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In vivo pharmacodynamic and pharmacokinetic effects of metformin mediated by the gut microbiota in rats

Bin Wu, ManYun Chen, YongChao Gao, JingLei Hu, MouZe Liu, Wei Zhang*, WeiHua Huang*

Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, Hunan, China
Institute of Clinical Pharmacology, Hunan Key Laboratory of Pharmacogenetics, Central South University, Changsha, Hunan, China
National Clinical Research Center for Geriatrics, Xiangya Hospital, Central South University, Changsha 410008, China

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ABSTRACT

Aims: The gut microbiota plays a crucial role in the efficacy of metformin in T2DM treatment. We evaluated whether the pharmacodynamics and pharmacokinetics of metformin are mediated by gut microbiota.
Main methods: We used conventional diabetic and pseudo-germ-free rats. After 6 weeks of metformin treatment, pharmacodynamic indexes were determined. Metformin concentrations were measured with a validated HPLC-MS/MS method after the first oral administration.
Key findings: Most endpoints were similar between vehicle-treated diabetic and vehicle-treated pseudo-germ-free diabetic rats. However, after 6 weeks of metformin treatment, compared with conventional diabetic rats, pseudo-germ-free diabetic rats exhibited significantly increased FBG, decreased oral glucose, reduced GSP, worsened insulin resistance, increased hyperlipidemia, and increased hepatic steatosis severity. Moreover, the Cmax of pseudo-germ-free rats increased significantly, while the t1/2α decreased significantly. These pharmacodynamic and pharmacokinetic changes were probably due to a decrease in Oct1 expression in the liver, resulting in altered hepatic uptake of metformin in vivo.
Significance: These results implied that the gut microbiota may play an important role in the pharmacodynamics and pharmacokinetics of metformin and that the changes in these properties are probably due to Oct1 down-regulation in the livers of pseudo-germ-free rats.

1. Introduction

Metformin is regarded as the first-line oral hypoglycemic drug for type 2 diabetes mellitus (T2DM) [1]. It is important to investigate factors and mechanisms that can impact the pharmacodynamics (PD) and pharmacokinetics (PK) of metformin.

Accumulating evidence indicates that the PD of metformin are not only determined by the activation of AMP-activated protein kinase (AMPK), which lowers hepatic glucose production [2], but also influenced by the gut microbiota [3]. For example, recent studies in both rodents and humans have suggested that gavage of *Akkermansia* spp., which are significantly more abundant after metformin treatment, can improve metabolic disorders [4–6]. However, it is still unclear whether the interaction between the gut microbiota and metformin is essential for therapeutic potency.

Metformin is absorbed through the intestinal tract and organic cation transporter 1 (Oct1) is responsible for the hepatic uptake of metformin [7,8]. Moreover, prior studies have shown that Oct1 expression is affected by the gut microbiota [9]. Thus, dysbiosis of the gut microbiota may change the distribution of metformin in vivo. However, few reports have explored the effect of the gut microbiota on the PK of metformin.

In our study, we compared the plasma and tissue concentrations of metformin in pseudo germ-free rats with those in treat-free diabetic rats to investigate the role of the gut microbiota in the PD and PK of metformin.

Abbreviations: ALT, serum alanine transaminase levels; AMPK, AMP-activated protein kinase; DM, diabetes mellitus group; FBG, fasting blood glucose; FSI, fasting serum insulin; GF, antibiotic germ-free diabetic group; GFMET, antibiotic germ-free diabetes + metformin group; GSP, glycated serum protein; HFD, high-fat diet; HOMA-IR, homeostasis model of assessment-insulin resistance; HPLC-MS/MS, high-performance liquid chromatography tandem mass spectrometry; LDL-C, serum low-density lipoprotein cholesterol; MET, diabetes + metformin group; Oct, organic cation transporters; OGTT, oral glucose tolerance test; OGTT-AUC, oral glucose tolerance test-area under the curve; PK, pharmacokinetics; STZ, streptozotocin; T2DM, type 2 diabetes mellitus; TC, total serum cholesterol; TG, serum triglyceride

* Corresponding authors at: Department of Clinical Pharmacology, Xiangya Hospital, Central South University, No.110, Xiangya Street, Changsha, Hunan 410078, China.
E-mail addresses: yjsd2003@163.com (W. Zhang), endeavor34852@aliyun.com (W. Huang).

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2. Materials and methods

2.1. Animals

Male rats (body weight: 150 ± 20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Certificate: SCXK2016-0002). All rats were raised in a specific pathogen-free (SPF) animal laboratory at the Experimental Animal Center of Central South University. Food and water were freely available for animals under a 12-hour light/dark cycle at humidity (50–60%) and constant temperature (25 °C). The Animal Ethics Committee of Central South University approved the methods used in this experiment (No. 2018sydw098).

