Strategies to Improve Outcome after Transplantation of Extended Criteria Donor Livers

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Pre- and Postconditioning Effects of Metformin in Rat Donor Livers

Submitted for publication

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Abstract

**Background:** Pre- or reconditioning of donor livers can improve organ quality prior to transplantation. The aim of this study was to investigate whether metformin as pre- or reconditioning agent is able to reduce preservation injury in rat donor livers and improve hepatobiliary function during *ex situ* normothermic machine perfusion (NMP).

**Methods:** To study the preconditioning effects of metformin, metformin was administered via oral gavage 12 and 2 hours before the hepatectomy. To assess the reconditioning effects of metformin, in 2 other groups, metformin was added to the NMP perfusion fluid in two different concentrations (30 and 300 mg/L). In the reference group, no pre- or reconditioning was carried out. In all groups, rat donor livers were preserved for 4 hours in preservation fluid on melting ice. Thereafter, NMP was performed for viability assessment.

**Results:** Preconditioning improved ATP production, hepatobiliary function (assessed by total bile production, biliary bilirubin and bicarbonate), and significantly lowered levels of lactate and glucose during NMP. On the other hand, metformin preconditioning did not reduce markers for hepatobiliary injury such as AST, ALT, LDH, caspase-3 activity, TBARS or biliary gamma-GT, and LDH. Reconditioning with metformin did not improve hepatobiliary function or reduce injury markers during NMP.

**Conclusion:** Preconditioning of rat donor livers with metformin improves hepatobiliary function but does not reduce preservation injury as assessed during 3 hours of NMP. Reconditioning with metformin showed no beneficial effects.
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 (1,2). Several animal and human studies have shown that administration of metformin, either before an ischemic event (preconditioning) or immediately at the beginning of reperfusion (reconditioning), reduces tissue injury and preserves cardiac function after myocardial infarction (2-4).

Although the exact mechanism of action of metformin is not fully known, one of the main actions of metformin is to partly and selectively block complex I of the mitochondrial respiratory chain (5). This inhibition of complex I has positive but also negative consequences. Positively, partial complex I inhibition reduces the level of cellular energy, which stimulates phosphorylation of the cellular energy sensor enzyme adenosine monophosphate protein kinase (AMPK) (6). An indirect consequence of AMPK activation is prevention of opening of the mitochondrial permeability transition pore (MPTP) in the mitochondrial inner membrane (7). When the MPTPs are closed, it is not possible to leak cytochrome c, which normally activates apoptotic death pathways (8-10). Another positive effect of partial complex I inhibition is a reduction in the production of reactive oxygen species (ROS) (11). Some ROS production normally accompanies oxidative phosphorylation and this increases during reperfusion. ROS avidly attack and degrade many cellular components including the mitochondria themselves (12). Inhibition of oxidative phosphorylation by metformin is coupled with lower ROS production and the mitochondria will be better protected against ROS-mediated injury (11). On the other hand, a negative effect of metformin and AMPK activation is the down regulation of the nuclear bile acid farnesoid X receptor (FXR) (13). 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 (CYP7A1), the rate-limiting enzyme in bile salt synthesis, and the bile salt export pump (BSEP), the major bile salt exporter (14).

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 (15). However, these ECD livers are more prone to I/R injury and are therefore related to lower graft survival rates (16). To enhance outcome after transplantation of ECD livers, quality of the organ can be improved before or after organ procurement with preconditioning or reconditioning strategies (17). Preconditioning of the donor can be carried out with pharmacological compounds administered to the donor during intensive care stay (18), while for reconditioning purposes machine perfusion can be used to “revitalize” the liver prior to transplantation (19).
The aim of the current study is to investigate whether metformin is able to reduce preservation injury of donor livers when applied as pre- or reconditioning agent. Hepatobiliary viability assessment was carried out during 3 hours of ex situ normothermic machine perfusion (NMP: perfusion at body temperature).

**Materials and methods**

**Animals**
Male Lewis rats (LEW/Han®Hsd) (270-300 g) were obtained from Harlan Laboratories (Boxmeer, the Netherlands). Animals received care according to the Dutch Law on Animal Experiments and the study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG).

**Experimental Design**
Rats were divided into three experimental groups and one reference group (n = 5-6 per group) (Figure 1). The three experimental groups were used to study the effects of preconditioning or reconditioning with metformin in rat donor livers. Metformin was purchased from Sigma-Aldrich (1,1-dimethylbiguanide hydrochloride, Sigma-Aldrich Inc., St. Louis, MO, USA). For assessment of the preconditioning effects, metformin (300 mg/kg body weight) was dissolved in saline (0.9% NaCl) and administered 12 and 2 hours before the hepatectomy directly into the stomach via oral gavage. Pretreatment with metformin in a concentration of 300 mg/kg body weight is in accordance with earlier rat studies in which serum levels were obtained that were within the therapeutic range used in humans (1-4 mg/L) (20,21). All other groups were pretreated with saline (0.9% NaCl) on the same time points before the hepatectomy.

In all study groups, rat livers were procured using a standard technique (see next paragraph) and subsequently preserved by static cold storage (SCS) in histidine-tryptophan-ketoglutarate (HTK) preservation solution (Custodiol, Essential Pharmaceuticals, Ewing, NJ, USA) for 4 hours. Prior to NMP, all livers were flushed

![Figure 1. Schematic representation of the experimental groups to examine the effects of pre- and reconditioning with metformin. All groups contained 5-6 rat livers.](image-url)
with 10 mL of cold saline. To study the reconditioning effects of metformin, metformin was added to the perfusion solution during NMP in 2 different concentrations: 300 mg/L and 30 mg/L. A concentration of 300 mg/L corresponds with 1.8 mM metformin. In a recent study with hepatocyte cell cultures, 2 mM of metformin was the lowest effective concentration to lower the degree of apoptosis induced by bile acids (22). However, 300 mg/L is a toxic concentration for humans (23). Therefore, we examined also the effects of 30 mg/L, which can be seen as a non-toxic concentration for humans (metformin toxicity > 40 mg/L) (23).

