Sex Differences in the Anticoagulant Effects of Warfarin

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ABSTRACT—Sex differences in the anticoagulant effects of warfarin were studied in rats. Warfarin was administered to rats from 7 days of gestation until 14 weeks of age. In male rats, the normal prothrombin level in the plasma was reduced, and the blood coagulation time was prolonged by treatment with warfarin at 4, 9 and 14 weeks of age. However, in female rats, the effects of warfarin on the prothrombin level and blood coagulation time were observed at 4 weeks to the same degree as in male rats, but these effects were reduced with aging, and at 14 weeks, no effect of warfarin was observed. Rats ovariectomized at 12 weeks of age and subsequently treated with warfarin for 2 weeks showed prolongation of blood coagulation time to the same level as in warfarin-treated male rats, which was inhibited by administration of 17β-estradiol (100 μg/kg /day for 4 days, i.m.). In male rats, treatment with 17β-estradiol also inhibited the anticoagulant effects of warfarin without changing the warfarin level in plasma. These results suggest that there is a sex difference in the anticoagulant effects of warfarin, and that this difference may be related to the estradiol level in plasma.

Keywords: Warfarin, Blood coagulation, Sex difference, 17β-Estradiol

Vitamin K is essential for γ-carboxylation of clotting factors II (prothrombin), VII, IX and X. When glutamic acid residues of these factors cannot be γ-carboxylated, the blood coagulation system does not work well as a result of the reduction in calcium ion binding properties of these factors (1-3). Dietary lack of vitamin K results in hemorrhagic conditions with a decrease in plasma prothrombin level (4-6). There have been several reports indicating that male rats are more sensitive to dietary vitamin K deficiency than female rats (7-10). Recently, the effects of a coumarin-related anticoagulant were reported to be different between male and female rats (11, 12), but whether estrogen is related to this sex difference was not clarified.

In the present study, we demonstrated the time course of the appearance of the sex difference in the effect of warfarin and investigated whether estradiol participates in this sex difference in rats.

MATERIALS AND METHODS

Animals

For all experiments, Sprague-Dawley rats (Clea Japan, Inc., Tokyo) were used. Animals were housed in stainless steel, wire-bottomed cages and kept in an air conditioned room at 24℃ with a 12-hr light-dark cycle. They were given free access to conventional food (CE-2, Clea Japan Inc.) throughout the experimental period. Rats in the warfarin-treated group were given warfarin solution (0.65 mg of warfarin per liter of water) instead of drinking water, while those in the normal control group were given distilled water. In experiments 2 and 3, the mean dose of warfarin, calculated from the body weight and water intake, was about 0.1 mg/kg body weight/day.

Materials

Warfarin potassium was synthesized by Eisai Co., Ltd. (Warfarin®, Tokyo). 17β-Estradiol was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and 0.2 mg of 17β-estradiol was dissolved in 1 ml of sesame oil.

Methods

Experiment 1: To evaluate the time course of the appearance of the sex difference in the effects of warfarin, this agent was given to pregnant rats. Pregnant rats (7-days gestation) were divided into two groups: normal control and warfarin-treated groups. Rats in the warfarin-treated group were given warfarin solution in their drinking water from 7 days of gestation until the pups were weaned. After weaning, the offspring were given the same warfarin solution for the experimental period. Rats in the normal control group were given distilled water. Blood coagulation activity in male and female rats was then measured at 4, 9 and 14 weeks of age (n=3-6).

Experiment 2: Twelve-week-old male and female rats were used. Female rats were divided into one normal group and 6 warfarin-treated groups (n=5-6). Rats in the warfarin-treated groups underwent a sham operation (one group) or bilateral ovariectomy (five groups) under pentobarbital anesthesia (Nembutal®, Abbott Laboratories, North Chicago, IL, USA). They were then given warfarin solution for 2 weeks from the day of operation. Male rats were also given the warfarin solution. Female rats in the normal group were given distilled water. Ovariectomized females treated with warfarin were intramuscularly injected with 100 μg/kg body weight of 17β-estradiol once or repeatedly (4 times, once every two days) during warfarin supplementation. After 2 weeks of warfarin treatment and at 6, 24 and 48 hr after single administration of 17β-estradiol or at 48 hr after the last of 4 treatments, blood coagulation activity was determined.

