


Mouse model of metformin-induced diarrhea

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ABSTRACT

Objective Metformin, an oral medication used for type 2 diabetes mellitus, is the most commonly prescribed drug with less economic burden of patients. Although metformin's efficacy and safety have long been recognized, approximately 5% of the patients treated with this drug develop severe diarrhea as an adverse effect and have to abandon treatment. Because there is no animal model to study metformin-induced diarrhea, it is hard to develop methods to maintain quality of life of patients prescribed with metformin.

Research design and methods Using mouse models, we tried to develop an evaluation system for metformin-induced diarrhea to improve diarrheal symptoms in patients with diabetes. Healthy (C57BL/6J) and diabetic obese (*db/db*) mice were subjected to a stepwise dose escalation of metformin (250 mg/kg/day (125 mg/kg twice daily oral dose)—1000 mg/kg/day (500 mg/kg twice daily oral dose)), and fecal moisture contents and their score were monitored. To evaluate anti-diarrheal medications, wood creosote (a traditional medicine) was tested. Several groups of enterobacteria in fresh feces were examined by using PCR.

Results 1000 mg/kg/day (four times maximal effective dose) of metformin significantly increased fecal moisture content. Although no symptoms of diarrhea were observed in healthy C57BL/6J mice, the same dose of metformin induced severe diarrhea in diabetic obese *db/db* mice. A reduction in PCR signals for the *Firmicutes* group was associated with metformin-induced diarrhea. Wood creosote reduced diarrhea (high water-content) without affecting metformin's efficacy or enterobacterial flora levels.

Conclusions We have created the first animal model of metformin-induced diarrhea using *db/db* mice, which will provide better quality of life for patients suffering from diarrhea caused by metformin.

INTRODUCTION

Metformin, the first-line drug for the treatment of type 2 diabetes mellitus, is the most frequently prescribed medication worldwide.¹ It attenuates mitochondrial electron transport, which increases the AMP/ATP ratio and lead to activation of AMP-activated kinase (AMPK).² Active AMPK inhibits gluconeogenesis and promotes glycolysis, improving impaired glucose tolerance. Despite the

Significance of this study

What is already known about this subject?

- ▶ Several risk factors of metformin-induced diarrhea have been proposed, such as obesity and liver dysfunctions.
- ▶ It is hard to provide a clue to reduce metformin-induced diarrhea.

What are the new findings?

- ▶ A combination of a moderately high dose of metformin and diabetic obese mice with liver dysfunctions reproduced diarrheal symptoms observed in human patients.
- ▶ The diarrheal symptoms in our mice models were ameliorated by the co-treatment with wood creosote, a traditional medicine commercially available for diarrhea.

How might these results change the focus of research or clinical practice?

- ▶ Our extrapolation model will provide new insights in metformin-induced diarrhea and better quality of life for patients with not only severe symptoms (5% of patients), but also mild symptoms (30% of patients).

well-established clinical benefits of treatment with metformin, digestive disorders, diarrhea and vomiting, are reported in approximately 30% of the patients who have been prescribed this drug.^{3–5} Additionally, 5% of patients suffered symptoms so severe they had to abandon the treatment.

Retrospective studies on metformin induced diarrhea have identified several risk factors, such as daily dose, age, sex, body mass index, and liver and biliary disorders.⁶ However, no digestive disorders have been observed in experimental animal models. Nevertheless, a few descriptions of diarrhea in experimental animals have been reported in interview forms for the approval of metformin.⁷ Animals administered with a semi-lethal dose of metformin had diarrhea with other adverse effects such as breathing difficulty, body weight loss, a decrease in a spontaneous activity, ataxia, and asitia (PubChem CID:



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14219). These backgrounds show that there is a lack of extrapolating animal models of metformin-induced diarrhea, which makes it difficult to develop methods to treat patients suffering from metformin-induced diarrhea.

Here we report that administration of a high dose (500 mg/kg twice daily oral dose) of metformin to healthy mice (C57BL/6J) increases their fecal moisture content. Diabetic obese mice C57BL/6J^{db/db} (*db/db*) with impaired liver function had severe diarrhea after the same treatment. The diarrheal symptom was ameliorated by the co-treatment with wood creosote, a traditional medicine commercially available for diarrhea. These results demonstrated that our study is the first experimental model for metformin-induced diarrhea, and the model may provide new mechanistic insights into metformin-induced digestive disorders and be used to design drugs to improve the quality of life of patients with diabetes.

RESEARCH DESIGN AND METHODS

Mouse experiments

C57BL/6J and *db/db* mice (10W male) were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under a 12-hour light/dark cycle (08:00–20:00) and received a standard rodent diet CE-2 (CLEA Japan, Tokyo, Japan).

Metformin hydrochloride (Combi-Blocks, San Diego, California, USA) and wood creosote⁸ were suspended in 0.5% Tween 80 (Kanto Kagaku, Tokyo, Japan). The drugs were orally administered to the mice twice daily at 18:00–20:00 and 8:00–10:00. Body weight was measured, and feces samples were collected in the evening following drug administration.

Measurement of fecal moisture content, scoring of feces, and blood biochemistry

Four to six mouse feces were collected into 1.5 mL sample tubes and weighed. The samples were dehydrated overnight and the weight was measured again. Scoring of feces are as follows; 0: normal, 1: stick to forceps, 2: easily collapsed, 3: unformed feces.

