Accumulation of Liver Lipids Induced by Vitamin B₆ Deficiency Was Effectively Ameliorated by Choline and, to a Lesser Extent, Betaine

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Summary Despite previous studies suggesting that choline and betaine ameliorate lipid accumulation in rat livers, the relative effectiveness of the two nutrients is unclear. We examined the efficacy of dietary supplementation with choline or betaine in ameliorating lipid accumulation induced by vitamin B₆ (B₆) deficiency in the rat liver. Male Wistar rats were fed control, B₆-deficient, choline-supplemented B₆-deficient, betaine-supplemented B₆-deficient, or both choline and betaine-supplemented B₆-deficient diets (all containing 9 g of l-methionine (Met)/kg) for 35 d. Two experiments were performed, i.e., one using 17 mmol/kg diet choline bitartrate, betaine anhydrous, and the combination and another using 8.5 mmol/kg diet. Rats fed a B₆-deficient diet developed lipid accumulation in the liver with a reduction of plasma lipids induced by the disruption of Met metabolism. However, the addition of 17 mmol/kg diet choline or betaine was sufficient to ameliorate the disruptions of lipid and Met metabolism. Additionally, 8.5 mmol/kg diet choline ameliorated liver lipid deposition, while the same amount of betaine had no significant effects on liver or plasma lipid profiles. Supplementation with choline resulted in a higher liver betaine than that found using the same amount of betaine alone, although the overall liver betaine content was reduced in B₆-deficient rats. Our findings indicate that choline is more effective than betaine in ameliorating B₆ deficiency-related disruptions in Met metabolism and liver lipid accumulation by increasing liver betaine levels.

Key Words vitamin B₆ deficiency, liver lipids, choline, betaine, methionine metabolism

Vitamin B₆ (B₆) is a cofactor in methionine (Met) metabolism. In previous studies, rats fed a B₆-deficient diet supplemented with l-Met (0.9%) developed lipid accumulation triggered by homocysteine (Hcy) accumulation in the liver (1–3). Hcy is a risk factor for cardiovascular disease (4, 5). In the transsulfuration pathway, Hcy is metabolized to cystathionine by cystathionine β-synthase (EC 4.2.1.22; CBS), and cystathionine is further metabolized to cysteine by cystathionine γ-lyase (EC 4.4.1.1; CGL), which requires pyridoxal 5′-phosphate (PLP) as a coenzyme (Fig. 1). Under B₆ deficiency, the activity levels of CBS and CGL are reduced due to decreased PLP levels (6), thus impairing Hcy metabolism (7). As a result, Hcy accumulates in the liver, leading to increased plasma Hcy levels. Moreover, Hcy accumulation induces an increase in liver S-adenosylhomocysteine (SAH) levels because the equilibrium between Hcy and SAH is shifted toward SAH (8). Accumulated SAH increases phosphatidylethanolamine (PE) levels via the inhibition of phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17; PEMT) (9). SAH and PE accumulate in the liver under B₆-deficient conditions (6, 10, 11). Phosphatidylcholine (PC) is an essential structural component of very low-density lipoprotein (VLDL) (12, 13). Additionally, PC synthesis from PE is important for hepatic synthesis and the secretion of VLDL (12–15). VLDL exports lipids, such as triglycerides (TG), from the liver by converting to very low-density lipoprotein (VLDL), which is exported to the arterial circulation.
Liver Lipid Accumulation from Vitamin B6 Deficiency

We previously revealed that PC, choline, and betaine ameliorate liver lipid accumulation in rats fed a 0.9% Met-supplemented B6-deficient diet (2, 3). Choline and betaine are anti-obesity factors (16–19) linked to Met metabolism, and betaine is produced by choline oxidation (20). In a previous study, the effective levels of choline and betaine were 4 g/kg diet (15.8 mmol/kg) and 4 g/kg diet (25.6 mmol/kg), respectively, indicating that choline seemed to be more effective than betaine (3). However, owing to the variation in experimental durations and conditions among studies, comparisons of the effects of choline and betaine supplementation are impossible. Therefore, the effects of supplementation with these compounds must be simultaneously examined in an experiment to clarify the effectiveness of choline as compared with betaine. This will also illuminate the mechanism involved in the amelioration of liver lipid accumulation by choline and betaine. Therefore, in this study, we examined the effects of choline and betaine supplementation on liver lipid accumulation in rats fed a B6-deficient L-Met-supplemented diet within the same experiment. The experimental design allows the determination of the efficacy of both compounds in ameliorating liver lipid accumulation and the mechanisms underlying their effects.

