Low Dietary Protein Impairs Blood Coagulation in BHE/cdb Rats\textsuperscript{1,2}

Yow-Ling Chang, Hee-Sook Sohn,\textsuperscript{*} Kung-Chi Chan,\textsuperscript{†} Carolyn D. Berdanier and James L. Hargrove\textsuperscript{3}

The Department of Foods and Nutrition, The University of Georgia, Athens, GA 30602-3622, \*Department of Food Science and Human Nutrition, Chonbuk National University, Chonju 561-756, Korea and \textsuperscript{7}Department of Foods and Nutrition, Providence University, Shalu, Taichung, Taiwan, ROC

ABSTRACT The influence of dietary protein on blood coagulation tests was evaluated in BHE/cdb rats. Three experiments were conducted in order to compare effects of diets with low (8 g/100 g diet) or high (38 g/100 g diet) protein, to establish values for coagulation tests at intermediate (12–30 g/100 g diet) concentrations of dietary protein, and to compare feeding identical quantities of diets with 8 g protein/100 g diet vs. 18 g protein/100 g diet. After 4 wk of feeding the semipurified diets, bleeding time exceeded 15 min in the groups fed low protein diets, compared to a range of 3–6 min for the groups fed high protein diets. Several in vitro tests of coagulation were abnormal in the rats fed low protein diets. For example, prothrombin time averaged 27 ± 8 s in rats fed 8 g protein/100 g diet plus beef tallow, but 17 ± 1 s in rats fed 38 g protein/100 g diet plus tallow. The coagulation deficit in rats fed low protein was not affected by fat source (tallow vs. menhaden oil), but fibrinogen was elevated in rats fed diets with menhaden oil. Conversely, no differences in coagulation tests were observed among rats fed 12–30 g protein/100 g diet. Bleeding times ranged from 7 to 9 min, and prothrombin time was 17–18 s. Significant differences in plasma fibrinogen concentration and prothrombin time were observed in rats fed 8 vs. 18 g protein/100 g diet at a fixed rate of 6 g/100 g body weight. Platelet and blood cell numbers were unaffected by dietary protein. The evidence for multiple deficits in the coagulation system suggests that hepatic function in BHE/cdb rats may become impaired when the rats are fed low protein diets of the composition used here. J. Nutr. 127: 1279–1283, 1997.

KEY WORDS: • dietary proteins • blood coagulation • fibrinogen • prothrombin time • rats

Blood coagulation is controlled by a balance between procoagulant and anticoagulant systems in the blood plasma, the platelets, the vascular wall, and tissue factors that are exposed by injury (Eastham and Slade 1992, Halkier 1991). Several nutrients affect coagulation, including vitamin K, which is needed by the liver for synthesis of prothrombin and other coagulation factors that are carboxylated post-translationally (Suttie 1991). In addition, the substitution of long chain, (n-3) polyunsaturated fatty acids for arachidonic acid in platelet membrane phospholipids can contribute to an abnormally prolonged bleeding time due to altered platelet function (Beswick et al. 1991, Chin 1994, Dyerberg et al. 1978). An increase in total dietary fat may affect coagulation by activating factor VII zymogen in the blood plasma (Miller et al. 1986, Miller 1992).

The effect of protein nutrition on coagulation has not been studied extensively. However, during a study of interactions between dietary protein and fat type on kidney function, Chan (1993) observed qualitatively that blood coagulated more rapidly in BHE/cdb rats fed 38 g protein/100 g diet compared to 8 protein/100 g diet. The BHE/cdb rat is a non-obese animal model of noninsulin-dependent diabetes mellitus (NIDDM)\textsuperscript{4} that develops diabetes as it ages (Berdanier 1991 and 1995) and has a defect in the mitochondrial ATPase gene (Mathews et al. 1995). Due to the design of the prior study (Chan 1993), it was not clear whether the high protein diet accelerated coagulation, or whether the low protein diet impaired coagulation. Thus, this study was conducted to compare effects on several laboratory tests of coagulation of feeding BHE/cdb rats low, moderate and high levels of dietary protein.

