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Sodium-Glucose Cotransporter-2 Inhibition Exacerbates Hepatic Encephalopathy in Biliary Cirrhotic Rats

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ABSTRACT

In liver cirrhosis, hepatic inflammation and abundant portal-systemic collaterals are indicated for the development of hepatic encephalopathy. Sodium-glucose cotransporter-2 (SGLT-2) inhibitors are a type of anti-diabetic agent which exert pleiotropic and anti-inflammatory effects. Diabetes and chronic liver disease often coexist, but the influence of SGLT-2 inhibition on liver cirrhosis and hepatic encephalopathy remains unknown. This study investigated the effect of SGLT-2 inhibition on cirrhotic rats. Biliary cirrhosis was induced in Sprague-Dawley rats via common bile duct ligation. A total of two weeks of treatment with the SGLT-2 inhibitor, empagliflozin 30 mg/kg/d, was applied. The motor activities, hemodynamics, biochemistry parameters, plasma levels of vascular endothelial growth factor (VEGF), and the severity of portal-systemic collateral shunts were measured. The hepatic histopathology and protein expressions were examined. We found that empagliflozin treatment did not affect hemodynamics, liver biochemistry, or blood glucose levels in cirrhotic rats. Empagliflozin did not affect hepatic inflammation and fibrosis. The protein expression of factors related to liver injury were not influenced by empagliflozin. However, empagliflozin decreased motor activities in cirrhotic rats and increased portal-systemic collateral shunts and VEGF plasma levels. In summary, SGLT-2 inhibition by empagliflozin did not ameliorate portal hypertension and hepatic inflammation in cirrhotic rats. In contrast, it exacerbated hepatic encephalopathy, which was evidenced by a decrease in motor activity. A possible mechanism could be an increase of portal-systemic shunts related to VEGF upregulation. Therefore, empagliflozin use should be cautious in cirrhotic patients regarding the development of hepatic encephalopathy.

SIGNIFICANCE STATEMENT

Sodium-glucose cotransporter-2 inhibition by empagliflozin did not ameliorate portal hypertension and hepatic inflammation in cirrhotic rats. In contrast, it exacerbated hepatic encephalopathy through increased portal-systemic shunts related to VEGF up-regulation.

Introduction

In liver cirrhosis, chronic inflammation and fibrosis increase intrahepatic resistance, which leads to portal hypertension and the formation of portal-systemic shunts. Hepatic encephalopathy (HE) is a neuropsychiatric complication of liver cirrhosis, presenting as altered mental status and decreased motor activities. HE is induced by circulatory toxins, especially ammonia, which bypass the liver via the portal-systemic collateral vessels to the central nervous system and which consequently induce astrocyte inflammation and brain edema (Wijdicks, 2016). Portal hypertension, overwhelming liver injury, and abundant portal-systemic shunts are major contributors to HE in cirrhotic patients.

Sodium-glucose cotransporter-2 (SGLT-2) inhibitors lower glucose plasma levels by reducing renal glucose reabsorption and promoting its excretion; they have been widely used for the treatment of patients with diabetes mellitus (Ferrannini, 2017). Recently, SGLT-2 inhibitors have attracted attention due to their cardiovascular benefits beyond their hypoglycemia effects. In addition to glycemic control, treatment with SGLT-2 inhibitors has been associated with a reduction in systemic blood pressure in diabetic patients, possibly via changes in plasma volume and decreased arterial stiffness (Fioretto et al., 2016; Marx and McGuire, 2016). The EMPA-REG OUTCOME trial showed the cardioprotective effect of empagliflozin, a type

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ABBREVIATIONS: Akt, protein kinase B; ALT, alanine transaminase; AST, aspartate aminotransferase; BDL, bile duct ligation; BW, body weight; CI, cardiac index; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; CO, cardiac output; eNOS, endothelial nitric oxide synthase; HE, hepatic encephalopathy; H&E stain, hematoxylin-eosin stain; HR, heart rate; iNOS, inducible nitric oxide synthase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; MAP, mean arterial pressure; NFκB, nuclear factor kappa B; PP, portal pressure; PVf, portal venous flow; SGLT-2, sodium-glucose cotransporter-2; SMAf, superior mesenteric artery flow; SVR, systemic vascular resistance; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

of SGLT-2 inhibitor, which lowered cardiovascular mortality in type 2 diabetic patients with established cardiovascular disease (Zinman et al., 2015). The mechanism by which empagliflozin exerts its beneficial cardiovascular effects is not fully understood. Given the minor difference in glycemic control and atherosclerosis changes observed with empagliflozin treatment compared with a placebo, these effects are unlikely to be the result of improved glycemic control and the atherosclerotic process. Potential mechanisms for the cardiovascular protection inferred by SGLT-2 inhibitors include a reduction in blood pressure, body weight, and diuretic, anti-oxidative, anti-inflammatory, and anti-apoptotic effects (Perrone-Filardi et al., 2017).

