Metformin Preconditioning Improves Hepatobiliary Function and Reduces Injury in a Rat Model of Normothermic Machine Perfusion and Orthotopic Transplantation

Andrie C. Westerkamp, MD, PhD,[1,2,3] Masato Fujiyoshi, MD, PhD,[1,2] Petra J. Ottens, BSc,[2] Maarten W.N. Nijsten, MD, PhD,[4] Daan J. Touw, PhD,[5] Vincent E. de Meijer, MD, PhD,[1,2] Ton Lisman, PhD,[1,2] Henri G.D. Leuvenink, PhD,[2] Han Moshage, PhD,[6] Tim A. Berendsen, MD, PhD,[1,2] and Robert J. Porte, MD, PhD[1,2]

Background. Preconditioning of donor livers before organ retrieval may improve organ quality after transplantation. We investigated whether preconditioning with metformin reduces preservation injury and improves hepatobiliary function in rat donor livers during ex situ normothermic machine perfusion (NMP) and after orthotopic liver transplantation. **Methods.** Lewis rats were administered metformin via oral gavage, after which a donor hepatectomy was performed followed by a standardized cold storage period of 4 hours. Graft assessment was performed using NMP via double perfusion of the hepatic artery and portal vein. In an additional experiment, rat donor livers preconditioned with metformin were stored on ice for 4 hours and transplanted to confirm postoperative liver function and survival. Data were analyzed and compared with sham-fed controls. **Results.** Graft assessment using NMP confirmed that preconditioning significantly improved ATP production, markers for hepatobiliary function (total bile production, biliary bilirubin, and bicarbonate), and significantly lowered levels of lactate, glucose, and apoptosis. After orthotopic liver transplantation, metformin preconditioning significantly reduced transaminase levels. **Conclusions.** Preconditioning with metformin lowers hepatobiliary injury and improves hepatobiliary function in an in situ and ex situ model of rat donor liver transplantation.

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INTRODUCTION

Metformin, a well-known drug for antihyperglycemic therapy in patients with type II diabetes mellitus, has also been shown to have beneficial effects in reducing ischemia-reperfusion (I/R) injury.[1] Several animal and human studies have shown that administration of metformin before an ischemic event (preconditioning) reduces tissue injury and preserves cardiac function after myocardial infarction.[2-4]

Although the exact protective mechanism of metformin during I/R injury is not fully known, one of the main actions of metformin is to partially and selectively inhibit complex I of the mitochondrial respiratory chain.[5] This

inhibition of complex I has positive and negative consequences. Positively, inhibition of complex I reduces the level of cellular energy, which stimulates phosphorylation of the cellular energy sensor enzyme AMP protein kinase (AMPK).[6] An indirect consequence of AMPK activation is prevention of opening of the mitochondrial permeability transition pore in the mitochondrial inner membrane.[7] When the mitochondrial permeability transition pores are closed, it is not possible to leak cytochrome c, which normally activates apoptotic death pathways.[8] Another positive effect of partial complex I inhibition is a reduction in the production of reactive oxygen species (ROS).[9] Some ROS production normally accompanies oxidative phosphorylation and this increases during reperfusion. ROS avidly attack and degrade many cellular components including the mitochondria themselves. Inhibition of oxidative phosphorylation by metformin is coupled with lower ROS production and the mitochondria will be better protected against ROS-mediated injury.[9] On the other hand, a negative effect of metformin and AMPK activation is the downregulation of the nuclear bile acid farnesoid X receptor (FXR).[10] FXR plays a central role in the control of bile salt homeostasis, maintaining the balance between bile salt synthesis and transport. FXR regulates expression of cholesterol 7α-hydroxylase (CYP7A10), the rate-limiting enzyme in bile salt synthesis, and the bile salt export pump (BSEP), the major bile salt exporter.[11] In addition, it has been shown that repression of the FXR transporter during ischemic conditions contributes to biliary epithelial injuries by disturbing bile acids transport in cholangiocytes after rat liver transplantation.[12]

One of the current challenges in liver transplantation is the shortage of available donor livers. A widely adopted strategy to increase the number of donors is the use of extended criteria donor (ECD) livers.[13] However, these ECD livers are more prone to I/R injury and are therefore related to lower graft survival rates.[13] To enhance outcome after transplantation of ECD livers, quality of the organ can be improved before procurement with preconditioning strategies.[14]

The aim of this study was to investigate whether metformin as a preconditioning agent is able to reduce preservation injury and improves hepatobiliary function. First, we administered metformin to rats before donation and performed hepatobiliary viability assessment using ex-situ normothermic machine perfusion (NMP, perfusion at 37°C). In an additional experiment, metformin preconditioned rat donor livers were transplanted and after 48 h, survival and hepatobiliary injury and function were evaluated. In both experiments, sham-fed rats served as controls.

MATERIALS AND METHODS

Animals

Male Lewis rats (LEW/HanHsd) (270–300 g) were obtained from Harlan Laboratories (Boxmeer, the Netherlands). Animals received care according to the Dutch Law on Animal Experiments. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC no. 6708A-C).
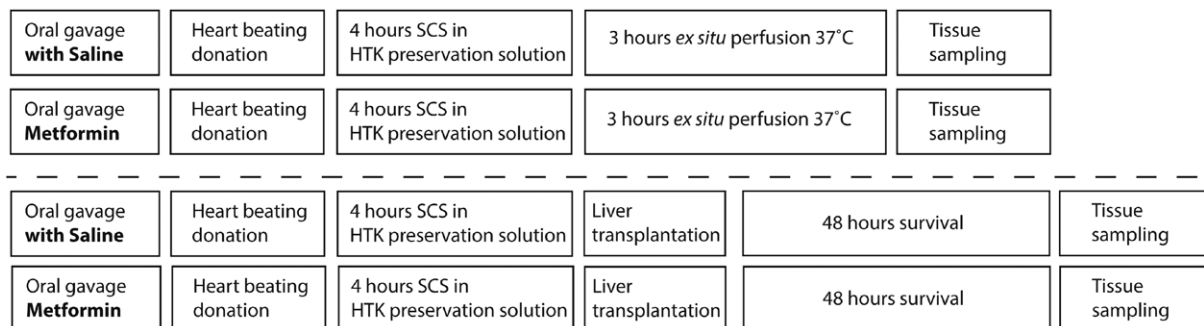
Experimental Design

The study consisted of 2 experimental groups and 2 reference groups (n = 4–6 per group) (Figure 1). The 2 experimental groups consisted of preconditioning with metformin, followed by NMP or transplantation and were compared with groups sham-fed with saline.

