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The effect of synthetic ovarian hormones on an *in vivo* model of thrombosis in the rat

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1 The effects of endogenous and exogenous, synthetic ovarian hormones on thrombus formation have been examined using an *in vivo* model in the rat.

2 Thrombus formation in female rats was greatest during the di-oestrus stage of the oestrous cycle.

3 Thrombus formation in both male and female rats was reduced following 6 weeks treatment with the oestrogen, ethynyl oestradiol whilst in females the progestogen, norethindrone acetate had no effect. These findings are in contrast to the increased risk of thromboembolic disorders reported in women taking oral contraceptives.

4 The inhibitory effect of ethynyl oestradiol was not due to changes in blood flow but was dependent on the heparin concentration, being greatest at the highest heparin concentration used.

5 Thrombus formation was greater in male rats than in females, a sex difference which is consistent with the higher incidence of cardiovascular disease in men than in women.

Introduction

Cardiovascular disease occurs more frequently in men than in premenopausal women, whilst in post-menopausal women the incidence of the disease is similar to that seen in men of the same age (Office of Population Censuses and Surveys, 1980). A protective role for ovarian hormones has been postulated to account for this phenomenon (Oliver, 1959) since the production of these hormones is reduced at the time of the menopause. The incidence of thromboembolic disease is, however, increased by the use of oral contraceptives which contain an oestrogen and a progestogen (Medical Research Council, 1967). The oestrogen component may be responsible for many of these adverse effects because an excess of cases has been reported in women using high oestrogen preparations (Inman *et al.*, 1970).

The progestogen component may, however, also play a role, for example by causing hypertension and by lowering high density lipoprotein cholesterol levels (Royal College of General Practitioners, 1974).

Investigations have been carried out in animals to determine the effects of contraceptive steroids on the vessel wall, constituents of the blood and rheological factors. In the rat aorta, for example, oestrogens have been shown to induce intimal thickening (Gammal, 1976) and to increase the production of the potent inhibitor of platelet aggregation, prostacyclin (Roncaglioni *et al.*, 1979; Karpati *et al.*, 1980). Furthermore, oestrogens have been demonstrated to influence the sensitivity of rat platelets, either depressing platelet responses (Johnson & Ramwell, 1974) or enhancing them (McGregor *et al.*, 1979) although the effect produced may depend upon the dose administered (Orchard & Botting, 1981). Platelet-associated changes in the coagulation system have also been observed following oestrogen/progestogen treatment (Lecompte & Renaud, 1973). Not only are the results of these animal studies often conflicting but it is also difficult to ascertain their significance *in vivo*, where thrombus formation occurs as a result of a complex series of interactions.

In the present study, which was reported briefly to the Pharmacological Society (Emms & Lewis, 1984), we have investigated the effect of endogenous female hormones and exogenous, synthetic oestrogen and progestogen on an *in vivo* model of thrombosis in the rat (Umetsu & Sanai, 1978; Smith & White, 1982).

Methods

Animals

Female Wistar rats, 240–290 g 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 female rats 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. Male Wistar rats, 270–300 g and 340–375 g were used for weight and age-matched comparisons, respectively.

Administration of steroids

Silastic capsules 9 × 25 mm were prepared and filled with approximately 50 mg crystalline ethynyl oestradiol (Sigma Chemical Co.) or norethindrone acetate (Sigma Chemical Co.) as described earlier (Henderson *et al.*, 1977). Rats were anaesthetized with 0.25 ml kg^{-1} i.m. Hypnorm (fentamyl base 0.2 mg ml^{-1}, fluanisone 10 mg ml^{-1}, Janssen Pharmaceutica) and the capsules implanted subcutaneously at the back of the neck. Male rats received 1 capsule filled with ethynyl oestradiol, whilst females received either 1 capsule containing ethynyl oestradiol or norethindrone acetate or 2 capsules, 1 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 after this regime, which remain fairly constant throughout the treatment period (Henderson *et al.*, 1977). These levels, approximately 5 times the normal pro-oestrus peak oestradiol level (Butcher *et al.*, 1974) are sufficient to inhibit ovulation in the rat and are comparable with the levels measured in women following a 50 μg dose of ethynyl oestradiol (Pasqualini *et al.*, 1977).