SGLT-2 inhibition has been documented to improve vascular function. It was shown that 8-week treatment with SGLT-2 inhibitors significantly lowered arterial stiffness and improved endothelial and smooth muscle dysfunction in diabetic mice (Lee et al., 2018). Chronic SGLT-2 inhibition enhances coronary vasodilation to nitric oxide donors in coronary arteries, but not in pulmonary arterial dilation in diabetic mice, suggesting that SGLT-2 inhibition regulates vascular relaxation differently depending on the type of vessel (Han et al., 2015). Acute administration of SGLT-2 inhibitors also improves systemic arterial and renal vascular function, occurring in the presence of stable blood glucose levels in diabetic patients (Solini et al., 2017). Together, emerging evidence has shown that SGLT-2 inhibitors have pleotropic effects when given via acute or chronic administration in various vasculatures.

Regarding the liver, SGLT-2 inhibitors have been shown to reduce hepatic steatosis in murine models (Kabil and Mahmoud, 2018). Four weeks of 10 mg/kg/d empagliflozin treatment attenuates hepatic inflammation and fibrosis in mice with non-alcoholic steatohepatitis (Jojima et al., 2016). Although the anti-inflammatory effects of SGLT-2 inhibition have been previously documented in non-alcoholic steatohepatitis (Mirarchi et al., 2022), the impact of SGLT-2 inhibition on liver cirrhosis and cirrhosis-related complications remains unclear. Therefore, in the current study, we tested the therapeutic effect of SGLT-2 inhibition by empagliflozin on biliary cirrhotic rats and also investigated its impact on hemodynamic changes, hepatic fibrosis, inflammation, the severity of portal-systemic shunts and HE.

Materials and Methods

Experimental Animal Model for Biliary Cirrhosis. Male Sprague-Dawley rats purchased from BioLASCO Taiwan Co., Ltd., weighing 300–320 g at the time of surgery, were used for all experiments. Rats were housed in plastic cages and allowed free access to food and water. All rats were fasted for 12 hours before the operation. Rats were randomly allocated to receive common bile duct ligation (BDL) to induce secondary biliary cirrhosis (Franco et al., 1979) or sham operation as surgical control. A high yield of secondary biliary cirrhosis was noted four weeks after the ligation (Cameron and Muzaffar Hasan, 1958). To avoid coagulation defects, BDL rats received weekly vitamin K injections (50 μg/kg intramuscularly). Survival surgery and hemodynamic study were performed under Zoletil (50 mg/kg, intramuscular injection) anesthesia. After experiments, rats were euthanized with potassium chloride (1–2 meq/kg) via venous injection. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals, 8th edition, 2011" published by the National Research Council, United States. This study was authorized by the Animal Committee of Taipei Veterans General Hospital in Taipei (approval no. IACUC 2020-070).

Study Design. Male Sprague-Dawley rats received BDL or sham operations. Two weeks post operation, the rats received the following treatments for two weeks: (1) sham-operated rats administered normal saline 0.2 ml/d (oral gavage, sham+vehicle, SV group); (2) BDL rats administered normal saline 0.2 ml/d (oral gavage, BDL+vehicle, BV group); (3) BDL rats administered 30 mg/kg/d empagliflozin in normal saline 0.2 ml (oral gavage, BDL+ empagliflozin, BE group). On the 29th day post operation, the motor activities of the rats were evaluated. Motor activities were used to indicate the severity of HE in the animals (Chang et al., 2017). Body weight and portal and systemic hemodynamic parameters were measured after the motor activity tests. The plasma levels of tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), fasting blood glucose, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and ammonia were measured at the end of the experiments. Hepatic protein expression levels were determined using Western blotting analysis. Histopathological changes to the liver were also examined. On parallel groups of sham-operated or BDL rats, the severity of portal-systemic shunts was measured using the color microsphere method. The study design is illustrated in Fig. 1.