2.2. T2DM model

After acclimating for 1 week, six rats were randomly separated as the normal control group (NC) and fed on a normal diet. The other rats were fed a high-fat diet (HFD, 10% lard oil, 20% sugar, 70% normal diet) for 4 weeks with sufficient food and water. Then, after 12 h of fasting, 40 mg/kg streptozotocin (STZ; Sigma-Aldrich, USA) dissolved in a 0.1 M citric acid/sodium citrate buffer at pH 4.5 was intraperitoneally injected. After 2 days, the animals' fasting blood glucose (FBG) levels were measured on several occasions. Rats with an average FBG ≥ 11.1 mmol/L were considered as T2DM models and selected for the next experiment.

2.3. Pseudo germ-free diabetic rats

To deplete the gut microbiota, a solution of neomycin (100 mg/kg), vancomycin (50 mg/kg), and amphotericin-B (1 mg/kg), which are non-absorbable antibiotics, were administered by antibiotic gavage twice per day. Fresh antibiotic concoction and ampicillin were prepared every day. Antibiotics were given from 2 weeks before the administration of metformin or placebo until the end of the study in the pseudo germ-free groups. The feces were collected weekly, and the amounts of the gut microbiota were evaluated by PCR of the bacterial 16S ribosomal RNA gene in feces (Fig. S1) [9].

2.4. Experimental design

Rats were randomly divided into the following five groups and processed accordingly: the normal control (NC) group and the diabetes mellitus (DM) group, which were treated with pure water; the pseudo germ-free diabetes (GF) group, which was treated with antibiotics to deplete the gut microbiota using the method described above; the metformin (MET) group, which was treated with metformin (200 mg/kg/day); and the pseudo germ-free + metformin (GFMET) group, which was treated with antibiotics and metformin (200 mg/kg/day). Rats received metformin by oral gavage using water as the vehicle daily for 6 weeks.

2.5. FBG levels

All rats were fasted for 12 h per week, and their FBG levels were measured by Accu-Chek Active (Accu-Check, Germany). Insulin resistance was measured with the homeostasis model of assessment-insulin resistance (HOMA-IR) and was calculated using the following formula: FBG (mmol/L) × fasting serum insulin (FSI) (mIU/mL)/22.5 [10].

2.6. Oral glucose tolerance test (OGTT)

After 6 weeks of administration, the rats fasted overnight (12 h) and were intragastrically administered sterilized glucose solution (2 g/kg, Sigma Aldrich, USA). The FBG levels of the blood samples collected from the tail vein were estimated at 0, 15, 30, 60, 120, and 180 min using an Accu-Chek Active (Accu-Chek, Germany).

2.7. Serum biochemical analyses

After the whole administration period, all animals were sacrificed under anesthesia with a combination of Zoletil (tiletamine-zolazepam, Virbac, France) and Rompun (xylazine-hydrochloride, Bayer, Germany) (1:1, v/v). Blood samples were collected from the central aorta and quickly transferred into a BD Vacutainer (BD, USA). Fresh blood samples were kept for 2 h at room temperature and centrifuged (3000 rpm for 20 min) to isolate the serum and then stored at −80 °C for further analysis. The serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), glycated serum protein (GSP) and alanine transaminase (ALT) levels were measured with reagent kits (Nanjing Jiancheng Bioengineering Institute, China). FSI levels were measured by a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Specific procedures were performed according to the kit manufacturer's instructions.

2.8. Organ histology

Organs were quickly excised and homogenized in a 4% paraformaldehyde fixation solution (Sangon Biotech, China). Paraffin sections of the livers were prepared and stained with hematoxylin and eosin (H&E) to evaluate histopathological changes [11].

2.9. Serum and tissue sample collection

All rats were fasted for at least 12 h before the oral administration of metformin (200 mg/kg). Five hundred microliters of blood samples from cannulated jugular veins were collected at the following times after dosing: 0, 0.17, 0.33, 0.5, 1, 2, 4, 6 and 24 h. The jugular vein cannulation method was employed as previously described [12].