Blood biochemistry

One to 2 μ L of blood was collected via the tail vein of the mice, and glucose levels were measured by using G-sensor (Arkray, Kyoto, Japan). Blood samples with glucose levels over 600 mg/dL were diluted with phosphate buffered saline (PBS) and the levels were measured by using the glucose assay kit-WST (Dojin, Tokyo, Japan). Serum bile acid and alanine aminotransferase (ALT) were measured with Total Bile Acids Assay Kit (Diazyme, Poway, California, USA) and ALT activity colorimetric kit (BioVision, Milpitas, California, USA), respectively.

Western blotting

For western blotting, a piece of liver was homogenized in PBS buffer with the protease inhibitor cocktail (Nacalai, Kyoto, Japan), and 50 μ g of total protein was suspended in Laemmli SDS sample buffer. Antibodies are

as follows: Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1E6D9, Proteintech, Rosemont, Illinois, USA), anti-AMPK (ABV10739, ABGENT, San Diego, California, USA), anti-phospho AMPK (pT172) (40H9, Cell Signaling Technology, Danvers, Massachusetts, USA), anti-total Protein Kinase B (AKT) (200–401 N98, Rockland, Limerick, Pennsylvania, USA), and anti-phospho AKT (pS473) (D9E, Cell Signaling Technology).

Analysis of enterobacteria

To analyze the enterobacteria in mouse feces, one drop of fresh feces was directly suspended in 600 μ L denaturation buffer associated with a DNA purification kit (GP1 buffer in Fast Gene Gel/PCR Extraction kit, Nippon Genetics Co., Tokyo, Japan). After the removal of insoluble residue by centrifugation at 12000 G for 20 min, 500 μ L of the supernatant was applied to a silica gel columns in accordance with the manufacturer's recommendation. To amplify the 16S rRNA of enterobacteria, Expand High Fidelity PCR System (Merck, Burlington, Massachusetts, USA) was used with 1 μ L of purified DNA solution and primer sets that had been previously validated⁹: Universal (926F), AACTCAAAGGAATTGACGG and (1062R), CTCACRRCACGAGCTGAC; *Betaproteobacteria* (Beta979F), AACGCGAAAAACCTTACCTACC and (Beta1130R), TGCCCTTTCGTAGCAACTAGTG; *Firmicutes* (Firm934F), GGAGYATGTGGTTTAATTGGAAGCA and (Firm1060R), AGCTGACGACAACCATGCAC; *Bacteroidetes* (Bac960F), GTTTAATTTCGATGATACGCGAG and (Bac1100R) TTAASCCGACACCTCACGG. To obtain amplicon images, the following PCR conditions were applied: 94°C, 20s; 55°C, 20s; 72°C, 20s (19 cycles for Universal, 20 cycles for *Firmicutes* and *Bacteroidetes*, and 25 cycles for *Betaproteobacteria*). To quantify the amplicons, quantitative PCR (qPCR) was used with Thunderbird Sybr qPCR Mix (Toyobo, Osaka, Japan) and MyiQ (BioRad, Hercules, California, USA). The signals for *Betaproteobacteria*, *Firmicutes*, and *Bacteroidetes* were normalized to that of Universal.

Antibiotics, penicillin (1000 U/mL), and streptomycin (1000 μ g/mL) (Nacalai Tesque, Kyoto, Japan), were dissolved in drinking water and provided for 5 days. For the final 2 days, metformin (2 \times 500 mg/kg/day) was also administered to the mice.

Statistical analysis

For all experiments, data are expressed as mean \pm SEM. One-way analysis of variance (ANOVA) was used to analyzed data of control, metformin, and wood creosote groups, and Student's t-test was used to compare data from the metformin and wood creosote group.

Data and resource availability

The data sets generated during the current study are available from websites (see Data Availability Statement). We use only commercially available materials except for wood creosote. To request the wood creosote, contact Dr. Masafumi Ito (masafumi.ito@seirogan.co.

jp, Department of Quality Assurance, Taiko Pharmaceutical Co., Osaka, 550-0005, Japan). For other requests, contact to corresponding author or The Organization for Research and Community Development, Gifu University (orchid@gifu-u.ac.jp).

RESULTS

Metformin induces severe diarrhea in obese diabetic mice

Hypoglycemic effects of metformin are observed at the dose of 250 mg/kg in mice.¹⁰ However, the median lethal dose, LD₅₀, of metformin in mice had been reported to be approximately 1500 mg/kg.¹¹ We first carried out a stepwise increase in administration of metformin to healthy mice (C57BL/6J), from 250 mg/kg to 1000 mg/kg. For clinical relevancy, metformin was orally administered twice per day, for 3 days with 2-day intervals. Although metformin at even 500 mg/kg twice daily (daily dose of 1000 mg/kg) did not cause diarrhea in mice, we observed

an increase in fecal moisture levels when metformin was administered at 250 mg/kg twice daily and 500 mg/kg twice daily (figure 1A). There was no significant change in the body weights of the mice (figure 1B). Unexpectedly, blood glucose levels were lowered only when a high dose (500 mg/kg twice daily) of metformin was administered under fed conditions.