MATERIALS AND METHODS

Animal care and diet composition. Specific pathogen-free, male BHE/cdb rats from the colony at the University of Georgia (Berdanier 1995) were used according to procedures for care and experimental protocols approved by the UGA Institutional Animal Care and Use Committee. The rats were 2–3 months old at the beginning of the experiments, and were housed individually in wire-bottom cages in a room controlled for temperature (21 ± 1°C), humidity (40–50%),

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\textsuperscript{3} To whom correspondence and reprint requests should be addressed, e-mail: jhargrov@fcs.uga.edu

\textsuperscript{4} Abbreviations used: APTT, activated partial thromboplastin time; NIDDM, noninsulin-dependent diabetes mellitus; PT, prothrombin time.

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and light (lights on, 0600–1800 h). Diets were isocaloric, with sucrose substituted to compensate for differences in protein (casein:lactalbumin, 1:1 by weight), and fat was contributed by 1 g corn oil/100 g diet and either 9 g beef tallow or 9 g menhaden oil/100 g diet, as indicated (Table 1).

**Experiment 1.** The first experiment was designed to identify interactions between protein concentration and fat type on coagulation. For one week, 32 rats were fed a semipurified diet that contained (per 100 g diet) 8 g casein:lactalbumin, 9 g beef tallow and 1 g corn oil, and food intakes were recorded. The rats were then randomly assigned to four groups (8 rats each) and fed diets containing 8 or 38 g protein/100 g diet, with fat provided by 1 g corn oil plus either 9 g beef tallow or 9 g menhaden oil/100 g diet. Different fat sources were used solely to duplicate the diets in the preliminary study (Chan 1993). Body weights and food and water intakes were measured weekly.

**Experiment 2.** The second experiment was conducted to establish norms for coagulation as a function of dietary protein concentration. One group of 7 rats was fed a nonpurified diet (Purina Rodent Chow, Richmond, IN) and four groups of 7 rats each were fed semipurified diets containing 12–30 g protein/100 g diet, with fat provided by 1 g corn oil and 9 g beef tallow/100 g diet.

**Experiment 3.** The last experiment was designed to compare effects of coagulation tests of feeding rats diets with either 8 or 18 g protein/100 g diet. However, rats in the low protein group initially consumed about 1 g more food per 100 g body weight per day than did the rats fed 18 g protein/100 g diet, when food was freely available. To equalize food intake, both groups were fed at the rate of 6 g diet/100 g body weight/day after the third week, when tests of bleeding time were carried out as described (Tschopp and Zucker 1972). Because the bleeding test could potentially activate the coagulation system, the rats were allowed to recover for the final two weeks. After a total of 5 wk, the rats were anesthetized with ketamine:xylazine (10:1), and blood samples for coagulation tests were collected from the inferior vena cava as described previously (Chan et al. 1993).

### Blood collection and coagulation tests.

Blood was collected from the inferior vena cava of anesthetized rats into syringes containing 0.5 mL of disodium citrate (0.16 mol/L) or a ratio of 9 volumes of blood:1 volume disodium citrate. To prepare platelet-rich plasma, whole blood was centrifuged at 190 × g for 15 min at 22°C; and the supernatant was collected. The recalcification test was carried out by adding one-tenth volume of 25 mmol calcium chloride/L to fresh platelet-rich plasma, and measuring the time until the initial clot was caused the bleeding test could potentially activate the coagulation system. Thromboplastin time was determined by mixing sample plasma with 25 mmol calcium chloride/L and a partial thromboplastin reagent (Automated APTT, Organon Teknika) and timing formation of the initial clot. The factor VII assay was carried out on the COAG-A-MATE™ XM fibrometer by mixing the diluted plasma with factor VII deficient plasma (Organon Teknika Co.), and timing clot formation after thromboplastin reagent (Thromboplastin with calcium, Sigma, St. Louis, MO) was added. Coagulation controls were performed using Level I normal human plasma (Sigma) for the PT, APTT, and fibrinogen tests. The tests of prothrombin time and activated partial thromboplastin time for experiments 1 and 2 were done at the same time using platelet-poor plasma that had been stored at −70°C. The tests were conducted by one operator using the same set of reagents.

Plasma total protein concentration was determined with a kit based on the bicinchoninic acid reagent with bovine serum albumin as a protein standard (Pierce Chemical, Rockford, IL). Albumin concentration was analyzed with a bromocresol green kit (Sigma Diagnostic kits 631-2). Glucose was assayed with kit 115-A based on glucose oxidase, and urea was quantified using kit 640-A based on urease (Sigma Diagnostics).

