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Sex and hormonal influences on platelet sensitivity and coagulation in the rat

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1 Platelet sensitivity to adenosine di-phosphate (ADP), thrombin, collagen, arachidonic acid and prostaglandin I_2 (PGI_2) and the activity of the coagulation system as measured by the activated partial thromboplastin time, prothrombin time, Russell's viper venom time and plasma fibrinogen have been examined in male and female rats, female rats during the oestrous cycle and female rats treated with oestrogen and a progestogen.

2 Male rat platelets were less sensitive to thrombin and more sensitive to inhibition by PGI_2 than those from females and fibrinogen levels in male rat plasma were approximately twice those seen in females.

3 During the oestrous cycle, platelets were more sensitive to ADP and less sensitive to thrombin at di-oestrus.

4 Following 6 weeks treatment with 17 β-oestradiol or ethynyl oestradiol, both platelet aggregation and release of granular ATP induced by collagen were significantly reduced. Platelet sensitivity to other agents, ADP, arachidonic acid, thrombin and PGI_2 was, however, unchanged following oestrogen treatment. Activation of factor X by Russell's viper venom was accelerated in rats treated with ethynyl oestradiol, although this enhancement was not reflected in the overall clotting times.

Introduction

The ovarian hormones, oestrogen and progestogen, are thought to influence the cardiovascular system; on the one hand by protecting against cardiovascular disease in lowering its incidence in women compared with men (Oliver, 1959) and on the other by increasing the risk in women taking oral contraceptives (Medical Research Council, 1967). To account for these effects, a number of human studies have been carried out on platelets and the coagulation system, comparing their activity in men and women (for example, Brakman *et al.*, 1966; Johnson *et al.*, 1975) and women taking oral contraceptives (for a review of the literature, see Poller, 1978), but the results have proved inconsistent.

Whilst many studies have been carried out in humans, few have investigated sex and hormonal influences on platelet sensitivity and coagulation in other species. In the guinea-pig (Hartley strain) no male: female differences were observed in the plasma levels of specific coagulation factors (Dodds *et al.*, 1977b) or in a number of clotting time tests (Dodds & Pickering, 1972). However, these authors did find higher plasminogen levels and shorter clot lysis times in females than in males. In Syrian hamsters, females had shorter 'one-stage prothrombin times', longer 'partial thromboplastin times' and higher levels of fibrinogen and plasminogen than males (Dodds *et al.*, 1977a). Using an *in vivo* model, we have previously shown thrombus formation to be greater in male rats (Wistar strain) than in females, to be greatest at the di-oestrous stage of the oestrous cycle and to be reduced, in both sexes, following 6 weeks treatment with a synthetic oestrogen (Emms & Lewis, 1985). The purpose of this study was to examine the platelet and coagulation characteristics of Wistar rats and to determine how these may be influenced by gender, the stage of oestrus and oestrogen, progestogen treatment. These *in vitro* findings may then be correlated with the results we obtained *in vivo*. Some of the results presented here have been previously reported to the Pharmacological Society (Emms & Lewis, 1984).

Methods

Animals

Male, 455 ± 12 g, and female, 291 ± 2 g, Wistar rats (matched approximately for age) were kept under controlled lighting conditions 12 h light (08 h 00 min–20 h 00 min) and 12 h dark (20 h 00 min–08 h 00 min) with food and water available *ad libitum*. When females in a known stage of the oestrous cycle

were used, they were monitored for at least 2 cycles by microscopic examination of vaginal smears taken daily between 09 h 00 min and 10 h 00 min.

Administration of steroids

Silastic capsules 9 × 25 mm were prepared and filled with approximately 50 mg crystalline ethynyl oestradiol, 17 β-oestradiol or norethindrone acetate as described previously (Henderson *et al.*, 1977). Female rats were anaesthetized with Hypnorm (0.2 mg ml^{-1} fentamyl base, 10 mg ml^{-1} fluanisone), 0.25 ml kg^{-1} i.m., and the capsules implanted subcutaneously in the interscapular region. Rats received either 1 capsule containing ethynyl oestradiol, 17 β-oestradiol or norethindrone acetate or 2 capsules, one containing ethynyl oestradiol and the other norethindrone acetate. Control rats received empty capsules.

Capsules were left *in situ* for 6 weeks. Plasma levels of 300–500 pg ml^{-1} oestradiol have been reported following this regime (Henderson *et al.*, 1977), which compare favourably with the peak plasma levels of 435 ± 144 pg ml^{-1} found in women 1 h after ingestion of 50 μg ethynyl oestradiol (Pasqualini *et al.*, 1977). In the rat, these levels are sufficient to inhibit ovulation and remain fairly constant throughout the treatment period.

