 $\pm$ 0.2</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>91 $\pm$ 3</td>
<td>92 $\pm$ 3</td>
</tr>
</tbody>
</table>
Platelet aggregation

Platelet aggregation in whole blood was measured using an impedance system; the aggregation time curves (see Figure 1, upper panel, for a collagen trace) were measured, as were the slopes of the curves (Figure 1, lower panel). Collagen (2 μg/ml) induced platelet aggregation in whole-blood samples. Neither the maximal degree of aggregation nor the maximal rate of aggregation was significantly different between blood samples from women on placebo or HRT (Figure 2). The nitric oxide synthase inhibitor L-NMMA (100 μM) by itself did not influence collagen-induced aggregation in samples from either the placebo or the HRT groups (Figure 3), with no significant differences in aggregation between the placebo and HRT groups being apparent after addition of L-NMMA (Figure 3).

Arachidonic acid (1 mM) induced platelet aggregation in whole-blood samples. Neither the maximal rate nor the maximal degree of platelet aggregation was significantly different between blood samples from the placebo and HRT groups (Figure 2).

Similarly, ADP (8 μM) induced platelet aggregation in whole-blood samples. Again, neither the maximal degree of aggregation nor the maximal rate of aggregation was significantly different between blood samples from the placebo and HRT groups, indicating no change in sensitivity in the HRT group (Figure 2).

DISCUSSION

We have studied the effects of combined oestradiol and norethisterone therapy on platelet aggregation using an impedance technique in whole blood (WBIA). This technique is considered to be a more physiological method than those using platelet-rich plasma [19]. WBIA also has advantages over the turbidometric method, including more rapid sample processing, retention of larger platelets (as there is no centrifugation step), smaller blood volumes are required, and lipaemia does not interfere with testing.

This technique produced similar results using the agonists collagen, arachidonic acid and ADP similar to those obtained using the optical method and with platelet-rich plasma [16], and has been shown to be reproducible [23]. In the present study, experimental conditions were optimized by dilution of the sample with isotonic saline, which improves accuracy [24]. The length of time that elapses after sample collection can also affect results, and therefore samples were tested within 3 h of collection, as reproducibility of results has been demonstrated up to 4 h after sample collection (L. Waring and A. Turner, unpublished work). The WBIA technique has been used in the clinical setting, and patients with diabetes and ischaemic heart disease have been reported to show increased platelet aggregation compared with controls [25,26].

The primary finding of the present study was that there were no significant differences between women receiving oral combined continuous oestradiol/norethisterone and those receiving placebo in either the rate or the maximum value of whole-blood platelet aggregation. These findings were observed in response to collagen (2 μg/ml), arachidonic acid (1 mM) and ADP (8 μM). The agonist
concentrations used were based on manufacturer’s recommendations, and were specifically applicable to whole-blood aggregation techniques, for which ADP concentrations in particular may be higher than those required in platelet-rich plasma [27]. Recent human cross-sectional and interventional studies completed by our group, based on identical aggregation methodology, have demonstrated significant effects of diet on platelet aggregation responses [21,22]. Those studies demonstrated platelet aggregation effects with agonist concentrations equivalent to and higher than those used in the present study. However, it is acknowledged that, as a single standard concentration of each agonist was used here, we cannot exclude the possibility of effects of HRT on platelet aggregation induced by lower agonist concentrations.

There are several lines of evidence suggesting that endogenous oestrogen can affect platelet function. Firstly, oestrogen receptors are present on platelet precursors, i.e. megakaryocytes [13]. Next, platelet aggregation is higher in pregnant women and lower in untreated postmenopausal women when compared with controls, and fluctuates during the menstrual cycle in line with oestrogen stimulation of aggregation [14]. Also, a cross-sectional study demonstrated that samples from premenopausal women had similar platelet adhesion and aggregation as those from postmenopausal women on HRT, while samples from postmenopausal controls had lower platelet reactivity than both of these groups with higher oestrogen levels [28].

Although studies focusing on the effects of HRT on platelet function are scarce, some workers have noted no changes in platelet aggregation following HRT administration [29], while others suggest that oestrogen may reduce platelet aggregation. A study by Chen et al. [11] noted that 6 months of combined cyclic HRT with oestradiol valerate and medroxyprogesterone acetate decreased platelet aggregation. Interestingly, the benefits were most notable after 1 month, but had decreased markedly by 6 months, with the study concluding that further research on long-term HRT was needed. An initial improvement in platelet function cannot be excluded in the present study. Aune et al. [30] suggested a decrease in the cellular activation of platelets, based on measurement of tissue factor activity, tissue necrosis factor and thromboxane B₂ levels, following 12 months of treatment with either transdermal oestrogen or oral combined oestradiol and medroxyprogesterone acetate. Finally, a decrease in adrenaline-induced platelet aggregation was seen in women on unopposed oestrogen, but these benefits were not observed in women on combined therapy containing medroxyprogesterone acetate [12].

In contrast, there are limited data from indirect studies suggesting that combined HRT may increase platelet aggregability. Mean platelet volume, a potential marker of platelet reactivity, appears to increase in response to HRT [31]. However, in the present study, no difference in mean platelet volume was noted between the two groups. It has also been reported that oestrogen replacement therapy reduced platelet-activating factor acetylhydrolase [32]. The present study did not demonstrate any significant difference between the two groups; however, there was a trend towards increased platelet aggregation in the HRT group.

The available data on the effects of HRT on platelet aggregation come from studies that incorporate many differences in hormonal preparations, duration, methodologies and study design, making it difficult to draw conclusions. It should be noted that no previous study has focused on the effects of norethisterone, a progestin with greater androgenic activity than medroxyprogesterone acetate [33]. Oestrogen alone may have beneficial effects, which are potentially attenuated or masked by additional progestin [12], an effect which may be progestin-subgroup-specific. However, following a review of the literature on the oral contraceptive pill, it was concluded that the increased platelet aggregation was mediated by oestrogen and not by progestin [8]. These issues could not be resolved in the present study, and further research is needed.

Oestrogen has been shown to increase nitric oxide production in many tissues [34]. Nitric oxide, acting via protein kinase G within platelets, has been shown to reduce platelet adhesion and aggregation [35], and is also cytoprotective to platelets [36]. While the principal source of nitric oxide is the vascular endothelium [37], platelets also synthesize nitric oxide, a process that is stimulated during platelet aggregation in response to aggregating agents [34]. It has in fact been hypothesized that the regulation of nitric oxide production may be a potential mechanism of oestrogen and progestin action within the platelet, a hypothesis supported by observations of platelet function following in vitro incubation with oestrogen [38]. The addition of a NO synthase inhibitor (l-NMMA) in vitro did not affect the platelet response to collagen in the present study. The absence of significant differences between women receiving oral combined continuous oestradiol/norethisterone and those receiving placebo in the rate and maximum value of whole-blood platelet aggregation persisted after addition of l-NMMA.

In conclusion, postmenopausal women on oral combined continuous HRT, consisting of oestradiol and norethisterone, showed no significant differences in the rate or the maximum level of whole-blood platelet aggregation in response to collagen, arachidonic acid or ADP, when compared with women receiving placebo therapy. In addition, no significant difference between the groups was noted in the response to collagen following the inhibition of nitric oxide synthase by l-NMMA. Any potential beneficial effects of oestrogen
may have been masked by the progestin; however, this could not be determined in the present setting. Further large controlled interventional studies are needed to clarify these findings.

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REFERENCES


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