In patients with diabetes, the incidence of metformin-induced diarrhea increases in the presence of risk factors such as body mass index. Therefore, we carried out the same dose-escalation study in diabetic obese mice. Feces moisture content did not change when *db/db* mice were administered low (125 mg/kg twice daily) to medium (250 mg/kg twice daily) doses of metformin (figure 1C). However, severe diarrhea was observed when a high dose (500 mg/kg twice daily) of metformin was administered (figure 1D, E). We observed that basal levels (in the absence of metformin treatment) of the moisture in

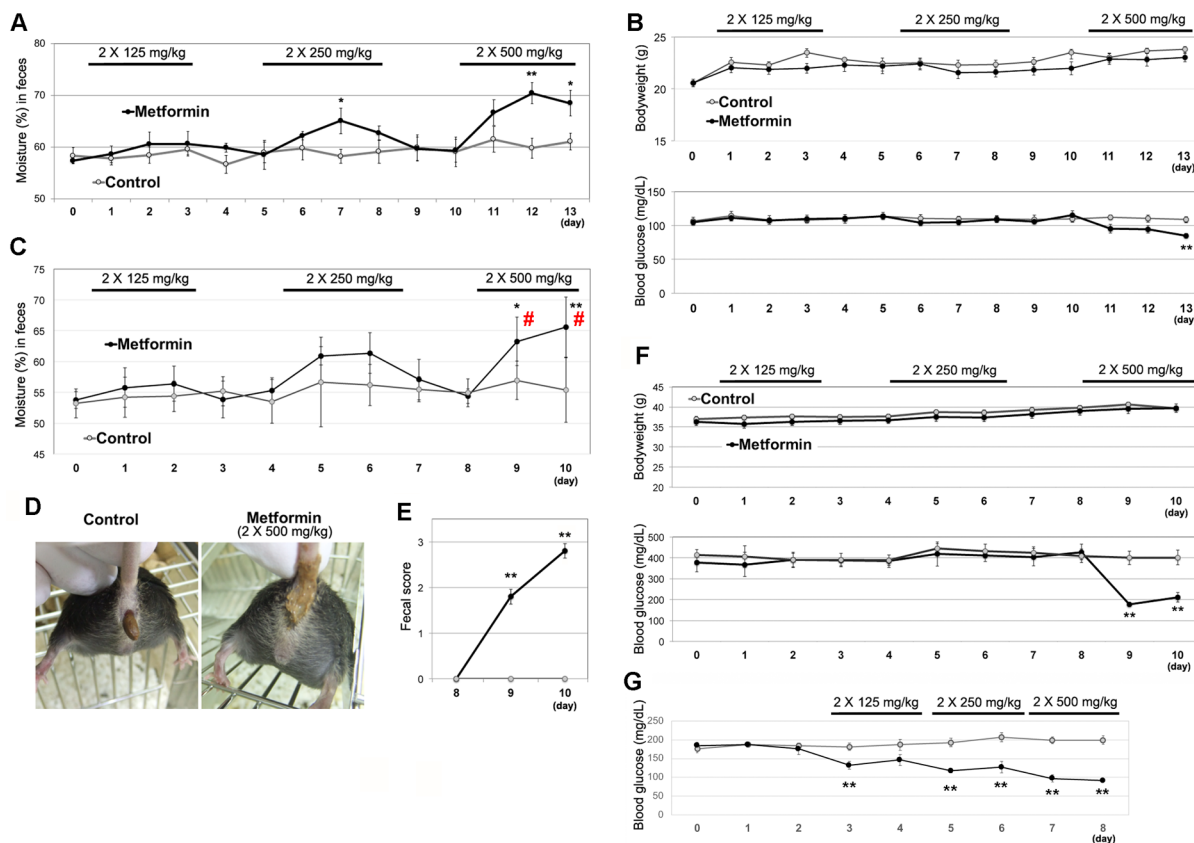


Figure 1 Metformin induces severe diarrhea in diabetic obese mice. (A) Ten-week-old male C57BL/6J mice (n=5) were orally administered metformin with a stepwise increase in dose from 125 mg/kg twice daily to 500 mg/kg twice daily, for 3 days with 2-days interval (without administration). Wet weight of feces, collected into sample tubes, was measured. The feces were dehydrated (90°C for overnight), and the dry weight was measured to determine the feces moisture content. Data are shown as means±SEM. *P<0.05, **p<0.01 between the control group (saline alone) and the metformin group. (B) Bodyweight (upper) and blood glucose (lower) changes of 10-week-old male C57BL/6J mice (in figure 1A) were monitored. P<0.05, **p<0.01 between the metformin group and the co-treatment group. (C) Ten-week-old male *db/db* mice (n=6) also received metformin with a stepwise increase in dose from 125 mg/kg twice daily to 500 mg/kg twice daily, for 2 days with 2-days interval. # indicates that the values (feces moisture content) are not accurate, due to severe diarrhea. (D) Representative image of the severe diarrhea. (E) Fecal score (at day 8–10) was calculated as follows. 0: normal, 1: stick to forceps, 2: collapsed by forceps, 3: unformed feces. (F) Bodyweight (upper) and blood glucose (lower) changes of 10-week-old male *db/db* mice (in figure 1B) were monitored. (G) Blood glucose levels after a 3-hour fast were monitored in a different group of *db/db* mice (10-week-old male mice, n=5).

the feces of *db/db* mice were lower than those in healthy C57BL/6J mice, suggesting the presence of unknown differences in the physiological environment of the *db/db* mice gut.