Metformin was purchased from Sigma-Aldrich (1,1-dimethyl biguanide hydrochloride, Sigma-Aldrich Inc., St. Louis, MO). For assessment of the preconditioning effects, metformin (300 mg/kg body weight) was dissolved in saline (0.9% NaCl) and administered 12 and 2 h before the hepatectomy directly into the stomach via oral gavage. Pretreatment with metformin in a dose of 300 mg/kg body weight is in accordance with earlier rat studies wherein adequate serum levels were obtained (metformin therapeutic range 1–4 mg/L).[15,16] All other groups were only fed with saline (0.9% NaCl) at the same time points (sham feeding) before the hepatectomy.

In all study groups, livers were procured from living donors (see next paragraph) and subsequently preserved by static cold storage (SCS) in histidine-tryptophan-ketoglutarate (HTK) preservation solution (Custodiol, Essential Pharmaceuticals, Ewing, NJ) for 4 h. Before NMP, all livers were flushed with 10 mL of cold saline.

As described before,[17,18] the basic NMP perfusion fluid consisted of 25 mL human red blood cell concentrate (final hematocrit 25%; Sanquin, Amsterdam, the Netherlands), 53.9 mL William's Medium E solution, 20 mL human albumin (200 g/L Albuman, Sanquin, Amsterdam, the Netherlands), 1 mL insulin (100 IE/mL Actrapid, Novo Nordisk, Alphen aan den Rijn, the Netherlands), and 0.1 mL unfractionated heparin (5000 IE/mL, Heparin LEO Pharma, Amsterdam, the Netherlands), adding up to a total volume of 100 mL.

FIGURE 1. Schematic representation of the experimental groups to examine the effect of metformin as preconditioning agent during normothermic machine perfusion (NMP) and after rat liver transplantation. All donor liver recipients survived the 48 h postoperative observation period without complications. HTK, histidine-tryptophan-ketoglutarate; SCS, static cold storage.

Procurement of Rat Livers From Living Donors During NMP

Procurement of rat livers from living donors is described previously.[17,18] In brief, inhalation anesthesia with isoflurane and oxygen was used before and during the procurement (2%–3% isoflurane). After the laparotomy, the large bile duct was cannulated and 1 mL 0.9% NaCl with 500 IU of heparin was administered via the dorsal penile vein. Moreover, a blood sample was taken via the dorsal vein for assessment of the serum level of metformin in situ (see next paragraph for analysis metformin). After heparinization, the hepatectomy was performed by ligation of the splenic vein, mesenteric artery, and mesenteric vein cannulation of the celiac trunk. After clamping the infra-hepatic vena cava and the portal vein, the portal vein was cannulated and via the portal vein cannula, the liver was flushed in situ with 10 mL 0.9% NaCl (37°C). Subsequently, the supra-hepatic vena cava was transected, followed by a cold flush out with 5 mL HTK preservation solution (4°C) via the portal vein cannula. The liver was removed and flushed with an additional 20 mL of cold (4°C) HTK via the portal vein cannula and 5 mL of cold (4°C) HTK via the hepatic artery (celiac trunk cannula) before preservation by SCS.

SCS and NMP

For SCS, livers were stored in bags with ice-cold HTK (4°C) on melting ice for 4 h. Ex situ NMP of rat donor livers was performed with a liver machine perfusion system that enabled dual perfusion via both the hepatic artery and the portal vein using a closed circuit (Figure 2). Our research group has already used this experimental set-up in earlier experiments.[17,18] In brief, the system was pressure-controlled by a computer algorithm (provided by Organ Assist, Groningen, the Netherlands) allowing autoregulation of blood through the liver, with a constant pressure at variable flow rates. In-line sensors monitored pressure and flow. Ex situ NMP was performed with a mean arterial pressure of 110 and 11 mmHg at portal side.

Orthotopic Liver Transplantation

Similar to the NMP experiment, donor rats were subjected to oral gavage with metformin 12 and 2 h before the hepatectomy or sham-fed with saline at the same time intervals. After the hepatectomy, donor livers were stored on ice for 4 h in HTK solution. Thereafter, these livers were orthotopically transplanted in weight-matched recipients as described previously without reconstruction of the hepatic artery.[19] In brief, the hepatectomy took 30 min, the anhepatic time was kept below 20 min and the reperfusion phase was performed within 20 min. After 48 h, a reoperation in the recipient was performed to obtain blood samples for determination of serum levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and bilirubin. Thereafter, the animals were terminated and liver tissue was harvested and stained with hematoxylin and eosin (H&E) for further pathological analysis.

