Vitamin K-Reversible Hypoprothrombinemia in Rats

I. Sex Differences in the Development of Hypoprothrombinemia and the Effects of Beta-Lactam Antibiotics

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Abstract—Male and female rats were fed an ordinary diet which contained about 500 ng vitamin K/g or a vitamin K-deficient diet containing less than 5 ng vitamin K/g. Hypoprothrombinemic changes such as prolongation of the prothrombin time (PT) and activated partial thromboplastin time (APTT) were detected in male rats within 4–6 days after feeding of the vitamin K deficient diet. Blood clotting factor VII and descarboxy prothrombin (PIVKA) levels changed rapidly, with maximum alteration at 2–4 days. Similar changes in factor VII and PIVKA levels were observed in female rats, but they appeared only after feeding of the K deficient diet for a long period. PT and APTT in female rats showed slight or no alteration even after 10 day feeding of the K-deficient diet. These results indicate that male rats are more susceptible to vitamin K deficiency than female rats. Administration of latamoxef led to a dose-dependent development of hypoprothrombinemia in vitamin K-deficient female rats. The hypoprothrombinemia in vitamin K-deficient female rats was caused by beta-lactam antibiotics with N-methyltetrazolethiol, thiadiazolethiol and methyl-thiadiazolethiol as the 3'-position substituent of the cepham nucleus.
effects of several beta-lactam antibiotics on the coagulation parameters.

This paper describes the hematological and pathological alterations in rats following vitamin K deficiency. Administration of various antibiotics, especially those with NMTT, TDT and MTDT as the 3'-position substituents of the cephem nucleus, to vitamin K-deficient rats enhances the vitamin K-deficient conditions.

Materials and Methods

Animals: Slc-Sprague Dawley rats, aged 7 weeks, were used for the experiments. The animals were kept in an air-conditioned room (23±2°C, 55±10% relative humidity) lighted 12 hr a day (8:00–20:00). Rats were fed an ordinary diet (CA-1: Clea Japan Inc., Tokyo) or a vitamin K-deficient diet (TD-81053, Teklad Co., Madison, WI, U.S.A.). The vitamin K content in the ordinary and the vitamin K-deficient diet was about 500 ng/g and less than 5 ng/g, respectively. During the experiments, the rats were housed in wire-bottomed cages (2 rats per cage) to prevent vitamin K intake by coprophagy.

Antibiotics: LMOX and CMD were obtained from Shionogi & Co. (Osaka), CTX from Hoechst Japan Co. (Tokyo), CEZ and CTZ from Fujisawa Pharmaceutical Co. (Osaka), CTZ and CMX from Takeda Chemical Industries (Osaka), CPZ from Toyama Chemical Co. (Tokyo), CTT from Yamanouchi Pharmaceutical Co. (Tokyo), CMZ from Sankyo Co. (Tokyo), CFX from Daiichi Pharmaceutical Co. (Tokyo) and CBPC from Pfizer Taito Co. (Tokyo).

Determination of blood coagulation parameters: The antibiotics were dissolved in distilled water at the concentration of 25% (w/v), and the resulting solution was injected intravenously, once daily for 2–10 days, at the speed of 2.0 ml/min. Animals in the control group were given physiological saline at 3.6 ml/kg/day, intravenously. The bleeding time of these animals was determined according to the method of Duke (14). At the end of the experiments, the animals were sacrificed by exsanguination from the abdominal aorta, and the liver was removed quickly. The liver PIVKA level was determined as described previously (15) using the microsomal fraction prepared from the middle lobe of the liver.

Pathological examination: When animals were sacrificed at the end of the experiments, heparinized blood samples were obtained. Biochemical analyses of the heparinized plasma samples were carried out with a Technicon auto analyzer SMA Plus Micro system (Technicon Instrument Co., New York, NY, U.S.A.). All animals were then examined macroscopically to check for pathological changes.

Statistical analysis: Statistical significance was analyzed using Student's t-test.

Results

Alteration of blood coagulation parameters in rats following feeding of vitamin K-deficient diet: Feeding of the vitamin K deficient diet caused changes in blood coagulation parameters in both male and female rats (Figs. 1 and 2). In male rats, PT and APTT became gradually more prolonged with feeding time, and a marked prolongation was detected after 6 days. Similar changing patterns were observed in the fibrinogen level and bleeding time. On the other hand, the plasma factor VII and the liver PIVKA levels changed rapidly, with maximum changes after 2–4 days. Although a decrease in the plasma level of clotting factor VII and an increase in the liver PIVKA content were observed also in female rats, they appeared later than in male rats. The PT, APTT and bleeding time in female rats showed only slight prolongations even after 10 days. Platelet number slightly varied during the
experiment, but no statistically significant alteration was observed in both male and female rats, suggesting that vitamin K deficiency does not affect platelet number.