Measurement of Motor Activities. The motor activities of BDL rats were determined using the Auto-Track Opto-Varimex activity monitoring system (Columbus Instruments, Columbus, OH, USA). This system is a position tracking system which detects the motor activities of small laboratory rodents (Chang et al., 2017). The rats were housed in a transparent cage (42.2 × 42.5 × 20.5 cm) and the system utilizes infrared beams to calculate the animals' movement and current position. The Opto-Varimex system can be configured by sensor pairs which consist of emitters and detectors. These sensor pairs detect the movements of experimental animals and record their resting time(s), ambulatory time(s), and stereotypic time(s). Resting time is equivalent to the rat's total time in the chamber minus their ambulatory and stereotypic time. To standardize the experimental condition concerning the influence exerted by acrophase, a dark environment was provided, the rats were rested for 30 minutes before experiment, the experiments were performed in the afternoon, and a control group was applied. During the activity measurements, animals had no access to food. All studies were performed under strictly standardized conditions in a dark room for 30 minutes. The resting, ambulatory, and stereotypic times were recorded separately to reflect the motor activities.

Measurement of Systemic and Portal Hemodynamics. The right femoral artery and superior mesentery vein were cannulated with PE-50 catheters that were connected to a Spectramed DTX transducer (Spectramed, Inc., Oxnard, CA, USA). Continuous recordings of mean arterial pressure (MAP), heart rate (HR), and portal pressure (PP) were performed on a multi-channel recorder (model RS 3400, Gould, Inc., Cupertino, CA, USA). Cardiac output (CO, ml/min) was measured via the thermodilution method, as previously described (Albillos et al., 1992). Cardiac index (CI, ml/min/100 g body weight)

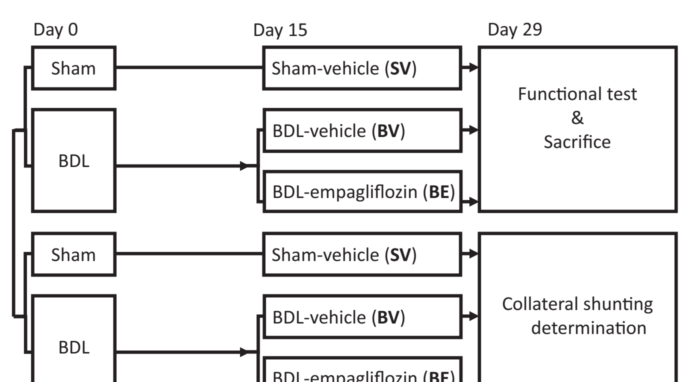

Fig. 1. The illustration of study design and sequences of events.

TABLE 1
Body weight, hemodynamic parameters and biochemistry data for BDL rats with or without empagliflozin treatment

Item	Sham+vehicle (n=9)	BDL+vehicle (n=9)	BDL+empagliflozin (n=9)
BW1 (g)	307 ± 9	312 ± 8	311 ± 13
BW2 (g)	411 ± 30	357 ± 59 [a]	341 ± 22 [a]
MAP (mmHg)	145 ± 12	120 ± 11 [a]	125 ± 21 [a]
PP (mmHg)	8.5 ± 0.7	16.3 ± 3.8 [a]	16.4 ± 3.6 [a]
HR (beats/min)	402 ± 33	397 ± 42	376 ± 50
PVf (ml/min/100g)	10.3 ± 2.3	10.3 ± 4.2	12.2 ± 2.7
SMAf (ml/min/100g)	6.9 ± 2.4	8.0 ± 2.9	9.1 ± 2.0
SVR (mmHg/ml/min/100g)	4.8 ± 0.7	3.8 ± 1.2 [a]	3.3 ± 0.6 [a]
CI (ml/min/100g)	30.6 ± 3.4	33.3 ± 8.3	38.3 ± 5.4 [a]
Ammonia (μmol/L)	83 ± 56	216 ± 67 [a]	241 ± 70 [a]
ALT (IU/L)	134 ± 59	309 ± 182 [a]	298 ± 142 [a]
TB (mg/dL)	0.03 ± 0.01	8.7 ± 2.1 [a]	7.7 ± 1.2 [a]
Cr (mg/dL)	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Glu (mg/dL)	113 ± 5	92 ± 11 [a]	87 ± 15 [a]

BW1, body weight before treatment; BW2, body weight after treatment; PaO_2, partial pressure of oxygen TB, total bilirubin; Cr, creatinine; Glu, fasting glucose;
[a]$p < 0.05$ compared with the Sham+vehicle group.

was calculated as CO per 100 g body weight (BW). Systemic vascular resistance (SVR, mmHg/ml/min/100 g BW) was calculated as MAP divided by the CI. Measurements of portal venous flow (PVf, ml/min) and superior mesenteric artery flow (SMAf, ml/min) were performed (Chang et al., 2019) using a non-constrictive perivascular ultrasonic transit-time flow probe (lRB, 1-mm diameter; Transonic Systems, Ithaca, NY, USA).

