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Antibiotics-Induced Disruption of Gut Microbiota Increases Systemic Exposure of Clopidogrel Active Metabolite in Type 2 Diabetic Rats[S]

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ABSTRACT

Gut microbiota play an important role in the pathophysiology of type 2 diabetic mellitus (T2DM) and biodisposition of drugs. Our previous study demonstrated that T2DM rats had the decreased plasma exposure of clopidogrel active metabolite (Clop-AM) due to upregulation of P-glycoprotein (P-gp). However, whether the change to clopidogrel (Clop) disposition under T2DM conditions is associated with gut microbiota needs to be elucidated. In the study, we used an antibiotic cocktail consisting of ampicillin, vancomycin, metronidazole, and neomycin to disrupt gut microbiota and observed its influence on pharmacokinetic profiles of Clop-AM. Antibiotic administration markedly alleviated T2DM rats' phenotype, including hyperglycemia, insulin resistance, oxidative stress, inflammation, hyperlipidemia, and liver dysfunction. Meanwhile, treatment with antibiotics significantly reversed the reduced systemic exposure of Clop-AM in T2DM rats relative to control rats, which was associated with the decreased intestinal P-gp level that might promote Clop absorption, resulting in more Clop transformation to Clop-AM. Fecal microbiome analysis exhibited a serious disruption of gut microbiota after antibiotic treatment with the sharply reduced microbial load and the altered microbial composition. Interestingly, an in vitro study showed that antibiotics had no influence on P-gp mRNA levels in SW480 cells, suggesting that the microbiome disruption, not the direct role of antibiotics on P-gp expression, contributes to the altered P-gp level and Clop disposition in T2DM rats. The findings add new insights into the potential impact of gut microbiota on Clop biodisposition.

SIGNIFICANCE STATEMENT

Antibiotics increase systemic exposure of Clop-AM in T2DM rats, which is associated with the downregulation of P-gp levels. Antibiotics-induced disruption of gut microbiota, not the direct effect of antibiotics on P-gp and cytochrome P450 expression, contributes to the altered Clop disposition. Antibiotics also alleviate the T2DM phenotype, including hyperglycemia, hyperlipidemia, insulin resistance, liver dysfunction, and inflammation.

Introduction

Clopidogrel (Clop), the first-line antiplatelet drug combined with aspirin, has become a gold standard for preventing atherothrombosis in patients with acute coronary syndrome or undergoing percutaneous coronary interventions (Nawarskas and Montoya, 2018). However, variable interindividual response to Clop represents a significant clinical limitation. Accumulated evidence indicates that patients with acute coronary syndrome and diabetes mellitus have a high risk of Clop resistance and are referred to have a poor or no response to Clop, which causes the increased incidence of recurrence of cardiovascular events and mortality compared with nondiabetic patients (Angiolillo et al., 2005; Serebruany et al., 2008; Shuldiner et al., 2009; Samoš et al., 2016).

Clop is an oral prodrug that requires an intestinal absorption and complex liver metabolism process to generate its active metabolite, Clop-AM (Savi et al., 2000). Clop is known as a substrate of P-gp, which affects the oral absorption and bioavailability of Clop (Taubert et al., 2006). Once delivered to the liver, 85% of Clop is hydrolyzed by carboxylesterase (CES)1 to clopidogrel acid (Clop-acid), an inactive metabolite, while the remaining is two-step oxidized to Clop-AM by cytochrome P450 (P450) 2C19, 1A2, 2B6, 2C9, and 3A4 (Kazui et al., 2010). Two recent clinical studies demonstrate that Clop resistance in patients with diabetes is mainly attributed to the decreased Clop-AM generation (Erlinge et al., 2008; Angiolillo et al., 2014). Similarly, our previous study found that T2DM rats had lower systemic exposure of Clop-AM than control rats, due to P-gp upregulation-caused reduction of Clop absorption (Yao et al., 2020). Further, the underlying mechanism needs to be understood.

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ABBREVIATIONS: ABX, Antibiotic mixture; CES, carboxylesterase; Clop, clopidogrel; Clop-acid, clopidogrel acid; Clop-AM, clopidogrel active metabolite; IL, interleukin; KF, potassium fluoride; LC-MS/MS, Liquid Chromatography-Mass Spectrometry and Liquid Chromatogarphy-Tandem Mass Spectrometry; LPS, lipopolysaccharide; MTZ, metronidazole; P450, cytochrome P450; P-gp, P-glycoprotein; PK, pharmacokinetic; PXR, pregnane X receptor; T2DM, type 2 diabetic mellitus; VAN, vancomycin.

Growing evidence suggests that gut microbiota are closely associated with the onset and development of T2DM (Blandino et al., 2016). Diabetic and nondiabetic persons had obvious differences in the amount and composition of gut microbiota (Larsen et al., 2010). Patients with T2DM exhibited gut microbial dysbiosis with the decreased butyrate-producing bacteria and the increased opportunistic pathogens (Qin et al., 2012), which can cause or aggravate the T2DM phenotype. In addition, gut microbiota play an important role in the pharmacokinetic process of drugs by regulating the expression of drug-metabolizing enzymes and transporters, consequently affecting individual response to drug treatment (Collins and Patterson, 2020). Based on the double effects of gut microbiota on T2DM development and drug disposition, it is worthwhile to explore whether gut microbiota contribute to the reduced Clop-AM plasma exposure in T2DM rats.

It is widely demonstrated that antibiotics reduce the total bacterial amount, as well as cause gut microbiota disturbance (Mu and Zhu, 2019). Antibiotic treatment is an effective method to study microbiota-host interaction in animal models. Due to the broad-spectrum antibacterial characteristics and a poor intestinal absorption after oral administration, an antibiotic cocktail consisting of ampicillin, neomycin, metronidazole, and vancomycin has been demonstrated to be powerful at disrupting or depleting gut microbiota (Rodrigues et al., 2017). Here, to explore the association between gut microbiota and Clop disposition, we selected the same antibiotic cocktail to disrupt gut microbiota and observed their role in pharmacokinetic profiles of Clop and its metabolites, Clop biotransformation in liver microsomes, and Clop-metabolizing enzymes and transporter expression in T2DM rats.

Materials and Methods

Chemicals and Reagents. Clopidogrel, Clop-acid, and the stable 3′-methoxyacetophenone derivative of Clop-AM were obtained from the Beijing Institute of Pharmacology (Beijing, China). P450 probe substrate and its metabolic product: CYP1A2, phenacetin, and acetaminophen; CYP2B, bupropion, and hydroxybupropion; CYP2C9-related protein, tolbutamide, and 4-hydroxytolbutamide; CYP2C19-related protein, (S)-mephenytoin, and 4-hydroxymephenytoin; CYP3A, midazolam, and α-hydroxymidazolam were purchased from Canspec Scientific&Technology Co. Ltd. G (Shanghai, China). Ampicillin was purchased from Suzhou Meilun Biotechnology Co., Ltd (Suzhou, China). Neomycin, metronidazole (MTZ), and vancomycin (VAN) were purchased from Beijing Coolaber Biotechnology Co., Ltd (Beijing, China). Antibodies to rat P-gp, pregnane X receptor (PXR), β-actin, and Histone H3 were purchased from Abcam (Cambridge, MA).

Animals and Treatment. Male Sprague-Dawley rats at 8 weeks of age were purchased from Liaoning Changsheng Biotechnology (Benxi, China). All rats were housed at 20–25°C and 50%–60% relative humidity with a light to dark cycle every 12 hours. T2DM rats were induced by the combination of high-fat diet feeding and low-dose streptozotocin injection according to the method described previously (Reed et al., 2000). Control and T2DM rats were orally administered with either vehicle or an antibiotic cocktail consisting of ampicillin (100 mg/kg), neomycin (100 mg/kg), metronidazole (100 mg/kg), and vancomycin (50 mg/kg) for 5 consecutive days. After the last administration, some rats were fasted overnight and orally gavaged with Clop at a dose of 30 mg/kg for pharmacokinetic study; the others were sacrificed, and the serum, liver, small intestine, or feces were collected for further analysis. All animal experiments were approved by the Ethics Committee of Jilin University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Jilin University.