2.10. Plasma concentration and tissue distribution of metformin

Metformin in plasma, liver, kidney, intestine, muscle, and heart tissue homogenate and metformin standards (50, 100, 200, 400, 800, 1600, 3200, 6400 and 12,800 ng/ml) were precipitated with acetonitrile containing phenformin hydrochloride (800 ng/mL) as an internal standard. Following centrifugation, the metformin concentration was determined with a validated high-performance liquid chromatography atmospheric pressure ionization tandem mass spectrometry (HPLC-MS/MS) method [13]. The HPLC system (Acquity Ultra Performance Liquid Chromatography, Waters) consisted of a binary pump, an autosampler and a Hypersil BDS C18 column (5 μm, 150 mm × 2.1 mm i.d., Thermo, USA) at 35 °C.

2.11. Western blot analysis

Stored liver tissues were homogenized in protein extraction solution (iNtRON Biotechnology, Inc., Gyeonggi-do, Republic of Korea) containing protease inhibitor (Sigma Aldrich, USA) and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA). Protein concentrations of the homogenates were determined using a BCA kit (Beyotime Institute of Biotechnology, Jiangsu, China).

The primary antibodies used were anyibodies against β-actin (Sigma, dilution 1:3000); Oct1 (Sigma, dilution 1:500); and Oct2 (Cell Signaling Technology, dilution 1:1000).

2.12. Data analysis

The PK parameters were calculated using DAS 2.0 (BioGuider Co., Shanghai, China) using noncompartmental analysis. Statistical analyses were analyzed using SPSS 23.0 (IMB Corp., USA) software. The significance of between-groups differences was analyzed by using two-way