Determination of Plasma VEGF and TNF-α Levels. The plasma levels of VEGF and TNF-α were measured by commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

Measuring the Degree of Portal-Systemic Collateral Shunting. The degree of portal-systemic shunting was determined using the technique previously described by Chojkier and Groszmann, substituting color for radioactive microspheres (Chojkier et al., 1981).

Hepatic Histopathological Examination of Inflammation and Fibrosis. Liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin-eosin (H&E). Sirius red staining was performed to determine the severity of liver fibrosis. Immunohistochemical staining with anti-CD68 antibodies (diluted 1:200, ab31630, Abcam, Cambridge, UK) was also performed to detect CD68-positive macrophages to measure intrahepatic inflammation (Hsu et al., 2020). The semi-quantitative counting of CD68-positive stained cells was measured. The H&E, Sirius red and CD68 stains were examined using a light microscope (Eclipse Ni-E, Nikon, Japan).

Western Blot Analysis. Liver tissues were frozen in liquid nitrogen and stored at -80°C prior to analysis. The blots were incubated with the following primary antibodies: endothelial nitric oxide synthase (eNOS; Cell Signaling Technology, Inc., Danvers, MA, USA, 32027S; 1:1000), inducible nitric oxide synthase (iNOS; Genetex Irvine, CA, USA, Gtx130246; 1:1000), cyclooxygenase 1 (COX-1; Cell Signaling Technology, Inc., Danvers, MA, USA, 4841S; 1:2000), cyclooxygenase 2 (COX-2; Cell Signaling Technology, Inc., Danvers, MA, USA, 12282S; 1:1000), nuclear factor kappa B (NFκB; Cell Signaling Technology, Inc., Danvers, MA, USA, 8242S; 1:3000), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα); Cell Signaling Technology, Inc., Danvers, MA, USA, 4814S; 1:3000), protein kinase B (Akt; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:500), and beta-actin (Genetex Irvine, CA, USA, Gtx629630; 1:5000). Then the blots were incubated for 90 minutes with secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG antibody; Merck KGaA, Darmstadt, Germany). Specific proteins were then detected via enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Merk Millipore Co., Billerica, MA, USA) and scanned with a computer-assisted video densitometer and digitalized system (BioSpectrum 600 Imaging System, Ultra-Violet Products, Ltd., Upland, CA, USA). Then the signal intensity (integral volume) of the appropriate band was analyzed.

Drugs. Empagliflozin was purchased from Boehringer Ingelheim and Eli Lilly, Germany. All solutions were freshly prepared on the day of the experiment.

Statistical Analysis. All results are expressed as the mean ± standard deviation. Statistical analyses were performed using an unpaired Student's t test or one-way ANOVA with the least significant difference test as appropriate. Survival curve analysis was performed using the log-rank test. Results were considered statistically significant with a two-tailed p value < 0.05.

Results

Mortality Rates of Empagliflozin- and Vehicle-Treated Rats. There was no significant difference in the mortality rate between empagliflozin- and vehicle-treated (control) BDL rats (empagliflozin versus control: 25% (3/12) versus 31% (4/13), $p>0.05$). All sham-operated rats survived during the experimental period.

BW, Hemodynamic, and Biochemistry Parameters. Table 1 shows the BW, hemodynamic, and biochemistry parameters of rats with or without empagliflozin treatment. The BW of BDL rats was significantly lower than the sham-operated rats after 2 weeks of treatment, but empagliflozin did not significantly reduce BW in the BDL rats (SV versus BV and BE, $p<0.05$; BV versus BE, $p>0.05$). In addition, BDL rats had significantly lower MAP, higher PP, higher CI and lower SVR compared with the sham-operated rats (MAP, PP, SVR: SV versus BV and BE, $p < 0.05$; BV versus BE, $p > 0.05$. CI: SV versus BE, $p < 0.05$; BV versus BE, $p > 0.05$). The SMAf and PVf were not significantly different among these 3 groups (SV versus BV versus BE, $p > 0.05$). Compared with the sham-operated rats, BDL rats had higher ammonia, ALT and total bilirubin levels (SV versus BV and BE, $p < 0.05$; BV versus BE, $p > 0.05$). Fasting glucose plasma levels were significantly lower in BDL rats with or without empagliflozin treatment compared with the sham-operated rats (SV versus BV and BE, $p < 0.05$; BV versus BE, $p > 0.05$).

TABLE 2
Motor activities in BDL rats with or without empagliflozin treatment

Time measure	Sham+vehicle (n=9)	BDL+vehicle (n=9)	BDL+empagliflozin (n=9)
Resting time (s)	485 ± 142	748 ± 297 [a]	1173 ± 103 [a, b]
Ambulatory time (s)	1098 ± 154	848 ± 231 [a]	526 ± 91 [a, b]
Stereotypic time (s)	195 ± 96	186 ± 93	90 ± 28 [a, b]

[a]$p < 0.05$ compared with the Sham+vehicle group, [b]$p < 0.05$ compared with the BDL+vehicle group.

