Newly Invented Micellized Vitamin K2 Recovered Prolonged Prothrombin Time under Obstructive Jaundice in Rats with Bile Duct Ligation

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Summary In cholestatic liver diseases, coagulopathy is induced by malabsorption of vitamin K. Supplementation of vitamin K has previously been shown to prevent coagulopathy. In this study, we tested the efficacy of a newly invented micellized vitamin K2 (m-vitK2) in treating coagulopathy, using a rat bile duct ligation (BDL) model. Experiment 1: m-vitK2 (0.3 mg/kg) or m-vitK2 (0.3 mg/kg) mixed with taurocholic acid (TA) (10 mg/body) was orally administered every day for 7 d from the fourth day after BDL (n=6 for each). Experiment 2: To evaluate absorption, m-vitK2 (0.3 mg/kg) with or without TA (10 mg/body) was orally administered on the fourth day after BDL and compared with the untreated control BDL (n=2 for each). These data were compared with sham-operated (n=6) and untreated control BDL rats (n=6). The m-vitK2 recovered prothrombin time (PT) in Experiment 1 (control 42.7±5.7 s vs. m-vitK2 24.0±9.3 s, p<0.05). Experiment 2 demonstrated that the mixture of m-vitK2 and TA enhanced absorption compared to m-vitK2 alone. Moreover, in Experiment 1, m-vitK2 mixed with TA completely recovered PT (control 42.7±5.7 s vs. m-vitK2+TA 14.9±1.2 s, p<0.01). Micelle sizes decreased with the m-vitK2 and TA treatment (m-vitK2 86.3±5.6 nm vs. m-vitK2+TA 71.9±4.7 nm, p<0.05). Orally administered, newly invented m-vitK2 recovered coagulopathy even under obstructive jaundice. TA decreased the mean micelle size and improved m-vitK2 absorption.

Key Words cholestasis, obstructive jaundice, cirrhosis, vitamin K, coagulopathy

Vitamin K is a fat-soluble vitamin essential for the function of numerous proteins within the body, such as the coagulation factors (II, VII, IX, X, protein C, and protein S), osteocalcin (a bone-forming protein), and matrix-Gla protein (MGP, an anti-calcification protein) (1). In cholestatic liver diseases, including patients with alcoholic liver disease (ALD), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC), vitamin K deficiency results in coagulopathy, as measured by prolongation of the prothrombin time (PT) due to malabsorption (2, 3). In obstructive jaundice, external biliary drainage (EBD) is performed in some cases, this reduces the amount of bile and decreases absorption of vitamin K. Use of broad-spectrum antibiotics also induces a shortage of vitamin K because of disturbances to the intestinal flora, which produces vitamin K2 (4, 5). Vitamin K supplementation is recommended in liver diseases (6). Intravenous or intramuscular vitamin K administration can recover PT in some cholestatic diseases, including cirrhosis (7, 8). However, vitamin K administration is not always sufficient, and the routine use of vitamin K is controversial (8). Bile replacement also improves PT in obstructive jaundice (9). Oral administration is a physiological route, and it may be more suitable for routine supplementation. In the neonatal period, vitamin K deficiency may lead to Vitamin K Deficiency Bleeding (VKDB) (10). For preventing neonatal VKDB, solubilized vitamin K with propylene glycol and Cremophor EL had been used for supplementation in the past. However, adverse effects, such as dermatitis or anaphylactic shock reactions, have been implicated in several cases (11, 12). Therefore, a mixed micelle solution has replaced these solubilizers. Mixed micelles composed of phospholipids and bile salts were first described by Hofmann and Borgstroem (13). However, mixed micelles also have a problem. They destabilize at gastric pH resulting in low bioavailability (12). Von Kries et al. reported that mixed micellar vitamin K did not significantly improve bioavailability compared to conventional oral vitamin K (14). The modulation of the micelles has been a key to improving vitamin
K absorption. There are no previous reports that demonstrate oral administration of micellized vitamin K in liver diseases and obstructive jaundice. We found a newly developed micelle technology invented by miVital AG (St. Gallen, Switzerland). We have investigated cholestatic liver cirrhosis using bile duct ligation (BDL) in rats (15–18). Using this model, we can avoid bile acid and precisely evaluate the efficacy of new micelle technology. Our aim was to evaluate the efficacy of this new micellized vitamin K using the animal model.