Thrombus formation in vivo

Thrombus formation (Smith & White, 1982) was induced in male and female rats anaesthetized with sodium pentobarbitone (May & Baker Ltd.) 40 mg kg^{-1} i.p. A longitudinal incision was made over the trachea; the right jugular vein and left carotid artery were located and the two ends of an extracorporeal shunt inserted into them. The shunt, composed of polyethylene and polyvinyl tubing, contained a 6.5 cm length of cotton thread secured in place by a silicone plug. The shunt was filled with 0.5 ml of heparin (Evans or Duncan Flockhart) 40 iu ml^{-1} producing a final blood concentration of approximately 0.8 iu ml^{-1}. Blood was allowed to flow over the thread for 15 min after which the flow was stopped and the central tubing containing the cotton thread was removed. A second central tube filled with saline and containing a second cotton thread was connected into the shunt. Heparin 0.2 ml of 20 iu ml^{-1}, was injected into the venous cannula prior to restarting blood flow. After a further 15 min period both threads were weighed. The weight of a 6.5cm length of cotton soaked in plasma (12.9 ± 0.3 mg) was subtracted from these weights to obtain the wet weight of the thrombi alone. The average weight of the thrombi deposited on the two threads was determined.

The effect of heparin on thrombus formation in steroid-treated rats

Thrombus formation was induced in control and ethynyl oestradiol-treated female rats in the presence of varying concentrations of heparin (0 to 0.8 iu ml^{-1}

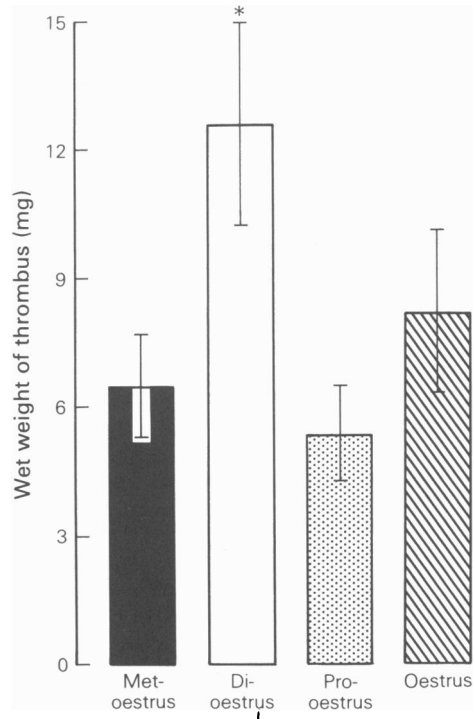

Figure 1 Thrombus formation in female rats during the four stages of the oestrous cycle, metoestrus (solid columns); di-oestrus (open columns); pro-oestrus, (stippled columns); oestrus (hatched columns). The difference between di-oestrus and pro-oestrus only was statistically significant (*$P < 0.05$); there were no significant differences between di-oestrus and oestrus or di-oestrus and metoestrus. Each column represents the mean of 5 animals and vertical lines show s.e.mean.

approximate blood concentration). Heparin 0 to 40 iu ml^{-1} was used to prime the shunt (0.5 ml) and 0.2 ml of a heparin concentration half that used to fill the shunt was injected into the venous cannula prior to restarting blood flow for the second 15 min period.

The effect of blood flow on thrombus formation in steroid-treated rats

Thrombus formation was induced in control and ethynyl oestradiol-treated female rats in an extracorporeal shunt modified by incorporating a pump on the arterial cannula. This enabled blood flow through the shunt to be maintained at a rate of 6 ml min^{-1} (the average rate of blood flow through the shunt without the pump). Systemic blood pressure was monitored during these experiments by an Elcomatic pressure transducer connected to a cannula placed in the femoral artery.