Biomolecular Assays

Samples of heparinized arterial blood during the different procedures were centrifuged (2700 g for 5 min at 4°C)
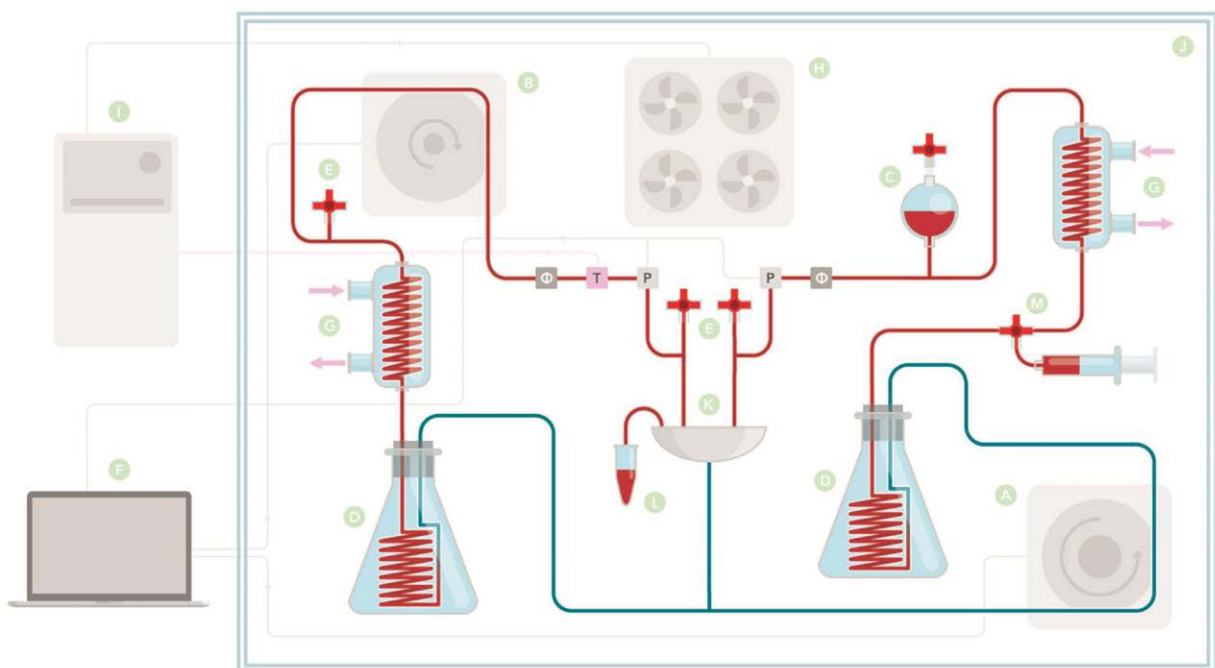
FIGURE 2. Ex situ rat liver machine perfusion system. Two roller pumps provide a continuous flow to the portal vein (A) and a pulsatile flow to the hepatic artery (B). Pulses in the portal flow were eliminated with elastic tubing and a pulse damper (C). Two tubular membrane oxygenators provide oxygenation of the perfusion solution, as well as removal of CO_2 (D). Several bubble traps (3-way connectors) were used to eliminate air bubbles in the perfusion solution (E). Flow (Φ) and pressure (P) were detected by in-line sensors and data were analyzed and displayed in real time on a connected laptop (F). The perfusion temperature was maintained constant by 2 heat exchangers (G) and a radiator/ventilator combination (H), all connected to the thermostat pump (I). For real time control of the perfusion temperature, 1 in-line temperature sensor (T) was connected to the thermostat pump. The isolated box encapsulated the perfusion system (J) preventing loss of warm or cold air. The rat liver was placed into an organ chamber (K). Bile was collected in Eppendorf tubes (L). By the 3-way connecter at the portal side, samples of the perfusion solution were taken every 30 min for analysis of the perfusate (M).

Gene	Sequence
18S	Forward: CGG CTA CCA CAT CCA AGG A
	Reversed: CCA ATT ACA GGG CCT CGA AA
	Probe: CGC GCA AAT TAC CCA CTC CCG A
CYP7A1	Forward: CAG GGA GAT GCT CTG TGT TCA
	Reversed: AGG CAT ACA TCC CTT CCG TGA
	Probe: TGC AAA ACC TCC AAT CTG TCA TGA GAC CTC C
BSEP	Forward: CCA AGC TGC CAA GGA TGC TA
	Reversed: CCT TCT CCA ACA AGG GTG TCA
	Probe: CAT TAT GGC CCT GCC GCA GCA

BSEP, bile salt exporter; RT PCR, real-time polymerase chain reaction.

and the supernatant was collected, frozen, and stored at −80°C for future additional analysis. Metformin was measured by a validated liquid chromatography–tandem mass spectrometry method in the laboratory of the Department of Clinical Pharmacy and Pharmacology of the University Medical Center Groningen. Insulin serum levels were analyzed using an ELISA kit (Rat/Mouse Insulin ELISA, Merck, Billerica, MA). Determination of glucose, lactate, AST, LDH, and bilirubin was performed using standard biochemical methods.

Bile production was measured gravimetrically at 30-min intervals by weighing Eppendorf tubes in which bile was collected from the biliary drain. The density of bile was defined as 1 mg/mL. The hepatic bile production was calculated as milliliter per gram liver. Biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile.[17,18] For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark). Biliary concentration of γ-glutamyl transferase (γ-GT) and LDH was measured as biomarkers of biliary epithelial cell injury,[17,18] and biliary bilirubin concentration was measured as biomarker of hepatocellular secretory function,[17,18] using standard biochemical methods. Total bile salt concentrations were measured as described previously.[20]

Thiobarbituric acid reactive substances were measured in perfusate samples after 2 h of reperfusion, as a marker for oxidative stress (degree of lipid peroxidation of membranes) as described before.[17]

Caspase-3 Enzyme Activity Assay

Directly after SCS and after 3 h of NMP, caspase-3 enzyme activity was measured in liver parenchyma as described previously.[21] In brief, the arbitrary fluorescence unit was corrected for total protein content. Protein concentration was determined using a commercially available kit (Bio-Rad, Veenendaal, the Netherlands).

RNA Isolation and Polymerase Chain Reaction

Immediately after SCS and after 3 h of NMP, hepatic mRNA expression of hepatocellular transporter protein BSEP and cholesterol 7α-hydroxylase (gene symbol CYP7A1) was determined by quantitative real-time polymerase chain reaction (PCR). RNA isolation, reverse transcription PCR, and quantitative PCR were performed as described previously,[22] and 18S mRNA levels were used as endogenous control. Primers and probes are provided in Table 1.