During feeding of the vitamin K-deficient diet, some pathological changes were detected in male rats, but not in female rats. Ten of the eighteen male rats died after 8–10 days of the treatment, and bloody urine, paralysis and weakness were observed in almost all of the surviving male rats. Hemorrhaging in the meninges, stomach, abdominal and thoracic walls, limbs, urinary bladder and other locations was observed in almost all of the male rats after 6 days, while only hemorrhaging in the lung was detected in 2 of 9 female rats after 10 days. Marked increases in plasma GOT, GPT and LDH levels were observed in male rats, but not in female rats, after 8 or 10 days (data not shown). The results suggest that long feeding of the vitamin K-deficient diet causes alterations not only in blood coagulation parameters but also in liver function.

Effect of latamoxef administration on blood coagulation parameters: With LMOX as a model of NMTT-containing antibiotics, the effect of LMOX on PT and APTT was studied in the vitamin K-deficient rats. Increases in PT and APTT were detected in female rats with successive administration of LMOX at 500 mg/kg/day for 6 days or more, with a marked alteration after 8 days (Fig. 3). The PIVKA level in both plasma and liver was elevated slightly in the vitamin K-deficient female rats after treatment with LMOX for 6–8 days (data not shown). When vitamin K-deficient male rats were given LMOX successively, PT and APTT tended to show prolongation, but the effect of LMOX was not clear because both parameters also showed prolongation with feeding of the vitamin K deficient diet alone as shown in Fig. 1. Enlargement of the caecum was observed in both LMOX-treated male and female rats, and hemorrhaging in the muscle of heads was detected in one of the six LMOX-treated female rats. No alteration in liver functions
was observed in both male and female rats (data not shown).

The vitamin K-deficient male and female rats were given intravenously various doses of LMOX for 4 and 10 days, respectively. Feeding of the vitamin K-deficient diet caused changes in blood coagulation parameters in both male and female rats, with severe changes in the male rats. Blood coagulation parameters, especially PT, APTT, clotting factor VII and PIVKA levels in female rats were further affected dose-dependently by LMOX administration, suggesting that the vitamin K deficient conditions were enhanced by LMOX administration (Figs. 4 and 5). The plasma prothrombin level seemed to decrease dose-dependently, and the fibrinogen level also tended to increase in female rats administered higher doses of LMOX. Although no hemorrhagic effect was detected in control female rats, hemorrhaging in the thoracic wall and the caecum was found in 2 of 9 and 5 of 9 female rats, respectively, after injection of the highest dose (2700 mg/kg/day) of LMOX. On the other hand, a slight or no effect of LMOX treatment was observed in male rats, but the effect of LMOX was not clear because of the high variability of the coagulation parameters in male rats caused by the feeding of vitamin K-deficient diet alone. Symptoms commonly observed in rats treated with a high

Fig. 2. Effect of vitamin K-deficient diet feeding on fibrinogen level, platelet number and bleeding time in rats. The same animals used for the experiments of Fig. 1 were used for the determination of parameters. Open and closed circles represent male and female rats, respectively. Data in the figure came from 9 rats, except that there were only 3 rats from the 8 and 10 day-feeding male group for the fibrinogen level and platelet number calculation. Bleeding time in 7- and 9-day-feeding male rats was determined with 7 and 6 rats, respectively. The values represent the mean and standard error. * and **, statistically different (P<0.05 and P<0.01, respectively) from the control.

Fig. 3. Effect of latamoxef administration on PT and APTT in vitamin K-deficient female rats. Female rats were given intravenously LMOX at 500 mg/kg/day, once daily for 2–8 days under vitamin K-deficient conditions, and blood samples for the determination of PT (circles) and APTT (triangles) were obtained 24 hr after the last administration of LMOX. Closed symbols in the figure represent PT and APTT values in rats fed the vitamin K-deficient diet without LMOX administration. The values in the figure represent the mean and standard error of 6 animals. * and **, statistically significant (P<0.05 and P<0.01, respectively) against the control.
A dose of antibiotics such as soft feces, diarrhea and enlargement of the caecum were detected similarly in both male and female rats. Abnormalities in liver functions were not detected under the treatment conditions employed here in both male and female rats even with the injection of 2700 mg/kg/day dose of the antibiotic.