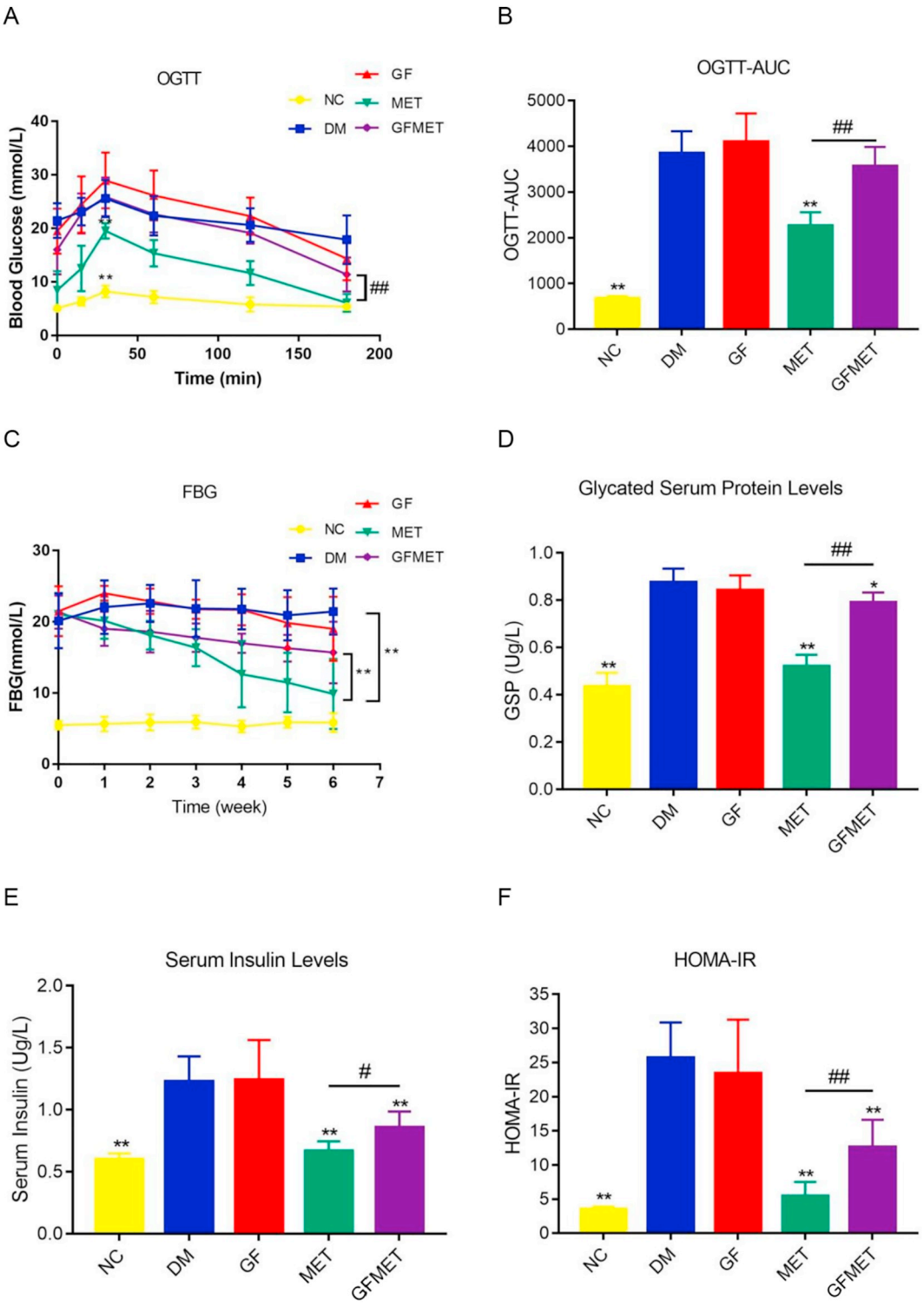

Fig. 1. Antihyperglycemic and insulin resistance ameliorating effects of the oral administration of metformin (200 mg/kg/day) or placebo in the normal control group (NC), diabetes mellitus rats (the MET group/the DM group) and pseudo germ-free diabetic rats (the GFMET group/the GF group). The plasma glucose level curve (**A**). The area under the curve (AUC) of the oral glucose tolerance test (OGTT) (**B**), The fasting blood glucose (FBG) levels (C), glycated serum protein (GSP) levels (**D**), fasting serum insulin (FSI) levels (**E**), and the homeostasis model of assessment-insulin resistance (HOMA-IR) (**F**). Significant differences between two groups were determined by using two way ANOVA analyses and Student's *t*-test. $^{**}P < 0.01$ vs. DM group. $^{*}P < 0.05$ vs. DM group. $\#\#P < 0.01$ vs. GFMET group. $\#P < 0.05$ vs. GFMET group. (n = 6).