Cell Culture. LO_2 (human normal hepatocyte) was purchased from Procell Life Science and Technology Co., Ltd (Wuhan, China). SW480 (human colon cancer cell line) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). The two cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Bacterial Culture. Rat feces were collected and suspended in thioglycolic acid medium. The suspension was plated on a nonselective LB agar plate (EMD Millipore) and cultured at 37°C for 24 hours to assess the amount of bacterial load (Lowe et al., 2018).

Biochemical Analysis. Fasting blood glucose levels were measured from the tail vein using a blood glucose meter (Sinocare, Changsha, China). The levels of alanine aminotransferase or aspartate aminotransferase, triglycerides, and cholesterol in the serum, liver, or small intestine were analyzed by commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China). The serum insulin level was analyzed by a Rat Insulin ELISA Kit (North Institute of Biotech Co., Ltd, China). The levels of malondialdehyde and glutathione in liver and small intestine were analyzed by a commercial assay kit (Solarbio, Beijing, China). The lipopolysaccharide (LPS) level in serum, liver and small intestine was analyzed by a Rat LPS Elisa Kit (Shanghai FANKEL Industrial Co.,Ltd, China). All results in tissues were normalized by the total protein concentration in each sample.

Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis. Total RNA was extracted from tissues or cell lines using a Total RNA Kit (OMEGA, Japan), and converted to cDNA using the PrimeScriptTM reverse-transcription reagent kit (TaKaRa Biotech, Japan), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was carried out with a LightCycler480 System (Roche, Switzerland) using cDNA template, SYBR green polymerase chain reaction Master Mix (TAKARA, Beijing, China), and gene-specific primers. The sequences of primers for rat *CYP1A2*, *CYP2B1/2*, *CYP2C11*, *CYP2C22*, *CYP3A2*, *Ces1*, *P-gp*, *PXR*, and *GAPDH* were described previously (Yao et al., 2019). Forward and reverse primers for rat *interleukin (IL)-1β*, *tumor necrosis factor (TNF)-α*, *IL-6*, or human *CES1*, *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *P-gp*, *PXR*, and *GAPDH* were listed in Supplemental Table 1. The relative mRNA expression was calculated using the comparative Ct (ΔΔCt) method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for normalization.

CES or P450s Activity Assays in Liver Microsomes. The enzymatic activities of CES and all studied P450s in rat liver microsomes were measured as described previously (Yao et al., 2019).

Clop Metabolism in Liver Microsomes. Clop biotransformation to Clop-AM and Clop-acid in rat liver microsomes in the absence or presence of potassium flouoride (KF) was investigated as described previously (Yao et al., 2020). The levels of Clop-AM and Clop-acid were analyzed by Liquid Chromatography-Mass Spectrometry and Liquid Chromatogarphy-Tandem Mass Spectrometry (LC-MS/MS).

Western Blot Analysis. The protein levels of membrane P-gp and nuclear PXR in the whole small intestine were evaluated as described previously (Yao et al., 2020).

Pharmacokinetic Study. The pharmacokinetic (PK) study of Clop, Clop-AM, or Clop-acid was performed in T2DM rats and control rats with or without antibiotic treatment. As described previously (Yao et al., 2019), rats were fasted overnight, and then serial blood samples were collected at 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3.5, 6, 10, and 24 hours after an oral dose of Clop 30 mg/kg. Plasma was treated with diazepam and 3′-methoxyphenacyl bromide for determination of Clop-AM, or with acetonitrile and diazepam for determination of Clop and Clop-acid by LC-MS/MS method. Noncompartmental pharmacokinetic parameters were estimated using software DAS3.0 (Drug and Statistics Software, Mathematical Pharmacology Professional Committee of China, Shanghai, China).

LC-MS/MS Analysis. The levels of plasma Clop, Clop-AM, and Clop-acid were analyzed by LC-MS/MS, as described previously (Yao et al., 2019). Also, the five metabolites of P450s probe drugs were simultaneously determined by LC-MS/MS, as described previously (Yao et al., 2019).

16S rRNA Sequence Analysis. DNA was extracted from fecal samples using a stool DNA isolation Kit (TianGen, China). The V3-V4 variable regions of the bacterial 16S rRNA genes was amplified with universal primers 343F and 789R (343F, 5′-TACGGRAGGCAGCAG-3′; 789R, 5′-AGGGTATVTAATCCT-3′). After purified with AMPure XP beads (Agencourt), amplicons were pooled for sequencing on an Illumina MiSeq platform at Shanghai OE Biotech. Co., Ltd. The normalized operational taxonomic unit tables were used for diversity and statistical analyses through multiple-step process of the raw sequencing data. The microbial alpha-diversity or beta-diversity was calculated with Chao 1 and Shannon indexes or weighted UniFrac, respectively. The microbial distances were used for principal coordinate analysis. The relative abundance of microbiota

at the phylum, family, and genus levels was visualized by the taxonomic summary bar plots.

Statistical Analysis. Data are displayed as mean ± S.D. Statistical analysis between two groups was done using the Student's *t* test. Comparisons with *P* value of less than 0.05 were considered statistically significant. All analyses were performed using SPSS 20.0 (IBM SPSS, Chicago, IL).

Results

Antibiotic Effects on Biochemical Parameters in T2DM Rats. T2DM rats were established by a combination of high fat diet and low dose of streptozotocin treatment, as described previously (Yao et al., 2020). The biochemical parameters in the serum, liver, and small intestine were monitored in T2DM and control rats treated with an antibiotic cocktail or not. As expected, T2DM rats exhibited a significant increase in the level of serum fasting blood glucose, insulin, triglycerides, cholesterol, alanine aminotransferase, aspartate aminotransferase, or LPS, as compared with control rats (Fig. 1, A–G). Interestingly, treatment with antibiotics significantly restored these changes in T2DM rats. Likewise, the increased triglycerides, cholesterol, malondialdehyde, IL-1β, IL-6, TNF-α, and LPS levels or the decreased glutathione level in both the liver and small intestine of T2DM rats were also significantly attenuated after 5 days of antibiotic exposure (Fig. 1, H–W). These data confirm that antibiotic treatment markedly alleviates T2DM phenotypes such as hyperglycemia, insulin resistance, oxidative stress, inflammation, hyperlipidemia, and liver dysfunction.

Antibiotic Effects on Pharmacokinetics of Clop and Its Metabolites in T2DM Rats. Next, antibiotic effects on PK profiles of Clop and its two metabolites were evaluated in T2DM rats (Fig. 2). The PK parameters were listed in Table 1. T2DM rats had lower plasma exposure of Clop, Clop-AM, and Clop-acid than control rats, consistent with our previous report (Yao et al., 2020). Surprisingly, after continuous treatment with antibiotics, the area under the curve (AUC) and C_{max} of Clop, Clop-AM, or Clop-acid were significantly elevated in T2DM rats, with $AUC_{0\text{-}t}$ increased by 9-, 3.1-, or 4.7-fold and C_{max} increased by 4-, 7.8-, or 14.4-fold, respectively.

Antibiotic Effects on Hepatic CES1 and P450s Levels in T2DM Rats. The generation of Clop-AM and Clop-acid is dependent on the relative expression of P450s and CES1. To investigate the altered systemic exposure of Clop metabolites in T2DM rats pre and pro antibiotic treatment, the mRNA levels of 6 rat-specific *Ces1e* and *P450*s genes were measured in the liver. As shown in Fig. 3, A–F, T2DM rats exhibited the decreased mRNA levels of *Ces1e*, *CYP2B1/2*, *CYP2C22*, and *CYP2C11* as compared with control rats, although antibiotic treatment significantly alleviated these changes. In contrast, the mRNA levels of *CYP1A2* and *CYP3A2* were significantly increased in T2DM rats, but with no obvious change after antibiotic treatment.

Similar trends were also found in enzymatic assay of liver microsomes (Fig. 3, G–L). T2DM rats had higher activities of CES1, CYP2B, CYP2C9-, and CYP2C19-related protein and lower activities of CYP1A2 or CYP3A than control rats. Antibiotic treatment markedly blunted the alteration of CES1, CYP2B, CYP2C9-, or CYP2C19-related protein activity ($P < 0.05$), but had no apparent impact on CYP1A2 or CYP3A activity.