Platelet aggregation studies

Rats were anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.p.). Blood was collected from the abdominal aorta, via a polythene canula, into plastic tubes containing either heparin (50 iu ml^{-1}) or 3.8% tri-sodium citrate (9 vol blood to 1 vol anticoagulant). Platelet-rich plasma (PRP) was prepared by centrifugation at 190 *g* for 12 min at 4°C. The PRP was removed and the remaining blood centrifuged at 4080 *g* for 20 min at 4°C to obtain platelet-poor plasma (PPP). The platelet count of PRP was determined using phase contrast microscopy and adjusted to 800 × 10^3 μl^{-1} with PPP (for studies comparing oestrogen-treated rat platelets with controls) or to 400 × 10^3 μl^{-1} with 50% PPP and 50% saline (for studies comparing male with female rat platelets and femal rat platelets over the oestrous cycle). PRP was stored at room temperature in tightly-capped plastic pots before use.

Platelet aggregation was carried out in a Born Mk III aggregometer. Dose-response curves to thrombin were carried out in citrated PRP following recalcification, whilst responses to other agents were determined in heparinised PRP. Six hundred microlitres of PRP were equilibrated at 37°C for 2 min before the addition of adenosine di-phosphate (ADP), collagen, arachidonic acid, thrombin (the addition of thrombin was preceded 1 min earlier by the addition of 15 μl 0.1 M $CaCl_2$) or prostaglandin I_2 (PGI_2) (the addition of PGI_2 was followed 1 min later by the addition of 0.68 μM ADP, final concentration). Responses to ADP and collagen were measured as the height of aggregation and expressed as a percentage of maximal aggregation induced by 50 μM ADP or 32.2 μg ml^{-1} collagen, respectively. The height of the aggregation response to maximal concentrations of ADP and collagen did not vary significantly between any of the groups studied. Responses to thrombin were measured as the initial rate of aggregation. The concentration of ADP, collagen or thrombin to produce 50% of maximal aggregation (EC_{50}) was determined. Responses to arachidonic acid were measured as the delay time before the onset of aggregation and the concentration producing a 30 s delay was determined. Responses to PGI_2 were measured as inhibition of ADP-induced aggregation and inhibition expressed as a percentage of the response to ADP in the presence of vehicle (Tris-HCl buffer 0.05 M pH 9 at 25°C) only. The concentration of PGI_2 producing 50% inhibition of ADP-induced aggregation (IC_{50}) was determined.

In some experiments, platelet aggregation and release of granular adenosine 5′-triphosphate (ATP) were monitored simultaneously using a Payton lumi-aggregation module. Three hundred and sixty microlitres of PRP were warmed to 37°C for 1 min in the aggregometer cuvette before the addition of the ATP monitor, followed 1 min later by the addition of collagen. The amount of ATP released was determined by comparison with a known amount of ATP (2.5 μM final concentration), added immediately after the secretion peak. Responses to collagen were expressed as the height of the aggregation response.

Coagulation studies

Rats were anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.p.) and blood collected from the abdominal aorta, via a polythene cannula, into plastic tubes containing 3.8% tri-sodium citrate (9 vol blood to 1 vol citrate). Blood was centrifuged at 10,000 *g* for 3 min to obtain PPP or at 190 *g* for 12 min to obtain PRP. The platelet count, determined using a Coulter Counter, was adjusted to give 800 × 10^3 μl^{-1} with PPP.

Coagulation assays were carried out in a Labor fibrin-timer II. The activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined in PPP using the reagents and methods of Dade Diagnostics Inc. The Russell's viper venom time (RVTT) was determined in PRP using the reagent and method of Wellcome Research Laboratories. Fibrinogen levels were determined using a modification of the sodium sulphite precipitation technique (Goodwin, 1961): 0.125 ml PPP (stored at −20°C until

Table 1 Sensitivity of platelets from male and female rats

	ADP EC_{50} (μM)	*Collagen* EC_{50} (μg ml^{-1})	*Arachidonic acid 30 s delay (mM)*	*Thrombin* EC_{50} (μ ml^{-1})	*PGI_2* IC_{50} (ng ml^{-1})
Male ($n = 4-6$)	0.28 ± 0.02	1.06 ± 0.14	0.15 ± 0.06	1.32 ± 0.12	2.05 ± 0.1
Female ($n = 16-21$)	0.25 ± 0.02	1.0 ± 0.07	0.20 ± 0.02	1.05 ± 0.05*	2.97 ± 0.13*