A loss in body weight was not observed during the experiment (figure 1F). Only *db/db* mice treated with the high dose (500 mg/kg twice daily) of metformin showed a decrease in the blood glucose levels. We surmised that the apparent lack of effect of the low and medium doses of metformin on blood glucose levels may have been a result of the fed condition of mice. Therefore, we examined blood glucose levels in mice after a 3-hour fast (figure 1G) and confirmed that the low dose (125 mg/kg twice daily) of metformin decreased the blood glucose level in *db/db* mice, indicating that 500 mg/kg twice daily metformin was four times higher than the effective dose for *db/db* mice.

Administration of wood creosote reduced metformin-induced diarrhea in mice

Next, we examined whether our mouse model was able to evaluate anti-diarrheal medications. We assessed the effects of wood creosote, a mixture that is used as an anti-diarrheal medication. First, we examined effective dose of wood creosote (figure 2A): 5 mg/kg twice daily of wood creosote decreased excess fecal moisture content in C57BL/6J mice administered 500 mg/kg twice daily of metformin. Additionally, wood creosote (5 mg/kg twice daily) had no effects on fecal moisture content (figure 2B) or fecal score (figure 2C) in animals, which did not receive metformin. We did not observe adverse effects such as weight loss (figure 2D), interferences with hypoglycemic effects (glucose tolerance (figure 2E)), changes in serum bile acid levels (figure 2F), and liver function (ALT (figure 2G)).

We also assessed the effect of wood creosote administration on metformin-induced diarrhea in diabetic obese mice. Co-administration of wood creosote (5 mg/kg twice daily) and metformin (500 mg/kg twice daily) significantly suppressed diarrhea (figure 3A, B). Mice co-administered wood creosote and metformin excreted feces that could be picked up with forceps (figure 3C). No adverse effects or interactions between metformin and wood creosote were observed (figure 4A–F). Moreover, wood creosote did not inhibit the effects of metformin on AMPK and AKT phosphorylation in the liver of *db/db* mice (figure 4G). These results suggest that our mouse model can be used to evaluate the efficacy of commercially available medications for metformin-induced diarrhea.

Reduced PCR signals for the Firmicutes phylum was partially associated with metformin-induced diarrhea

Diarrhea symptoms are often associated with changes in the enterobacterial flora, which alter the metabolic environment of the gut.^{12 13} Because the gastrointestinal microbiome modulator improved gastrointestinal intolerance in human patients treated with metformin, the

modification of enterobacterial flora has been proposed to be a cause of the gastrointestinal intolerance.¹⁴ In addition, in mice, the genetic background, including obesity and diabetic phenotypes (such as *db/db* mice), and metformin treatment are known to induce the modification of enterobacterial flora.^{15–18} Therefore, we examined the differences in enterobacterial flora in metformin-induced diarrhea.

As the flora of *Betaproteobacteria* and *Firmicutes* were found to change in *db/db* or metformin-treated mice,¹⁸ we measured the ratio of these groups. *Bacteroidetes*, the major enterobacterial group, is often used for the normalization of the target enterobacterial flora group. To forcibly modify the flora, mice (healthy, C57BL/6J) were treated with penicillin and streptomycin for 3 days. Fresh feces were directly suspended in denaturation buffer, and fecal DNA was purified and amplified by using 16S rRNA from each primer set. To quantify the amplicons, qPCR analysis followed by normalization to signals from the Universal primer set was used.

As shown in figure 5A, B, the signals for *Bacteroidetes* were low in *db/db* mice feces, and those for *Firmicutes* were slightly high. The penicillin and streptomycin treatment also reduced the signals for *Bacteroidetes*. To determine if these differences were associated with metformin-induced diarrhea, C57BL/6J mice were treated with metformin (500 mg/kg twice daily) for 2 days after the 3 days of penicillin and streptomycin treatment (figure 5C, D). Interestingly, the level of the signals for *Bacteroidetes* was restored by the metformin treatment; in contrast, the level of the signals for *Firmicutes* was significantly lowered. Fecal moisture (figure 5E) and score (figure 5F) were also increased by the metformin treatment in mice pretreated with penicillin and streptomycin, suggesting that the reduction in *Firmicutes* group levels may be associated with the elevation of fecal moisture in the metformin-treated mice.

Finally, enterobacterial flora was examined in *db/db* mice treated with both metformin and wood creosote (figure 5G, H). Although the levels of *Betaproteobacteria* were partially restored after treatment with the medium dose of metformin (250 mg/kg twice daily), the levels were restored to those of the untreated *db/db* mice (control) by treatment with the high dose of metformin (500 mg/kg twice daily), suggesting that different subspecies in the *Betaproteobacteria* group may contribute to the discrepancies between the modulate and high doses of metformin.

Similar to the antibiotic treated mice, the *Firmicutes* levels were suppressed in mice treated with the high dose of metformin, and the mice showed diarrhea symptoms. However, the treatment of mice (both *db/db* and C57BL/6J) with wood creosote did not alter the levels for these enterobacterial groups. These results suggested that the reduced levels of *Firmicutes* may be associated with diarrhea symptom, but that this may not be the only factor.