MATERIALS AND METHODS

Animals. Wistar male rats (11 wk of age) approximately 250 g were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and maintained in a room at a controlled temperature of 24±2 °C with a 12-h light-dark cycle. There is no influence of sex difference on the results of the study. Animals were given a standard pellet chow containing vitamin K3, which is metabolized to vitamin K2, and water ad libitum. BDL was carried out as previously described (19). All surgery was performed under an anesthetic mixture of medetomidine, midazolam, and butorphanol, and all efforts were made to minimize suffering. As controls (n=6), sham-operated rats were also sacrificed. Sham-operated and control BDL rats were supplemented with standard amounts of vitamin K from a standard pellet chow. Blood was collected from the inferior vena cava. Serum samples were frozen and stored at −30 °C until analysis. Liver specimens were fixed in 10% buffered formalin and embedded in paraffin for histological analysis. Micellized Vitamin K: We purchased micellized vitamin K2 (menaquinone-4) (m-vitK2) from miVital AG. The new micelle type is entirely transparent and extremely stable, even at 100 °C (Fig. 1A). In this study, we selected vitamin K2 because it is commonly used as a medicine in clinical settings. The micelle was made from Gum Arabic. Although this has been commonly used as an emulsion, the form is unstable. The patented method is to stabilize the micelles by covering the melted Gum Arabic with water at the same temperature (Patent CH2007000455). According to their announcement, it enables micelle very long-term stable (>24 mo) with no critical micellization concentration (cmc) limit.

Study protocols. Rats were randomly assigned to the control and m-vitK2 group after BDL. Experiment 1: m-vitK2 (0.3 mg/kg) or m-vitK2 (0.5 mg/kg) mixed with TA (10 mg/body) adjusted to 1 mL was orally administered every day for 7 d from the fourth day after BDL (n=6 for each) (Fig. 1B). The leading time was determined according to the half-life of coagulation factor II (100 h). Experiment 2: To evaluate absorption, m-vitK2 (0.3 mg/kg) with or without taurocholic acid (TA) (10 mg/body) was orally administered on the fourth day after BDL and compared with control BDL (n=2 for each). Among the bile acid components, TA was selected with the expectation of improving vitamin K absorption according to the report by Yamanashi et al. (20). Blood samples were obtained via the inferior vena cava 2 h after administration (Fig. 1C). The dosage of m-vitK2 was calculated according to the standard human dosage (20 mg daily) divided by 60 kg. All the compounds were administered directly into the stomach by using a flexible sonde. TA was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The dosage of TA was determined according to the bile acid concentration in the upper jejunum (50 mM) (21). One-third of the bile acid consists of TA (MW 515.7 g/mol), and two-thirds consist of glycocholic acid (MW 465.6 g/mol). TA was calculated as approximately 8 mg/mL. The m-vitK2 and TA were diluted in 1 mL of saline. In Experiment 1, the data was compared with sham-operated rats (n=6) and control BDL (n=6). These rats were sacrificed at the end of the experiments, and liver and blood samples were obtained. Total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), vitamin K2, and prothrombin time (PT) were analyzed by Sanritsu zelkova, a commercial laboratory in Kanna-gawa, Japan. Vitamin K concentration was analyzed using high performance liquid chromatography (HPLC) by SRL Inc., a commercial laboratory in Tokyo, Japan.
We selected only models which achieved total bilirubin >3 mg/dL for analysis. All animal experiments were carried out in accordance with the Animal Experimentation Guidelines of Tottori University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Tottori University (20-Y-12).

**Histological analysis.** Sections (4-μm) of formalin-fixed, paraffin-embedded livers were processed according to Hematoxylin-Eosin and Masson’s Trichrome Staining. Micelle size measurement: Nanoparticle tracking analysis (NTA) of m-vitK2 (100 μg/mL), TA (500 μg/mL), and m-vitK2 (100 μg/mL) with TA

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|                  | Sham-operated (n=6) | BDL (n=6)          | BDL+m-vitK2 (n=6) | BDL+m-vitK2+TA (n=6) |
|------------------|---------------------|-------------------|------------------|-------------------|
| T-bil (mg/dL)    | 0.07±0.05           | 8.6±2.5*          | 9.8±6.1*         | 6.8±2.5*          |
| AST (IU/L)       | 67.5±7.3            | 465.2±254.3*      | 626.5±404.3*     | 385.8±112.8*      |
| ALT (IU/L)       | 47.0±9.5            | 96.2±31.0*        | 114.2±80.1*      | 84.3±24.8*        |
| GGT (IU/L)       | 1.0±0.0             | 24.3±7.9*         | 42.8±37.2*       | 62.3±36.8*        |

*p<0.01 compared with the Sham-operated. Values are the means±SD.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligation; GGT, γ-glutamyl transpeptidase, m-VitK2, micellized vitamin K2; TA, taurocholic acid; T-Bil, total bilirubin.
sections was performed in vitro with a NanoSight system (NanoSight NS300, Malvern Panalytical) (n=6 for each).