Clop Metabolism in Liver Microsomes. The Clop metabolism in rat liver microsomes was evaluated to verify the impact of CES1 and P450s on systemic exposure of Clop-acid and Clop-AM.

Clop-AM generation was significantly enhanced in T2DM rat microsomes incubated with Clop in the absence and presence of KF, an esterase inhibitor, although the increased Clop-AM level was slightly attenuated in antibiotics-treated T2DM rat microsomes (Fig. 4A), in

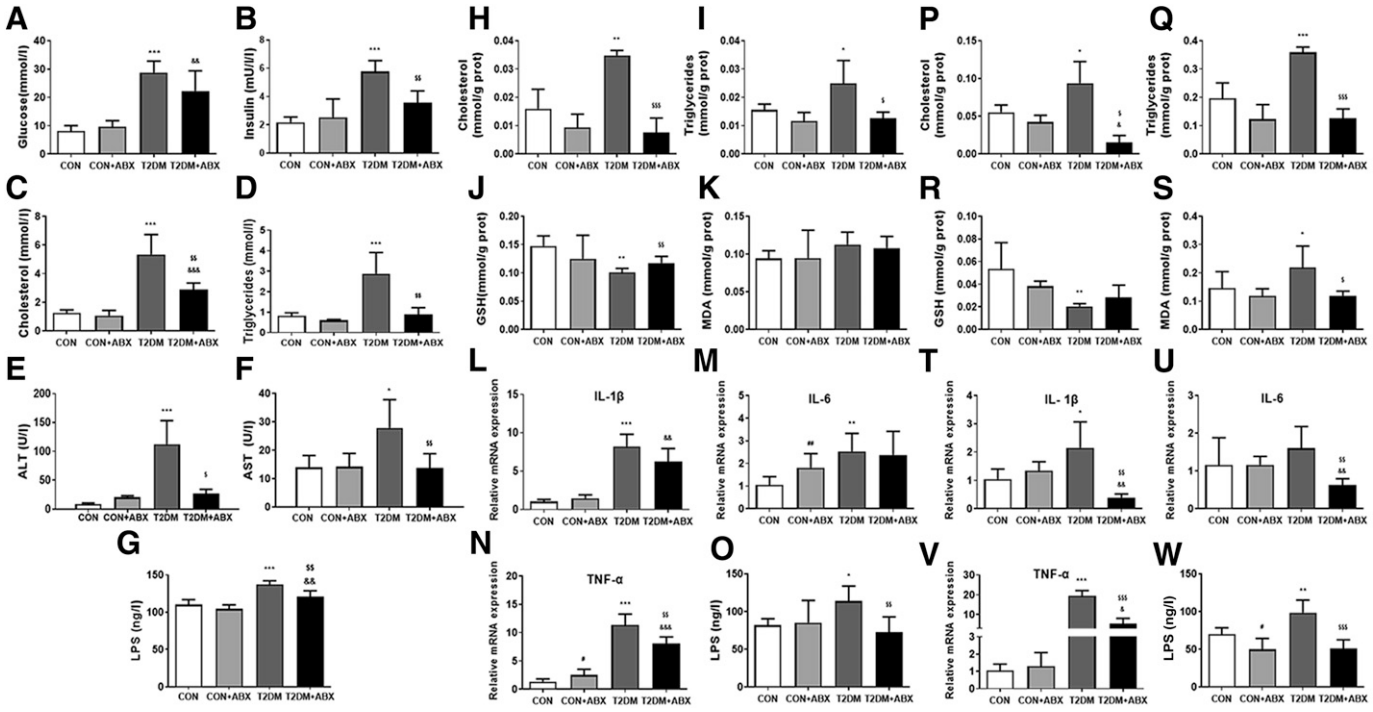

Fig. 1. The physical and biochemical parameters in CON, T2DM, CON+ABX, and T2DM+ABX rats. (A) Glucose, (B) Insulin, (C) Cholesterol, (D) Triglycerides, (E) alanine aminotransferase (ALT), (F) aspartate aminotransferase (AST), and (G) LPS levels in serum. (H) Cholesterol, (I) Triglycerides, (J) glutathione (GSH), (K) malondialdehyde (MDA), (L) IL-1β mRNA, (M) IL-6 mRNA, (N) TNF-α mRNA, and (O) LPS levels in liver. (P) Cholesterol, (Q) Triglycerides, (R) GSH, (S) MDA, (T) IL-1β mRNA, (U) IL-6 mRNA, (V) TNF-α mRNA, and (W) LPS levels in small intestine. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (T2DM versus CON group); $^{\#}P < 0.05$ (CON+ABX versus CON group); $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ (T2DM+ABX versus T2DM group); $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ (T2DM+ABX versus CON+ABX group). CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

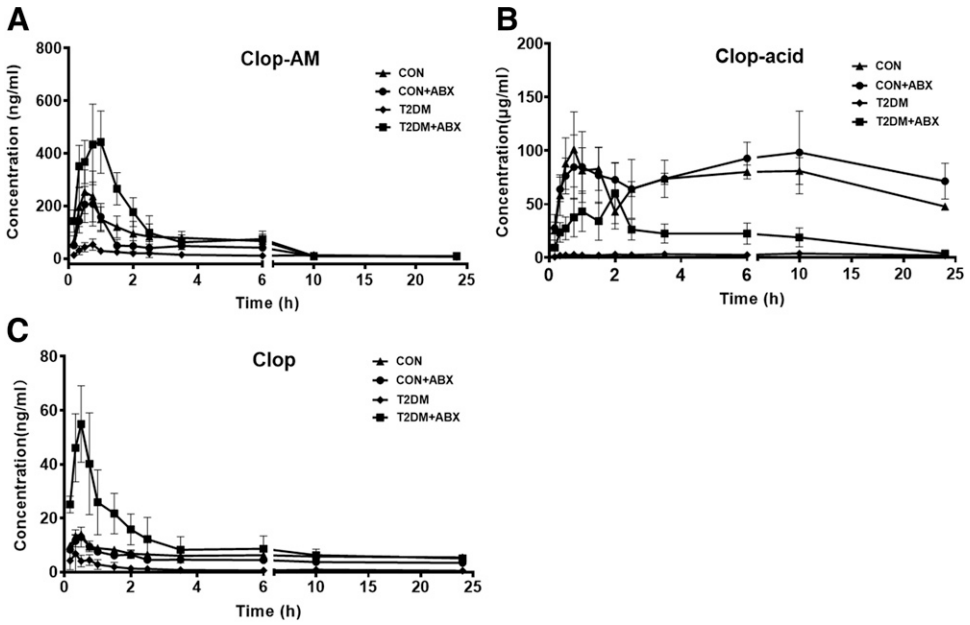

Fig. 2. Plasma concentration-time curves of (A) Clop-AM, (B) Clop-acid, and (C) Clop in CON, T2DM, CON+ABX, and T2DM+ABX rats after single oral 30mg/kg doses of Clop. CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

contrast to in vivo results. This suggests that Clop hepatic metabolism might not be responsible for the elevated plasma exposure of Clop-AM in T2DM rats after antibiotic treatment.

As for Clop-acid, there was significantly lower Clop-acid generation in liver microsomes of T2DM rats than in that of control rats, whereas antibiotic treatment reversed the change, especially in the presence of KF, consistent with in vivo results (Fig. 4B). An explanation may be that antibiotic treatment attenuated the downregulated CES1 levels in T2DM rats. Meanwhile, this suggests that CES1-based Clop hydrolyzation is attributed, at least partly, to the change in Clop-acid exposure in T2DM rats pre and pro antibiotic treatment.

Antibiotic Effects on Intestinal P-gp and PXR Levels in T2DM Rats. To explore whether intestinal absorption contributes to the alteration of systemic exposure of Clop and its metabolites in antibiotics-treated T2DM rats, the mRNA and protein levels of P-gp and its transcription factor, PXR, in small intestine were measured (Fig. 5). T2DM significantly elevated the mRNA and protein levels of both P-gp and PXR in the small intestine, whereas antibiotics treatment reversed these changes. According to our previous studies demonstrating that P-gp is the key factor affecting the oral absorption and bioavailability of Clop (Yao et al., 2020), it is considered that antibiotics-caused reduction of P-gp level promotes Clop absorption, causing more Clop biotransformation to Clop-AM.