*Denotes a significant difference compared with male ($P < 0.05$).

assayed) was warmed to 37°C and 0.25 ml sodium sulphite (15 w/v) added to precipitate fibrinogen. The turbidity of the solution was measured in a Factor 1 fibrinogen monitor (Malin Electronics). Functional antithrombin III (ATIII) levels were determined in PPP using the automated method described by Scully & Kakkar (1977). The authors would like to thank Dr M.F. Scully of King's College Hospital, London, for carrying out these determinations. The levels of ATIII are expressed as a percentage of that obtained for pooled control female rat plasma. Using this method, the normal range for functional ATIII levels in human plasma is 100 ± 20% (± 2 standard deviations) (Thaler & Lechner, 1981).

Statistics

All values are expressed as mean ± s.e.mean. Differences between the means of any two groups were evaluated using the unpaired Student's *t* test and a probability (*P*) of less than 0.05 was taken as being significant.

Results

Platelet sensitivity and coagulation parameters in male and female rats, ex vivo

Table 1 shows the sensitivity of platelets from male rats and from female rats, where the results obtained for the four stages of the oestrous cycle (Table 3) have been combined to provide an 'average' female value. Platelets from male and female rats were equally sensitive to ADP, collagen and arachidonic acid. However, male rat platelets were less sensitive to thrombin and more sensitive to inhibition by PGI_2 (Table 1).

The activity of the coagulation system, determined by measuring the APTT, PT and RVTT, was similar in both sexes. However, fibrinogen levels in male rat plasma were approximately twice those seen in females (Table 2). For this male: female comparison, females were taken regardless of the stage of the oestrous cycle as this was not found to influence coagulation activity (Table 4).

Platelet sensitivity and coagulation parameters in female rats over the oestrous cycle, ex vivo

The sensitivity of platelets from female rats to collagen, arachidonic acid and PGI_2 was unchanged over the oestrous cycle, whereas their responses to ADP and thrombin varied. Female rat platelets were more sensitive to ADP during di-oestrus compared with oestrus but less sensitive to thrombin at di-oestrus compared with metoestrus or oestrus (Table 3).

No differences in any of the parameters of the coagulation system were found in female rats during the oestrus cycle (Table 4).

Table 2 Activity of the coagulation system in male and female rats

	APTT (s)	*PT* (s)	*RVVT* (s)	*Fibrinogen* (g l^{-1})
Male ($n = 4$)	17.8 ± 1.0	16.9 ± 0.7	21.2 ± 4.0	2.67 ± 0.11
Female ($n = 4$)	16.5 ± 1.0	15.7 ± 1.0	19.5 ± 0.7	1.29 ± 0.1***

***Denotes a significant difference compared with male ($P < 0.001$).
Abbreviations used in this and following tables: APTT = activated partial thromboplastin time, PT = prothrombin time, RVVT = Russell's viper venom time.

Table 3 The influence of the oestrous cycle on the sensitivity of rat platelets

	ADP EC_{50} (μM)	*Collagen* EC_{50} (μg ml^{-1})	*Arachidonic acid* *30s delay* (mM)	*Thrombin* EC_{50} (μ ml^{-1})	*PGI_2* IC_{50} (ng ml^{-1})
Metoestrus (n = 4/5)	0.29 ± 0.05	1.12 ± 0.18	0.21 ± 0.04	0.94 ± 0.09*	3.1 ± 0.19
Di-oestrus (n = 3–6)	0.19 ± 0.01	1.0 ± 0.13	0.18 ± 0.06	1.28 ± 0.11	3.1 ± 0.6
Pro-oestrus (n = 4–6)	0.24 ± 0.03	0.9 ± 0.12	0.22 ± 0.05	1.11 ± 0.07	2.9 ± 0.2
Oestrus	0.27 ± 0.03*	1.02 ± 0.19	0.2 ± 0.04	0.91 ± 0.07*	2.8 ± 0.2

*Denotes a significant difference compared with di-oestrus ($P < 0.05$).

Platelet sensitivity and coagulation parameters in female rats following 6 weeks treatment with oestrogen and/or progestogen

Platelets collected from female rats treated for 6 weeks with 17 β-oestradiol did not differ in their sensitivity to ADP, arachidonic acid, thrombin or PGI_2, but were less sensitive to collagen when compared with controls (Table 5).

A similar decrease in platelet sensitivity to collagen was observed when female rats were treated with the oestrogen, ethynyl oestradiol; both platelet aggregation and release of granular ATP induced by collagen were found to be reduced compared with controls, as shown in Figure 1.