Materials and Methods

Animals. Four-week-old male Wistar rats weighing 80–100 g were obtained from Japan SLC, Inc. (Hammatsu, Japan). The rats were housed individually in stainless steel wire-bottomed cages in an animal room at 23±1˚C under an inverted 12 h light-dark cycle (lights on at 06:00). Prior to each feeding experiment, the rats were acclimatized to an AIN-76 diet for 5 d. Animal experiments were approved by the Committee for Animal Research and Welfare of Gifu University on the Proper Use of Laboratory Animals (Approval numbers 15037 for Exp. 1 and 15079 for Exp. 2).

Diets and experimental procedures. In experiment 1 (Exp. 1), the rats were randomly divided into five groups (n=7 each) and fed one of the following experimental diets in addition to 9 g L-Met/kg: AIN-76-based control diet (C), B6-deficient diet (D), and B6-deficient diets supplemented with betaine (2 g of betaine anhydrous [MW 117.15]/kg diet, equivalent to 17.1 mmol/kg diet; B2D), choline (4.3 g of choline bitartrate [MW 253.25]/kg diet, equivalent to 17.1 mmol/kg diet; Ch4D), or both betaine and choline (2 g of betaine anhydrous and 4.3 g of choline bitartrate/kg diet; B2Ch4D).

In experiment 2 (Exp. 2), the rats were randomly divided into five groups (n=7 each) and fed one of the following experimental diets in addition to 9 g L-Met/kg: AIN-76-based control diet (C), B6-deficient diet (D), and B6-deficient diets supplemented with betaine (1 g of betaine anhydrous/kg diet, equivalent to 8.54 mmol/kg diet; B1D), choline (2.15 g of choline bitartrate/kg diet, equivalent to 34.0 mmol/kg diet; Ch2.15D), or both betaine and choline (1 g of betaine anhydrous/kg diet, equivalent to 8.54 mmol/kg diet; B1Ch4D).
equivalent to 8.49 mmol/kg diet; Ch2D), or both betaine and choline (1 g of betaine anhydrous and 2.15 g of choline bitartrate/kg diet; B1Ch2D).

The compositions of the experimental diets are shown in Table 1. The D group had free access to the B6-deficient diet, and the other groups were pair-fed. After 35 d of feeding, the rats were anesthetized by intraperitoneal injection of pentobarbital (Somnopentyl; Kyoritsu Seiyaku Co., Tokyo, Japan).

In both experiments, blood was collected via the abdominal aorta using a heparinized syringe. Livers were excised immediately, rinsed in ice-cold 0.25 m sucrose, blotted on paper towels, and frozen immediately in liquid nitrogen. Plasma was obtained from heparinized whole blood by centrifugation at 2,000 g for 15 min at 4˚C. The plasma and liver were stored at −20˚C until analyses.

Materials. Vitamin-free casein, gelatinized cornstarch, cellulose powder, the AIN-76 vitamin mixture and the AIN-76 mineral mixture were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Choline bitartrate and L-Met were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Retinol acetate was purchased from Sigma-Aldrich Japan (Tokyo, Japan). S-Adenosylmethionine (SAM), SAH, L-a-phosphatidylcholine from egg yolk (type XI-E), and L-a-phosphatidyl ethanolamine from egg yolk (type III) were purchased from Sigma-Aldrich (St. Louis, MO). N-Acetyl-L-cysteine, 4-fluoro-7-sulfobenzofurazan ammonium salt, and 1-heptanesulfonic acid sodium salt were purchased from Wako Pure Chemical Industries. Methanol and acetonitrile (HPLC grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Betaine anhydrous was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), unless otherwise indicated.

Measurement of lipid concentrations in the plasma and liver. Total lipids were extracted from the liver following the methods of Folch et al. Concentrations of total lipids and total cholesterol (TC) in the extract were measured by a conventional gravimetric method, i.e., the Zak-Henly method, and concentrations of TG were determined by the Triglyceride G-Test (Wako Pure Chemical Industries).