ATP Extraction and Measurement

Liver tissue biopsies after 3 h of NMP were immediately frozen in liquid nitrogen and were processed later for ATP measurement, as described before.[17]

Statistical Analysis

Continuous variables were presented as median with interquartile range (IQR) and were compared using the Mann-Whitney U test. The level of significance was set at $P < 0.05$. All statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Metformin Preconditioning Resulted in Equal Levels of Glucose and Insulin But Higher Levels of Lactate Before Hepatectomy

Oral administration of metformin caused serum levels of ca. 5 mg/mL before hepatectomy (Figure 3A). Glucose and insulin levels before the hepatectomy were comparable between the metformin preconditioning group and sham-fed group (saline) (Figure 3B and C). In the group with metformin preconditioning, significantly higher levels of lactate were found compared with the group with saline preconditioning before hepatectomy (Figure 3D).

### Metformin Preconditioning Improved Hepatobiliary Function During NMP

Hepatic ATP concentrations (Figure 4A) after 3 h of NMP and bile production (Figure 4B) during the whole period of NMP were significantly higher in the metformin preconditioning group in comparison with saline preconditioning. Bilirubin concentration in bile after 3 h of NMP was also significantly higher after metformin preconditioning compared with the group fed with saline (Figure 4C).

Bicarbonate concentration (a marker for cholangiocyte function) was measured in bile after 1.5 h of NMP (Figure 4D). The levels displayed a pattern comparable to the biliary bilirubin levels. Metformin preconditioning yielded a significantly higher concentration of bicarbonate in bile compared with the group with saline as preconditioning agent. Biliary pH levels after 1.5 h of NMP were similar between both groups (Figure 4E). Perfusate levels of lactate and glucose during NMP are presented in Figure 5A and B. In the group with metformin preconditioning, lactate and glucose levels were significantly lower during all the different time intervals of NMP, in comparison with saline preconditioning.

Metformin Preconditioning Lowered Total Concentration of Bile Salts and Reduced BSEP and CYP7A1 mRNA Expression

Concentrations of total bile salts after 1.5 h of NMP are presented in Figure 6A. The group with metformin
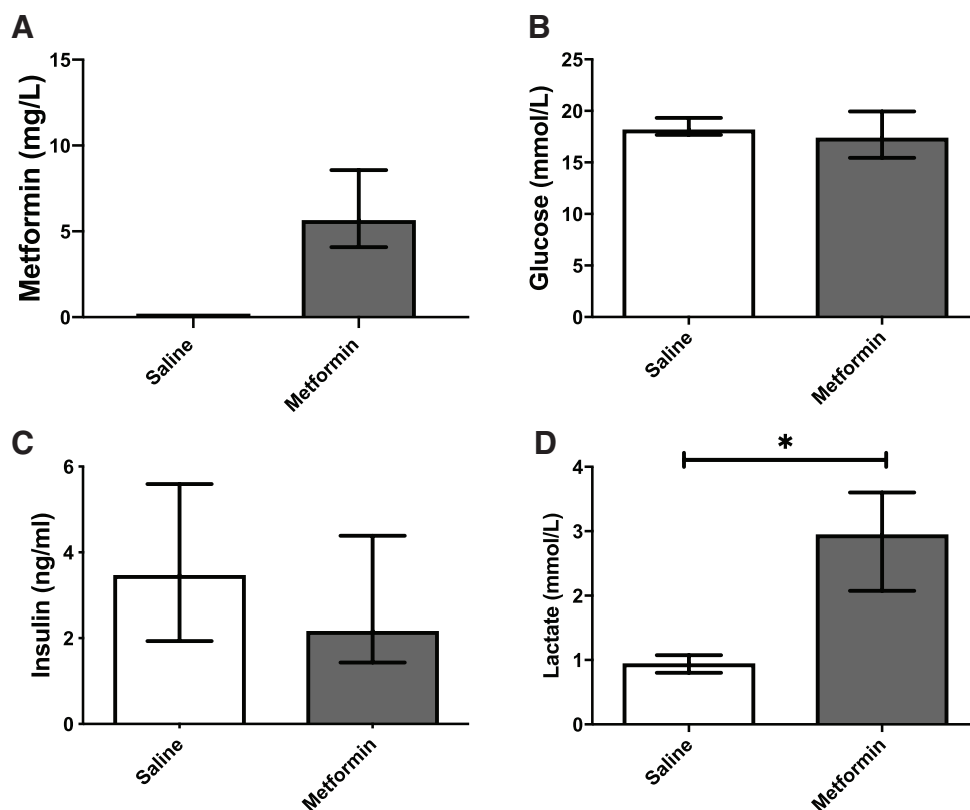
FIGURE 3. Serum levels of metformin, glucose, insulin, and lactate before hepatectomy. A, Serum levels of metformin before hepatectomy, the median value was 5.6 mg/L (metformin therapeutic range 1–4 mg/L). In the group without preconditioning, metformin levels were undetectable. B, Serum glucose levels before hepatectomy. No significant differences observed. C, Serum insulin levels before hepatectomy. No significant differences observed. D, Serum lactate levels before hepatectomy. The median level of lactate was significantly higher in the group with metformin preconditioning compared with the group with saline preconditioning (*$P < 0.05$). Data are represented as medians with interquartile range (IQR; error bars).

preconditioning displayed a significantly lower concentration of total bile salts after 1.5 h of NMP compared with saline preconditioning.

Relative mRNA expression of CYP7A1 and BSEP immediately measured after SCS (thus before NMP) and after 3 h of NMP are presented in Figure 6B and C. Before NMP metformin preconditioning, significantly decreased mRNA levels of CYP7A1 and BSEP were observed compared with the groups with saline preconditioning. In addition, after 3 h of NMP, mRNA levels of CYP7A1 and BSEP were again significantly lower in the group with metformin preconditioning compared with the saline preconditioning.