The biochemistry data were compatible with jaundice and liver injury in the BDL rats, and this was not significantly affected by empagliflozin treatment. Furthermore, BDL rats had a lower fasting blood glucose level compared with the sham-operated rats, which was not influenced by empagliflozin.

Motor Activity of Sham-Operated and BDL Rats. Table 2 presents the motor activities of sham-operated and BDL rats with or without empagliflozin treatment. Increased resting time and decreased ambulatory time were observed in BDL rats compared with the control group, indicating decreased motor activities in BDL rats (resting time and ambulatory time: SV versus BV, both $p < 0.05$). In addition, empagliflozin further decreased motor activities in BDL rats (resting time, ambulatory time, and stereotypic time: BV versus BE, all $p < 0.05$).

Histopathological Changes in Liver and Intrahepatic CD68-Positive Stained Cells. Fig. 2 depicts the histopathological changes in sham-operated and BDL rats with or without empagliflozin treatment. Compared with the sham-operated rats, hepatic H&E staining of BDL rats showed increased mononuclear cell infiltration and bile duct proliferation, indicating inflammatory changes in the livers of BDL rats. This intrahepatic inflammation was not ameliorated by empagliflozin treatment in BDL rats. Sirius Red staining revealed obvious fibrosis of the liver in BDL rats, which was not attenuated by empagliflozin. In addition, many CD68-positive stained cells infiltrated the livers of BDL rats. Empagliflozin treatment did not reduce the number of CD68-positive stained cells in the liver (BV versus BE: 36 ± 20 versus 29 ± 18 cell numbers/high-power field, $p > 0.05$).

TNF-α and VEGF Plasma Levels. Fig. 3 depicts the plasma levels of TNF-α and VEGF in BDL rats with or without empagliflozin treatment. The plasma level of VEGF significantly elevated after empagliflozin treatment, but TNF-α level was not significantly affected by empagliflozin (BV versus BE: VEGF = 10.4 ± 1.6 versus 12.0 ± 1.4 pg/ml, $p = 0.03$; TNF-α = 12.4±5.9 versus 15.9 ± 6.0 pg/ml, $p > 0.05$).

Degree of Portal-Systemic Shunting in BDL Rats. Fig. 4 shows the degree of portal-systemic shunting in BDL rats with or without empagliflozin treatment (BV versus BE: $n = 8, 8$). The number of portal-systemic shunts significantly increased after empagliflozin treatment in BDL rats (BV versus BE: 39 ± 7 versus 67 ± 10%, $p < 0.001$).

Hepatic Protein Expression in BDL Rats. Fig. 5 reveals hepatic protein expression in BDL rats with or without empagliflozin treatment (BV versus BA: $n = 7, 7$). NFκB, IκBα, eNOS, iNOS, COX-1, COX-2, and Akt protein expression were not significantly influenced by empagliflozin treatment (NFκB/β-actin = 0.65 ± 0.15 versus 0.62 ± 0.10; IκBα/β-actin = 0.83 ± 0.12 versus 0.80 ± 0.07; eNOS/β-actin = 0.70 ± 0.10 versus 0.72 ± 0.10; iNOS/β-actin = 0.33 ± 0.12 versus 0.35 ± 0.12; COX-1/β-actin = 0.73 ± 0.21 versus 0.68 ± 0.09; COX-2/β-actin = 0.60 ± 0.24 versus 0.67 ± 0.07; Akt/β-actin = 0.84 ± 0.09 versus 0.83 ± 0.09; all $p > 0.05$).

Discussion

In the present study, SGLT-2 inhibition via 2-week empagliflozin treatment did not exert an obviously hypoglycemic effect