The concentrations of plasma TC, high-density lipoprotein cholesterol (HDL-C), TG, and phospholipids (PL) were determined by enzymatic methods using the Cholesterol E-Test, HDL-cholesterol E-Test, Triglyceride G-Test, and Phospholipid C-Test (Wako Pure Chemical Industries), respectively. Differences between TC and HDL-C concentrations are expressed as the VLDL-Low-density lipoprotein (VLDL-LDL)-cholesterol ((VLDL-LDL)-C) concentration. The concentration of apolipoprotein B100 (apoB100) was determined by an enzyme-linked immunosorbent assay (ELISA) using an ELISA kit for apoB100 (Usen Life Science Inc., Wuhan, China).

Measurement of PC and PE in the liver microsomal fraction. The concentrations of PC and PE in the liver microsomal fraction were measured by isocratic high-performance liquid chromatography (HPLC) with ultraviolet detection (210 nm). The liver was homogenized in four volumes (v/w) of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. An aliquot (12 mL) of the resulting homogenate was centrifuged at 10,000 ×g for 12 min at 4˚C, and the resulting supernatant was further centrifuged at 105,000 ×g for

| Table 1. Compositions of the experimental diets (g/kg diet) (Exps. 1 and 2). |
| Group | Exps. 1 and 2 | Exps. 1 | Exps. 2 |
| Ingredients | C1 | D2 | B2D3 | Ch4D4 | B2Ch4D5 | B1D6 | Ch2D7 | B1Ch2D8 |
| Vitamin-free casein | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| L-Methionine⁹ | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Gelatinized cornstarch | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 |
| Sucrose | 494 | 494 | 492 | 490 | 488 | 493 | 492 | 491 |
| Cellulose powder | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Soybean oil | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| AIN-76 mineral mixture | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 |
| AIN-76 vitamin mixture | 10 | — | — | — | — | — | — | — |
| Vitamin B₆-free vitamin mixture¹⁰ | — | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline bitartrate | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Supplemental choline bitartrate | — | — | 4.3 | 4.3 | — | 2.15 | 2.15 | 2.15 |
| Supplemental betaine anhydrous | — | — | 2 | 2 | 2 | 1 | 1 | 1 |

¹ C: Control, 2 D: B₆-deficient diet, 3 B2D: 2 g (17.1 mmol) betaine anhydrous/kg diet, 4 Ch4D: 4.3 g (17.0 mmol) choline bitartrate/kg diet, 5 B2Ch4D: 2 g (17.1 mmol) betaine anhydrous and 4.3 g (17.0 mmol) choline bitartrate/kg diet, 6 B1D: 1 g (8.54 mmol) betaine anhydrous/kg diet, 7 Ch2D: 2.15 g (8.49 mmol) choline bitartrate/kg diet, 8 B1Ch2D: 1 g (8.54 mmol) betaine anhydrous and 2.15 g (8.49 mmol) choline bitartrate/kg diet.
² L-Methionine content was three-fold higher than that in the AIN-76 diet.
³ Pydoxine hydrochloride was omitted from the AIN-76 vitamin mixture.
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The method of She HPLC with ultraviolet detection (254 nm) according to the resulting supernatant was used for the Hcy analysis. The obtained microsomes were resuspended in homogenization buffer and stored at −80°C until PC and PE analyses.

The analytical conditions were as follows: column, TSKgel Silica-60 (4.6 mm I.D.×25 cm); mobile phase, acetonitrile/methanol/85% phosphoric acid (180/4/3); flow rate, 1.0 mL/min; standards, l-α-phosphatidylcholine from egg yolk (type XI-E) and l-α-phosphatidylethanolamine; PL, phospholipid; TC, total cholesterol; TG, triglycerides; (VLDL+LDL)-C, (very low-density lipoprotein+low-density lipoprotein)-cholesterol.