Metformin Preconditioning Did Not Reduce Hepatobiliary Injury During NMP

Markers for hepatocellular injury, such as AST and LDH, measured hourly during NMP were not significantly different between the groups with metformin preconditioning or saline preconditioning.

Figure 7A presents caspase-3 activity, a marker for apoptosis, measured directly after SCS (thus before NMP) and after 3 h of NMP. Before NMP, caspase-3 activity was significantly lower in the group with metformin preconditioning compared with the group with saline preconditioning. This difference disappeared after 3 h of NMP.

Levels of thiobarbituric acid reactive substances, a marker for oxidative stress, were not significantly different between both groups after NMP (Figure 7B).

Markers for cholangiocyte injury, LDH, and γ-GT, measured in bile after 3 h of NMP are provided in Figure 7C and D. The concentrations of biliary LDH and γ-GT were not significantly different between the groups with metformin preconditioning and saline preconditioning.

Metformin Preconditioning Reduced Hepatocellular Injury After Rat Liver Transplantation

Posttransplant serum levels of AST, a marker for hepatocellular injury, were significantly lower in the group of rats, that received a rat donor liver preconditioned with metformin compared with rats, that received a rat donor liver preconditioned with saline after 48 h of survival (Figure 8A). No significant differences were found between posttransplant serum levels of LDH (Figure 8B). Also, posttransplant serum levels of bilirubin, a hepatocellular function marker, were not significantly different in recipients with a preconditioned metformin donor liver in comparison to recipients with a donor liver without metformin preconditioning (data not shown). Both study groups achieved 100% survival after 48 h. Figure 9 shows liver parenchyma of both groups stained with H&E after 48 h of survival. There were no pathological differences observed such as hepatocyte injury and/or necrosis between metformin preconditioning or without.
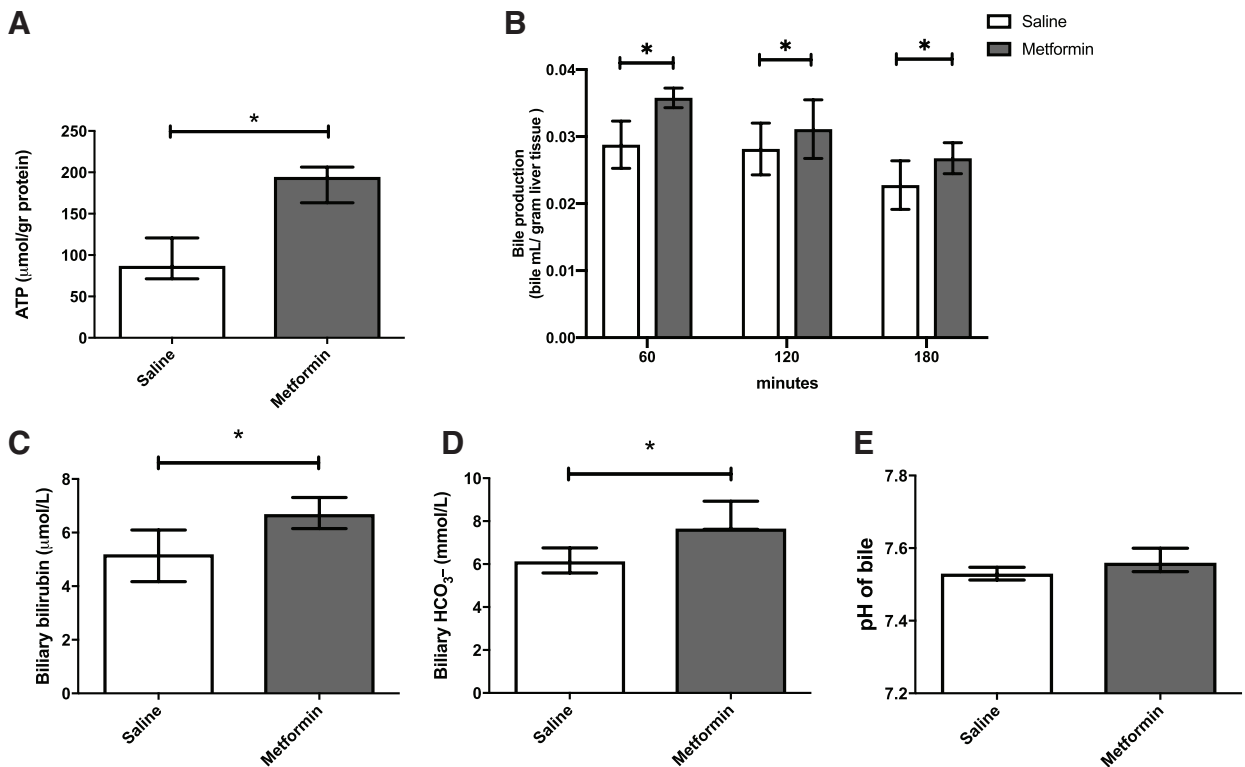
FIGURE 4. Metabolic parameters for hepatobiliary function during normothermic machine perfusion (NMP). A, Hepatic ATP concentrations after 3h of NMP. Hepatic ATP content was significantly higher in the group with metformin preconditioning compared with saline preconditioning (*$P < 0.05$). B, Bile production during 3h of NMP at different time intervals. The group with metformin preconditioning displayed a significantly higher bile production during all the specific time periods, respectively 60, 120, and 180 min (*$P < 0.05$). C, Biliary bilirubin after 3h of NMP. Significantly higher concentration of bilirubin was measured in the group with metformin preconditioning (*$P < 0.05$). D, Biliary bicarbonate concentrations after 1.5h of NMP. A significantly higher concentration of bicarbonate was measured in the bile samples in the group with metformin preconditioning vs the group with saline preconditioning (*$P < 0.05$). E, Biliary pH levels after 1.5h of NMP. Biliary pH was not significantly different between both groups. Data are represented as medians with interquartile range (IQR; error bars).