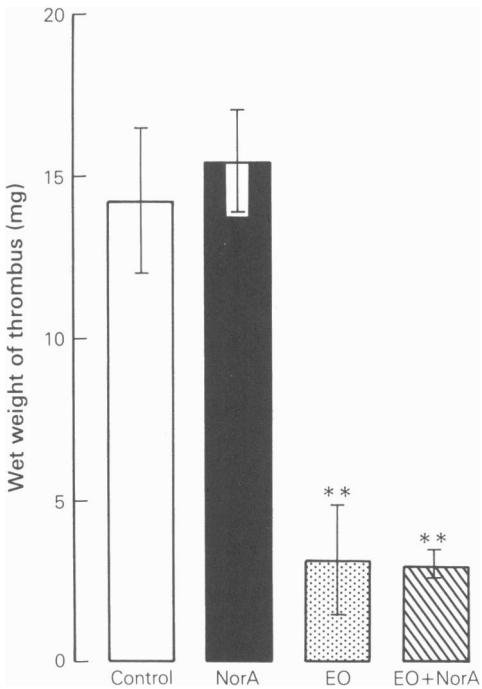

Figure 2 Thrombus formation in female rats treated for six weeks with ethynyl oestradiol or norethindrone acetate either alone or in combination. Control (open column); norethindrone acetate (Nor A, solid column); ethynyl oestradiol (EO, stippled column); ethynyl oestradiol plus norethindrone acetate (EO + Nor A, hatched column). Treatment with ethynyl oestradiol significantly reduced thrombus deposition in female rats (**$P<0.01$) whilst norethindrone acetate had no effect either alone or on the inhibition produced by ethynyl oestradiol. Each column represents the mean of 5 or 6 animals and vertical lines show s.e.mean.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (s.e.mean). Statistical comparisons between the means of any two groups were made using Student's *t* test and a probability (*P*) of less than 0.05 was taken as being significant.

Results

Thrombus formation in female rats over the oestrous cycle

Thrombus formation in female rats (Figure 1) was greater during di-oestrus compared with that of pro-oestrus ($P<0.05$) but there were no significant differences between any of the other stages. In subsequent experiments, unless otherwise stated, control female rats were used at any stage of the oestrous cycle except di-oestrus.

Thrombus formation in steroid-treated female rats

Ethynyl oestradiol, either alone or in combination with norethindrone acetate, reduced thrombus deposition in female rats by 78 ± 12% and 79 ± 3% respectively ($P<0.01$). Norethindrone acetate had no effect either alone or on the inhibition produced by ethynyl oestradiol (Figure 2).

Thrombus formation in male and female rats

Thrombus formation was induced in female rats (at any stage of the cycle) and in weight- and age-matched male rats (Figure 3). Thrombus deposition in weight-matched males was almost three times that seen in female rats ($P<0.01$) and was also greater in age-matched males compared with the females (although this difference was not statistically significant). Thrombus deposition in the 2 groups of males was not significantly different.

Thrombus formation in male rats treated with ethynyl oestradiol

Thrombus formation in male rats treated with ethynyl oestradiol was reduced by 86 ± 6% ($P<0.05$) compared with age-matched controls and by 65 ± 15% ($P<0.05$) compared with untreated weight-matched male rats (Figure 4). Weight-matched males were included for comparison since oestrogen-treated rats failed to gain weight at the same rate as animals of the same age and sex. This effect was most marked in male rats where control animals gained weight rapidly: at the end of the six week treatment period the control group weighed

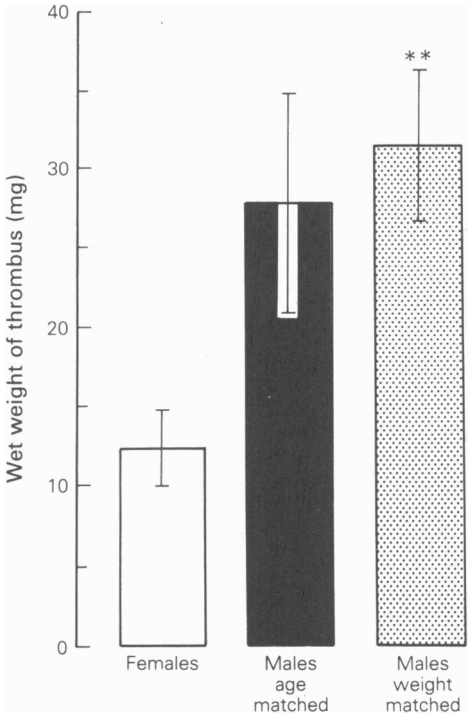

Figure 3 Thrombus formation in male and female rats. Females (open column); males matched to the females by age (solid column); males matched to females by weight (stippled column). Thrombus formation in weight-matched males was significantly greater than that in females (**$P<0.01$) and whilst not statistically significant, thrombus formation was also greater in age-matched males compared with the females. In this experiment 25 iu ml^{-1} heparin was used to prime the shunt and 0.2 ml of 12.5 iu ml^{-1} heparin was administered prior to the second 15 min period. Each column represents the mean of 5 animals and vertical lines show s.e.mean.

approximately 185 g more than the oestrogen-treated male rats.