Measurement of methionine metabolites in the plasma and liver. Plasma and liver total Hcy levels were measured by an isocratic HPLC system equipped with a fluorescence detector (excitation 380 nm, emission 525 nm) according to the methods of Yamaguchi et al. (24). Each liver was homogenized in three volumes (v/w) of 10 mm potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 ×g for 15 min at 4°C, and the resulting supernatant was used for the Hcy analysis.

Liver SAM and SAH levels were measured by an isocratic HPLC with ultraviolet detection (254 nm) according to the method of She et al. (25). Each liver sample was homogenized in four volumes (v/w) of 0.4 m phosphoric acid. The homogenate was centrifuged at 10,000 ×g for 20 min at 4°C, and the resulting supernatant was used for SAM and SAH analyses.

Liver betaine contents were measured by isocratic HPLC with ultraviolet detection (254 nm) according to the method of Laryea et al. (26). Each liver sample was prepared by the method described for the liver Hcy analysis.

Measurement of vitamin B6 in the plasma and liver. PLP levels in the liver and plasma were determined by isocratic HPLC with fluorescence detection (excitation 320 nm, emission 420 nm) according to the method of Tsuge (27).

Statistical analysis. Data are expressed as means ± standard error (SE). Statistical analyses were performed using Excel 2012 for Windows (Social Survey Research Information, Tokyo, Japan). Differences in means among groups were considered significant at p<0.05, according to one-way ANOVA followed by the Tukey-Kramer test or Kruskal-Wallis analysis followed by the Steel-Dwass multiple comparison test. ApoB100, apolipoprotein B100; HDL-C, high-density lipoprotein-cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; TC, total cholesterol; TG, triglycerides; (VLDL+LDL)-C, (very low-density lipoprotein+low-density lipoprotein)-cholesterol.

Results and Discussion. The final body weight and total food intake did not differ among the five experimental groups in Exps. 1 and 2 (Tables 2 and 3). The liver weight and total lipid, TC, and TG levels in the liver were significantly higher in the D group than in the C group in Exps. 1 and 2. Plasma TC, (VLDL+LDL)-C, and PL concentrations were lower in the D group than in the C group (Table 2), and plasma HDL-C and TG concentrations did not differ significantly among groups. The secretion of TG-rich VLDL from the liver was impaired, and lipids accumulated in the liver because of B6 deficiency.

Table 2. Growth parameters and liver and plasma lipid concentrations in rats fed the experimental diets (Exp. 1).

|            | C        | D        | B2D      | Ch4D     | B2Ch4D   |
|------------|----------|----------|----------|----------|----------|
| Initial body weight (g) | 124±3    | 123±3    | 123±3    | 122±3    | 123±3    |
| Final body weight (g)   | 181±3    | 175±4    | 178±5    | 175±6    | 173±5    |
| Body weight gain (g/35 d) | 57.6±3.2 | 51.2±1.8 | 55.0±3.1 | 52.6±3.5 | 50.8±3.1 |
| Total food intake (g/35 d) | 310±5    | 308±5    | 310±5    | 310±5    | 310±5    |
| Liver weight (g/100 g BW) | 2.79±0.12b | 3.50±0.25a | 2.96±0.14ab | 2.81±0.10b | 2.89±0.09ab |
| Liver Total lipids (mg/g) | 45.0±2.3b | 110±16a  | 42.1±2.1b | 41.4±1.3b | 45.9±2.7b |
| TG (mg/g)   | 4.50±0.70b | 25.8±4.3a | 6.08±1.23b | 4.80±0.61b | 4.27±0.85b |
| PC (mg/g)   | 6.76±0.53ab | 21.1±3.5a | 6.25±0.34b | 6.53±0.39b | 7.64±0.81b |
| PE (μg/mg microsomal protein) | 248±8b | 177±7a | 198±14b | 202±14b | 213±9b |
| PC/PE       | 7.84±3.5a | 82.3±6.1a | 71.4±7.1a | 67.2±3.9a | 67.6±2.7a |
| Plasma TC (mg/dL)        | 47.8±1.9a | 34.7±2.3b | 49.2±4.0a | 46.9±2.3a | 54.3±2.6a |
| HDL-C (mg/dL)           | 20.9±1.1a | 18.7±1.0a | 19.6±0.8a | 18.4±0.8a | 20.4±1.4a |
| (VLDL+LDL)-C (mg/dL)    | 26.9±1.6a | 16.0±1.9b | 29.6±3.6a | 28.5±1.8a | 34.0±1.6a |
| TG (mg/dL)             | 28.1±4.8a | 20.1±2.3a | 29.4±6.1a | 29.9±4.8a | 30.1±7.5a |
| PL (μg/dL)             | 104±3.4a | 76.9±4.5b | 104±4a | 105±4a | 111±6a |
| ApoB100 (μg/dL)        | 489±70a | 540±79a | 587±83a | 574±78a | 504±64a |

Values are presented as means ± SE (n=7).