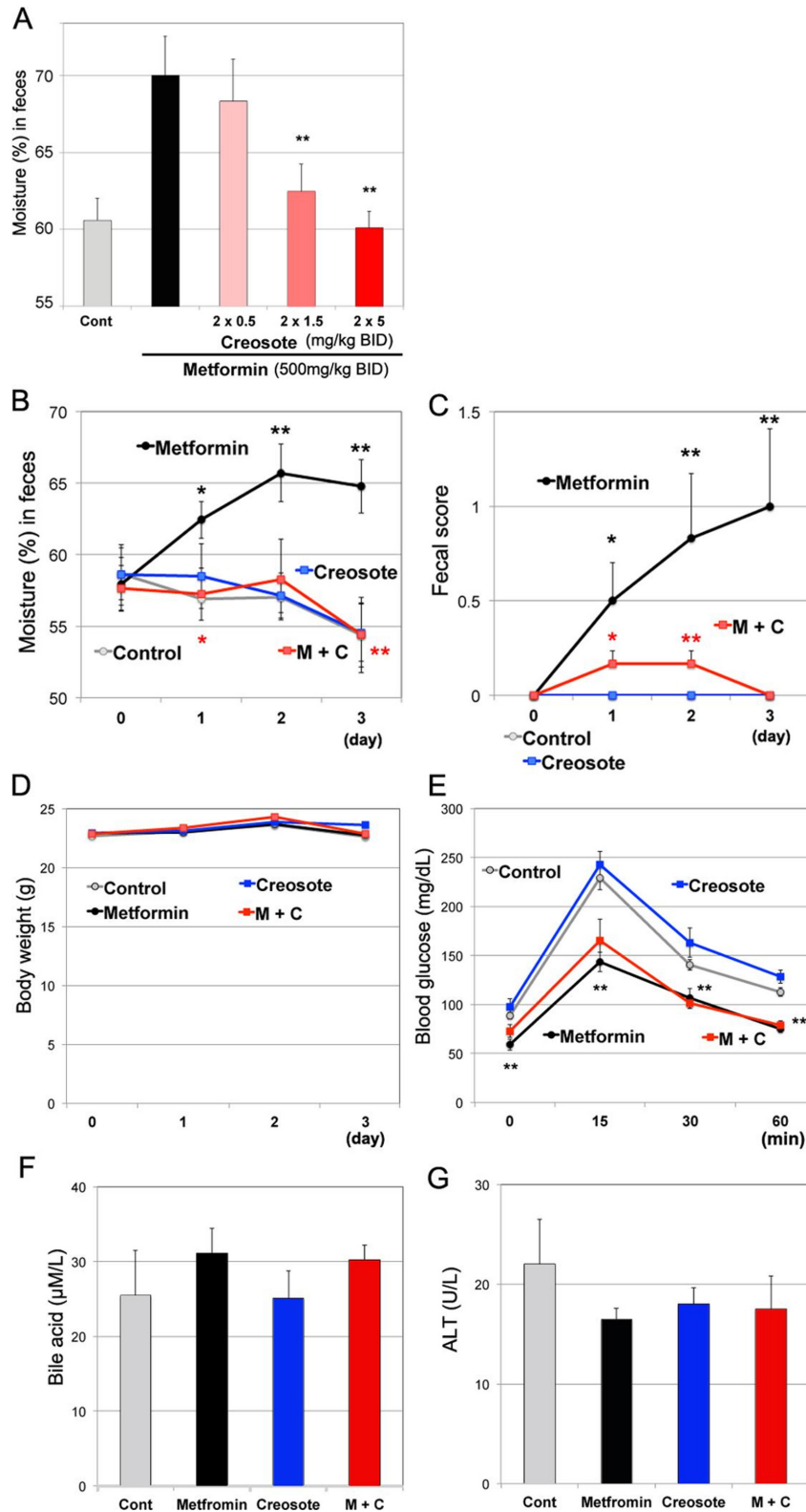


Figure 2 Effects of wood creosote on healthy mice treated with metformin. (A) Ten-week-old male C57BL/6J mice ($n=4$) were orally administered metformin (500 mg/kg twice daily) and different dose of wood creosote (<5 mg/kg twice daily). At 8 hours after the second treatment, feces were collected into sample tubes. Bars indicate mean fecal moisture (%) \pm SEM. * $P<0.05$, ** $p<0.01$ between the metformin group and the co-treatment group. Ten-week-old male C57BL/6J mice ($n=6$) received metformin (500 mg/kg twice daily) with or without wood creosote (5 mg/kg twice daily) for 3 days. The changes of fecal moisture (B), fecal score (C), and body weight (D) were monitored. (E) Mice were fasted for 3 hours after the final treatment. One g/kg glucose was administered via orally. One to 2 μL of blood was collected via tail vein at indicated time points, and blood glucose was measured using GlucoSensor. At day 4, blood was collected from the ventricle from anesthetized animals. The levels of serum bile acids (F) and alanine aminotransferase (ALT) (G) were measured. M+C, metformin+creosote.