Experiment 3: Twelve-week-old male rats were given warfarin solution for 8 days and were then once intramuscularly treated with 100 μg/kg body weight of 17β-estradiol. At 6, 24 and 48 hr after the treatment with 17β-estradiol, blood coagulation activity and warfarin level in the plasma were determined.

Measurements

Under pentobarbital anesthesia, 4.5 ml of blood was taken from the abdominal aorta with a syringe containing 0.5 ml of 3.8% (w/v) sodium citrate, and plasma was then separated by centrifugation at 3,000 r.p.m. for 10 min at 4℃. Blood coagulation time was measured using a Hepaplastintest® kit (Eisai Co., Ltd.). Normal prothrombin in plasma was determined by the method of Shah et al. (13) as follows: To prepare barium sulfate ($BaSO_4$)-adsorbed plasma, 400 mg of $BaSO_4$ was added to 1 ml of plasma and then shaken for 20 min at room temperature. Untreated and $BaSO_4$-adsorbed plasma samples were diluted 561- and 51-fold with 0.1 M Tris buffer (pH 8.7), respectively. Echis carinatus venom 0.1 ml (10 ng/ml, Sigma) was added to 0.5 ml of each diluted plasma sample and incubated at 37℃ for 10 min. Then 0.1 ml of substrate (3 mM, tosylglycyl-L-prolyl-L-arginine-*p*-nitroanilide acetate; Sigma) was added and incubated at 37℃. After 10 min, the reaction was stopped by adding 0.1 ml of 20% (w/v) lauryl sulfate, and the absorbance at 405 nm was measured. The results for untreated and $BaSO_4$-adsorbed plasma represented the total prothrombin level and abnormal prothrombin level (PIVKA-II, protein induced by vitamin K absence or antagonist-II), respectively. Normal prothrombin was calculated as the difference between total prothrombin and PIVKA-II levels. Warfarin in the plasma was extracted and measured by high performance liquid chromatography (HPLC) according to the procedure shown in Fig. 1. Column: YMC-ODS (6×150 mm); elution buffer: acetonitrile/0.1 M acetate buffer (55/45, v/v); flow rate: 1.0 ml/min; column temperature: 35℃; pump: Shimadzu LC-6A (Kyoto); detection: UV 320 nm, Waters Lambda-Max model 481 (Milford, MA, USA).

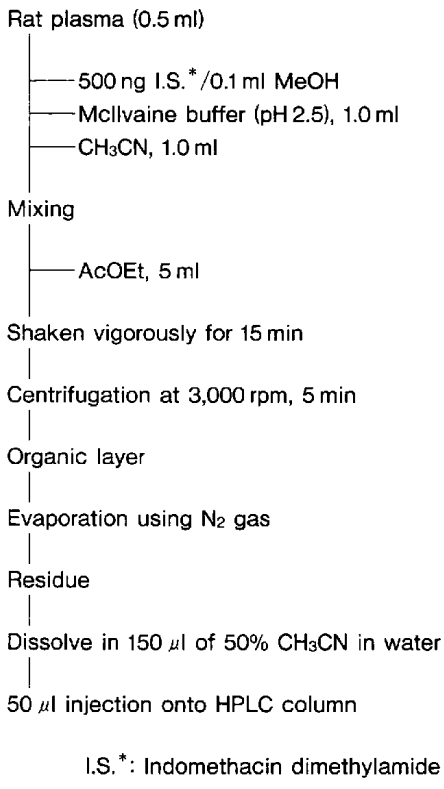

Fig. 1. Procedure for determination of warfarin in rat plasma.

Statistical analyses

All data are expressed as the mean±standard error of the mean (S.E.M.). Analysis of variance was performed, and the significance of differences was determined by Student's *t*-test or Dunnett's multiple-comparison test.