Means not sharing the same superscript within a row are significantly different at p<0.05, as determined by one-way ANOVA followed by the Tukey-Kramer test or Kruskal-Wallis analysis followed by the Steel-Dwass multiple comparison test. Differences in means among groups are considered significant at p<0.05, according to one-way ANOVA followed by the Tukey-Kramer test or Kruskal-Wallis analysis and the post-hoc Steel-Dwass multiple comparison tests. The correlations between liver total lipid content and liver Hcy content were examined using Pearson correlation coefficients and considered significant at p<0.05.
With respect to Met metabolism, the concentrations of Hcy in the liver and plasma and SAH in the liver were higher, and the liver SAM/SAH ratio, an index of methylation, was significantly lower in the D group than in the C group in Exps. 1 and 2 (Tables 4 and 5). These abnormalities in Met metabolism under B6-deficiency are consistent with previous results (1, 6, 8, 10, 28, 29). The level of betaine in the liver was significantly lower in the D group than in the C group in Exp. 2 (Table 5). In another Hcy metabolic pathway, the re-methylation pathway, betaine is an important substrate of betaine-homocysteine methyltransferase (EC 2.1.1.5; BHMT) and an alternative methyl donor contributing to re-methylation (Fig. 1). Betaine may be utilized for the catabolism of accumulated Hcy in B6 deficiency (32, 33). We found that the liver microsomal PC level was lower, the PE level was higher, and the PC/PE ratio was lower, the PE level was higher, and the PC/PE ratio was lower. The inhibition of PEMT (3). Accumulated SAH decreases PC and increases PE by lowering PE/PH ratio. We found that the liver microsomal PC level was lower, the PE level was higher, and the PC/PE ratio was lower.

Table 3. Growth parameters and liver and plasma lipid concentrations in rats fed the experimental diets (Exp. 2).

|            | C          | D          | B1D        | Ch2D       | B1Ch2D     |
|------------|------------|------------|------------|------------|------------|
| Initial body weight (g) | 114 ± 3    | 114 ± 2    | 114 ± 2    | 114 ± 2    | 114 ± 3    |
| Final body weight (g) | 187 ± 6    | 181 ± 6    | 179 ± 5    | 178 ± 6    | 174 ± 6    |
| Body weight gain (g/35 d) | 73.2 ± 5.0 | 67.2 ± 5.2 | 65.3 ± 4.0 | 64.1 ± 4.6 | 60.6 ± 5.2 |
| Total food intake (g/35 d) | 366 ± 10   | 367 ± 10   | 358 ± 8    | 365 ± 9    | 365 ± 9    |
| Liver weight (g/100 g BW) | 3.07 ± 0.05 | 3.86 ± 0.12 | 3.54 ± 0.10 | 3.35 ± 0.12 | 3.36 ± 0.12 |

Liver

| Total lipids (mg/g) | 48.3 ± 2.4 | 82.7 ± 10.8 | 64.9 ± 4.6 | 48.9 ± 4.1 | 47.1 ± 2.1 |
| TG (mg/g) | 8.45 ± 0.81 | 21.2 ± 1.3 | 18.3 ± 1.3 | 11.2 ± 1.4 | 11.5 ± 0.91 |
| TC (mg/g) | 8.06 ± 0.36 | 13.8 ± 1.8 | 10.1 ± 1.0 | 9.18 ± 0.37 | 9.46 ± 0.47 |
| PC (μg/mg microsomal protein) | 209 ± 9 | 174 ± 12 | 186 ± 9 | 196 ± 8 | 194 ± 10 |
| PE (μg/mg microsomal protein) | 76.2 ± 1.8 | 91.3 ± 3.2 | 92.8 ± 4.6 | 87.0 ± 3.4 | 80.8 ± 4.1 |
| PC/PE | 2.76 ± 0.16 | 1.92 ± 0.14 | 2.03 ± 0.15 | 2.28 ± 0.16 | 2.44 ± 0.18 |