Coagulation parameters in control and treated female rats are shown in Table 6. The APTT, PT and fibrinogen levels in female rat plasma were unaltered following 6 weeks treatment with ethynyl oestradiol or norethindrone acetate either alone or in combination. However, treatment with ethynyl oestradiol, alone or in combination with norethindrone acetate, but not norethindrone acetate alone, significantly shortened the RVVT. The levels of functional ATIII were unchanged following treatment with ethynyl oestradiol compared with controls. Levels of ATIII were not measured in plasma from rats treated with noreth-

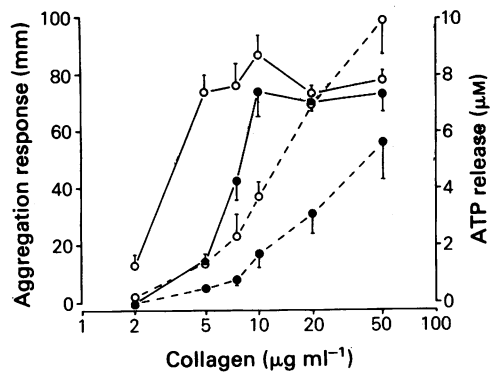

Figure 1 Sensitivity of platelets from control (○) and ethynyl oestradiol-treated (●) female rats to collagen *ex vivo*. Platelet aggregations (———) and release of adenosine 5'-triphosphate (ATP) (– – – –) were monitored simultaneously by a lumi-aggregation module. Both aggregation and release of ATP induced by collagen were significantly reduced in platelets from female rats treated with ethynyl oestradiol, compared with controls. In this experiment, higher concentrations of collagen were required than previously used here, due to an unavoidable delay between platelet preparation and aggregation. Each point represents the mean of 6 animals and vertical lines show s.e.mean where greater than the symbols.

Table 4 Activity of the coagulation system in female rats during the oestrous cycle

	APTT (s)	*PT* (s)	*RVVT* (s)	*Fibrinogen* (g l^{-1})
Metoestrus (n = 5)	16.3 ± 0.8	17.9 ± 0.4	16.5 ± 1.5	0.93 ± 0.08
Di-oestrus (n = 5)	14.9 ± 0.2	17.2 ± 0.4	15.5 ± 0.7	0.93 ± 0.08
Pro-oestrus (n = 5)	16.3 ± 0.7	18.1 ± 0.3	15.1 ± 0.5	1.19 ± 0.11
Oestrus (n = 5)	17 ± 1	18.5 ± 0.4	18.5 ± 0.4	1.18 ± 0.12

Table 5 The effect of 17 β-oestradiol on platelet sensitivity *ex vivo*

	ADP EC_{50} (μM)	*Collagen* EC_{50} (μg ml^{-1})	*Arachidonic acid* 30 s delay (mM)	*Thrombin* EC_{50} (μ ml^{-1})	*PGI_2* IC_{50} (g ml^{-1})
Control ($n = 5/6$)	0.28 ± 0.02	0.71 ± 0.07	0.50 ± 0.13	1.49 ± 0.17	0.77 ± 0.06
17 β-oestradiol ($n = 6$)	0.32 ± 0.03	1.43 ± 0.16**	0.67 ± 0.08	1.49 ± 0.11	0.93 ± 0.10

** Denotes a significant difference compared with control ($P < 0.01$).

Table 6 The effect of ethynyl oestradiol and norethindrone acetate alone or in combination on the activity of the coagulation system

	APTT (s)	*PT* (s)	*RVTT* (s)	*Fibrinogen* (gl^{-1})	*ATIII* (% of normal pool)
Control ($n = 4$)	16.5 ± 1.0	15.7 ± 1.0	19.5 ± 0.7	1.29 ± 0.1	93 ± 1.0 ($n = 8$)
Ethynyl oestradiol ($n = 4$)	14.8 ± 0.6	16.9 ± 1.3	7.7 ± 0.6**	0.83 ± 0.14	94 ± 2.0 ($n = 6$)
Norethindrone acetate ($n = 5$)	18.4 ± 1.9	18.9 ± 0.4	17.7 ± 0.4	N.D.	N.D.
Ethynyl oestradiol + norethindrone acetate ($n = 4$)	14.9 ± 0.4	17.2 ± 0.6	8.4 ± 1.0	1.24 ± 0.25	N.D.

** Denotes a significant difference compared with control ($P < 0.01$). N.D. denotes not determined.
ATIII = antithrombin III.

indrone acetate or in the combined oestrogen/progestogen group.