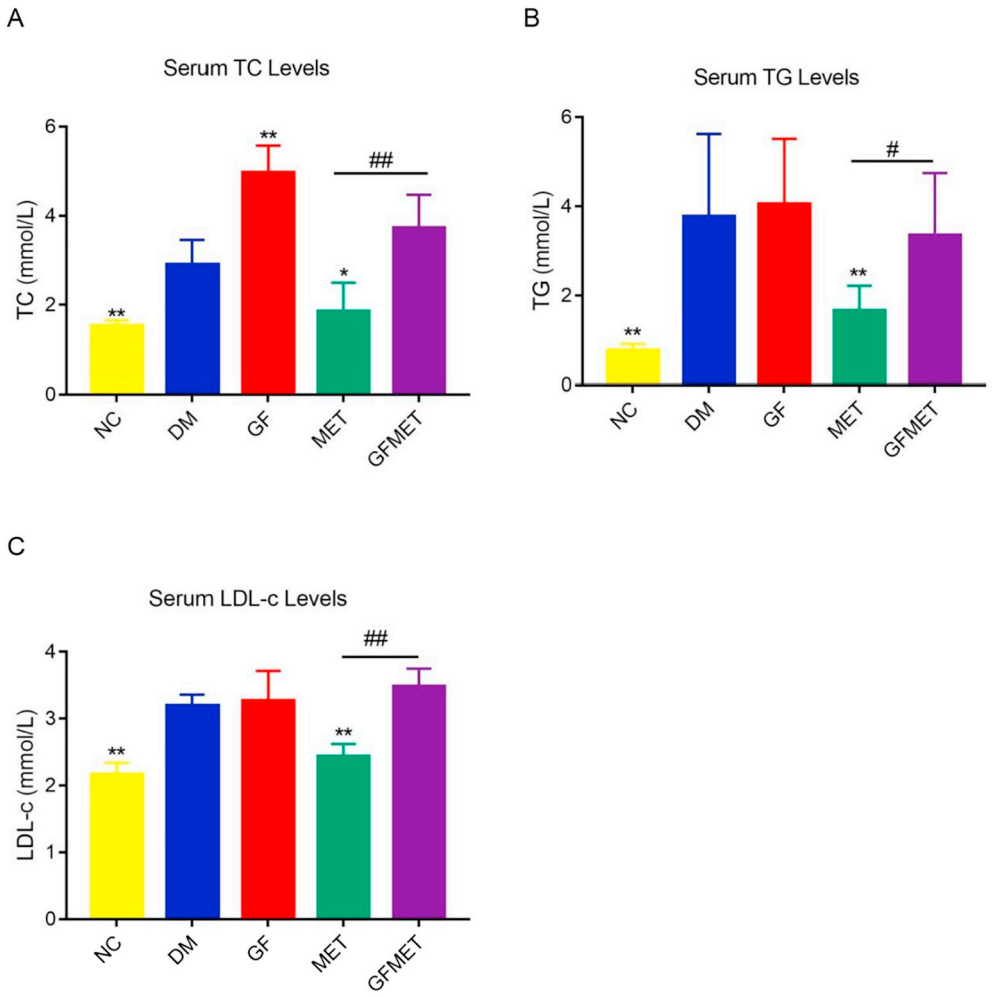

Fig. 2. After 42 days of administration, serum total cholesterol (TC) (**A**), serum triglyceride (TG) (**B**) and serum low-density lipoprotein cholesterol (LDL-C) (**C**) levels were significantly lower in the NC/MET group than in the other groups (DM group, GF group, GFMET group). Significant differences between two groups were determined by using two way ANOVA analyses and Student's *t*-test. $^{**}P < 0.01$ vs. DM. $^{*}P < 0.05$ vs. DM. $\#\#P < 0.01$ vs. GFMET. $\#P < 0.05$ vs. GFMET. (n = 6).

ANOVA analyses and Student's *t*-test. In all cases, a difference was considered significant at $P < 0.05$.

3. Results

3.1. Reduction in intestinal bacteria by antibiotics

In the pseudo germ-free rats (the GF and GFMET groups), the gut microbiota was decreased to less than approximately 10% of the gut microbiota found in the DM and MET groups after antibiotic treatment and remained at a low concentration for 2 days after the cessation of treatment (Fig. S1).

3.2. Antihyperglycemic effect of metformin in pseudo germ-free rats

FBG level was estimated at 4 time points in the 5 groups of rats with different treatments (the NC, DM, GF, MET, and GFMET groups) (Fig. 1C). Not surprisingly, the FBG levels of the NC/MET/GFMET group but not of the GF group were significantly lower than that of the DM group after two weeks of metformin treatment. The FBG levels were gradually reduced to 41.3% and 70.9% of the original level in six weeks with continuous metformin treatment in both the MET and GFMET groups, respectively (Fig. 1C). Interestingly, significantly less reduction was observed between the MET and GFMET groups ($P < 0.05$).

The results of the OGTT, oral glucose tolerance test-area under the curve (OGTT-AUC) and GSP levels are presented in Fig. 1(A, B, D). The GSP level and OGTT-AUC for the entire 180 min test decreased significantly in the MET group but not in the GFMET group. All results indicated that the antihyperglycemic efficacy of metformin was weakened when the gut microbiota was depleted.

3.3. Insulin resistance in pseudo germ-free rats

Fig. 1E shows that the FSI levels of the MET and GFMET groups were significantly lower than those of the DM group after six weeks of metformin treatment, although the degree of decline differed (45.8% for the MET group ($P < 0.01$) vs. 30.3% for the GFMET group ($P < 0.01$)). As shown in Fig. 1F, HOMA-IR values were decreased in the MET group (87.2%, $P < 0.01$) and the GFMET group (54.6%, $P < 0.01$) compared with those in the DM group, and these values significantly differed between the MET group and the GFMET group ($P < 0.01$).

3.4. Antihyperlipidemic effect of metformin in pseudo germ-free rats

As shown in Fig. 2, metformin reduced the levels of serum TC, TG and LDL-C by 36.2% ($P < 0.05$), 56.1% ($P < 0.01$) and 23.4% ($P < 0.01$), respectively, in the MET group, but these levels were not impacted in the GFMET group.

