INTRODUCTION

Vitamin K contributes to maintaining normal levels of clotting factors II, VII, IX and X. They are synthesized in the liver as inactive pro-proteins (Bloom and Brandt, 2001). In order to activate these coagulation factors, the posttranslational modification that converts glutamate residues in pro-proteins to gamma-carboxyglutamate residues is required, and it is catalyzed by a vitamin K-dependent carboxylation reaction (Marcus and Coulston, 1996). Vitamin K is commonly found in plant and animal tissues which are the sources of vitamin K for human beings. Additionally, intestinal bacteria can synthesize vitamin K, and thus vitamin K-deficiency is extremely rare in adults (Sakurakawa, 1985). On the other hand, newborns are vulnerable to vitamin K-deficient bleeding, because vitamin K is poorly delivered to the fetus via the placenta during pregnancy and the intestinal flora in newborns lacks microorganisms that synthesize the vitamin (Marcus and Coulston, 1996). There are several reports of neonatal hemorrhage in infants born to mothers treated with a barbiturate antiepileptic drug, phenobarbital (PB), a CYP2B inducer, during pregnancy (Moslet and Hansen, 1992). However, there are no reports of effects of PB-administration to dams on the coagulation system of newborn pups in laboratory animals. Recently we reported that prolongation of coagulation time and increased ATIII concentration were observed in Sprague-Dawley rats which were treated with PB (Mochizuki et al., 2008). There are almost no reports of the effects of PB on coagulation time in rabbits which are widely used for toxicological studies.

Changes in blood coagulation-related parameters in phenobarbital-treated rabbits

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ABSTRACT — The effects of repeated administration of phenobarbital (PB) on blood coagulation time were examined using male Japanese white SPF rabbits, which are widely used for toxicological studies. PB was administered to the rabbits by oral gavage for 2 weeks, at dose levels of 0, 12.5, 25 and 50 mg/kg/day. Blood was collected on Days 8 and 14 after each day’s dosing to perform blood coagulation examination. The liver was excised, weighed and examined histopathologically. Activated partial thromboplastin time (APTT) was prolonged at dose levels of 12.5 mg/kg/day or more and Thrombotest® (TBT) was prolonged at 50 mg/kg/day on Day 8. APTT was prolonged at dose levels of 12.5 mg/kg/day or more, TBT was prolonged at 25 mg/kg/day or more and factor IX activity decreased at 50 mg/kg/day on Day 14. At pathological examination, liver weight increased at dose levels of 25 mg/kg/day or more, and a ground-glass appearance of the hepatocytes was observed in the central and middle parts of lobules at 12.5 mg/kg/day or more. However, changes in factor VII or X activity or prolongation of prothrombin time (PT) were not observed. Therefore, prolongation of blood coagulation time by PB administration in rabbits was considered to be due to PB’s effect on the endogenous pathway alone. Moreover, an increase in antithrombin III (ATIII) concentration was noted at 50 mg/kg/day; however, no change was noted at dose levels of 25 mg/kg/day or less. This suggests that the contribution of ATIII to the PB-induced prolongation of coagulation time in rabbits was small.

Key words: Phenobarbital, Vitamin K, APTT, ATIII, Rabbit

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min K is accelerated by PB administration in rabbits in the same manner as rats (Wilson and Park, 1984). In this study, we focused on rabbits to investigate effects on blood coagulation time by PB administration including detailed measurements of factors VII, IX and X activities. In addition, males were used in this study since effects of 2,2',4,4',5,5' hexachlorobiphenyl (HxCB), a CYP2B inducer, on the coagulation system are more apparent in males than females (Bouwman et al., 1999). All procedures on this study were conducted in compliance with “Guidelines for Proper Conduct of Animal Experiments” (Science Council of Japan, June 1, 2006) and according to the Protocol approved by the Animal Care and Use Committee at BOZO Research Center Inc. All efforts were made to minimize animal suffering.

MATERIALS AND METHODS

Animals

A total of 24 male Japanese white SPF rabbits (Kbl:JW, Kitayama Labes Co., Ltd., Nagano, Japan, Kanagawa, Japan) were used at 23 to 26 weeks of age. The animals were kept individually in aluminum cages in an air-conditioned animal room (temperature: 23 ± 3.5°C; humidity: 50 ± 20%; air ventilation: 10-15 times/hr; lighting: 12 hr/12 hr light/dark cycle). The animals were allowed free access to solid feed (RC4, Oriental Yeast, Co., Ltd., Tokyo, Japan) and tap water during the experimental period.