Antibiotic Effects on the Expression of CES1, P450s, P-gp, or PXR in Cell Lines. Although it is widely demonstrated that the selected four antibiotics have a poor or no absorption after oral administration (Reikvam et al., 2011; Zarrinpar et al., 2018), the direct role of antibiotic on Clop-metabolizing enzyme and transporter levels cannot be ignored. Next, we explored the impact of antibiotics on the expression of CES1, P450s, P-gp, or PXR in cell lines. The four antibiotics each at a concentration of less than 100 μM or their combination [Antibiotic mixture (ABX)] at 1:8 or 1:16 dilution had no significant effects on cell viabilities in both LO_2 and SW480 cells using MTT assay (Supplemental Fig. 1). However, there was a significant decrease in the mRNA level of *CYP1A2*, *CYP2B6*, or *CYP2C19* after treatment with four antibiotics either alone or their combination at different concentrations (Fig. 6, B, C, and E). Also, the *CYP2C9* level was reduced by MTZ at both concentrations or ABX at 1:8 dilution (Fig. 6D). In contrast, the *CYP3A4* expression was enhanced by neomycin (100 μM), VAN (25 μM), and ABX at 1:16 dilution (Fig. 6F). Of interest, antibiotic mixture at both dilutions did not affect the mRNA level of *CES1,* as well as *P-gp* and *PXR,* although the *P-gp* level was increased by VAN at both concentrations and the *PXR* level was decreased by MTZ at 100 μM (Fig. 6 A, G, and H).

Then, effects of antibiotics on mRNA, protein, or enzymatic level of Clop-metabolizing enzymes and transporters were compared between

TABLE 1

PK parameters of Clop, Clop-AM, and Clop-acid after oral administration of Clop

Parameter Unit		AUC_{0-t} (mg/l*h)	$AUC_{0-\infty}$ (mg/l*h)	C_{max} (mg/l)	T_{max} (h)	$t_{1/2}$ (h)
Clop-AM	CON	0.86 ± 0.37	0.93 ± 0.4	0.25 ± 0.08	0.75 ± 0.1	8.1 ± 7.2
	CON+ABX	0.62 ± 0.11	0.72 ± 0.12	0.21 ± 0.08	0.5 ± 0.1	9.6 ± 8.5
	T2DM	0.30 ± 0.13**	0.49 ± 0.21*	0.05 ± 0.02**	0.75 ± 0.1	18.2 ± 14.3**
	T2DM+ABX	1.23 ± 0.5$^{\$\$,\&\&}$	1.33 ± 0.6$^{\$\$,\&\&}$	0.44 ± 0.12$^{\$\$,\&\&}$	1.0 ± 0.1	7.0 ± 5.9$^{\$\$}$
Clop-Acid	CON	1653.6 ± 486.1	1727.8 ± 654.1	101.2 ± 35.1	0.75 ± 0.1	22.3 ± 15.3
	CON+ABX	2024.2 ± 732.8	1945.7 ± 654.3	98.4 ± 33.1	10 ± 5.1	41.7 ± 18.3*
	T2DM	72.6 ± 392.7**	187.2 ± 110.9**	3.9 ± 1.8**	10 ± 4.2	38.1 ± 19.2*
	T2DM+ABX	414.7 ± 197.3$^{\$\$,\&\&}$	459.1 ± 218.4$^{\$\$,\&\&}$	60.2 ± 18.8$^{\$\$,\&}$	2.0 ± 1.2$^{\$,\&}$	7.7 ± 7.1$^{\$\$,\&\&}$
Clop	CON	0.15 ± 0.02	0.14 ± 0.02	0.02 ± 0.01	0.5 ± 0.1	41.8 ± 16.4
	CON+ABX	0.1 ± 0.02	0.1 ± 0.07	0.02 ± 0.01	0.5 ± 0.1	46.6 ± 14.2
	T2DM	0.02 ± 0.01**	0.04 ± 0.03**	0.01 ± 0.01	0.33 ± 0.1	27.5 ± 18.2*
	T2DM+ABX	0.2 ± 0.08$^{\$\$}$	0.4 ± 0.02$^{\$\$,\&}$	0.05 ± 0.01	0.5 ± 0.1	26.3 ± 21.2$^{\&}$

AUC, area under the curve.
*P < 0.05; **P < 0.01 (T2DM versus CON group); $^{\$}P$ < 0.05; $^{\$\$}P$ < 0.01 (T2DM+ABX versus T2DM group); $^{\&}P$ < 0.05; $^{\&\&}P$ < 0.01 (T2DM+ABX versus CON+ABX group).

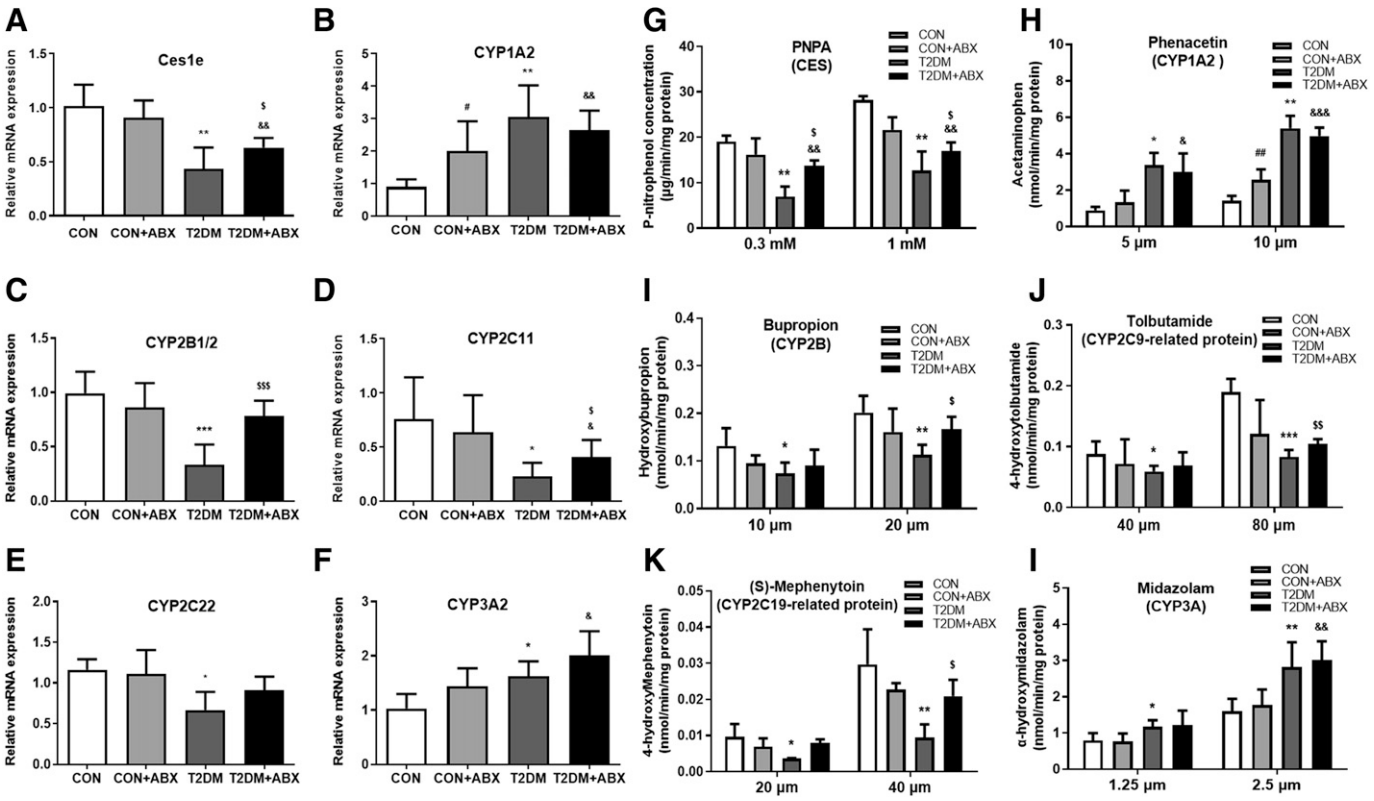

Fig. 3. Hepatic mRNA levels of (A) Ces1, (B) CYP1A2, (C) CYP2B1/2, (D) CYP2C11, (E) CYP2C22, and (F) CYP3A2 in CON, T2DM, CON+ABX, and T2DM+ABX rats. Hepatic microsomal activities of (G) CES, (H) CYP1A2, (I) CYP2B, (J) CYP2C9-related protein, (K) CYP2C19-related protein, and (L) CYP3A in CON, T2DM, CON+ABX, and T2DM+ABX rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (T2DM versus CON group); $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ (CON+ABX versus CON group); $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ (T2DM+ABX versus T2DM group); $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ (T2DM+ABX versus CON+ABX group). CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

in vivo and in vitro, as shown in Table 2. There were different patterns of change in the mRNA and protein levels of P-gp and PXR between T2DM rats and SW480 cells after antibiotic treatment. Also, inconsistent change was observed in the mRNA or enzymatic levels of Clop-metabolizing enzymes between T2DM rats and LO_2 cells. This suggests that antibiotics-caused change of P-gp and P450s levels in vivo might not be the results of the direct role of antibiotics on P-gp and P450s expression.