Effect of various antibiotics on blood coagulation parameters in rats: Animals fed the vitamin K-deficient diet were given various antibiotics intravenously at 900 mg/kg/day for 10 days, and the coagulation parameters were measured 1 day after the last injection (Table 1). The dose of antibiotics (900 mg/kg) employed here, suitable for detecting the hypoprothrombinemic effects of drugs, was decided upon from the above results (Figs. 4 and 5). Feeding of the vitamin K-deficient diet (control group in the table) caused changes in the parameters compared with the animals fed the ordinary diet as seen in Table 1. Administration of NMTT-containing antibiotics (LMOX, CMD, CPZ, CTT, CMZ and CMX) resulted in

Fig. 4. Effect of various doses of latamoxef on blood coagulation parameters in vitamin K-deficient rats. Male (M) and female (F) rats were fed the vitamin K-deficient diet and were given LMOX intravenously at 100, 300, 900 and 2700 mg/kg/day, once daily for 4 and 10 days, respectively. Blood samples for the determination of coagulation parameters were obtained 1 day after the last injection. The parameters of rats fed the ordinary diet are also shown in the figure for comparison. The values in the figure represent the mean and standard error of 9 rats. * and **, statistically significant (P<0.05 and P<0.01, respectively) against the control (vitamin K deficient diet alone).

Fig. 5. Effect of latamoxef administration on plasma prothrombin, PIVKA and fibrinogen levels in vitamin K-deficient rats. Blood samples were obtained from LMOX-treated vitamin K-deficient rats as described in Fig. 4. The values in the figure represent the mean and standard error of 9 rats. * and **, statistically significant (P<0.05 and P<0.01, respectively) against the vitamin K-deficient control rats.
Table 1. Effect of various antibiotics on blood coagulation parameters in vitamin K-deficient female rats

| Exptl group | Treatment of rat | PT (sec) | APTT (sec) | Factor VII (%) | Prothrombin (U/ml) | PIVKA Plasma (U/ml) | PIVKA Liver (U/mg protein) |
|-------------|-----------------|----------|------------|---------------|-------------------|-------------------|------------------------|
| 1           | Control         | 21.2±2.4 | 41.2±4.6   | 75.4±13.3     | 35.5±3.6          | 7.8±0.6           | 0.76±0.02              |
|             | LMOX            | 48.8±7.9**| 88.1±11.4**| 16.2±1.9**    | 18.8±1.7**        | 12.1±1.0**        | 1.04±0.09*             |
|             | CMD             | 37.5±3.2**| 84.2±6.0** | 22.5±3.0**    | 20.4±2.1**        | 10.8±0.5**        | 0.99±0.05**            |
|             | CTX             | 18.1±1.4  | 38.1±2.6   | 57.9±10.1     | 42.8±5.4          | 8.0±0.6           | 0.76±0.03              |
|             | CBPC            | 16.8±1.4  | 33.9±2.1   | 88.3±15.5     | 57.2±8.5*         | 7.1±0.2           | 0.74±0.03              |
|             | Ordinary        | 10.7±0.2**| 17.2±0.3** | 272.9±42.2**  | 264.5±3.3**       | 2.4±0.4**         | 0.11±0.01**            |
| 2           | Control         | 16.2±1.2  | 35.2±3.4   | 53.5±8.4      | 42.8±5.5          | 5.9±0.5           | 0.84±0.07              |
|             | LMOX            | 21.0±1.7* | 43.7±3.5   | 33.0±5.3*     | 31.4±3.7          | 7.2±0.6           | 0.82±0.05              |
|             | CPZ             | 22.0±0.7**| 48.6±1.9** | 28.1±3.1*     | 27.7±1.9*         | 7.8±0.3**         | 0.87±0.02              |
|             | CTT             | 23.4±1.7**| 58.5±8.4*  | 21.9±2.9*     | 24.1±1.6**        | 7.5±0.2**         | 0.89±0.02              |
|             | CMZ             | 20.2±2.1  | 46.2±4.7   | 31.9±5.1*     | 34.1±3.7          | 7.5±0.2**         | 0.85±0.02              |
|             | CEZ             | 68.0±14.2**| 157.8±31.0**| 9.9±1.3* | 19.9±1.2**     | 14.9±1.3**        | 1.02±0.02              |
|             | CTZ             | 79.0±27.7 | 120.2±34.9 | 13.8±4.8**    | 45.5±20.2         | 11.7±1.8*        | 0.86±0.09              |
|             | CTM             | 12.9±0.7* | 25.5±1.5*  | 86.0±12.1     | 91.7±12.4**       | 4.9±0.5           | 0.73±0.04              |
|             | CFX             | 13.5±0.5* | 26.6±1.4*  | 73.0±10.4     | 67.5±6.1*         | 4.3±0.2**         | 0.76±0.02              |
|             | Ordinary        | 11.0±0.2**| 17.5±0.3** | 214.3±21.6**  | 240.2±7.8**       | 0.9±0.2**         | 0.12±0.01**            |