Statistical analysis. The Kruskal-Wallis test was applied for comparing three independent groups. The Mann-Whitney test was applied for post hoc multiple comparisons. All statistical tests were performed using StatFlex (Windows ver. 6.0; Artech, Osaka, Japan). Values are expressed as median (range) or mean with a standard deviation of the mean (SD). Statistical significance was set at p<0.05.

RESULTS

Obstructive jaundice by BDL was achieved successfully: the ligated common bile duct was dilatated, and the liver demonstrated cholestasis. Serum total bilirubin (sham-operated 0.07±0.05 vs. BDL 8.6±2.5 mg/dL, p<0.01), AST (sham-operated 67.5±7.3 vs. BDL 465.2±254.3 IU/L, p<0.01), ALT (sham-operated 47.0±9.5 vs. BDL 96.2±31.0 IU/L, p<0.01), and GGT (sham-operated 1.0±0.0 vs. BDL 24.3±7.9 IU/L, p<0.01) were significantly increased in BDL rats compared with sham-operated rats (Table 1). PT was significantly prolonged in BDL rats compared with sham-operated rats (sham-operated 15.4±0.9 vs. BDL 42.7±5.7 s, p<0.01). Histology also demonstrated bile duct proliferation in the liver of BDL rats, indicating cholestasis, and there was no evidence of fibrosis compared with the control rats (Figs. 2A, B, C, D).

In Experiment 1, m-vitK2 could recover PT (control 42.7±5.7 s vs. m-vitK2 24.0±9.3 s, p<0.05). The result was encouraging; however, it was not a complete recovery. On the other hand, m-vitK2 mixed with TA could completely recover PT (control 42.7±5.7 s vs. m-vitK2+TA 14.9±1.2 s, p<0.01) using an 11-d BDL model (Fig. 2E). Then, we conducted a further experiment to check absorption efficacy. In Experiment 2, the absorption of vitamin K2 was higher in TA mixture than m-vitK2 alone (m-vitK2 0.13±0.07 vs. m-vitK2+TA 0.35±0.15 ng/mL) (Fig. 2F). The measured mean
micelle size was $86.3 \pm 3.1$ nm, and 90% of particles were $128$ nm or smaller (Fig. 3A). There was no difference between m-vitK2 (86.3 $\pm$ 5.6 nm) and TA (88.9 $\pm$ 12.3 nm) in micelle size. The micelle size of m-vitK2 mixed with TA significantly decreased relative to the micelle size in the absence of TA (m-vitK2 86.3 $\pm$ 5.6 nm vs. m-vitK2+TA 71.9 $\pm$ 4.7 nm, $p=0.007$) (Figs. 3A–D). There were no differences detected in the other biochemical parameters among control and administered BDL rats. The m-vitK2 did not influence liver function tests; it only affected the recovery of PT. There was no statistical difference in ALT between sham-operated (sham) and m-vitK2 administered BDL rats. The m-vitK2 did not influence liver function tests; it only affected the recovery of PT. There was no statistical difference in ALT between sham-operated (sham) and m-vitK2 administered BDL rats because of the broad statistical dispersion (Table 1).

**DISCUSSION**

In this study, we demonstrated that orally administered m-vitK2 could recover coagulopathy even under obstructive jaundice. To our knowledge, this is the first report of PT recovery using an artificial micelle treatment. Moreover, we showed that TA decreased the micelle size and improved vitamin K absorption.

The efficacy of routine supplementation of vitamin K has been controversial for liver diseases (7, 22). There are several studies that aimed to evaluate the efficacy of vitamin K supplementation in liver diseases. In 1987, Nambu et al. demonstrated that 60 mg daily vitamin K1 administration could not improve hepaplastin test (HPT) outcomes in decompensated cirrhotic patients; however, they also reported that the combination of vitamin K1 and ursodeoxycholic acid (UDCA) could significantly improve their HPT (23). Another study also demonstrated no improvement in PT in cirrhotic patients by subcutaneous vitamin K1 administration (24).