Antibiotic Effects on Community Structure and Diversity of Gut Microbiota. To investigate the influence of the antibiotic cocktail on gut microbiota, feces samples were collected and cultured in aerobic conditions after the last administration. There was no apparent difference in the number of fecal aerobic bacteria population in both rats, whereas antibiotic treatment achieved a decrease of 99.1% or 96.5% in T2DM rats or control rats, respectively (Fig. 7A), indicating a serious disruption or depletion of gut microbiota.

Then, 16S rRNA sequencing analysis was performed to determine the bacterial composition in fecal samples of different groups. As indicated by Chao1 and Shannon indices, T2DM rats had lower richness and diversity of gut microbiota than control rats, whereas antibiotic treatment caused their significant decrease in both rats (Fig. 7, B–C). The principal component analysis plot showed the clear separation of microbiota composition among T2DM rats, control rats, or antibiotics-treated rats (Fig. 7D).

Further, the structure and composition of gut microbiota at phylum, family, or genus levels were shown in Fig. 7, E–G. The microbial

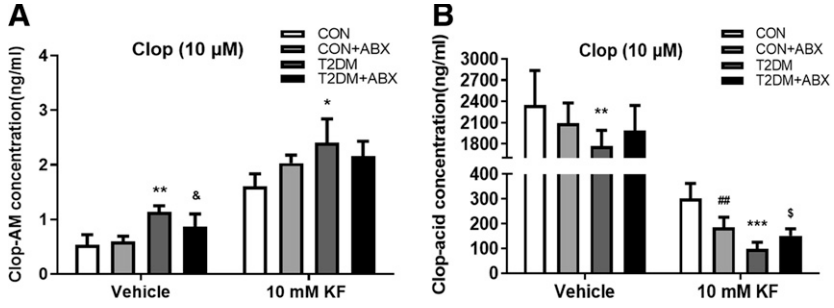

Fig. 4. (A) Clop-AM and (B) Clop-acid levels in liver microsomes of CON, T2DM, CON+ABX, and T2DM+ABX rats incubated with Clop at concentrations of 10μM for 100 minutes in the presence or absence of 10 mM KF. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (T2DM versus CON group); $^{\#\#}P < 0.05$ (CON+ABX versus CON group); $^{\$}P < 0.05$ (T2DM+ABX versus T2DM group); $^{\&}P < 0.05$ (T2DM+ABX versus CON+ABX group). CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

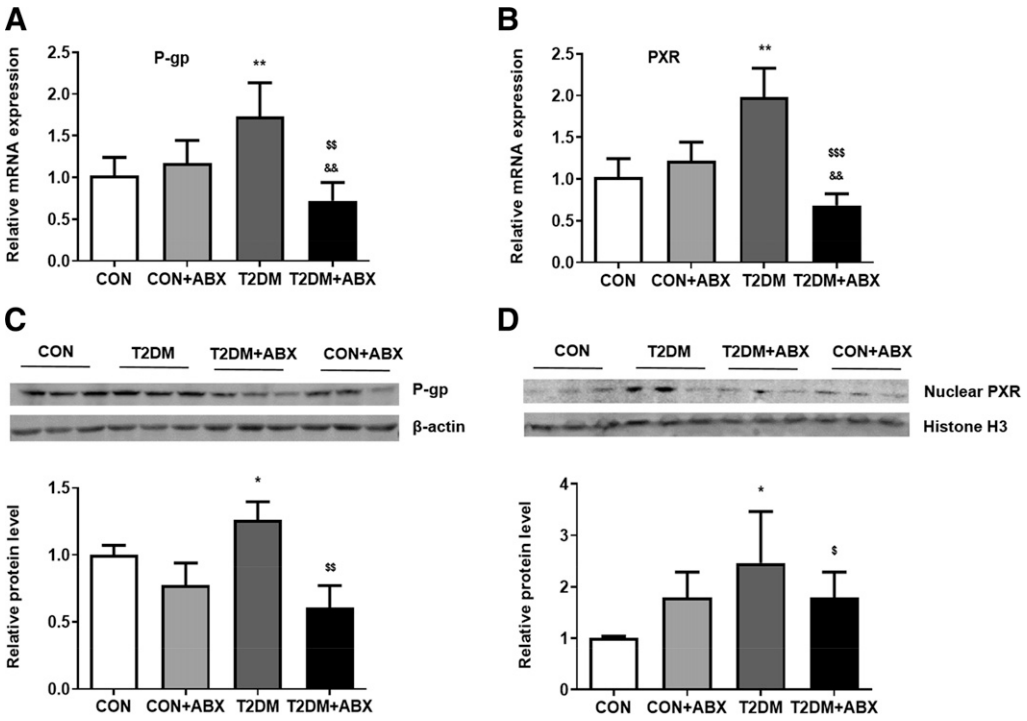

Fig. 5. Intestinal mRNA and protein levels of P-gp and PXR in CON, T2DM, CON+ABX, and T2DM+ABX rats. (A) P-gp and (B) PXR mRNA levels in small intestines; (C) P-gp and (D) PXR protein levels in small intestines. $^{*}P < 0.05$, $^{**}P < 0.01$ (T2DM versus CON group); $^{\#}P < 0.05$, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ (T2DM+ABX versus T2DM group); $^{\&\&}P < 0.01$ (T2DM+ABX versus CON+ABX group). CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

structure in T2DM rats was obviously different from that in control rats. T2DM rats showed considerably elevated proportions of Bacteroidetes or Proteobacteria and reduced proportions of Firmicutes at phylum level compared with control rats (Fig. 7E). Likewise, the decreased relative abundance of Muribaculaceae, Lachnospiracea, and Ruminococcaceae and the increased relative abundance of Enterobacteriaceae and Prevotellaceae at family level were also observed in T2DM rats (Fig. 7F). At genus level, there was a predominant increase in the relative abundance of Klebsiella, Prevotella-9, and Lachnospiraceae_NK4A136_group and a decrease in the relative abundance of Ruminococcaceae_UCG-014, Ruminococcus_1, and Ruminococcaceae_UCG-005 in T2DM rats (Fig. 7G). Antibiotics had a profound effect on microbiota composition, with Proteobacteria, Enterobacteriaceae, or Klebsiella being the dominant phylum, family, or genus, respectively (>90%), in T2DM rats and control rats.

Discussion

Antibiotic-caused disruption of gut microbiota has been widely used to study microbiota- host interaction in pathologic conditions (Rakoff-Nahoum et al., 2004; Shen et al., 2015; Sampson et al., 2016). The current study explored the influence and underlying mechanism of an antibiotic cocktail on Clop disposition in T2DM rats. The results showed that antibiotic administration significantly increased the systemic exposure of Clop, Clop-AM, and Clop-acid in T2DM rats, which was associated with the downregulation of P-gp in the small intestine.