DISCUSSION

The aim of the current study was to examine whether metformin preconditioning was able to reduce preservation injury and improves hepatobiliary function during NMP as well as after transplantation of donor rat livers. Our study demonstrated that preconditioning with metformin during NMP significantly improved hepatobiliary function as reflected by an increased ATP content, improved bile, bilirubin. and biliary bicarbonate production. In addition, metformin preconditioning lowered apoptosis (less caspase-3 activity) and glucose and lactate production during NMP. In the additional transplantation experiment, a significantly lower level of the injury marker AST was measured in rats that received a donor liver preconditioned with metformin compared with rats that received a saline preconditioned donor liver. Combining the results of NMP and transplantation model, we suggest that preconditioning with metformin can reduce preservation injury
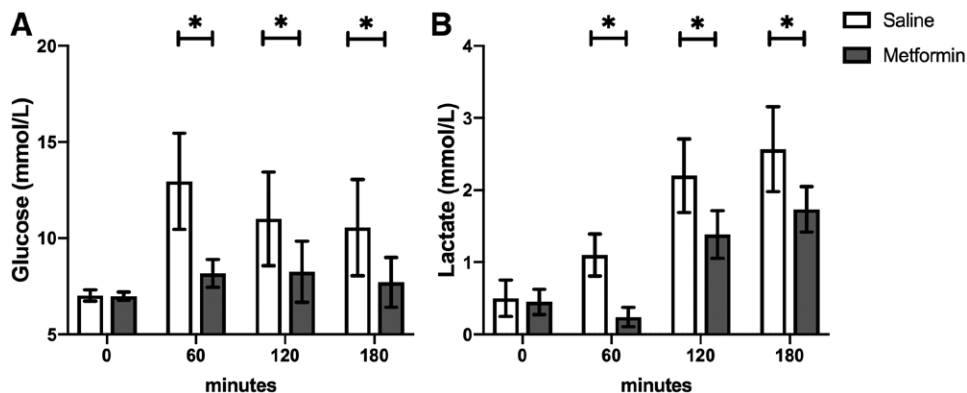
FIGURE 5. Serum lactate and glucose concentrations during normothermic machine perfusion (NMP). A, Serum lactate concentrations during 60, 120, and 180 min of NMP. B, Serum glucose concentrations during 60, 120, and 180 min of NMP. In the group with metformin preconditioning, serum lactate and glucose levels were significantly lower at 60, 120, and 180 min of NMP in comparison with the saline preconditioning (*$P < 0.05$). Data are represented as medians with interquartile range (IQR; error bars).
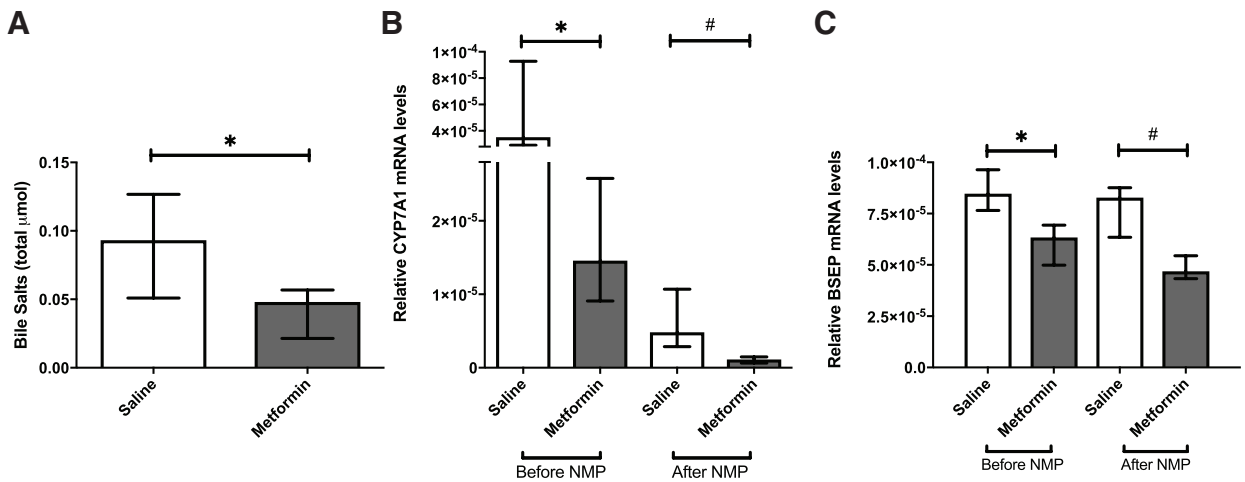
FIGURE 6. Total concentration of bile salts and expression of CYP7A1 and BSEP during NMP. A, concentrations of bile salts after 1.5 h of NMP. The group with metformin preconditioning displayed a significantly lower concentration of bile salts after 1.5 h of NMP (*P < 0.05). B, Relative mRNA expression of CYP7A1. C, Relative mRNA expression of BSEP. mRNA expression of CYP7A1 and BSEP in biopsies directly after SCS (thus before NMP) showed that metformin preconditioning