The effect of heparin on thrombus formation in oestrogen-treated female rats

The extent of inhibition of thrombus formation produced by ethynyl oestradiol was dependent on the heparin concentration used (Figure 5). At 0.8, 0.5 and 0.25 iu ml^{-1} blood concentration of heparin, thrombus deposition was reduced by $78\pm12\%$ ($P<0.01$), $48\pm14\%$ ($P<0.05$) and $33\pm12\%$ ($P<0.05$), respectively. In the absence of heparin thrombus formation was inhibited by $32\pm3\%$ ($P<0.01$).

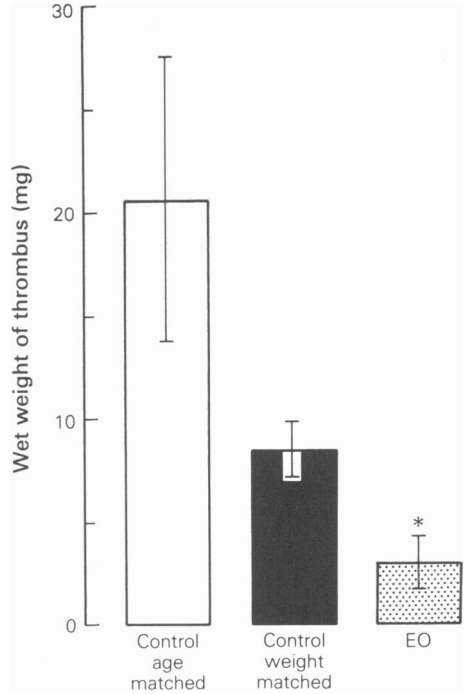

Figure 4 Thrombus formation in male rats treated for six weeks with ethynyl oestradiol. Control matched for age (open column); control matched for weight (solid column); ethynyl oestradiol (stippled column). Treatment with ethynyl oestradiol significantly reduced thrombus deposition in male rats compared with both age-matched and weight-matched controls (*$P<0.05$). Each column represents the mean of 5 or 6 animals and vertical lines show s.e.mean.

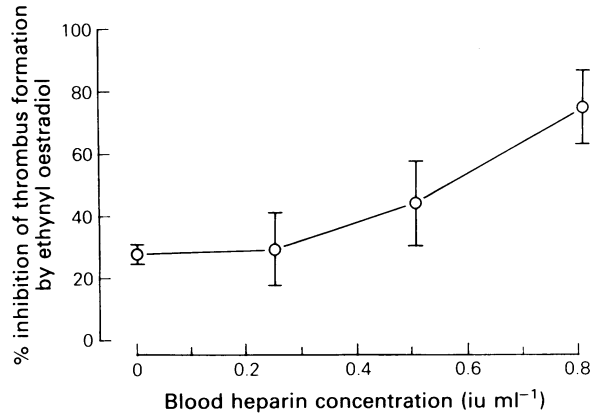

Figure 5 Thrombus formation at different heparin concentrations in female rats treated for six weeks with ethynyl oestradiol. The horizontal axis represents the blood heparin concentration calculated on the basis of a blood volume 10% of body weight. The vertical axis represents the reduction in thrombus weight produced by ethynyl oestradiol expressed as % of control values. The inhibitory effect of ethynyl oestradiol was greatest at the highest heparin concentration used. Each point represents the mean of 5 or 6 treated animals and their controls and vertical lines show s.e.mean.

The influence of blood flow on the inhibition of thrombus formation by ethynyl oestradiol

Thrombus deposition in this model is influenced by the rate of blood flow through the shunt, such that an increase in flow produces an increase in the weight of thrombus deposited (Smith & White, 1982). The possibility therefore existed that the inhibitory effect of ethynyl oestradiol was mediated by changes in blood flow. To investigate this, blood flow through the shunt was maintained at a rate of 6 ml min^{-1} throughout the period of thrombus deposition.