Discussion

We have previously demonstrated (Emms & Lewis, 1985) enhanced thrombus deposition in male rats compared with females. The results presented here show that, in the rat, this is not attributable to differences in platelet sensitivity (at least when measured *ex vivo*) since male and female rats were equally responsive to ADP, collagen and arachidonic acid. Furthermore, male rat platelets were less sensitive to thrombin and more sensitive to inhibition by PGI_2 compared with females, suggesting that male rat platelets may, in fact, be less reactive. These findings are in agreement with those of Orchard & Botting (1981) who showed that the sensitivity of male and female rat platelets to ADP was similar, but disagree with those of Johnson & Ramwell. (1974)) who found that male rat platelets were 10 times more sensitive to ADP than female rat platelets. No sex differences could be detected in the activity of the intrinsic or extrinsic pathways of the coagulation system, determined by measuring the APTT and PT respectively, or in factor X activation, measured by the RVTT. The levels of fibrinogen in male rat plasma, determined here using a modification of the sodium sulphite precipitation technique, were comparable with those previously found in male Wistar rats by Raymond & Dodds (1975) using a modification of the Ratnoff & Menzie method (1951). In the present study, fibrinogen levels in male rat plasma were found to be approximately twice those seen in females. As fibrinogen levels may be an important risk factor for stroke and myocardial infarction (Wilhelmsen *et al*, 1984) it seems possible that this sex difference in plasma fibrinogen could be responsible for the greater thrombus deposition we have observed in male rats compared with females.

The four well-recognized stages of the oestrus cycle: di-oestrus (degenerative phase), pro-oestrus (follicular or proliferative phase), oestrus (ovulation) and metoestrus (luteal or secretory phase) are accompanied by marked changes in the circulating levels of gonadotrophins and ovarian steroids (Butcher *et al.*, 1977). These fluctuations in endogenous hormones were not, however, found to influence the sensitivity of rat platelets to collagen, arachidonic acid or PGI_2, *ex*

vivo. Their sensitivity to ADP and thrombin did vary, although not consistently since, at di-oestrus, platelets were more sensitive to ADP compared with oestrus and less sensitive to thrombin compared with oestrus and metoestrus. It is difficult to link these differences in platelet sensitivity with the levels of any one hormone. The levels of progesterone, for example, are low during di-oestrus and whilst β-oestradiol levels rise during this stage, they do not reach their peak until pro-oestrus (Butcher *et al.*, 1977). The activity of the coagulation system and plasma fibrinogen levels were not found to alter during the oestrous cycle.

The effect of exogenous oestrogen on platelets and coagulation was also examined in female rats. Rats were not ovariectomized before treatment in order to mimic the situation in women receiving oral contraceptives as closely as possible. Six weeks treatment of female rats with the oestrogen 17 β-oestradiol had no effect on platelet sensitivity to ADP, arachidonic acid, thrombin or PGI_2. Treatment with either 17 β-oestradiol or ethynyl oestradiol did, however, significantly lower platelet sensitivity to collagen; both aggregation and adenine nucleotide release induced by collagen were reduced. In the rat, we have previously demonstrated that oestrogen treatment in fact reduces experimentally-induced thrombus formation (Emms & Lewis, 1985). Thus it would appear that the inhibition of thrombus deposition by oestrogen in the rat may be mediated by changes in platelet reactivity and in particular by an inhibition of the platelet release reaction. No changes in the overall activity of the coagulation system, measured by the APTT and PT or plasma fibrinogen levels were seen in female rats following treatment with ethynyl oestradiol or norethindrone acetate, alone or in combination. Plasma levels of functional ATIII were also unchanged following treatment with ethynyl oestradiol alone. However, the RVVT, which activates factor X and the final common pathway between the intrinsic and extrinsic systems, was shortened following oestrogen treatment. Whilst a similar shortening of the RVVT has previously been demonstrated in oestrogen/progestogen treated rats (McGregor *et al.*, 1979), its significance is difficult to ascertain since not only were the overall clotting times unchanged but also thrombus deposition induced *in vivo* has previously been shown to be reduced following treatment (Emms & Lewis, 1985).

In conclusion, a reduction in platelet sensitivity and in particular the platelet release reaction as induced by collagen was demonstrated in female rats following oestrogen treatment. This reduced reactivity may be involved in the inhibition of thrombus deposition previously reported after such treatment. In addition, sex differences in plasma fibrinogen were observed which may contribute to the greater thrombus deposition seen in male rats compared with females.

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