Chemicals

PB was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PB was dissolved in water for injection (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) immediately before use, and the concentration was adjusted to 2.5, 5 and 10 mg/ml.

Experimental design

A preliminary study using 2 animals orally treated with a single dose (100 mg/kg) of PB resulted in hyposthenia and staggering gait from 30 min after dosing, and these signs were observed until the next day. Therefore, 50 mg/kg/day was selected as the highest dose level, and a total of 3 dose levels, including 25 and 12.5 mg/kg/day, were provided. PB is commonly administered once or twice at 50 to 100 mg/man/day. Therefore, 50 mg/kg is equivalent to approximately 12-50 times the quantity. Each group consisted of 6 animals and they were given PB by oral gavage for 14 days. A control group (6 animals) was also provided and animals received water for injection in the same manner. During the administration period, animals were weighed daily and observed for general conditions 3 times a day: before dosing, approximately 0.5 hr and 2 hr after dosing. Blood was collected on Day 8 (approximately 2 hr after dosing) and on the day following the end of the administration period (Day 14), and blood coagulation examination was conducted. After blood sampling, all animals were sacrificed by exsanguination from the abdominal aorta under anesthesia by injection of 6 w/v% pentobarbital sodium solution (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) into the auricular vein.

Blood coagulation tests

Blood was collected via the auricular vein into test tubes containing 3.8% sodium citrate (Venoject® II, VP-C052K, Terumo Corporation, Tokyo, Japan). The samples were centrifuged (approximately 1,600 × g, 10 min) to obtain plasma. Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were measured by a coagulometer, ACL 100 (Instrumentation Laboratory, Lexington, MA, USA), while anti-thrombin III (ATIII) concentration was measured by a clinical chemistry autoanalyzer, TBA-120FR (Toshiba Corporation, Tokyo, Japan). Thrombotest® (TBT: Sankou-Junyaku Co., Ltd., Tokyo, Japan) was performed manually. Moreover, factor VII, IX and X activities were measured using HemosIL Factors VII, IX and X (Instrumentation Laboratory) by a coagulometer, ACL Elite (Instrumentation Laboratory).

Pathological examination of the liver

The liver was excised from each animal and weighed at necropsy, and then fixed in 10% phosphate-buffered formalin. Four-μm paraffin sections were stained with hematoxylin and eosin (HE) for histopathological examination.

Statistical analysis

The significance of differences from the control group was tested after calculating Mean ± S.D. in each group. Homogeneity of variance was assessed by Bartlett’s test (Snedecor and Cochran, 1989). For homogeneous data (level of significance: 1%, two-tailed), values were compared between the control group and each dose group by Dunnett’s test (level of significance: 1 and 5%, two-tailed) (Dunnett, 1955, 1964). For heterogeneous data, any group mean rank difference was compared between the control group and each dose group by Steel’s test (level of significance: 5% and 1%, two-tailed) (Steel, 1959).
RESULTS

Clinical findings
The results of clinical observation are shown in Table 1. At the dose level of 50 mg/kg/day, staggering gait was observed from Day 1. Thereafter, soft feces, decrease in feces or no feces, hyposthenia and lateral position were observed, and one animal died on each of Days 12 and 13.

In the other dose groups, neither deaths nor abnormal clinical signs were observed in any animal.

Body weight gain
Body weight gains are shown in Fig. 1. At the dose level of 50 mg/kg/day, body weight gain was lower than that in the control group from Day 11 to the day of necropsy.

Findings of the blood coagulation tests
Changes in blood coagulation parameters on Day 8 are shown in Table 2. APTT was prolonged or tended to be prolonged (at dose levels of 12.5 mg/kg/day or more (19 to 49% longer than that in the control group) and TBT tended to be prolonged at 50 mg/kg/day (30% longer than that in the control group).