Plasma

| TC (mg/dL) | 68.9 ± 1.5 | 56.6 ± 4.3 | 62.2 ± 3.6 | 72.6 ± 4.3 | 77.9 ± 4.2 |
| HDL-C (mg/dL) | 21.7 ± 0.9 | 22.9 ± 1.4 | 21.7 ± 1.6 | 21.9 ± 2.2 | 24.8 ± 2.2 |
| (VLDL+LDL)-C (mg/dL) | 47.1 ± 1.2 | 33.6 ± 3.5 | 40.5 ± 2.8 | 50.7 ± 3.8 | 53.1 ± 2.4 |
| TG (mg/dL) | 28.2 ± 4.3 | 28.4 ± 4.4 | 31.5 ± 2.4 | 34.8 ± 3.8 | 41.0 ± 2.8 |
| PL (mg/dL) | 108 ± 4.9 | 89.8 ± 5.3 | 98.5 ± 4.1 | 110 ± 8.4 | 127 ± 6.6 |

Values are presented as means ±SE (n = 7).

Means not sharing the same superscript within a row are significantly different at p < 0.05, as determined by one-way ANOVA followed by the Tukey-Kramer test or Kruskal-Wallis analysis followed by the Steel-Dwass multiple comparison test.

Table 4. Liver and plasma levels of methionine metabolites and PLP in rats fed the experimental diets (Exp. 1).

|            | C          | D          | B2D        | Ch4D       | B2Ch4D     |
|------------|------------|------------|------------|------------|------------|
| Hcy (nmol/g) | 0.602 ± 0.13 | 17.8 ± 11.2 | 0.949 ± 0.25 | 0.917 ± 0.15 | 0.68 ± 0.07 |
| SAM (nmol/g) | 25.4 ± 3.9 | 54.8 ± 17.9 | 25.2 ± 3.1 | 28.7 ± 5.2 | 21.1 ± 3.6 |
| SAH (nmol/g) | 21.5 ± 3.7 | 114 ± 42 | 38.1 ± 15.2 | 27.9 ± 5.0 | 24.7 ± 6.4 |
| SAM/SAH ratio | 1.34 ± 0.27 | 0.537 ± 0.05 | 0.991 ± 0.16 | 1.11 ± 0.14 | 0.991 ± 0.13 |
| Betaine (μmol/g) | 5.93 ± 0.85 | 3.98 ± 0.25 | 5.29 ± 0.59 | 6.00 ± 0.64 | 6.47 ± 0.56 |
| PLP (nmol/g) | 14.3 ± 1.7 | 5.35 ± 0.49 | 7.28 ± 0.84 | 6.82 ± 0.84 | 6.20 ± 0.72 |

Plasma

| Hcy (μM) | 11.6 ± 2.5 | 327 ± 81 | 52.3 ± 24.7 | 26.1 ± 11.0 | 14.4 ± 3.3 |
| PLP (nm) | 152 ± 13 | 12.5 ± 1.5 | 9.24 ± 0.70 | 7.14 ± 0.91 | 7.00 ± 0.73 |

Values are presented as means ±SE (n = 7).

Means not sharing the same superscript within a row are significantly different at p < 0.05, as determined by one-way ANOVA followed by the Tukey-Kramer test or Kruskal-Wallis analysis followed by the Steel-Dwass multiple comparison test.

Hcy: homocysteine; PLP: pyridoxal 5’-phosphate; SAH; S-adenosylhomocysteine; SAM: S-adenosylmethionine.
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Furthermore, the plasma concentration of apoB100 did not differ among the five experimental groups (Table 2). These results suggest that the secretion of apoB100 itself was not impaired; rather, the incorporation of lipid components into VLDL was potentially impaired via decreased PC biosynthesis from PE, resulting in the accumulation of liver lipids in the D group. However, further investigations are needed to clarify the factors involved in VLDL secretion.