RESULTS

Anticoagulant effects of warfarin in male and female rats at different ages (Experiment 1)

The normal prothrombin level in the plasma and blood coagulation time in 4-, 9- and 14-week-old male and female rats given warfarin are shown in Figs. 2 and 3, respectively. In male rats, the normal prothrombin level in the plasma was significantly lower in warfarin-treated rats than in normal control rats at all time points. In female rats, normal prothrombin levels in the plasma at 4, 9 and 14 weeks of age in the warfarin-treated groups were 48.5%, 73.6% and 118.6% of those of the respective nor-

mal control groups. Thus, the plasma prothrombin level increased age-dependently. The effects of warfarin on blood coagulation time were almost the same as those on normal prothrombin level. That is, in male rats, the blood coagulation time was significantly prolonged by warfarin treatment compared with that in the normal control group at all time points observed. In female rats, the same effects of warfarin as in male rats were observed at 4 weeks of age, but at 9 weeks, the effects of warfarin were weakened, 28.8±1.0 sec in warfarin-treated rats vs 27.9±0.6 sec in normal control rats; and at 14 weeks, the blood coagulation time in warfarin-treated rats was shorter than that in normal control rats with no sex-related differences.

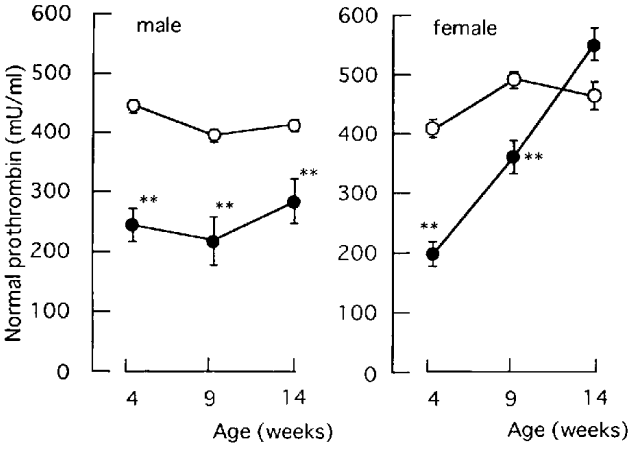

Fig. 2. Time course of normal prothrombin level in warfarin-treated male and female rats. Warfarin solution (0.65 mg/l) was given to rats in their drinking water from 7 days of gestation. Each point and bar represents the mean±S.E.M. of 3 to 6 rats. **P<0.01, compared with normal rats at the respective point. ○: Normal controls, ●: Warfarin-treated rats.

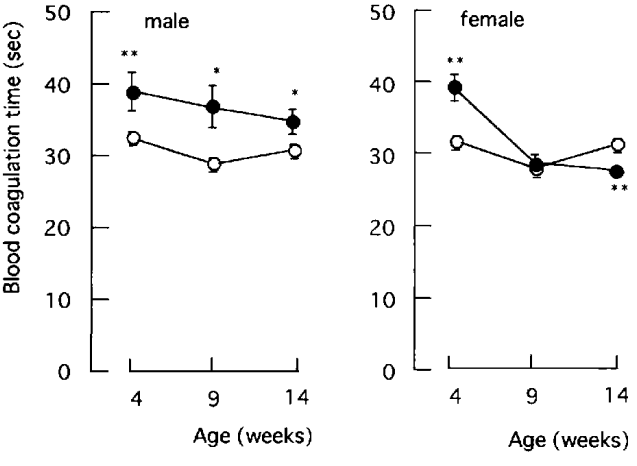

Fig. 3. Time course of blood coagulation time in warfarin-treated male and female rats. Conditions were the same as those described in Fig. 2. Blood coagulation time was determined by the Hepaplastintest®. Each point and bar represents the mean±S.E.M. of 3 to 6 rats. *P<0.05, **P<0.01, compared with normal rats at the respective time point. ○: Normal controls, ●: Warfarin-treated rats.