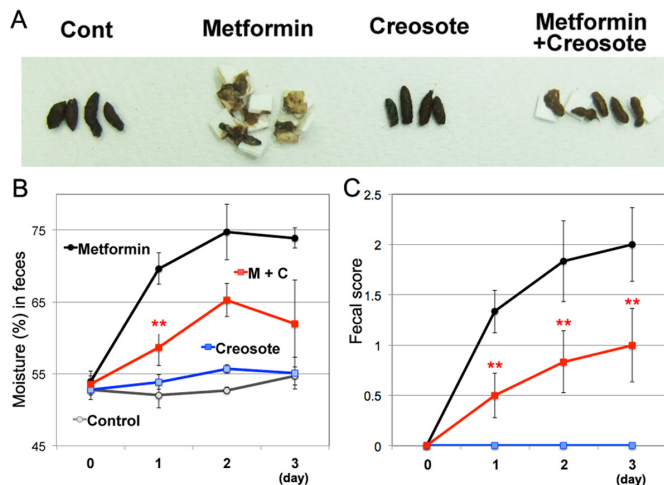


Figure 3 Wood creosote reduces diarrhea induced by metformin in obese diabetic mice. (A) Collected feces from the different experimental groups. (B) Effects of metformin (500 mg/kg twice daily) and wood creosote (5 mg/kg twice daily) co-administration on feces moisture content was examined (n=6). Red ** indicates $p < 0.01$ between the metformin group and the co-administered group (M+C). (C) Fecal score. Data are shown as mean \pm SEM. * $P < 0.05$, ** $p < 0.01$ between the control group (saline alone) and the metformin group. M+C, metformin+creosote.

DISCUSSION

Descriptions about metformin-induced diarrhea in model animals have only been documented as preclinical information (eg, National Library of Medicine, Toxicology Data Network). Diarrhea has been described

(PubChem CID: 14219), along with other adverse effects such as reduced spontaneous activity and ataxia, in healthy mice that received an extremely high dose of metformin (≥ 1500 mg/kg, (lethal dose is ≥ 1920 mg/kg)).¹⁹ Administration of metformin over the lethal dose (≥ 2280 mg/kg) to rats also caused diarrhea. In rabbits and dogs, only 100 mg/kg and 250 mg/kg of metformin, respectively, induces diarrhea.²⁰ However, this dose has been shown to lead to a number of adverse effects such as breathing difficulty, ataxia, asitia, and, in some case, death, suggesting that these diarrheal symptoms may not reflect those in human patients with diabetes.

A moderately high dose of metformin administered to *db/db* mice may be a clinically representative model. Studies suggest that in patients with diabetes, age, sex, body mass index, and liver and biliary disorders can increase the incidence of metformin-induced diarrhea.⁶ Patients with more than five risk factors definitely developed diarrhea when they were initially treated with metformin at a high dose (750 mg/kg). The model we have developed using *db/db* mice can be used to study metformin-induced diarrhea in patients with diabetes and obesity as well as impaired liver function.

The fluctuations in enterobacterial flora are even found to be associated with diarrhea that is not caused by pathogenic bacteria, such as in patients with irritable bowel syndrome (IBS).¹² The *db/db* mice used in this study were subjected to analysis of their enterobacterial flora and the effect of metformin treatment. The population of the three major groups of enterobacteria (*Betaproteobacteria*,

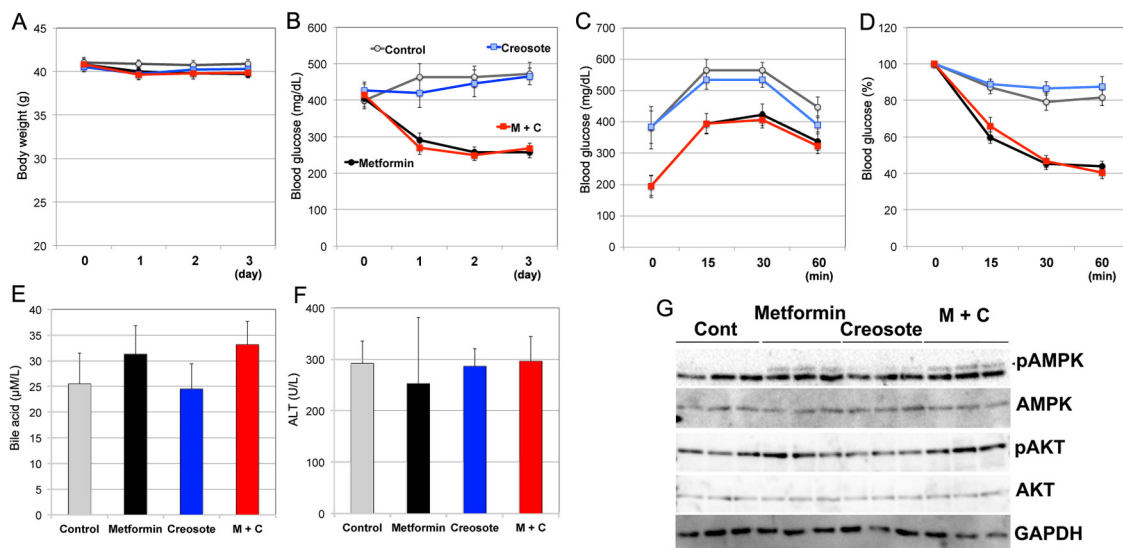


Figure 4 Effects of wood creosote on diabetic obese mice treated with metformin. Wood creosote does not affect efficacy of metformin. Weight change (A) and blood glucose levels (fed condition) (B) were examined during the experiment in figure 3. Data are shown as means \pm SEM. No significant differences between the metformin group and the metformin with wood creosote (M+C) group were observed. Mice were fasted for 3 hours after the final administration of drugs. (C) One g/kg glucose was administered via oral gavage. One to 2 μ L blood was collected via tail vein at indicated time points, and blood glucose was measured by using GlucoSensor. (D) At day 4 of figure 3, mice were fasted for 2 hours and then injected intraperitoneally with 36 μ g/kg insulin. Blood glucose levels are shown as % initial. At day 5 in figure 3, blood was collected and measured serum bile acid levels (E) and alanine aminotransferase (F) were measured. (G) Western blots were carried out using liver protein (50 μ g) to detect levels of phospho-AMPK and phospho-AKT. AKT, Protein Kinase B; AMPK, AMP-activated kinase.