### Statistics.

Data were analyzed with the Tukey-Kramer multiple comparisons after 2-way ANOVA using the Statistical Analysis System (SAS 1989, SAS/STAT Version 6, SAS Institute, Cary, NC) in Experiment 1, and Dunnet’s multiple comparisons test in Experiment 2 (Steel & Torrie, 1960). Because the variances for PT and APTT were not equal in Experiment 1, means were compared with the Wilcoxon signed rank test (Steel & Torrie, 1960). Means were compared using Student’s t test in Experiment 3. Variations in data is expressed as means ± SD (n = 7–10). Differences were considered significant at P ≤ 0.05.

### RESULTS

**Blood coagulation in relation to dietary protein and fat type.** Rats fed the low protein diets consumed more food than did the high protein groups [6.26 ± 0.68 vs. 5.28 ± 1.37 g/100 g body weight (±d), respectively], but weighed significantly less at autopsy (Table 2). Bleeding time was significantly longer in the low protein diet groups compared to the high protein diet groups regardless of fat source. In vitro measures of coagulation that differed in the low protein diet groups included clotting time (as tested by recalcification of platelet-
Effects of two dietary protein levels and two fat sources on body weight and the blood coagulation system in BHE/cdb rats

**Table 1**

<table>
<thead>
<tr>
<th>Diet groups</th>
<th>Body weight</th>
<th>Bleeding time</th>
<th>PRP2</th>
<th>PPP</th>
<th>PT</th>
<th>APTT</th>
<th>Fibrinogen</th>
<th>Factor VII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet groups</td>
<td>g/L</td>
<td>s</td>
<td>g/L</td>
<td>s</td>
<td>g/L</td>
<td>s</td>
<td>g/L</td>
</tr>
<tr>
<td>80 g/kg P + BT</td>
<td>328 ± 17</td>
<td>17 ± 7</td>
<td>380 g/kg</td>
<td>348 ± 29</td>
<td>6 ± 3</td>
<td>380 g/kg P</td>
<td>334 ± 12</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>380 g/kg P + BT</td>
<td>403 ± 16</td>
<td>218 ± 98</td>
<td>293 ± 17</td>
<td>27 ± 8</td>
<td>36 ± 13</td>
<td>1.64 ± 0.63</td>
<td>34 ± 2</td>
<td></td>
</tr>
<tr>
<td>380 g/kg P + MO</td>
<td>440 ± 8</td>
<td>203 ± 59</td>
<td>17 ± 1</td>
<td>21 ± 0.1</td>
<td>1.95 ± 0.38</td>
<td>24 ± 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P values:

- Protein: 30.291 0.020 0.0001 0.0001 0.005 24 0.172 0.019
- Fats: 0.668 0.984 0.841 0.350 0.715 0.0004 0.032
- Interaction: 0.874 0.793 0.199 0.733 0.356 0.511 0.514

1. Values are means ± SD, n = 7. Significant differences are defined at P < 0.05. Diet groups as defined in Table 1.
2. Abbreviations: PRP, the recalci®cation test in platelet-rich plasma; PPP, the recalci®cation test in platelet-poor plasma; PT, prothrombin time; APTT, activated partial thromboplastin time.
3. Effects of protein or fat type, respectively, or their interactions, on differences in the variables.
4. Results of ANOVA were invalid due to different variances of sample means. P values resulting from the Wilcoxon signed rank test were: PT and APTT for groups fed 80 g/kg P + BT vs. 380 g/kg P + BT, 0.028 ± 0.018, respectively; for 80 g/kg P + MO vs. 380 g/kg P + MO 0.003 and 0.018, respectively.