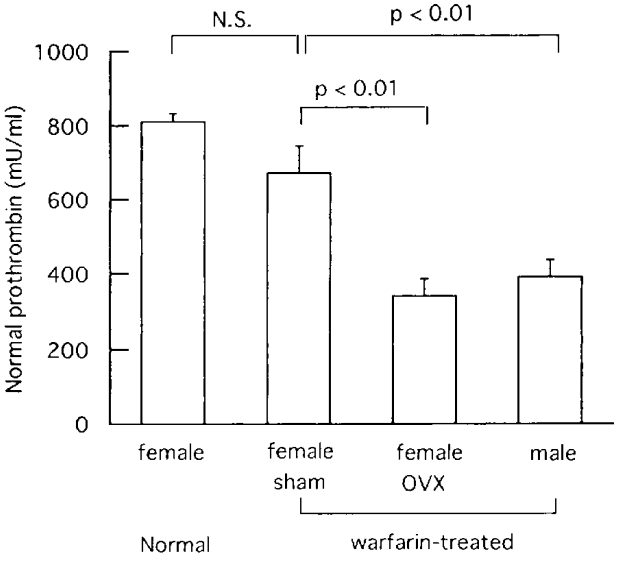

Fig. 4. Effects of ovariectomy on the normal prothrombin level in warfarin-treated female rats. Female rats were bilaterally ovariectomized at 12 weeks of age and given warfarin solution (0.65 mg/l) in their drinking water for 2 weeks. Each column and bar represents the mean±S.E.M. of 5 to 7 rats. N.S.: not significant.

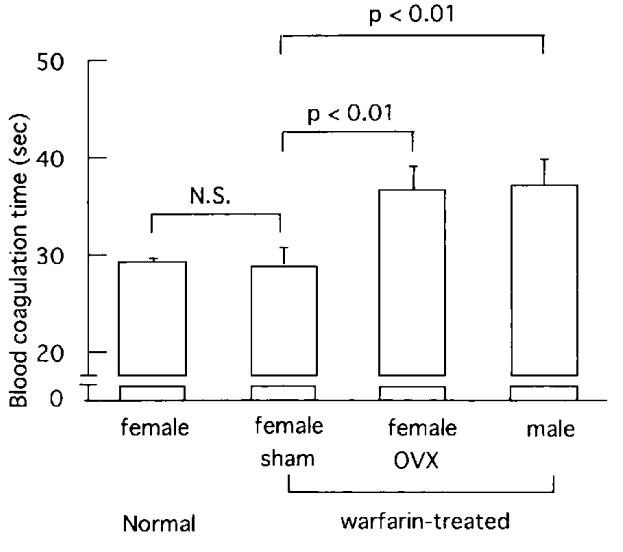

Fig. 5. Effects of ovariectomy on blood coagulation time in warfarin-treated female rats. Conditions were the same as those described in Fig. 4. Blood coagulation time was determined by the Hepaplastintest®. Each column and bar represents the mean± S.E.M. of 5 to 7 rats. N.S.: not significant.

Effects of ovariectomy and 17β-estradiol administration on anticoagulant effect of warfarin in female rats (Experiment 2)

The effects of ovariectomy on the normal prothrombin level in the plasma are shown in Fig. 4. The normal prothrombin level in the plasma in warfarin-treated females was significantly reduced by ovariectomy to the same degree as in warfarin-treated male rats. As shown in Fig. 5, the blood coagulation time in sham-operated female rats was 29.0±1.7 sec, similar to that in normal female rats (29.1±0.4 sec) despite the warfarin treatment. Ovariectomy in warfarin-treated female rats resulted in the prolongation of blood coagulation time to the same degree as in warfarin-treated male rats. Significant differences in blood coagulation time were observed between ovariectomized and sham-operated animals ($P<0.01$).

Ovariectomized warfarin-treated rats were administered 17β-estradiol (100 μg/kg, i.m.) once or repeatedly.