Changes in blood coagulation parameters on Day 14 are shown in Table 3. APTT was prolonged at dose levels of 12.5 mg/kg/day or more (18 to 27% longer than that in the control group) and TBT was prolonged or tended to be prolonged at dose levels of 25 mg/kg/day or more (7 or 36% longer than that in the control group). Moreo-

Table 1. Clinical observation during 2-week PB administration

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
<th>Day of dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>- - - - - - - - - -</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>- - - - - - - - - -</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>- - - - - - - - - -</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>- - - - - - - - - -</td>
<td></td>
</tr>
</tbody>
</table>

-: No abnormality, A: decrease in feces, B: hyposthenia, C: staggering gait, D: soft feces, E: no feces, F: lateral position, +: dead
ver, ATIII concentration increased at 50 mg/kg/day (35% higher than that in the control group). In measurement of coagulation factors, the coagulation time of factor IX was prolonged at 50 mg/kg/day (22% longer than that in the control group), confirming a decrease in factor IX activity.

In addition, a decrease in fibrinogen with no clear dose-dependency was noted at dose levels of 12.5 and 25 mg/kg.

Table 2. Effects of PB administration on coagulation-related parameters on Day 8 of administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (sec)</td>
<td>7.0 ± 0.0</td>
<td>7.1 ± 0.1</td>
<td>7.0 ± 0.0</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>20.7 ± 1.8</td>
<td>24.7 ± 1.7*</td>
<td>24.7 ± 3.9</td>
<td>30.9 ± 10.0*</td>
</tr>
<tr>
<td>TBT (sec)</td>
<td>33.1 ± 1.1</td>
<td>35.6 ± 2.9</td>
<td>34.6 ± 1.4</td>
<td>42.9 ± 14.0</td>
</tr>
<tr>
<td>AT III (%)</td>
<td>129.8 ± 10.9</td>
<td>109.3 ± 6.3</td>
<td>117.3 ± 8.4</td>
<td>126.3 ± 25.4</td>
</tr>
<tr>
<td>FIB (mg/dl)</td>
<td>451.7 ± 137.7</td>
<td>297.0 ± 49.5*</td>
<td>277.0 ± 71.4*</td>
<td>311.0 ± 128.4</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6 male rabbits.

*p < 0.05: Significantly different from the control group mean (Dunnett’s test).

Table 3. Effects of administration on coagulation parameters on Day 14 of administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (sec)</td>
<td>7.2 ± 0.2</td>
<td>7.7 ± 0.2*</td>
<td>7.2 ± 0.1</td>
<td>8.0 ± 0.9</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>21.4 ± 1.3</td>
<td>25.3 ± 1.8*</td>
<td>26.7 ± 2.3**</td>
<td>27.2 ± 3.0**</td>
</tr>
<tr>
<td>TBT (sec)</td>
<td>34.0 ± 1.0</td>
<td>34.4 ± 3.2</td>
<td>36.4 ± 1.4*</td>
<td>46.2 ± 18.6</td>
</tr>
<tr>
<td>AT III (%)</td>
<td>129.2 ± 7.1</td>
<td>126.0 ± 16.6</td>
<td>134.3 ± 15.3</td>
<td>175.0 ± 32.1**</td>
</tr>
<tr>
<td>FIB (mg/dl)</td>
<td>407.0 ± 47.6</td>
<td>317.2 ± 89.6</td>
<td>290.8 ± 55.4*</td>
<td>659.5 ± 368.3</td>
</tr>
<tr>
<td>Factor VII (sec)</td>
<td>15.7 ± 0.5</td>
<td>15.8 ± 0.7</td>
<td>16.1 ± 0.7</td>
<td>16.2 ± 1.4</td>
</tr>
<tr>
<td>Factor IX (sec)</td>
<td>37.3 ± 1.3</td>
<td>39.4 ± 2.6</td>
<td>39.1 ± 2.2</td>
<td>45.6 ± 11.4*</td>
</tr>
<tr>
<td>Factor X (sec)</td>
<td>13.7 ± 0.4</td>
<td>14.0 ± 0.7</td>
<td>14.0 ± 0.7</td>
<td>14.2 ± 1.5</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6 male rabbits (the mean ± S.D. of 4 male rabbits in the 50 mg/kg group).