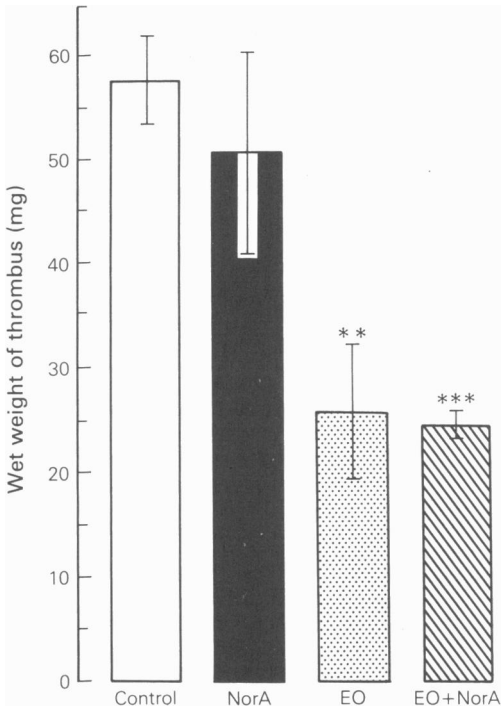

Figure 6 Thrombus formation in a controlled flow system in female rats treated for six weeks with ethynyl oestradiol. Thrombus formation was induced in a shunt modified to enable blood flow over the cotton thread to be maintained at the same rate (6 ml min^{-1}) in both control and treated rats. Control (open column); ethynyl oestradiol (EO, stippled column); norethindrone acetate (Nor A, solid column); ethynyl oestradiol + norethindrone acetate (EO + Nor A, hatched column). Treatment with ethynyl oestradiol alone (** $P < 0.01$) or in combination with norethindrone acetate (*** $P < 0.001$) reduced thrombus deposition even when the flow rate was standardized. In this experiment 25 iu ml^{-1} heparin was used to prime the shunt and 0.2 ml of 12.5 iu ml^{-1} heparin was administered prior to the second 15 min period. Each column represents 5 or 6 animals and vertical lines show s.e.mean.

Inhibition of thrombus formation following treatment with ethynyl oestradiol was not abolished when flow through the shunt was controlled (Figure 6). Thrombus deposition under these modified conditions was greater in all groups of rats and this was thought to be due to the action of the pump itself. Systemic blood pressure was unchanged by the pump and was similar in all groups of rats.

Discussion

In an *in vivo* model in the rat, thrombus formation has been found to be influenced by the oestrous cycle with enhanced deposition occurring during the di-oestrus stage. However, it is difficult to link this elevated thrombus deposition directly to the levels in any one hormone since several hormones show cyclical fluctuations. Furthermore, plasma levels of luteinizing hormone, follicle stimulating hormone, prolactin and progesterone are low during di-oestrus and whilst oestradiol levels are fairly high, they do not reach their peak until pro-oestrus (Henderson *et al.*, 1977).

Treatment of female or male rats with ethynyl oestradiol produced a significant inhibition of thrombus deposition. In female rats norethindrone acetate had no effect. These findings confirm and extend previous observations (Uzunova *et al.*, 1976) where oestradiol was shown to decrease thrombus weight in male rats whereas in females the effect was not significant. In the present experiments the inhibitory effect of oestradiol is unlikely to be due to changes in blood flow since the effect was still seen when flow through the shunt was maintained at 6 ml min^{-1} in both control and treated animals. However, the influence of heparin on the extent of inhibition produced by ethynyl oestradiol would suggest an action via the platelets. A greater reduction in thrombus deposition was observed at the higher heparin concentrations tested when the platelets might be expected to exert their maximum influence. Further investigations are in progress to examine this possibility.

Thrombus deposition in male rats was almost 3 times that seen in females in this experimental model where the thrombus formed possesses both arterial and venous characteristics (Smith & White, 1982). This sex difference has been previously demonstrated in rats and rabbits in a model which produces an arterial thrombus (Uzunova *et al.*, 1976). The greater susceptibility of male rats compared with females is consistent with the higher incidence of cardiovascular disease in men. In the rat, at least, this difference may be due to an inhibitory effect of oestrogen since exogenous oestradiol, administered for a period of 6 weeks, was found to reduce thrombus deposition. These observations however, con-

trast with the increased risk of thrombo-embolic disease reported in women taking oral contraceptives.

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