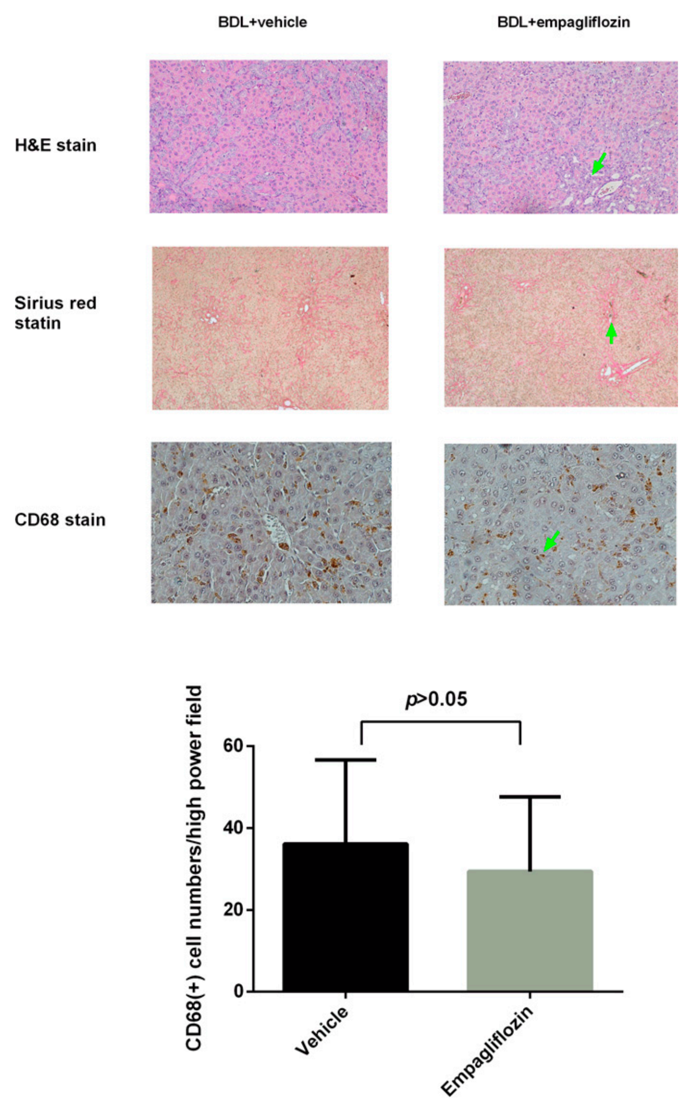

Fig. 2. Hepatic histopathology in BDL rats with or without empagliflozin treatment. The representative H&E staining image of BDL rats shows many inflammatory cells and bile duct proliferation (green arrow, magnification 200x). Sirius Red staining reveals obvious fibrosis of the liver (green arrow, magnification 40x). In addition, there are many CD68-positive stained cells (green arrow) in the liver of BDL rats, indicating an increase in intrahepatic macrophages (magnification 200x). Empagliflozin treatment neither ameliorated hepatic inflammation and fibrosis nor significantly reduced the number of CD68-stained macrophages.

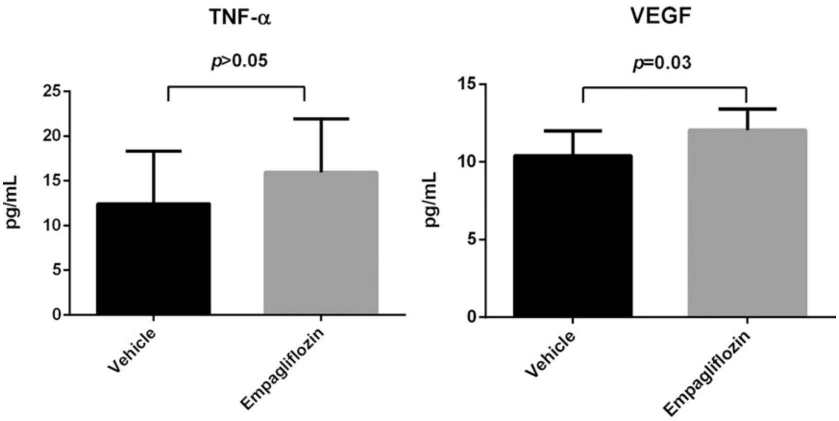

Fig. 3. TNF-α and VEGF plasma levels in BDL rats with or without empagliflozin treatment. The plasma level of VEGF significantly increased after empagliflozin treatment. The plasma level of TNF-α was not significantly affected by empagliflozin treatment.

on cirrhotic rats, although cirrhotic rats had a lower level of fasting blood glucose compared with sham-operated rats. In addition, empagliflozin did not increase the mortality rate of cirrhotic rats. Regarding the safety of SGLT-2 inhibitor treatment in cirrhotic patients, a single dose of ipragliflozin, a type of SGLT-2 inhibitor, is well tolerated in cirrhotic patients with moderate hepatic impairment (Child-Pugh score 7–9) (Zhang et al., 2013). Similarly, our data indicated that two weeks of empagliflozin treatment did not induce hypoglycemia or increase mortality rates in cirrhotic rats. BDL-induced biliary cirrhotic rats had significant jaundice, portal hypertension, and hyperdynamic circulation compared with the sham-operated rats, which was in accordance with our previous findings (Huang et al., 2021). Our data revealed that 2-week empagliflozin treatment neither improved portal hypertension nor ameliorated hepatic injury in biliary cirrhotic rats. However, inhibition of SGLT-2 by empagliflozin significantly increased portal-systemic shunts, elevated plasma VEGF levels, and decreased motor activities of cirrhotic rats.