**Table 2**

<table>
<thead>
<tr>
<th>Diet groups</th>
<th>Week 0</th>
<th>Week 5</th>
<th>Bleeding time</th>
<th>PRP2</th>
<th>PPP</th>
<th>PT</th>
<th>APTT</th>
<th>Fibrinogen</th>
<th>Factor VII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>min</td>
<td>s</td>
<td>g/L</td>
<td>s</td>
<td>g/L</td>
<td>s</td>
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<td>17 ± 7</td>
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**Discussion**

This study supports the hypothesis that the coagulation system in the diabetes-prone BHE/cdb rat becomes impaired when the rats are fed 8 g protein/100 g diet. Conversely, the data fail to demonstrate any effect of dietary protein within the range of 12 to 38 g casein-lactalbumin/100 g diet. Experiment 2 provided normal values for several coagulation tests in BHE/cdb rats. Compared to these values, only the two groups fed 8 g protein/100 g diet in Experiment 1 differed greatly; the bleeding time, clotting time, PT, APTT, and fibrinogen concentration were significantly lower in the low protein group (95 ± 15 s, P < 0.005). The coagulation test with recalculated, platelet-rich plasma was slightly longer in samples from the low protein group (95 ± 15 s, P < 0.005). The coagulation test with recalculated, platelet-poor plasma did not differ. In addition, APTT, plasma total protein and albumin concentrations, and numbers of red blood cells, white blood cells, and platelets did not differ between groups (not shown). Plasma glucose concentration was significantly higher in the low protein group (7.3 ± 1.5 vs. 6.5 ± 0.6 mmol/L, but the difference was not significant. Plasma urea concentration was significantly lower in the rats fed low protein diet than in those fed high protein diet (1.32 ± 0.40 vs. 2.44 ± 0.52 mmol/L, respectively, P < 0.005).
be consistent with the observation that coagulation was normal at all higher concentrations of dietary protein.

The prolonged bleeding time paralleled several in vitro tests of coagulation, including: 1) abnormal coagulation after recalcification of either platelet-rich or platelet-poor blood plasma; 2) decreased fibrinogen concentration; 3) prolonged prothrombin time and activated partial thromboplastin time; and 4) a significant increase in factor VII activity. The results of the in vitro tests of coagulation suggest that the primary defect in coagulation persisted in platelet-poor plasma, and was attributable to altered activity of the soluble coagulation system. Affected factors included fibrinogen, prothrombin, thromboplastin and factor VII. Thus, the low protein diet produced a general coagulopathy, rather than specifically altering one component of this system. Although fish oils reduce the rate of platelet aggregation and prolong bleeding time in humans (Chi 1994) and rats (Song and Wunder 1991), the low protein group had prolonged bleeding time even when fed tallow as a fat source. It was surprising that no significant difference in bleeding time was noted between rats fed diets with beef tallow as compared to menhaden oil, but the tail bleeding test is not a sensitive indicator of platelet function.

The content of high-quality protein that will maintain health in a normal, mature rat is approximately 6 g/100 g diet when other constituents are present at recommended levels (NRC 1978). The protein concentration of 8 g/100 g diet used in this study was substantially higher than the 0–5 g/100 g diet protein concentrations used in attempts to produce experimental protein deficiency in rodents (Anthony and Edozien 1975, Lago et al. 1993). At protein concentrations below 5 g/100 g diet, food intake is reduced and no growth is observed, in contrast to the increased food intake and moderate growth noted here in rats fed protein at 8 g/100 g diet. In addition, plasma albumin and plasma total protein were normal in these rats, and while weight gain was modest, there were no signs of frank protein deficiency. Thus it appears that BHE/cdb rats develop hepatic dysfunction as indicated by reduced concentrations of plasma coagulation factors when fed diets containing 8 g protein/100 g diet, with other constituents as described.

The BHE/cdb strain is a non-obese model of NIDDM that develops abnormal glucose tolerance and lipemia at approximately 300 d of age (Berdanier 1995). This disorder can be elicited at younger ages by feeding diets with high sucrose concentrations (65 g/100 g), which increase synthesis of fatty acids and liver triglyceride (Berdanier 1991). The livers of BHE/cdb rats develop fatty infiltration under the influence of high carbohydrate diets (Marshall et al. 1969), consistent with altered mitochondrial metabolism (Berdanier 1995). Noninsulin-dependent diabetes mellitus alters liver function and blood coagulation in human patients (Donders et al. 1993), and the data of Experiment 3 suggest that the rats used here were beginning to develop poor glucose tolerance (plasma glucose concentration, 7.3 ± 1.5 vs. 6.5 ± 0.6 mmol/L in the 8 and 18 g protein/100 g diet groups, respectively; P = 0.11). It should be noted that a coagulation deficit is an important general sign of liver dysfunction (Munoz et al. 1988). Consistent with this idea, a subsequent study showed that BHE/cdb rats fed diets containing 6 g protein/100 g diet supplemented with 0.25 g choline bitartrate/100 g diet develop midzonal necrosis (J. L. Hargrove, unpublished observation).