Increasing evidence indicates a complex relationship between gut microbiota and T2DM (Sikalidis and Maykish, 2020). In the present study, T2DM rats exhibited gut microbial disturbance with the decreased abundance of Firmicutes, the increased abundance of Bacteroidetes or Proteobacteria, and the increased ratio of Bacteroidetes to Firmicutes, all of which have also been observed in patients with diabetes (Qiao et al., 2013). Interestingly, we found that these microbial changes could be associated with some T2DM phenotypes, as follows. First, a previous study found that the ratio of Bacteroidetes to Firmicutes was associated positively with blood glucose levels (Larsen et al., 2010), consistent with our study. Second, it was demonstrated that the decreased prevalence of Firmicutes in T2DM rats produced a low level of butyrate (Macfarlane and Macfarlane, 2003). Importantly, sodium butyrate treatment markedly ameliorated diabetic inflammation in db/db mice (Xu et al., 2018). It is considered that the decreased abundance of Firmicutes and its two families, Lachnospiracea and Ruminococcaceae, in T2DM rats might be associated with the enhanced levels of LPS and proinflammatory factors in our study. Third, Proteobacteria is shown to play an important role in promoting the occurrence of low-grade inflammation in patients with diabetes (Larsen et al., 2010), suggesting that the increased prevalence of Proteobacteria in the study contributes, at least in part, to the inflammation status in T2DM rats. Finally, our results also showed that T2DM rats had a predominant increase in the abundance of *Prevotella-9* genus, which has been demonstrated to have a close association with insulin resistance (Pedersen et al., 2016).

Antibiotics alone or in combination have been widely used to investigate the role of gut microbiota in vivo (Mu and Zhu, 2019). Here, we selected an antibiotic cocktail to disrupt gut microbiota and observed their role in T2DM phenotype and Clop disposition. The results showed that 5 days of antibiotic treatment sharply reduced the microbial amount (>95%) and dramatically altered the microbial structure and composition with Proteobacteria, Enterobacteriaceae, or Klebsiella being the dominant phylum, family, or genus, respectively (each >90%), indicating a serious disruption of gut microbiota. Interestingly, antibiotics-caused microbial disruption restored T2DM phenotype. Similar results were also observed in the other two T2DM animal models. One study showed that removing gut microbiota with norfloxacin and ampicillin significantly decreased plasma glucose, insulin, LPS, and triglycerides levels in ob/ob mice (Chou et al., 2008). The other study showed that after two weeks of treatment with ceftazidime, Zucker Diabetic Fatty

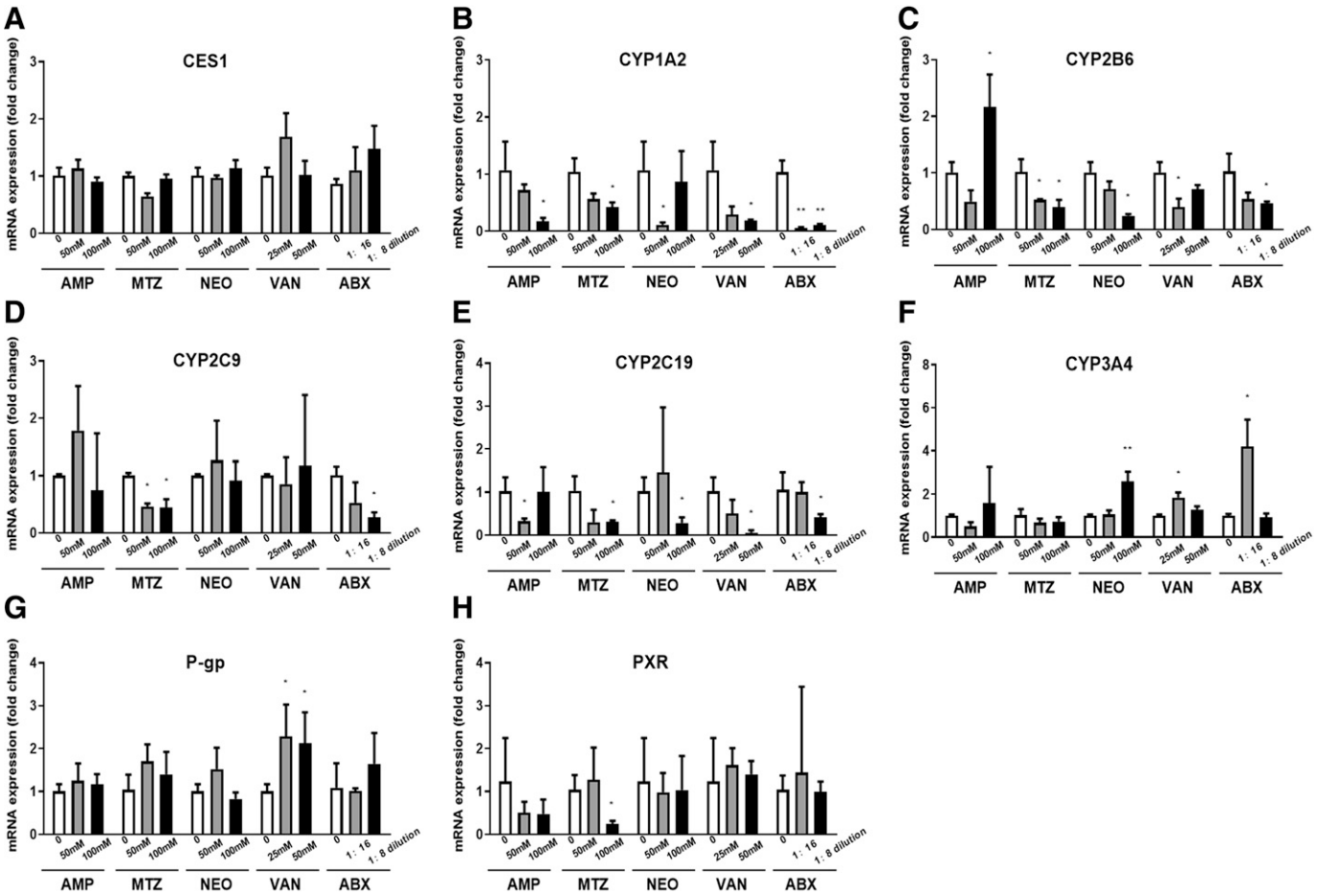

Fig. 6. The mRNA levels of Clop-metabolizing enzymes and transporters in vitro. Cells were treated with vehicle, Ampicillin (AMP), Metronidazole (MTZ), Neomycin (NEO), or Vancomycin (VAN) alone and their combination, including AMP (100 mM), MTZ (100 mM), NEO (100 mM), and VAN (50 mM) at different concentrations or dilutions for 24h. (A) CES1, (B) CYP1A2, (C) CYP2B6, (D) CYP2C9, (E) CYP2C19, and (F) CYP3A4 mRNA levels in LO_2 cells; (G) P-gp and (H) PXR mRNA levels in SW480 cells. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control groups.

(ZDF) rats showed markedly lower plasma glucose levels than control rats (Rajpal et al., 2015). Similarly, Zhou et al. (2016) found that treatment with antibiotic mixture (ampicillin, metronidazole, and neomycin) for 7 days exerted glucose-lowering action in high fat diet (HFD)-fed mice, as metformin did. Then, metformin in combination with antibiotics had a stronger role in improving glucose intolerance than metformin or antibiotics alone (Zhou et al., 2016), implying that antibiotics could reduce the dosage of metformin during glucose-lowering therapy. However, whether the phenomenon also occurs in diabetic or hyperlipemia patients has not been reported. It seems that translating this strategy to humans is not the best option because long-term use of antibiotics in clinics has some drawbacks, such as antibiotic resistance, gut microbiota dysbiosis, and body weight gain. We also found that antibiotic treatment had a similar disruption of gut microbiota in control rats, but had no apparent role in serum glucose, insulin, triglycerides, cholesterol, alanine aminotransferase, aspartate aminotransferase, and LPS levels, which has also been observed in healthy human males and chow-fed C57BL6 mice (Pang et al., 2013; Mikkelsen et al., 2016), but not in healthy Swiss Webster mice or normal C57BL/6 mice (Rodrigues et al., 2017; Zarrinpar et al., 2018). The debatable results might be due to differences in antibiotic ingredients, dosage, and duration. Taken together, these results suggest that antibiotic-induced microbiome depletion alleviates metabolic disorders in pathologic conditions, but has debatable effects on normal physiology.