significantly decreases mRNA levels of CYP7A1 and BSEP compared with the groups with saline preconditioning. In addition, after 3 h of NMP, mRNA levels of CYP7A1 and BSEP were significantly lower in the group with metformin preconditioning (#P < 0.05). Data are represented as medians with interquartile range (IQR; error bars). BSEP, bile salt export pump; NMP, normothermic machine perfusion.
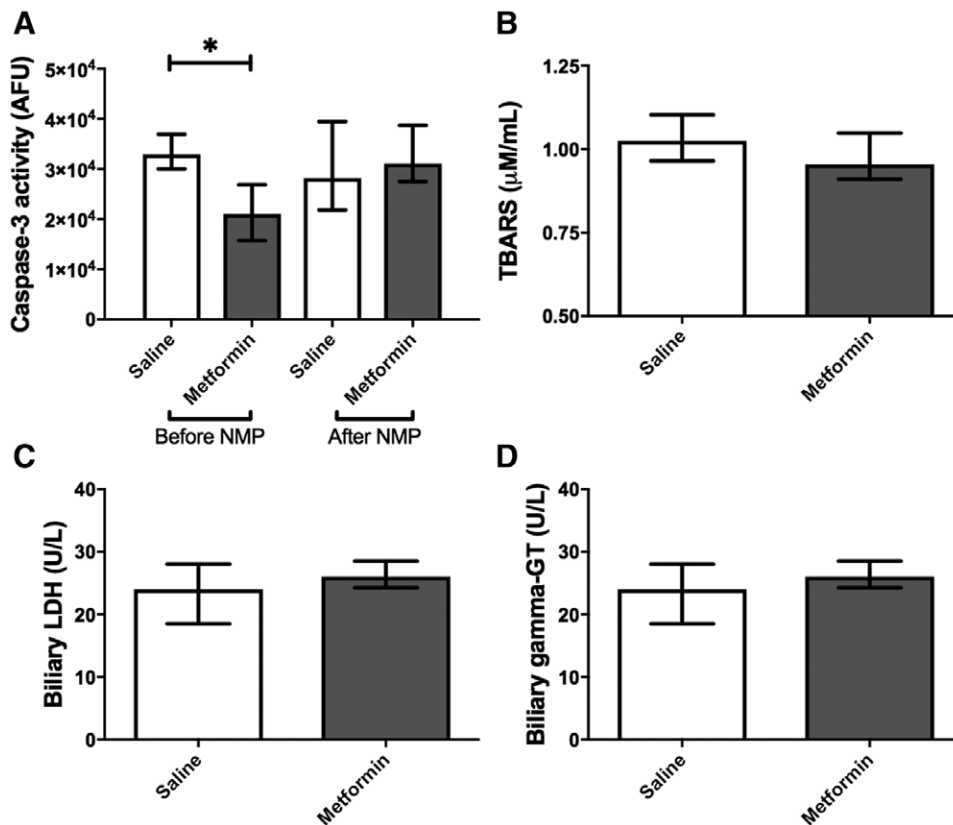
FIGURE 7. Markers of hepatobiliary injury during NMP. A, Caspase-3 activity measured in liver parenchyma after static cold storage (SCS; before NMP) and after 3 h of NMP. Caspase-3 activity was significantly lower in the group with metformin preconditioning compared with the group with only saline preconditioning before NMP (*P < 0.05). However, after 3 h of NMP, no significant differences were found in caspase-3 activity between both groups. B, Perfusate TBARS levels after 3 h of NMP. TBARS levels were not significantly different between both groups. C, Biliary LDH after 3 h of NMP. D, Biliary γ-GT after 3 h of NMP. The concentrations of biliary LDH and γ-GT were not significantly different between the groups with metformin preconditioning and saline preconditioning. Data are represented as medians with interquartile range (IQR; error bars). γ-GT, γ-glutamyl transferase; LDH, lactate dehydrogenase; NMP, normothermic machine perfusion; TBARS, thiobarbituric acid reactive substances.
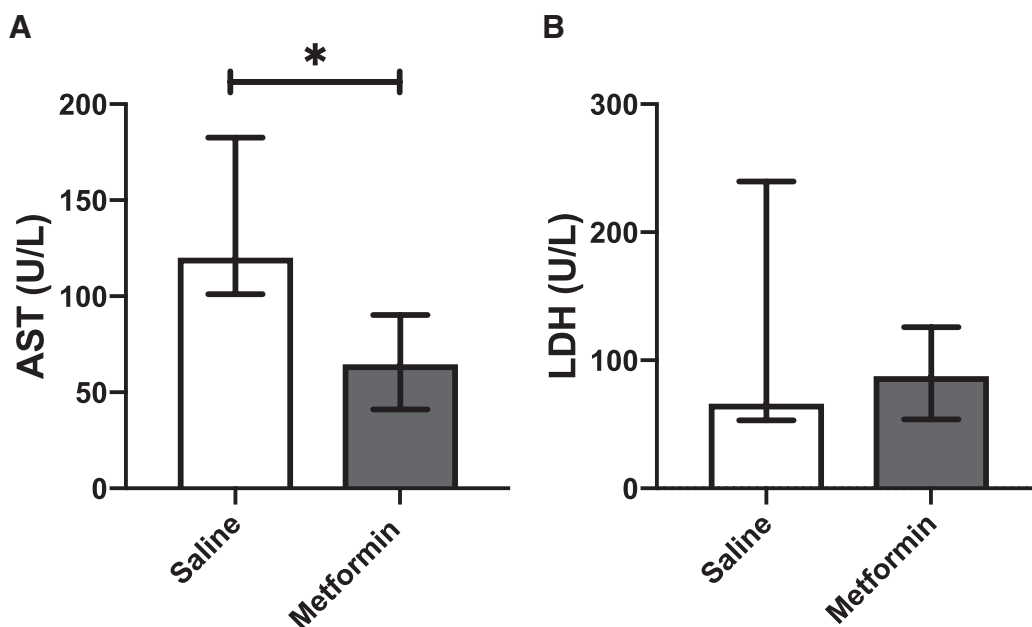
FIGURE 8. Serum markers of hepatocellular injury after rat liver transplantation. A, Posttransplant serum AST levels after 48 h of survival. Recipients of a metformin preconditioned donor liver showed significantly lower serum levels of AST in comparison with recipients without metformin preconditioned donor liver (*P < 0.05). B, Posttransplant serum LDH levels after 48 h of survival. No significant differences observed. Data are represented as medians with interquartile range (IQR; error bars). AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