Table 1. Effects of 17β-estradiol on warfarin-induced hypoprothrombinemia in ovariectomized female rats

Treatment	n	Blood coagulation time (sec)	Normal prothrombin (mU/ml)
Sham-operated	6	29.0±1.7	672±72
Ovariectomized			
Control	5	36.7±2.3†	343±44††
17β-Estradiol treatment			
6 hr after single treatment	5	42.0±3.4	285±48
24 hr after single treatment	6	42.5±5.1	344±77
48 hr after single treatment	6	31.6±1.2	465±58
48 hr after 4 treatments	6	28.0±0.7	612±61*

Rats were sham-operated or bilaterally ovariectomized at 12 weeks of age and then given warfarin solution (0.65 mg/l) in their drinking water for 2 weeks. During warfarin supplementation, 17β-estradiol at the dose of 100 μg/kg was intramuscularly administered to rats once or 4 times (once every two days). After 2 weeks of warfarin treatment and at 6, 24 and 48 hr after a single administration of 17β-estradiol or at 48 hr after the last of 4 administrations, blood samples were taken. Each value represents the mean±S.E.M. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, compared with sham-operated rats (Student's *t*-test). *$P<0.05$, compared with ovariectomized control rats (Dunnett's multiple comparison test).

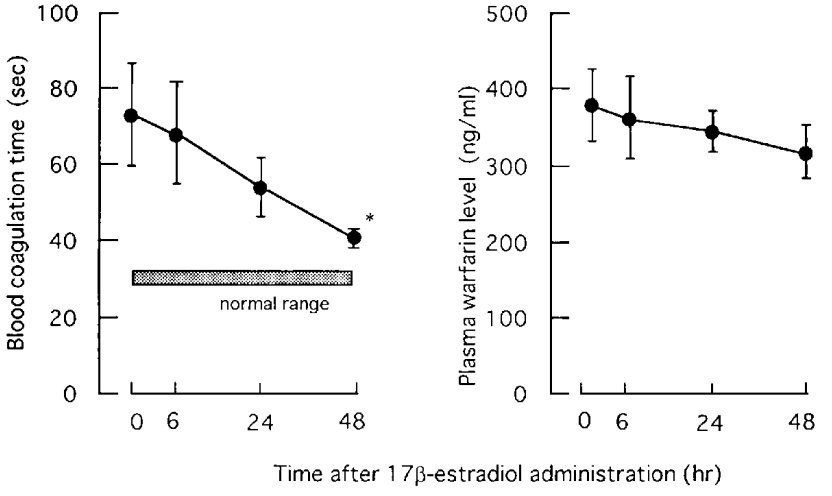

Fig. 6. Effects of 17β-estradiol on blood coagulation time and warfarin level in plasma in warfarin-treated male rats. Male rats at 12 weeks of age were given warfarin solution (0.65 mg/l) in their drinking water for 8 days. Then 17β-estradiol (100 μg/kg, i.m.) was administered to these animals, and blood samples were taken after 6, 24 and 48 hr. Each point and bar represents the mean±S.E.M. of 4 to 5 rats. *$P<0.05$, compared to the pre-administration value obtained in warfarin-treated rats.

As shown in Table 1, at 48 hr after the single administration, both the blood coagulation time and normal prothrombin level tended to show amelioration, but this was not significant. At 48 hr after the last of 4 administrations, these parameters recovered to the same levels as those in the sham-operated rats.

Effects of 17β-estradiol on the anticoagulant effects of warfarin and warfarin levels in plasma in male rats (Experiment 3)

The effects of 17β-estradiol on blood coagulation time and warfarin level in the plasma of warfarin-treated male rats are shown in Fig. 6. Warfarin treatment for 8 days caused a significant increase in blood coagulation time in male rats (73.1±13.3 sec) compared with the normal range (31.8±0.4 sec). Administration of 17β-estradiol resulted in a progressive decrease in blood coagulation time from 6 to 48 hr after administration; blood coagulation time was significantly shortened to within the normal range, and the normal prothrombin level in the plasma was significantly increased (293±25 mU/ml) compared to the pretreatment value (122±22 mU/ml), while the warfarin level in the plasma did not change 48 hr after 17β-estradiol administration.