TABLE 2

Effect of antibiotics on Clop-metabolizing enzymes and transporter level in vitro and in vivo

ABX in T2DM Rats		ABX in LO_2 or SW480 Cells	
mRNA/Activity		mRNA	
CES1/CES	↑	CES1	—
CYP1A2/CYP1A2	—	CYP1A2	↓
CYP2B1/2/CYP2B6	↑	CYP2B6	↓
CYP2C11 /CYP2C9	↑	CYP2C9	↓
CYP2C22/CYP2C19	↑	CYP2C19	↓
CYP3A2 / CYP3A4	—	CYP3A4	↑
mRNA/Protein		mRNA	
P-gp/P-gp	↓	P-gp	—
PXR/Nuclear PXR	↓	PXR	—

ABX, an antibiotic cocktail including ampicillin, metronidazole, neomycin and vancomycin.

Although antibiotics are well known to cause alteration in gut microbiota, there is a lack of understanding about their effects on Clop disposition. Our results first found that antibiotic treatment significantly reversed the reduced plasma exposure of Clop, Clop-AM, or Clop-acid in T2DM rats relative to control rats. Further, to elucidate the underlying mechanisms, we analyzed the hepatic Clop-metabolizing enzyme levels and fulfilled the Clop biotransformation in liver microsomes. As for T2DM rats, antibiotic treatment significantly restored the decreased expression or activity of CES, CYP2B, CYP2C9-, or 2C19-related protein, but had no effects on the change of CYP1A2 and CYP3A.

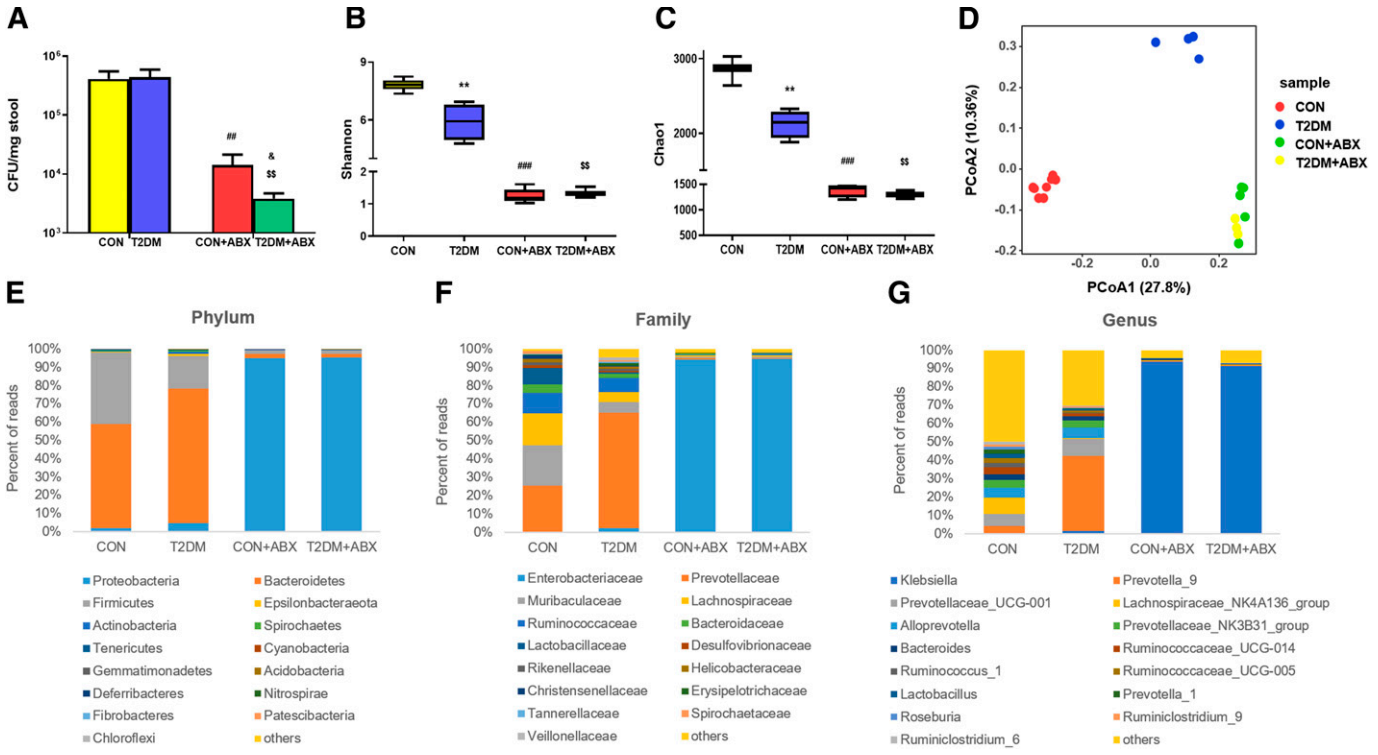

Fig. 7. (A) Colony-forming units (CFUs) were quantified from stool of CON, T2DM, CON+ABX, and T2DM+ABX rats. 16S rRNA sequencing analysis of isolated fecal DNA across the different groups (B) Shannon index; (C) Chao1 index; (D) principal coordinates analysis (PCoA); Taxonomic plots showing mean bacterial abundance at the (E) phylum, (F) family, and (G) genus levels. $^{**}P < 0.01$ (T2DM versus CON group); $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$ (CON+ABX versus CON group); $^{\$\$}P < 0.01$ (T2DM+ABX versus T2DM group). CON, control group; CON+ABX, antibiotics-treated control group; T2DM, T2DM group; T2DM+ABX, antibiotics-treated T2DM group.

Meanwhile, liver microsome metabolism assay showed that antibiotic treatment reversed the reduced Clop-acid generation in T2DM rats compared with control rats, consistent with the in vivo results, suggesting that CES1-based Clop hydrolyzation is attributed, at least partly, to the change in plasma Clop-acid exposure in T2DM rats pre and pro antibiotic treatment. Antibiotic treatment only slightly inhibited the enhanced Clop-AM generation in T2DM rat microsomes with or without KF, in contrast to the in vivo results, suggesting that Clop hepatic metabolism might not contribute to the altered plasma Clop-AM level in T2DM rats treated with antibiotic or not.

Further, to reveal the potential influence of absorption on the altered Clop disposition in T2DM rats pre and pro antibiotic treatment, the intestinal P-gp and PXR levels were measured. T2DM rats had higher mRNA and protein levels of P-gp and nuclear PXR than control rats, although antibiotic treatment significantly reversed these changes. In combination with our previous study demonstrating that T2DM rats had the decreased Clop-AM exposure due to the upregulation of P-gp (Yao et al., 2020), we suppose that the decreased P-gp level after antibiotic treatment promotes Clop absorption, then enhances the systemic exposure of Clop and its two metabolites.

We observed the effect of the microbial depletion on the altered P-gp level. However, the current data have some limitations in providing more information to find specific bacteria that play a direct role in P-gp regulation, which would be performed by fecal microbiota transplantation experiment in combination with metagenomic analysis and metabolomic analysis. Interestingly, the decreased prevalence of butyrate-producing Firmicutes phylum was observed in the feces of T2DM rats. Meanwhile, 16s rRNA-amplicon-based prediction of microbiome function showed the reduced biosynthesis of secondary bile acid (data not shown). It has been reported that the two classes of microbiota-derived metabolites affect P-gp expression and function in vitro and in vivo (Foley et al., 2021). We suppose that some butyrate- or secondary bile acid-producing bacteria might be associated with the altered P-gp level and Clop-AM exposure in T2DM rats, which needs to be demonstrated in the future.

It has been demonstrated that ampicillin, vancomycin, neomycin, and metronidazol are broad-spectrum antibiotics that have a poor or no absorption in the gut, and thus with no obvious systemic effects (Reikvam et al., 2011; Zarrinpar et al., 2018). Thus, it is considered that the orally administered antibiotics have little or no direct effect on P450s and P-gp expression in vivo. In particular, there were different patterns of change in the mRNA levels or catalytic activities of Clop-metabolizing enzymes and transporters between T2DM rats and LO_2/SW480 cells after antibiotic treatment. This suggests the changes of P450s and P-gp level in antibiotics-treated T2DM rats might not be associated with the direct role of antibiotics. Due to their lower level and activity of P450s, hepatic cell lines have some limitations to surrogate primary hepatocyte for P450 induction and inhibition study (Guo et al., 2011). Thus, the results in LO_2 cells should be verified in primary hepatocytes in the future.