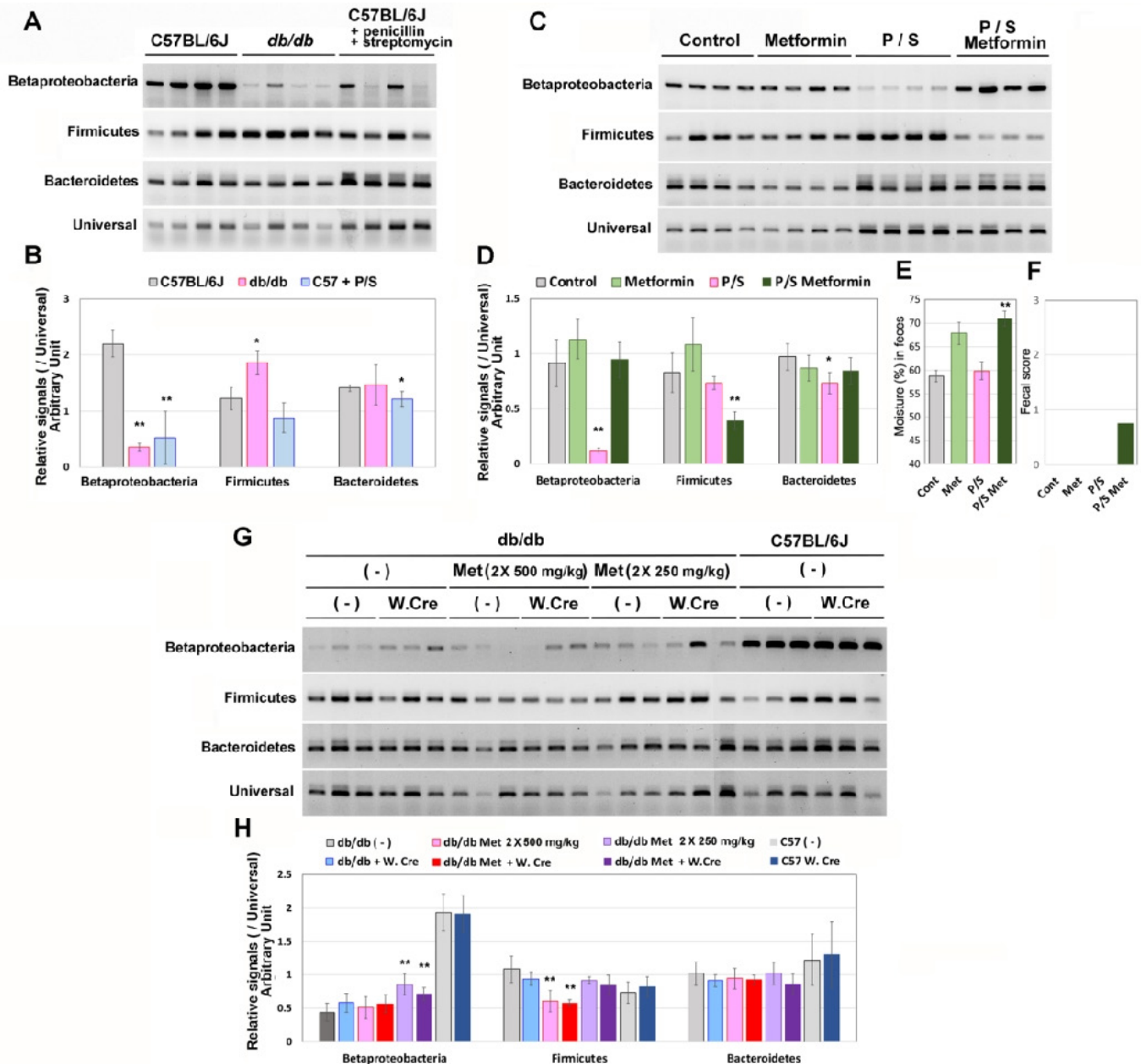


Figure 5 Difference in enterobacterial flora in *db/db* mice and metformin-treated mice. Enterobacterial DNA was prepared from the fresh feces of healthy (C57BL/6J), *db/db*, and healthy mice treated with antibiotics (penicillin and streptomycin) treatment for 3 days (10-week-old male mice, $n=4$, each). The DNA was analyzed by PCR, gel electrophoresis (A), and qPCR (B). The bars indicate the SD; * and ** indicate $p<0.05$ and $p<0.01$, respectively. C57BL/6J mice ($n=4$) that had been treated with the antibiotics (P/S: penicillin/streptomycin) for 3 days were treated with or without metformin (500 mg/kg twice daily) for 2 days with the antibiotics. The enterobacterial flora were analyzed by PCR (C and D). Fecal moisture (E) and score (F) were also analyzed on the final day. *db/db* mice (10-week-old male mice, $n=6$) were treated with metformin (Met: 250 mg/kg twice daily for 2 days followed by 500 mg/kg twice daily for 2 days) with or without wood creosote (W. CRE: 5 mg/kg twice daily). (-) indicates without metformin or without wood creosote, as appropriate. Diarrhea was observed in *db/db* mice treated only with metformin (500 mg/kg twice daily). (G) and (H) show the results of PCR analyses of the enterobacterial flora.