DISCUSSION

Warfarin, a coumarin-related anticoagulant, is widely used clinically in patients with myocardial infarction or cerebral embolism. There are some reports that infants whose mothers had received oral anticoagulant therapy during the pregnancy exhibited hemorrhagic complications and hypoplastic nose (14–16). Thus in experiment 1, to evaluate when the sex difference in the effect of warfarin occurs, 0.65 mg/l of warfarin solution was given to pregnant rats. The effect of warfarin on the blood coagulating system in 14-week-old female or male rats, which were given warfarin from day 7 of gestation, was similar to that of 14-week-old rats given warfarin from 12 weeks of age, indicating that the sensitivity of rats to warfarin was not affected by the treatment of warfarin from gestation. Moreover, the degree of hypoprothrombinemia induced by warfarin depended on the concentration of warfarin in solution: when 0.65 mg/l of warfarin solution was given to male rats, the normal prothrombin level in plasma was 60% of that in untreated male rats from 4 to 14 weeks of age and administration of 1.5 mg/l of warfarin solution to male rats for 9 days induced severe hypoprothrombinemia in which the normal prothrombin level in the plasma was 12% of that in normal control animals (17). From these results, different degrees of hypoprothrombinemia, in which inter-individual variation is very low, are easily produced by administration of warfarin solution in drinking water for about 1 week.

From the present observation of the constant anticoagulant effects of warfarin from 4 to 14 weeks of age in male rats, while the effect in female rats disappeared with age, it is obvious that there is a sex difference in the response to this agent, and that this difference is associated with aging. There have been many reports indicating that male rats are more sensitive to the effects of vitamin K deficiency than female rats (7–10). The findings of the present study are in good agreement with these previous reports. Data regarding the time course of the appearance of sex differences in the anticoagulant effects of warfarin have not been reported previously. As the maturation of reproductive ability is from about 10 weeks of age (18), the age when sex differences appear may be related to the age when the estrogen level in the plasma increases.

Warfarin exerted its anticoagulant effects in ovariectomized female rats to the same degree as in male rats (Figs. 4 and 5), and administration of 17β-estradiol to ovariectomized females and to male rats ameliorated the hypoprothrombinemia induced by warfarin (Table 1 and Fig. 6). These results suggest that 17β-estradiol participates in the sex-related differences in the response to warfarin. Previous studies showed that the fall in the activity of clotting factors induced by dietary vitamin K deficiency in male rats was not ameliorated by administration of estradiol (7) and that pretreatment with 17β-estradiol also did not influence the effects of warfarin (11), in which observations were performed at 6 or 24 hr after administration, respectively. In the present study, the effects of 17β-estradiol were not significant at 24 hr, but developed at 48 hr after treatment. The observation time after treatment in previous studies may, therefore, have been too short to detect the effect of estradiol, and thus these previous findings are not in conflict with those of the present study.

Warfarin inhibits vitamin K-epoxide reductase and vitamin K-reductase in the vitamin K cycle (19), resulting in the inhibition of the γ-carboxylation of prothrombin precursors and a decrease in the normal prothrombin levels in the plasma. In the present study, administration of 17β-estradiol normalized the blood coagulation time accompanied with an increase in plasma normal prothrombin level in warfarin-treated rats (Table 1 and Fig. 6). It has been reported that the rate of biosynthesis and the half-life of normal prothrombin in female rats are almost equivalent to those in male rats (7), suggesting that estradiol does not influence these parameters. There are several possible mechanisms of the action of estradiol observed in the present study: estradiol may influence absorption, metabolism and excretion of warfarin; estradiol may influence the susceptibility of the site of warfarin-

mediated inhibition in the vitamin K cycle; or estradiol may increase vitamin K levels in the liver. Administration of estradiol to male rats resulted in a shortening of the blood coagulation time at 48 hr, but did not affect the plasma warfarin level (Fig. 6), suggesting that estradiol does not influence absorption, metabolism and excretion of warfarin. Onset of the inhibitory effect of estradiol requires 48 hr, while vitamin K exerts its inhibitory effect only 2 hr after administration (17). These results suggest that estradiol affects neither the inhibitory site of warfarin in the vitamin K cycle nor the γ-carboxylation per se. Although we did not determine the vitamin K concentration in the plasma and liver in the present study, we speculate that estradiol influences absorption, metabolism or excretion of vitamin K, thus resulting in an increase in vitamin K concentration in the liver, which in turn, may reduce the anticoagulant effects of warfarin. This speculation is supported by the finding that administration of estradiol to rats augmented the uptake of vitamin K_1 from the intestine (20).