Emerging reports show that SGLT-2 inhibitors protect neurovascular units (Pawlos et al., 2021). In addition, SGLT-2 inhibition ameliorates cognitive dysfunction in obese and type 2 diabetic mice (Rizzo et al., 2022). An interesting study showed that empagliflozin alleviated neuronal apoptosis-induced cerebral ischemia/reperfusion injury and reduced the size of brain infarctions in a murine model through upregulation of hypoxia-inducible factor 1-alpha and its downstream mediator VEGF (Abdel-Latif et al., 2020). However, in the present study, we found that 2-week empagliflozin treatment did not exert neuroprotective effects; instead, it decreased motor activity in cirrhotic rats, although we also found that empagliflozin upregulated VEGF plasma levels. The motor activity detected by the Auto-Track Opto-Varimex activity monitoring system can reflect the severity of HE, that is, less motor activity count indicates severer HE (Chang et al., 2017). The motor activity is synchronous with the circadian rhythm of rodents. Emerging data show that motor activity of rats is highest in the midnight, and the circadian periodicity can regulate cerebral blood perfusion (Boakes and Wu, 2021; Wauschkuhn et al., 2005). Therefore, we conducted the motor activity study under strictly standardized conditions by setting a dark environment, resting the rats for 30 minutes before experiment, and performing experiments in the afternoon to mimic the acrophase of the rodents. Our data showed that 2-week empagliflozin treatment decreased motor activity of BDL rats, indicating the exacerbation of HE.

Two major pathologic factors of HE are overwhelming hepatic failure and abundant portal-systemic shunting vessels. The injured liver fails to "detoxify" ammonia and neurotoxins, then the portal-systemic shunts bypass these noxious agents from the liver to the systemic circulation and the central nervous system. In the present study, ammonia plasma levels were not affected by empagliflozin treatment. In addition, liver inflammation and fibrogenesis-related protein expressions were not influenced by empagliflozin. Thus, the major factor leading to aggravation of HE in empagliflozin-treated cirrhotic rats could be the increased portal-systemic shunts. Angiogenesis driven by VEGF-dependent signaling pathways is a major contributor to portal-systemic shunt formation and development (Fernandez et al., 2004). It could therefore be that the increased VEGF level after empagliflozin treatment observed in the current study might induce an increase in portal-systemic collaterals and contribute to adverse events.

The present study showed that SGLT-2 inhibition by empagliflozin did not alter portal hypertension and systemic hemodynamics in cirrhotic rats. Our data showed that cirrhotic rats had a significantly hyperdynamic circulation, presenting with higher CI and lower SVR. The effect of empagliflozin on hyperdynamic circulation was limited in cirrhotic rats; in addition, empagliflozin did not reduce portal pressure in cirrhotic rats. Portal pressure is determined by three main factors: intra-

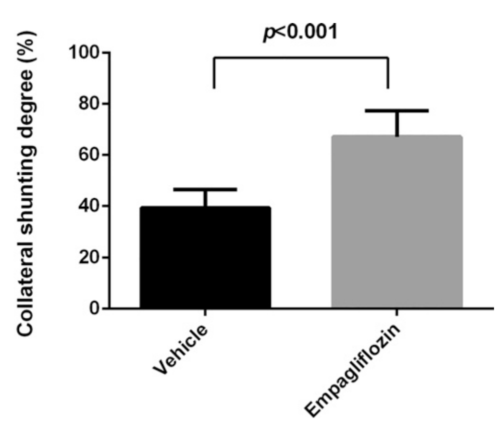

Fig. 4. Portal-systemic collateral shunts of BDL rats with or without empagliflozin treatment. The degree of collateral shunting significantly increased after empagliflozin treatment in BDL rats.