Firmicutes, and *Bacteroidetes*) were analyzed approximately by qPCR and it was found that the ratio of PCR signals for *Firmicutes*/Universal was lower in diarrhea conditions (*db/db* mice treated with a high dose of metformin).

Interestingly, although penicillin and streptomycin treatment did not decrease enterobacterial levels (as detected by the primer set for Universal), the mice excreted soft feces after metformin treatment, which

was associated with the reduced *Firmicutes* levels. In addition, metformin-induced diarrhea in *db/db* mice was also associated with reduced *Firmicutes* levels. The *Firmicutes* group is composed of a variety of enterobacterial species; some are decreased in patients with IBS, and others are increased.¹²

In experiments with mice, the ratio of *Firmicutes* group has been reported to be increased by anti-diarrheal

reagents or natural materials¹³. These reports agree with our observation of metformin-induced diarrhea. However, wood creosote, which we choose as the anti-diarrheal material, did not modulate enterobacterial flora, including *Firmicutes*, suggesting that the reduced ratio of the *Firmicutes* group may be a risk factor for metformin-induced diarrhea, but not the only factor.

Excess bile acid has been shown to be correlated with the incidence of diarrhea. Approximately 25%–33% of patients with functional diarrhea may show dysregulation of bile acid homeostasis.²¹ Studies have shown that AMPK, the major target of metformin, upregulates bile acid synthesis in the liver by disrupting a negative feedback for *CYP7a* (bile acid synthase) gene expression.²² Although the *db/db* mice had liver dysfunction, they did not have biliary disorders. In addition, the population of *Firmicutes* flora has been reported to be increased by excess bile acids in the rat gut²³; however, it was decreased in our model, suggesting that the excess bile acids did not cause metformin-induced diarrhea in our mouse model. In case of patients with diabetes, chronic biliary disorders may be associated with liver dysfunctions.⁴

A conflicting observation in rats has been reported that AMPK inhibits Cl⁻ channel that possibly leads to water uptake from the colon lumens.²⁴ Cholera toxin-mediated and bile acid-mediated cAMP signal results in fluid accumulation in isolated jejunal loops, which is countered by treatment with AMPK agonists,^{24–25} suggesting that metformin-induced diarrhea may not be caused by AMPK activation.

A low concentration of metformin (subthreshold-activating AMPK) has been found to stimulate the release of 5-hydroxytryptamine (5-HT) in tissue biopsy specimens of human duodenal mucosa,²⁶ which can explain diarrhea and vomiting via central nervous system. However, 5-HT₃ receptor antagonists show no effect on the metformin-stimulated 5-HT release in the specimens or on diarrheal symptoms in patients prescribed metformin.²⁷ In contrast, wood creosote inhibits not only 5-HT₃ receptors, but also 5-HT₄ receptors,²⁸ suggesting the relevance of the parasympathetic nerve system. Moreover, it has been reported that wood creosote does not modulate enterobacterial flora,²⁹ which we also found in this study. In contrast, a gastrointestinal microbiome modulator (a mixture of purified ingredients of foods) improves diarrhea symptoms induced by metformin and lowered fasting blood glucose levels.¹⁴ These evidences suggest that wood creosote does not act directly in the gut or alter the metformin effects or glucose tolerance.

Using our mouse model, we evaluated the anti-diarrheal effects of wood creosote on metformin-induced diarrhea. Understanding the mechanism by which wood creosote improves the symptoms of metformin-induced diarrhea may provide new insights into how metformin causes diarrhea. In addition, correlations between metabolites that are produced by enterobacteria and the severity of diarrheal symptom induced by metformin should be examined. Especially, human patients consume a

variety of diets, which may contribute to the difference in the severity of diarrheal symptom. Therefore, other risk factors for metformin-induced diarrhea in human patients, age, sex, as well as dietary patterns (for example, a high-fat diet), should also be examined in mouse models.

Contributors AH, KI, MI, and MT performed experiments. HM, and TM analyzed data. HT wrote this manuscript. HM, TM, KO, and TS designed the overall study and analyzed the data. HT is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Competing interests We declare that MI, MT, HM, TM, KO, and TS are employed by Taiko Pharmaceutical Co., Ltd. AH and KI have nothing to declare.

Patient consent for publication Not required.

Ethics approval All animal experimental procedures were approved by the Animal Committee in Gifu University (H30-040 and 2019–192).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data Sharing: Original data is available on the web (DOI: 10.13140/RG.2.2.28403.68645: https://www.researchgate.net/publication/338655326_Data_original. and DOI: 10.13140/RG.2.2.35953.43365: https://www.researchgate.net/publication/338649294_Entero_PCR).

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