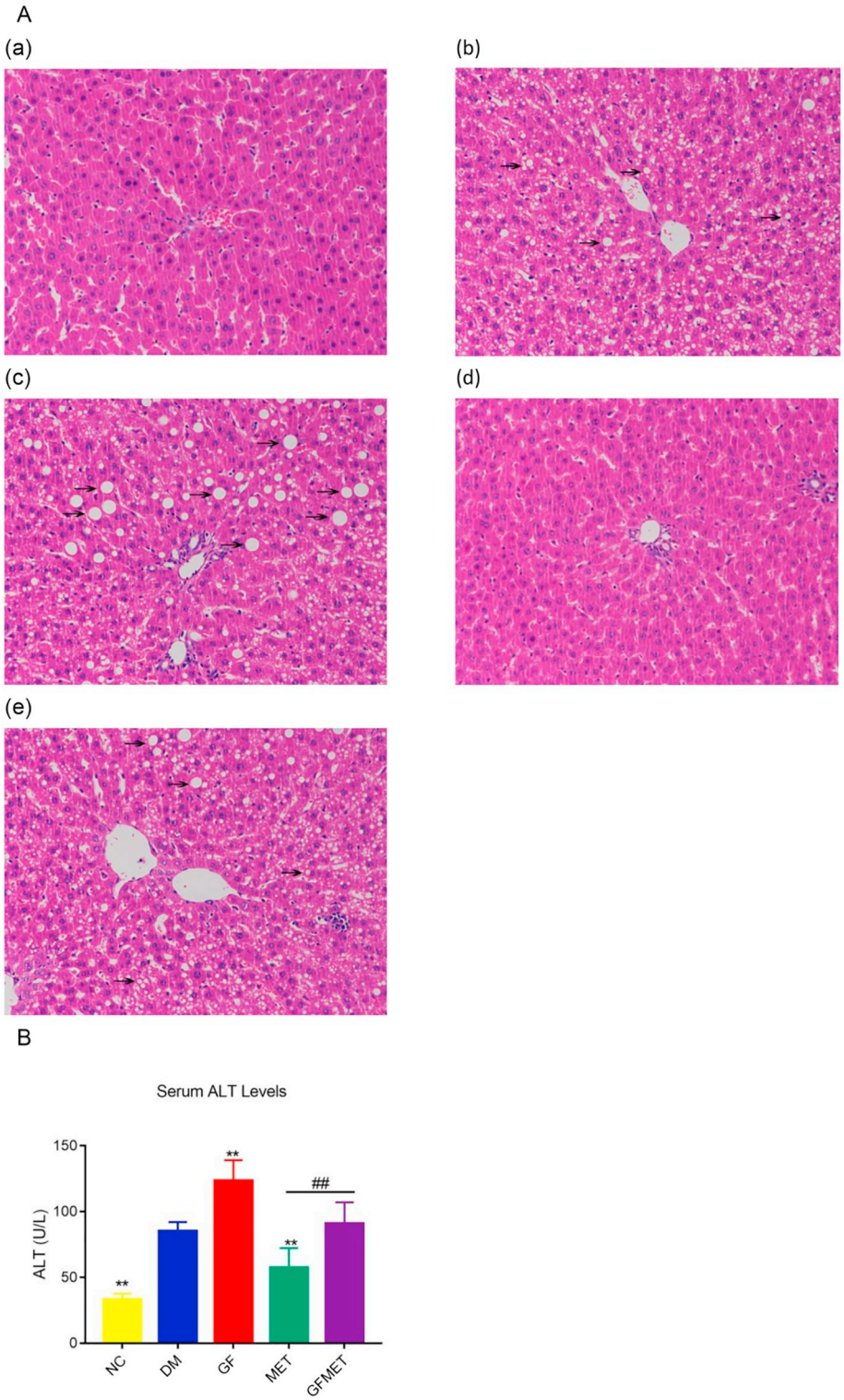

Fig. 3. Histopathological studies of the liver in rats were evaluated by staining with hematoxylin-eosin (H&E, 200×) (**A**). (a) NC group liver. (b) DM group liver. (c) GF group liver. (d) MET group liver. (e) GFMET group liver. Serum alanine transaminase (ALT) levels were measured with reagent kits (**B**). Significant differences between two groups were determined by using two way ANOVA analyses and Student's *t*-test. $^{**}P < 0.01$ vs. DM. $^{*}P < 0.05$ vs. DM. $\#\#P < 0.01$ vs. GFMET. $\#P < 0.05$ vs. GFMET. (n = 6).

3.5. More severe hepatic steatosis in pseudo germ-free rats

As shown in Fig. 3, H&E histological staining showed significant hepatic steatosis (arrows) in the DM, GF and GFMET groups (Fig. 3A). However, the hepatic lipid deposition of the MET group was decreased significantly in response to metformin treatment. Serum ALT levels were lower for the MET group than for the other groups (Fig. 3B). It is worth mentioning that the ALT values and the steatosis degree of the liver in the GF group were the highest.