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REFERENCES

1 Stenflo J, Fernlund P, Egan W and Roepstorff P: Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proc Natl Acad Sci USA **71**, 2730–2733 (1974)

2 Nelsestuen GL, Zytkovicz TH and Howard JB: The mode of action of vitamin K. Identification of γ-carboxyglutamic acid as a component of prothrombin. J Biol Chem **249**, 6347–6350 (1974)

3 Shah DV and Suttie JW: The vitamin K dependent, in vitro production of prothrombin. Biochem Biophys Res Commun **60**, 1397–1402 (1974)

4 Dam H, Schønheyder F and Tage-Hansen E: CLV. Studies on the mode of action of vitamin K. Biochem J **30**, 1075–1079 (1936)

5 Hara K, Akiyama Y, Kato Y, Takizawa K and Tajima T: Effect of vitamin K_2 on hypoprothrombinemia induced by antibiotic administration in vitamin K deficient rats. Vitamins (Japan) **62**, 235–240 (1988) (Abstr in English)

6 Harauchi T, Takano K, Matsuura M and Yoshizaki T: Liver and plasma levels of descarboxyprothrombin (PIVKA-II) in vitamin K deficiency in rats. Jpn J Pharmacol **40**, 491–499 (1986)

7 Matschiner JT and Bell RG: Effect of sex and sex hormones on plasma prothrombin and vitamin K deficiency. Proc Soc Exp Biol Med **144**, 316–320 (1973)

8 Matschiner JT and Willingham AK: Influence of sex hormones on vitamin K deficiency and epoxidation of vitamin K in the rat. J Nutr **104**, 660–665 (1974)

9 Uchida K, Shike T, Kakushi H, Takase H, Nomura Y, Harauchi T and Yoshizaki T: Effect of sex hormones on hypoprothrombinemia induced by *N*-methyltetrazolethiol in rats. Thromb Res **39**, 741–750 (1985)

10 Matsuura M, Satoh S, Takano K, Hirauchi T, Yoshizaki T, Kobayashi F, Matsubara T and Uchida K: Vitamin K-reversible hypoprothrombinemia in rats I. Sex differences in the development of hypoprothrombinemia and the effects of beta-lactam antibiotics. Jpn J Pharmacol **46**, 303–310 (1988)

11 Winn MJ and Park BK: The effects of 17β-oestradiol or testosterone on the response to S-warfarin in castrated male rats. J Pharm Pharmacol **39**, 958–960 (1987)

12 Winn MJ, Clegg JAD and Park BK: An investigation of sex-linked differences to the toxic and to the pharmacological actions of difenacoum: studies in mice and rats. J Pharm Pharmacol **39**, 219–222 (1987)

13 Shah DV, Swanson JC and Suttie JW: Abnormal prothrombin in the vitamin K-deficient rat. Thromb Res **35**, 451–458 (1984)

14 Pettifor IM and Benson R: Congenital malformations associated with the administration of oral anticoagulants during pregnancy. J Pediatr **86**, 459–462 (1975)

15 Shaul WL, Emery H and Hall JG: Chondrodysplasia punctata and maternal warfarin use during pregnancy. Am J Des Child **129**, 65–67 (1975)

16 Hall JG, Pauli RM and Wilson KM: Maternal and fetal sequelae of anticoagulation during pregnancy. Am J Med **68**, 122–140 (1980)

17 Akiyama Y, Hara K, Kato Y, Takizawa K, Tajima T and Shino M: Comparison of the effect on blood coagulation activity between menaquinone-4 and menaquinone-7 in hypoprothrombinemic rats induced by warfarin. Vitamins (Japan) **61**, 605–609 (1987) (Abstr in English)

18 Labhsetwar AP: Ageing changes in pituitary-ovarian relationships. J Reprod Fertil Supp **12**, 99–117 (1970)

19 Whitlon DS, Sadowski JA and Suttie JW: Mechanism of coumarin action: Significance of vitamin K epoxide reductase inhibition. Biochemistry **17**, 1371–1377 (1978)

20 Jolly DW, Craig C and Nelson TE Jr: Estrogen and prothrombin synthesis: effect of estrogen on absorption of vitamin K_1. Am J Physiol **232**, H12–H17 (1977)