and can improve hepatobiliary function during rat liver transplantation.

Our study results are in line with recent animal experiments and an observational human study where metformin preconditioning reduced I/R injury after myocardial infarction.[2-4] These studies demonstrated that metformin administration before myocardial infarction decreased infarction size, preserved cardiac function, and improved survival.[2-4] To our knowledge, we are the first who report the beneficial effects of metformin as preconditioning agent in a model of NMP and transplantation. To date, only Chai et al[23] showed that metformin, in particular only as postconditioning agent, reduces hepatocyte injury after 12 h of ex situ hypothermic machine perfusion (4°C) in rat donor livers. However, their results were not confirmed in a transplantation model. Moreover, the perfusion time they used may be debatable because the current clinical application of hypothermic machine perfusion consists of 2 h of perfusion.[24] Therefore, it would be interesting to known whether metformin can have its effect in a short perfusion time period and especially during hypothermic conditions, in which reduced metabolic conditions occur. We have also

studied the effect of metformin as postconditioning agent, in which metformin was added to the NMP solution (data not shown). Here we did not find any positive effect. We observed mainly lactate acidosis, low levels of ATP, and low bile production. Therefore we suggest that metformin should be intracellularly available before the ischemic event, which is in line with other clinical studies.[25,26]

As described before, metformin is able to phosphorylate and subsequently activate AMPK. An effect of AMPK activation is suppression of FXR transcriptional activity.[10] FXR plays a central role in bile acid hemostasis, maintaining the balance between bile acid formation and transport.[11] An impaired function of FXR can lead to an intracellular accumulation of toxic bile acids and subsequently bile acid–mediated biliary injury.[12] Normally, FXR mainly induces BSEP expression and inhibits CYP7A1 expression.[27,28] In our study, we observed that metformin preconditioning before and after NMP reduces mRNA levels of both CYP7A1 and BSEP. Consequently, our study could not confirm the association between AMPK activation by metformin and FXR suppression. A possible explanation for this might be the low concentration of metformin used in
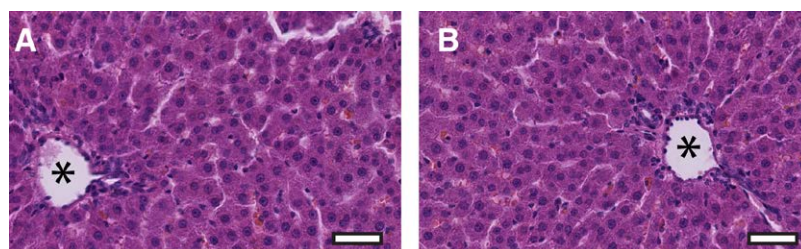
FIGURE 9. Histological evaluation of liver parenchyma after rat liver transplantation. A, Liver parenchyma of livers preconditioned with metformin. B, Liver parenchyma of livers preconditioned without metformin (controls). No pathological differences were observed. Coupes were stained with hematoxylin and eosin. The central vein is marked with an asterisk. Scale bars indicate 50 μm.

our experiment during preconditioning. With preconditioning, we obtained circulating metformin levels of 6 mg/L in situ, whereas in experiments with cultured hepatocytes concentrations of 165 mg/L were used and FXR suppression was observed.[29] Therefore, in our study, we did not find evidence that 2 therapeutic doses of metformin prior transplantation are related to biliary injury during NMP and after transplantation.

Another interesting finding of our study was the relatively low concentration of bile salts and high bile production in the group with metformin preconditioning versus the group with saline preconditioning. The question arises if bile in the group with metformin preconditioning was diluted. A possible explanation may be the osmotic effect of bicarbonate and water. Bile flow into the bile canaliculi is dependent on the osmotic gradients caused by bile salts (bile salt–dependent bile flow) and other solutes (bile salt–independent bile flow).[30] With respect to the bile salt–independent bile flow, it has been shown that biliary bicarbonate as solute increases the osmotic gradient, allowing water to passively follow into the canaliculi.[31] In the group with metformin preconditioning significantly more bicarbonate was secreted into bile during 3 h of NMP. As a result, the higher biliary bicarbonate production could play a role in the diluted bile production in the group with metformin preconditioning.

Surprisingly, in the NMP experiment, we did not find a significant reduction of levels of hepatocellular injury markers (in particular AST) between the group of metformin preconditioning and saline preconditioning. However, in the transplantation model, serum levels of the hepatocellular injury marker AST were significantly different between metformin preconditioning and the control group, without metformin preconditioning. A possible explanation could be the time period of reperfusion between both experiments. The perfusion period of NMP was 3 h in comparison with the transplantation model where 48 h of reperfusion occurred. Therefore, the NMP perfusion period might be too short to observe a significant difference in the levels of hepatocellular injury markers between metformin preconditioning and saline preconditioning.

As mentioned in the Introduction, metformin protects against I/R injury by modulation of complex I of the respiratory chain in the mitochondria. Recently, a part of our study group (Moshage, Geng et al) has described evidence that metformin directly induces superoxide dismutase 2 (SOD2) expression.[32] The SOD2 enzyme is an important enzyme in the reduction of apoptosis and cell death. Activation of SOD2 reduces ROS production during I/R with less cell death as consequence.[32]

Further research should be undertaken to translate our findings to the clinical situation. First, our perfusion and transplantation experiment should be performed with an animal model with more severe hepatobiliary cellular injury at baseline, for example, the usage of longer SCS times or a model with donation after circulatory death, to assess if metformin is also able to reduce cellular injury during ex situ NMP in these situations. Moreover, in human liver transplantation, it would be interesting to study outcome after transplantation of donor livers from diabetic donors who were using metformin medication, versus those who did not. These data could be obtained from a large data cohort such as the Eurotransplant database. Study endpoints could be serum levels of hepatobiliary injury markers and liver function markers the first week postoperative after transplantation.

In conclusion, the current study suggests that metformin preconditioning has a beneficial effect on donor rat livers, evidenced by improved hepatic function (increased bile production, higher biliary bicarbonate and bilirubin levels, and lower lactate levels), optimization of cellular energy (ATP production), and reduced hepatocyte injury as reflected by significantly lower levels of AST posttransplantation. Our study results may form a basis for further clinical research, whereby the beneficial effects of metformin in lowering I/R injury can be studied in human liver transplantation with ECD livers.

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