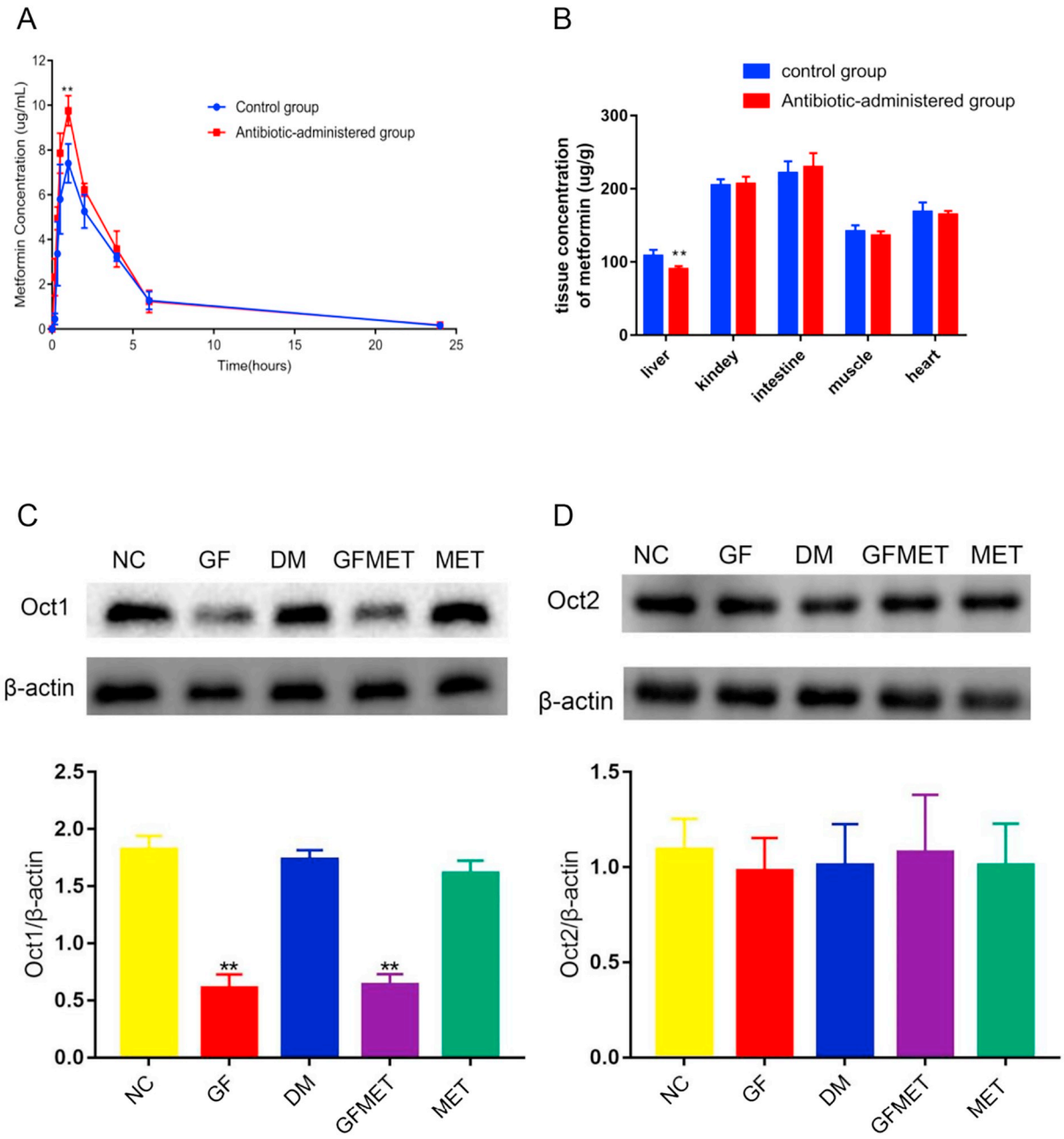

Fig. 4. The plasma concentration-time curve (**A**) and tissue distribution (**B**) of metformin in diabetic rats (the control group) and rats administered antibiotics (the antibiotic-administered group). Relative expression of Oct1 (**C**) and Oct2 (**D**) in liver tissues for each group (n = 6). Significant differences between two groups were determined by using two way ANOVA analyses and Student's *t*-test. $^{**}P < 0.01$ vs. DM. $^{*}P < 0.05$ vs. DM. $\#\#P < 0.01$ vs. GFMET. $\#P < 0.05$ vs. GFMET. (n = 6).

3.6. Metformin PK

The PK profiles of metformin are shown in Fig. 4A, and the mean PK parameters of metformin are listed in Table 1. C_{max} was significantly increased and $t_{1/2\alpha}$ was significantly decreased.

3.7. Tissue distribution of metformin in pseudo germ-free rats

The liver concentration of metformin was significantly lower in the antibiotic-administered group than in the control group (Fig. 4B). In contrast, metformin distributions in other tissues were almost identical for these two groups (Fig. 4B).

3.8. Oct1 and Oct2 expression in Rats Liver tissue

To investigate the reasons for the decreased hepatic uptake of metformin in the antibiotic-administered group, we used Western blotting to measure protein levels of Oct1 and Oct2, the main cationic transporters of metformin in liver. Oct1 protein was significantly decreased in liver tissues of pseudo germ-free rats (Fig. 4C). However, no significant changes in the expression of Oct2 protein were observed (Fig. 4D).

4. Discussion

Most previous studies on the PD and PK of metformin have focused

Table 1
Pharmacokinetic parameters of metformin after single oral administration (300 mg/kg) in diabetic rats (Control group) and germ-free diabetic rats (after antibiotics treatment), (n = 6).