Our data suggested that micellized vitamin K can be absorbed as water-soluble vitamins. However, additional factors likely affect vitamin K absorption. Van Hasselt et al. demonstrated that the polymeric micelles are incapable of inducing measurable absorption by themselves; however, vitamin K absorption in BDL rats was fully restored by duodenal polymeric administration of micelles together with bile acids (25). The authors speculated that bile acids outside the micelle might extract the encapsulated vitamin K and act as a shuttle to transport vitamin K to the endothelial lining. Combining their results with our data, micellization is just one aspect of the function of bile acid. Bile itself may also have a role of a shuttle for fat-soluble vitamins via transporters.

Recently, Takada et al. revealed that Niemann–Pick C1-Like 1 (NPC1L1) is a crucial regulator of intestinal vitamin K absorption (26). In vivo, a vitamin K absorption study revealed that the intestinal absorption of vitamin K1 in NPC1L1 KO mice was dramatically reduced to less than 30% of that in wild type mice (27). This study indicated that NPC1L1 may play a significant role in vitamin K absorption as well as cholesterol absorption. NPC1L1 is also known as a transporter of vitamin D and E (28). Reboul et al. indicated that vitamin D intestinal absorption was not occurring by a simple passive diffusion process only: some membrane transporters such as Scavenger Receptor class B type I (SR-BI), Cluster Determinant 36 (CD36), or NPC1L1 are involved (29). SR-BI is a receptor for high-density lipoprotein (HDL), and CD36 binds and internalizes oxidized low-density lipoprotein (OxLDL) (30, 31). These are all related to cholesterol transportation. These receptors may be recognized by cholesterol metabolites such as bile acids. Ezetimibe, the NPC1L1 inhibitor, enhances the anticoagulant effect of warfarin after co-administration (26). This indicates that the transporting mechanism protected by ezetimibe is also important to vitamin K transportation. Ezetimibe hinders the interaction of the NPC1L1/cholesterol complex with the AP2-clathrin complex (32). The crystal structure of the cysteine-rich N-terminal domain of NPC1 exposed a sterol-binding pocket (33). This pocket can bind with sterol structure.

Interestingly, an in vitro study using NPC1L1-overexpressing Caco-2 cells showed that micellar TA could increase NPC1L1-mediated cholesterol uptake in a concentration-dependent manner (20). TA has a sterol structure; therefore, it can bind with the pocket of NPC1L1. On this basis, we conducted co-administration of m-vitK2 with TA. In the components of bile acid, TA would be an essential molecule that accelerates the absorption of vitamin K via NPC1L1. The micelle size measurement demonstrated the size reduction when m-vitK2 combined with TA. According to previous work that evaluated the relationship between micelle size and absorption (34), different ratios of 1-palmitoyl-sn-glycero-3-phosphocholine (MPCC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) concentration, in combination with bile acid altered the micelle size. This study also demonstrated that smaller micelles enabled Caco-2 cells to increase beta-carotene uptake. We speculate that the new micelle made of Gum Arabic will decrease in size with TA and increase the absorption of vitamin K. NPC1L1 is also expressed in the liver (35–37). Ezetimibe reduced plasma cholesterol by inhibiting NPC1L1 function in the intestine and liver (36). We speculate that vitamin K with TA can more efficiently be absorbed through the intestine than vitamin K alone, resulting in increased uptake into the liver.

In patients with cholestatic liver diseases, including PBC, osteoporosis is a known complication (38). The bone mineral density (BMD) decreased with age at more than the expected rate of decline in female patients with PBC (39). Vitamin K supplementation can also reduce bone loss (40). This newly invented micelle technology has the potential of breakthrough technology in human vitamin K supplementation for improving coagulopathy and BMD.

In conclusion, orally administered m-vitK2 could recover the coagulopathy even under obstructive jaundice in rats. TA decreased micelle size and improved m-vitK2 absorption. The newly invented m-vitK2 can be stably micellized in a liquid such as oral nutrient for
liver failure patients for long-term with very low concentration. Furthermore, TA co-administration may improve vitamin K absorption under cholestatic liver diseases.

**Authorship**

Research conception and design: TS, YH, YM, TN, TK, TM, and HI; experiments: YH, TS, SI, RT, TT, TI, MU, MO, FO, and MK; statistical analysis of the data: TS; interpretation of the data: TS; writing of the manuscript: TS and JO. YH and TS contributed equally.

**Disclosure of state of COI**

No conflicts of interest to be declared.

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