*p < 0.05. **p < 0.01: Significantly different from the control group mean (Dunnett’s test).
Effects of phenobarbital on the blood coagulation system in rabbits

kg/day on Day 8 and at 25 mg/kg/day on Day 14. In these groups, there were no severe liver damages observed. Prolongation of PT was also noted at 12.5 mg/kg/day on Day 14, but it was not a dose-related change.

Pathological findings of the liver
Changes in liver weight are shown in Table 4. Relative liver weight increased at dose levels of 25 mg/kg/day or more (22 or 38% higher than that in the control group). Moreover, as shown in Fig. 2, ground-glass appearance of hepatocytes was observed in the central and middle parts of the hepatic lobules at dose levels of 12.5 mg/kg/day or more.

Table 4. Effects of 2-week PB administration on liver weights

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute liver weight (g)</td>
<td>105.5 ± 19.5</td>
<td>115.9 ± 18.7</td>
<td>130.8 ± 22.3</td>
<td>124.4 ± 12.2</td>
</tr>
<tr>
<td>Relative liver weight (g%)</td>
<td>2.73 ± 0.42</td>
<td>3.00 ± 0.34</td>
<td>3.34 ± 0.30*</td>
<td>3.76 ± 0.65**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6 male rabbits (the mean ± S.D. of 4 male rabbits in the 50 mg/kg group).
*p < 0.05, **p < 0.01: Significantly different from the control group mean (Dunnett’s test).

DISCUSSION

In this study, effects of PB-treatment on blood coagulation-related parameters as well as factor VII, IX and X activities were examined using rabbits. In the liver of the PB-treated groups, an increase in the weight of the liver and a ground-glass appearance of hepatocytes in the central to middle parts of hepatic lobules were observed, and these suggest the induction of drug metabolizing enzymes (Harada and Maita, 2000). In blood coagulation examination, APTT was prolonged at dose levels of 12.5 mg/kg/day or more. Additionally, TBT was prolonged at dose levels of 25 mg/kg/day or more. In this study, Throm-

Fig. 2. Photomicrographs of the liver of control (a), 12.5 mg/kg/day (b), 25 mg/kg/day (c) and 50 mg/kg/day groups (d). Ground glass appearance of hepatocytes was observed in a dose-dependent manner in the central and middle parts of hepatic lobules. HE, × 100. Bar = 20 μm.
botest® was employed as it specifically measures the activities of vitamin K-dependent coagulation factors II, VII, IX and X. It is a comprehensive indicator for the activity of factors II, VII, IX and X including an effect of proteins induced by vitamin K absence or antagonism-II (PIVKA-II), which inhibits coagulation factors, and is used as a monitoring assay for the diagnosis of vitamin K deficiency. Similar changes were observed in rats in our previous study (Mochizuki et al., 2008). Bouwman et al. (1999) reported that administration of HxCB, to germfree WAG/Rij rats which were kept on a vitamin K-deficient casein diet resulted in CYP2B induction, increase of vitamin K 2,3-epoxide reductase activity and decrease of factor VII. Furthermore, Wilson and Park (1984) reported that metabolism of vitamin K was accelerated by PB administration in rabbits as well as in rats. Therefore, APTT prolongation observed in rabbits was considered to be caused by decreases in vitamin K-dependent coagulation factors through CYP2B-induction, increased vitamin K turnover and accelerated vitamin K metabolism similar to the situation in rats.

However, in measurement of activities of vitamin K-dependent coagulation factors VII, IX and X, factor IX activity decreased at 50 mg/kg/day, whereas no changes were detected in factor VII or X activity and no prolongation of PT was observed. Therefore, the prolongation of blood coagulation time by PB administration in rabbits was considered to be due to PB’s effect on the endogenous pathway alone.

In addition, an increase in ATIII concentration was noted at the dose level of 50 mg/kg/day on Day 14. ATIII gradually forms an irreversible complex with thrombin and makes it inactive, and then inhibits factors such as IXa, Xa, XIa and XIIa, which accelerate the reaction by binding with heparin (William, 2005). In this study, however, although prolongation of APTT was observed, there were no changes in ATIII concentration at dose levels of 12.5 and 25 mg/kg/day. In addition, a decrease in factor IX activity was observed at the dose level of 50 mg/ kg/day, but there was no difference in factor X activity. These findings suggest that the contribution of ATIII to the PB-induced prolongation of coagulation time is small in rabbits.

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REFERENCES