Parameters		Control group	Germ-free group	*P* value
C_{max}	ug/mL	7.41 ± 0.79	9.78 ± 0.60**	< 0.01
T_{max}	h	1.00 ± 0.00	0.92 ± 0.19	0.34
AUC_{24h}	μg·h/mL	36.65 ± 3.31	41.53 ± 5.37	0.11
AUC_{∞}	μg·h/mL	37.55 ± 3.29	42.15 ± 6.07	0.17
$t_{1/2\alpha}$	h	1.92 ± 0.77	0.72 ± 0.50*	< 0.05
$t_{1/2\beta}$	h	2.81 ± 1.42	2.47 ± 0.67	0.64
MRT	h	5.07 ± 1.03	4.49 ± 0.86	0.36

C_{max}: maximum plasma concentration; T_{max}: time to reach Cmax; AUC_{24h} or AUC_{∞}: area under the plasma concentration-time curve from zero to 24 h or infinity; $t_{1/2\alpha}$: half-life of drug distribution; $t_{1/2\beta}$: half-life of drug elimination; MRT: mean residence time. Each value represents the mean ± SD.

* $P < 0.05$ vs. Control group.

** $P < 0.01$ vs. Control group.

on systemic-based mechanisms only, and the importance of the gut microbiota has been ignored. In this study, we hypothesized that the PD and PK of metformin would change in pseudo germ-free diabetic rats, most likely due to the expression or function of Octs in the liver, metformin's primary target organ. Therefore, we determined Oct1 protein levels in the liver as well as the PK and PD of metformin after antibiotic treatment in diabetic rats. To our knowledge, this investigation is the first study evaluating the PK of metformin, hepatic concentration of metformin and expression of the Oct1 gene in pseudo germ-free rats.

In our study, an HFD and a low-dose injection of STZ were used to induce T2DM in rats [14]. Then, the gut microbiota was depleted using antibiotics to construct a pseudo germ-free diabetic model: this treatment did not influence the physiological condition of the rats [15] and has been used widely in conjunction with, or instead of, the use of germ-free rats to investigate the role of the gut microbiome in certain pathological conditions [16–18]. As the results showed, metformin had an antihyperglycemic effect; the MET and GFMET groups showed a 60.6% ($P < 0.01$) and 31.7% ($P < 0.01$) reduction, respectively, in FBG levels, and there is a significant difference between the two groups ($P < 0.05$) (Fig. 1C). Additionally, these data implied the gut microbiome was responsible for 47.7% of the maximal glucose-lowering effect of metformin using FBG as an indicator. Histopathological observations also showed that the hepatoprotective effect of metformin was weakened in the absence of the gut microbiota. All these results suggested that metformin's efficacy was significantly weakened without gut microbiota mediation. It must be pointed out that the severity of diabetes in the GFMET group was slightly less than that of the DM group, which indicated that the antidiabetic effect of metformin in the GFMET group did not completely disappear. This is probably because the antibiotic-induced depletion of the gut microbiome was not complete. In addition, metformin may achieve antidiabetic effects through other mechanisms, such as mitochondrial function [19], AMPK [20], and glucagon receptor-stimulated adenylate cyclase in the liver and skeletal muscle [21–23]. The gut microbiome complements these metabolic organs, forming an intertwined system that modulates energy balance [24].

The increased C_{max} and decreased $t_{1/2\alpha}$ of metformin observed in the pseudo germ-free rats may be attributable to a decrease in Oct1 expression in the pseudo germ-free rat liver, which changes the hepatic uptake of metformin and thereby alters the shape of the drug-time curve. This phenomenon may increase the frequency of side effects, suggesting that we need to be more cautious when prescribing the combined use of antibiotics and metformin in patients with diabetes. However, the changes in AUC_{∞}, AUC_{24}, T_{max}, $t_{1/2\beta}$ and MRT have not been detected, which indicated that the bioavailability of metformin was unchanged. Previous studies reported that increased metformin exposure did not directly increase the antidiabetic effect of metformin [25,26], suggesting that the reduced antidiabetes activity in the GFMET group was unlikely to be due to a decrease in the bioavailability of metformin.

5. Conclusion

To our knowledge, this article is the first report that evaluates changes in the PD and PK of metformin in pseudo germ-free diabetic rats. Our data provide substantial evidence that the gut microbiota plays a vital role in the process of metformin's action, not just as an incidental participant, and the detailed mechanisms require further exploration.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.009.

Conflict of interest

The authors declare that they have no conflicting interests.

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