In addition, diabetes has been shown to alter the expression of P450s and P-gp via some factors such as glucose, insulin, nitric oxide, cytokines, gut microbiota, and its metabolites (Lam et al., 2010; Kobori et al., 2013; Chen et al., 2018). We found that antibiotic treatment disrupted gut microbiota accompanied with the attenuation of T2DM phenotype. Especially, there were synchronous changes in P-gp and glucose/insulin/LPS/cytokines levels in T2DM rats pre and pro antibiotic treatment, implying that besides gut microbiota disruption, T2DM phenotype attenuation might also be responsible for the reduced P-gp

level and the enhanced Clop-AM exposure after antibiotic administration. Thus, it is considered that there is a close relationship between gut microbiota, T2DM phenotype, and Clop disposition.

In conclusion, the results demonstrated that antibiotics-induced disruption of gut microbiota increased systemic exposure of Clop-AM and alleviated diabetic phenotype in T2DM rats. Gut microbiota modulation might be an effective therapeutic strategy to increase Clop-AM generation under T2DM conditions.

Acknowledgments

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Authorship Contributions

Participated in research design: Gu, Guo.

Conducted experiments: Chen, Liu, Yao.

Contributed new reagents or analytic tools: Liu, Yao.

Performed data analysis: Chen, W. Song.

Wrote or contributed to the writing of the manuscript: Chen, Y. Song, Guo.

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Antibiotics-induced disruption of gut microbiota increases systemic exposure of clopidogrel active metabolite in type 2 diabetic rats

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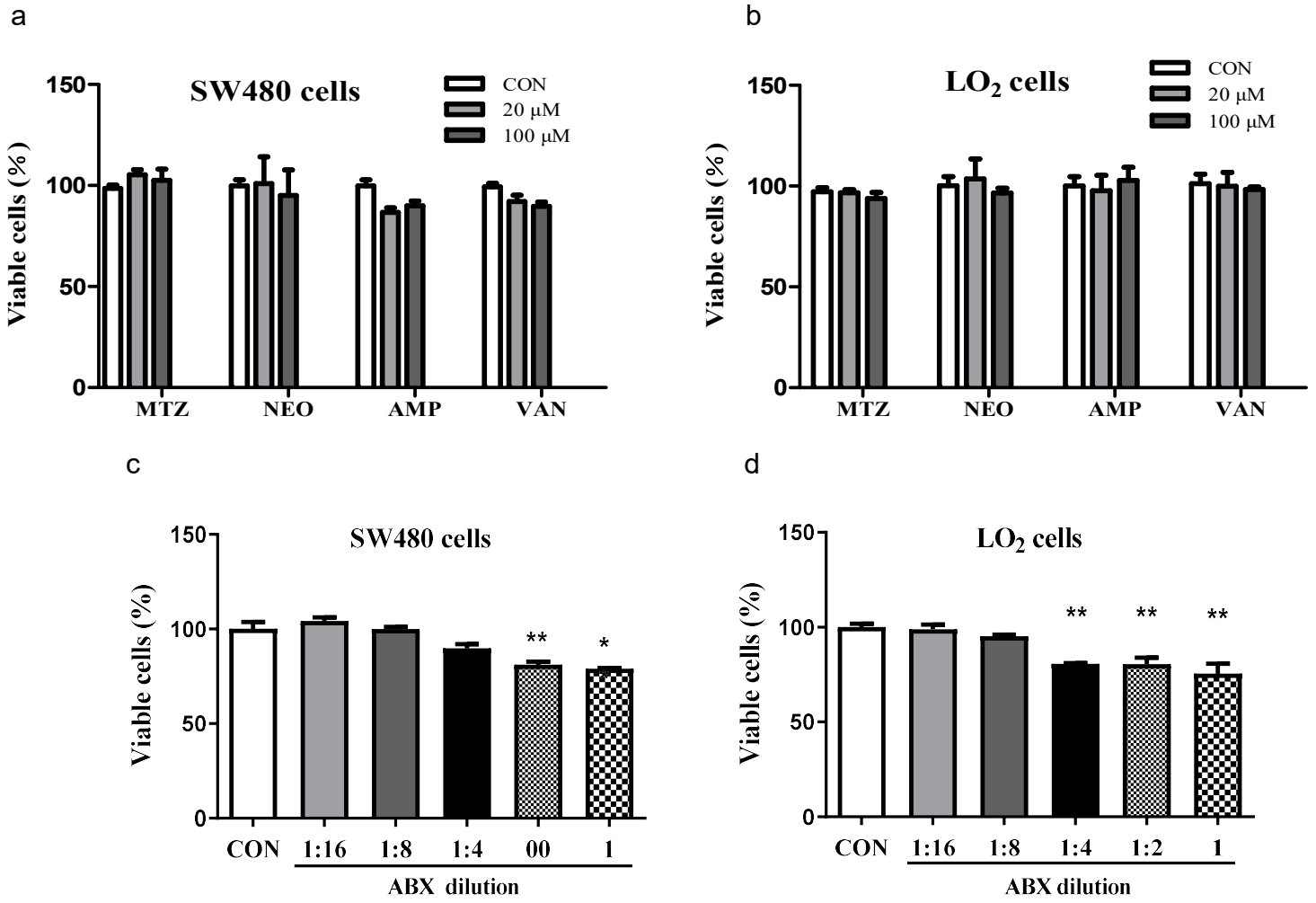
Drug Metabolism and Disposition

DMD-AR-2022-000906

Supplementary Fig.1



Supplementary Fig.1 The cell viability was detected by MTT assay. (a, c) SW480 or (b, d) LO2 cells were treated with vehicle, Ampicillin (AMP), Metronidazole (MTZ), Neomycin (NEO) or Vancomycin (VAN) alone and their combination including AMP (100 mM), MTZ (100 mM), NEO (100 mM), and VAN (50 mM) at different concentrations or dilutions for 24h. $^{**}P < 0.01$ compared to the control groups. ABX: an antibiotic cocktail including ampicillin, metronidazole, neomycin and vancomycin.

Antibiotics-induced disruption of gut microbiota increases systemic exposure of clopidogrel active metabolite in type 2 diabetic rats

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Drug Metabolism and Disposition

DMD-AR-2022-000906

Supplementary Table 1. Primer sequences used for qRT-PCR analysis

	Primer sequences	
Human gene		
CES1	F 5'-AGAGGAGCTCTTGGAGACGAC-3'	R 5'-ACTCCTGCTTGTTAATTCCGAC-3'
CYP1A2	F 5'-CCTCCTTCTTGCCCTTCACC-3'	R 5'-GGATGTAGAAGCCATTCAGCG-3'
CYP2B6	F 5'-CATCATCCCCAAGGACACAG-3'	R 5'-AAATCCGCTTCCCTAAGGAG-3'
CYP3A4	F 5'-TGCAGGAGGAAATTGATGCA-3'	R 5'-GTCAAGATACTCCATCTGTAGCA-3'
CYP2C9	F 5'-CGGATTTGTGTGGGAGAAGCCC	R 5'-GCGGCACAGAGGCAAATCCAT-3'
CYP2C19	F 5'-CCACATGCCCTACACAGATG-3'	R 5'-GGTCCTTTGGGTCAATCAGA-3'
P-gp	F 5'- GGGCACAAACCAGACAAC -3'	R 5'- TCCGCTCTTCACCTTCAGAT -3'
PXR	F 5'-CAAGCGGAAGAAAAGTGAAC-3	R 5'-TGAAATGGGAGAAGGTAGTG-3'
GAPDH	F 5'-CCCATCACCATCTTCCAGGAG-3'	R 5'-GTTGTCATGGATGACCTTGGC-3'
Rat gene		
IL-6	F 5'-TCTCTCCGCAAGAGACTTCCA-3'	R 5'-ATACTGGTCTGTTGTGGGTGG-3'
IL-1β	F 5'-GTGGCAGCTACCTATGTCTTGC-3'	R 5'-CCACTTGTTGGCTTATGTTCTGT-3'
TNF-α	F 5'-CCACCACGCTCTTCTGTCTACTG-3'	R 5'-GGGCTACGGGCTTGTCACT-3'