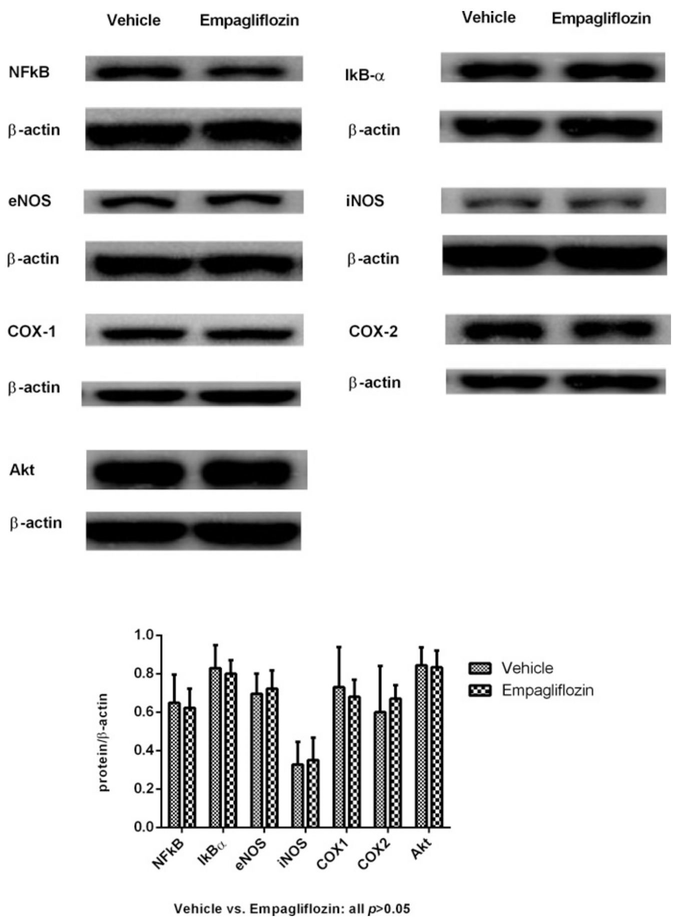

Fig. 5. Hepatic protein expression of inflammation-related factors in BDL rats with or without empagliflozin treatment. NFkB, IkBα, eNOS, iNOS, COX-1, COX-2, and Akt protein expression were not significantly influenced by empagliflozin treatment.

hepatic resistance, splanchnic blood flow as reflected by SMAf and PVf, and portal-systemic collateral vascular resistance. The unaltered severity of liver fibrosis (influencing the intrahepatic resistance), indicates that SMAf and PVf might be the main factors driving the neutral effects of empagliflozin on portal pressure in rats with BDL-induced cirrhosis.

Several previous studies have shown that empagliflozin treatment can improve liver function and ameliorate liver fibrosis in non-alcoholic fatty liver disease (Kuchay et al., 2018; Shimizu et al., 2019). Furthermore, an interesting study showed that SGLT-2 inhibition could attenuate non-alcoholic steatohepatitis and hepatocellular carcinoma in mice (Shiba et al., 2018). However, in the present study, 2-week empagliflozin treatment did not improve liver biochemistry, hepatic inflammation, or liver fibrosis in BDL-induced cirrhotic rats. Possible explanations for this finding could be the relatively short treatment period with empagliflozin in the present study and the use of different animal models. A longer treatment protocol for cirrhotic animals could be tried to evaluate the therapeutic effect of empagliflozin. On the other hand, BDL-induced chronic liver inflammation and biliary cirrhosis are severe and relatively non-modifiable, and thus not easily reversed. In contrast, animal models for non-alcoholic steatohepatitis have less prominent inflammation and fibrosis of the liver. Therefore, the discrepant results might be related to the use of different experimental models.

Empagliflozin has been shown to reduce macrophage accumulation within white adipose tissue and the liver, and to lower TNF-α plasma levels in a mouse model (Xu et al., 2017). However, our data showed that empagliflozin did not decrease intrahepatic CD68-positive staining in macrophages or the TNF-α plasma level in cirrhotic rats. We further tested the protein expression of inflammation-related factors, including NFκB, IκBα, eNOS, iNOS, COX-1, COX-2, and Akt. However, the expression of these proteins was not affected by empagliflozin treatment. According to our results, 2-week empagliflozin treatment did not alleviate intrahepatic inflammation and macrophage recruitment in cirrhotic rats.

In conclusion, our data showed that 2-week SGLT-2 inhibition by empagliflozin treatment did not affect the hemodynamics, hepatic inflammation, or liver fibrosis of cirrhotic rats. Nevertheless, it increased portal-systemic shunts with decreased motor activities in cirrhotic rats, indicating an exacerbation of HE. To our knowledge, this is the first study that demonstrates that empagliflozin treatment exacerbates HE in biliary cirrhotic rats. Therefore, clinicians should be cautious when considering the use of empagliflozin for cirrhotic patients and further clinical trials to investigate its impact on HE are warranted.

Authorship Contributions

Participated in research design: Hsu, H.-C. Huang, Chang.

Conducted experiments: Pun, Chuang.

Contributed new reagents or analytic tools: Pun, Chuang.

Performed data analysis: Y.-H. Huang, Hou, Lee.

Wrote or contributed to the writing of the manuscript: Chang, H.-C. Huang.

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