In the B2D, Ch4D, B2Ch4D, and B1Ch2D groups, B6-deficiency-induced lipid levels were lower than those in the D group (Tables 2 and 3). Supplementation with at least 17 mmol/kg diet choline and/or betaine ameliorated the liver lipid accumulation induced by B6 deficiency. Liver TG levels were lower in the Ch2D group than in the D group in Exp. 2; however, liver lipid parameters were not recovered in the B1D group compared to those in the Ch2D group. For 8.5 mmol/kg diet choline, more effective than betaine in ameliorating liver lipid accumulation induced by vitamin B6 deficiency. Reductions of plasma lipid concentrations were recovered by 17 mmol/kg diet choline in the B1D group than in the D group. Further, these reductions were not attenuated in the B1Ch2D group (Table 3). Therefore, choline was more effective than betaine in ameliorating plasma lipids reduced by B6 deficiency.

With respect to Met metabolism, the B6-deficiency-induced increase in Hcy levels was lower in the B2D, Ch4D, B2Ch4D, and B1Ch2D groups than in the D group (Tables 4 and 5). Supplementation with at least 17 mmol/kg diet choline and/or betaine ameliorated Hcy levels. Since liver Hcy contents were positively correlated with liver total lipid content in Exps. 1 and 2 with Pearson correlation coefficients of 0.701 and 0.377, respectively. Hcy metabolism played an important role in the deposition of total lipids in the liver. These results suggest that betaine, which is derived not only from the diet but also from the oxidation of choline, promoted the resynthesis of Met and reduced Hcy levels (30, 31). In the B1D and Ch2D groups, we found that supplementation with 8.5 mmol/kg diet betaine or choline tended to reduce liver Hcy and SAH levels in Exp. 2, although the effect was not statistically significant. Further, the liver SAM/SAH ratio was significantly higher in the Ch4D group than in the D group in Exp. 1. Based on these findings, the improvement of disturbed Met metabolism requires at least 17 mmol/kg diet betaine or choline supplementation. The level of betaine in the liver was significantly higher in the Ch2D and B1Ch2D groups than in the D group in Exp. 2 (Table 5). Surprisingly, choline supplementation increased liver betaine levels more than betaine supplementation at 8.5 mmol/kg diet (Exp. 2). The PC/PE ratio was significantly higher in the B2D, Ch4D, and B2Ch4D groups than in the D group (Table 2). Betaine and/or choline supplementation at 17 mmol/kg diet ameliorated the B6-deficiency-induced accumulation of SAH and improved the PC/PE ratio by recovering PC synthesis from PE, which is suppressed by SAH, a PEMT inhibitor (6). The recovery of PC synthesis from PE contributed to the improved secretion of VLDL from the liver. Further, PC produced from choline via the CDP-choline pathway is reported to be necessary for lipoprotein synthesis (34). Accordingly, supplemental choline may contribute to VLDL secretion by the improvement of Met metabolism via the supply of betaine as well as by PC synthesis from choline.

In this study, we examined the effects of betaine and/or choline supplementation in ameliorating B6 deficiency-induced metabolic abnormalities. With respect to the accumulation of liver lipids and reduction of plasma lipids, choline supplementation was more effective than betaine. Supplementation with 8.5 mmol/kg diet choline was effective, whereas 17 mmol/kg diet betaine was needed to demonstrate an improvement. Regarding the amelioration of abnormal Met metabolism and reduction of the microsomal PC/PE ratio, choline also appeared to be more effective than betaine. Interestingly,
liver betaine levels reduced by B<sub>6</sub> deficiency were significantly increased by choline supplementation. Based on our results, we conclude that choline supplementation is more effective than betaine in ameliorating liver fat accumulation and the disruption of Met metabolism by providing an efficient supply of betaine and PC in the livers of B<sub>6</sub>-deficient rats.

Authors’ contributions
Erina Kitagawa and Takashi Hayakawa designed the study. Erina Kitagawa drafted the manuscript, and Erina Kitagawa and Takashi Hayakawa completed the manuscript. Tomoyuki Nakagawa reviewed the manuscript. Yuki Ota and Maki Hasegawa took part in the analyses. All authors reviewed and approved the final manuscript.

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