Animals were maintained on the vitamin K-deficient diet for 10 days, except that the ones in the ordinary group were fed the ordinary diet. They were given antibiotics intravenously at 900 mg/kg/day for 10 days. The values in the table represent the mean±S.E. of 6 or 4 rats in Exptl groups 1 and 2, respectively. * and **, statistically significant (P<0.05 and P<0.01, respectively) against the control.
further enhancement of these changes. Interestingly, CEZ and CTZ, which do not contain NMTT in their molecules but do have MTDT and TDT, respectively, as the 3'-position substituent of the cephem nucleus, showed severe hypoprothrombinemic effects, although the results were not statistically significant for PT and APTT with CTZ-treated rats. Other antibiotics not containing NMTT, MTDT or TDT in their molecules showed no hypoprothrombinemic effect.

Discussion

Recently, beta-lactam antibiotic-induced coagulopathy has been a subject of discussion (16–18), with claims that NMTT causes hypoprothrombinemia. Subsequent experiments have demonstrated that the hypoprothrombinemic effects of these antibiotics were detectable only in vitamin K-deficient rats, and not in animals fed an ordinary diet (6, 8). In the present study, we also found the hypoprothrombinemic effect of various antibiotics in vitamin K-deficient rats. However, we detected no in vivo effect of antibiotics in male rats when the animals were fed the diet containing less than 1% of vitamin K compared with the ordinary diet (Figs. 4 and 5). Uchida et al. examined the in vivo effect of the NMTT-containing antibiotics and NMTT itself on male rats fed a diet that was vitamin K-deficient but contained a moderate amount (about 30 ng/g) of vitamin K (6, 8, 13). Under these conditions, the hypoprothrombinemic effect of antibiotics or NMTT was detected only in male rats, and not in female rats, and also not found in castrated male rats (13). These and the present findings suggest that the development of antibiotic-induced hypoprothrombinemia is closely related to the vitamin K level in the body, and male rats are more susceptible to vitamin K deficiency. When a severe vitamin K-deficient condition developed in male rats by feeding of the vitamin K-free or -deficient diet as in the present experiment, the NMTT-containing antibiotic LMOX did not cause further enhancement of vitamin K deficient states (Figs. 4 and 5).

Male rats are known to respond more severely to vitamin K deficiency than female rats (11–13). This sex-related difference is now considered to be due to sex hormones, although its mechanism has not been fully clarified. Jolly et al. reported that absorption of vitamin K increased with estrogen injection (19); but in another study, no sex difference was observed in the absorption of 14C-vitamin K, and 14C-menadione from the small intestine and colon loop (Yamada, H., unpublished results). Gustafsson et al. (20) and Matschiner and Bell (21) demonstrated a less requirement of vitamin K in female rats than in male rats. The present results shown in Figs. 1 and 2 agree with these previous results (20, 21), but further studies are needed to explain the exact mechanism.

As mentioned above, the antibiotics containing NMTT as the side chain moiety cause the development of hypoprothrombinemic changes in vitamin K-deficient female rats (Table 1). Administration of NMTT itself produced the same effect as its parent antibiotics (6–8, 13). Interestingly, the in vivo effects were also observed when CEZ or CTZ were administered (Table 1): they do not have NMTT, but MTDT and TDT, respectively, as the 3'-position substituent. Creedon and Suttie (22) revealed that the antibiotic administration caused inhibition of liver microsomal vitamin K epoxide reductase, which was thought to follow the depressed synthesis of vitamin K-dependent blood clotting factors under vitamin K-deficient conditions. Recent studies demonstrated that liver vitamin K epoxide reductase was inhibited in vivo not only by NMTT-containing antibiotics but also by MTDT- or TDT-containing antibiotics (23). These reports support the present results which indicate hypoprothrombinemic effects of the antibiotics containing NMTT, MTDT or TDT (Table 1). When rats were maintained on the ordinary diet containing a sufficient amount of vitamin K, these antibiotics caused no hypoprothrombinemic changes in blood coagulation parameters, suggesting that the biosynthetic reaction of blood clotting factors is not modified by the antibiotics when a sufficient amount of vitamin K is present. Several investigators found no inhibition of liver microsomal gamma-glutamylcarboxylase, an essential enzyme for the biosynthesis of blood coagulation factors, in rats treated...
with the NMTT-containing antibiotics (6–8, 24). These results support the above assumption.

We can conclude from these previous reports and the present experiment that the antibiotics having NMTT, MTDT and TDT as the 3'-position substituent enhance vitamin K deficiency via the same mechanism as warfarin and various drugs that inhibit vitamin K epoxide reductase.

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