The finding that the low protein diet produced a coagulation deficit is similar to reports that the soluble coagulation system is usually normal in human subjects with marasmic protein-energy malnutrition, but abnormal in kwashiorkor (Hassanein and Tankovsky 1973, Kalala et al. 1983). Bleeding disorders are not a constant feature of protein-energy malnutrition (Keys et al. 1950, Torun and Chew 1994, Viteri et al. 1968), and would not be expected unless the liver or platelets were affected. Two signs of impaired liver function in human patients with protein-energy malnutrition are fatty infiltration and edema (Flores et al. 1974, Truswell et al. 1969). These symptoms suggested to Jackson (1990) that kwashiorkor represents a failure of the liver to adapt to malnutrition and other environmental stressors, whereas marasmus represents a condition of better adaptation. The data presented here show that an imbalanced diet with low protein and high carbohydrate also impairs liver function in the BHE/cdb rat in the absence of environmental stressors such as enteritis. Because the concentration of albumin and other plasma proteins declines in protein deficiency (Waterlow 1992, Young and Marchini 1990), it is reasonable to expect that concentrations of coagulation factors may be depressed as well. Similarly, Meghelli-Bouchenak et al. (1987) have shown that low protein diets alter lipoprotein secretion, a feature that coincides with or precedes steatosis. Nutritional liver injury is produced when rats are fed diets that contain elevated sucrose (Bacon et al. 1984), imbalanced amino acid profiles (Newberne et al. 1969), or insufficient methyl donors (Zaki et al. 1963). Given this background, it seems likely that the coagulation deficits noted here represent one symptom of a general loss of liver function, rather than a specific effect of protein deficiency. If this conclusion is correct, other markers of hepatic injury should be affected in addition to coagulation. We are now testing the hypothesis that low protein intake promotes

**TABLE 3**

Effects of dietary protein on the blood coagulation system in BHE/cdb rats

<table>
<thead>
<tr>
<th>Dietary protein1</th>
<th>Bleeding time2</th>
<th>PRP3</th>
<th>PPP</th>
<th>PT</th>
<th>APTT</th>
<th>Fibrinogen concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g</td>
<td>min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>g/L</td>
</tr>
<tr>
<td>12</td>
<td>8 ± 3</td>
<td>100 ± 17</td>
<td>172 ± 40</td>
<td>18 ± 1.0</td>
<td>21 ± 0.3</td>
<td>2.02 ± 0.32</td>
</tr>
<tr>
<td>18</td>
<td>8 ± 4</td>
<td>91 ± 18</td>
<td>151 ± 65</td>
<td>17 ± 0.7</td>
<td>21 ± 0.2</td>
<td>1.99 ± 0.36</td>
</tr>
<tr>
<td>24</td>
<td>7 ± 3</td>
<td>80 ± 21</td>
<td>177 ± 65</td>
<td>17 ± 0.7</td>
<td>21 ± 0.2</td>
<td>2.40 ± 0.47</td>
</tr>
<tr>
<td>30</td>
<td>9 ± 4</td>
<td>102 ± 25</td>
<td>167 ± 42</td>
<td>18 ± 0.8</td>
<td>21 ± 0.1</td>
<td>2.34 ± 0.36</td>
</tr>
</tbody>
</table>

1 Each diet contained the amount of casein-lactalbumin shown in column 1, with fat provided by 9 g beef tallow and 1 g corn oil/100 g diet.
2 Values are means ± sd, n = 7.
3 Abbreviations: PRP, the recalcification test in platelet-rich plasma; PPP, the recalcification test in platelet-poor plasma; PT, prothrombin time; APTT, activated partial thromboplastin time.
DIETARY PROTEIN AND BLOOD COAGULATION

liver dysfunction in metabolically normal rats without necessarily producing steatosis.

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LITERATURE CITED