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 (Life Technologies Europe, Bleiswijk, the Netherlands), 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, LEO Pharma A/S, Ballerup, Denmark) adding up to a total volume of 100 mL.

**Procurement of Rat Donor Livers**

Procurement of rat livers has been described previously (24). 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 containing 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 vivo. After heparinization, the hepatectomy was performed by ligation of the splenic vein, mesenteric artery, and mesenteric vein. Thereafter, the celiac trunk was cannulated. After clamping of 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.

**Static Cold Storage and Normothermic Machine Perfusion**

For SCS, livers were stored in bags with ice-cold HTK (4°C) on melting ice for 4 hours. 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). Two roller pumps (Ismatec ISM404 + ISM719 and MS-2/6-160; IDEX Health & Science, Wertheim-Mondfeld, Germany) provided pulsatile flow through the hepatic artery and continuous flow through the portal vein. Continuous flow through the portal vein was achieved by the combination of elastic tubing and a pulse damper to remove pulses from the
Figure 2. *Ex vivo* 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 CO₂ (D). Several bubble traps (three-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 displayed in real-time on a connected laptop (F). The perfusion temperature was maintained constant by two heat exchangers (G) and a radiator/ventilator combination (H), all connected to the thermostat pump (I). For real time control of the perfusion temperature, one 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 three-way connector at the portal side, samples of the perfusion solution were taken every 30 min for analysis of the perfusate (M).

A roller pump. Two tubular membrane oxygenators provided oxygenation of the perfusion solution and removal of CO₂. Perfusion box and perfusate temperature were maintained stable at 37 degrees Celsius using a thermostat pump (Huber, Offenburg, Germany) and radiator/ventilator combination (Freezing Hardware, Losser, the Netherlands). The system was pressure-controlled by a computer algorithm allowing autoregulation of blood flow through the liver, with constant pressure at variable flow rates. In-line sensors monitored flow, pressure, and temperature and data were analyzed by and displayed in real-time on a connected laptop. NMP was performed with a mean arterial pressure of 110 mmHg and 11 mmHg at the portal vein. The perfusion fluid was oxygenated with 100% O₂ (1 L/min) and the pO₂ was 60-80 kPa (450-600 mmHg), as described in earlier machine perfusion experiments (25,26).

Analysis of Metformin and Biochemical Markers of Function and Injury

Samples of heparinized arterial blood during hepatectomy and perfusate before the start of NMP and after every 30 min of NMP were centrifuged (2700 g for 5 min at 4°C) and the supernatant was collected, frozen and stored at -80°C for future additional analysis. Metformin was measured by a validated liquid chromatography–mass spectrometry method in the laboratory of the Department of Clinical Pharmacy and Pharmacology of the University Medical Center Groningen. Serum insulin levels were analyzed using an ELISA kit (Rat/Mouse Insulin ELISA, Merck, Billerica, MA, USA). Determination of glucose, lactate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), was performed using standard biochemical methods.

Bile production was measured gravimetrically at 30-minute 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 expressed as mL/gram liver. Biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile (27). For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark). Biliary concentration of gamma-glutamyl
transferase (gamma-GT) and LDH were measured as markers of biliary epithelial cell injury (28), and biliary bilirubin concentration was measured as marker of hepatocellular secretory function (28), using standard biochemical methods. Total bile salt concentrations were measured as described before (29).

Thiobarbituric acid reactive substances (TBARS) were measured in perfusate samples after 2 hours of reperfusion, as a marker for oxidative stress (degree of lipid peroxidation of membranes). Detailed description of the method has been described previously (30).

**Caspase-3 Enzyme Activity Assay**
Immediately after SCS and after 3 hours of NMP, caspase-3 enzyme activity was measured in liver homogenates using a fluorimetric assay and expressed in arbitrary fluorescence units (AFUs) (31). Caspase-3 enzyme activity was corrected for the total protein content. The total protein concentration was determined using a commercially available kit (Bio-Rad, Veenendaal, the Netherlands).

**RNA Isolation and Polymerase Chain Reaction (PCR)**
Immediately after SCS and after 3 hours of NMP, hepatic mRNA expression of hepatocellular transporter protein bile salt export pump (BSEP) and cholesterol 7α- hydroxylase (gene symbol CYP7A1) was determined by quantitative real-time PCR. RNA isolation, reverse transcription PCR, and quantitative PCR (qPCR) was performed as described previously (32) and 18S mRNA levels were used as endogenous control. Primers and probes are provided in Table 1.

**Adenosine triphosphate (ATP) Extraction and Measurement**
The hepatic ATP content was used as an indicator of the energy status of the liver tissue. For this purpose, liver tissue biopsies after 3 hours of NMP were immediately frozen in liquid nitrogen and were processed later for ATP measurement, as described previously (30).

**Histological Evaluation of Liver Parenchyma**
After 3 hours of NMP, biopsies were taken from liver parenchyma and preserved with 10% formaldehyde. After inclusion in paraffin, slides were stained with periodic acid-Schiff (PAS) for evaluation of the glycogen content.

**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 a p-value of < 0.05. All statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).
Table 1. Primer-probe Sets Used for RT PCR Analysis

<table>
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<th>Gene</th>
<th>Forward</th>
<th>Reversed</th>
<th>Probe</th>
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<tbody>
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<td>18S</td>
<td>CGG CTA CCA CAT CCA AGG A</td>
<td>CCA ATT ACA GGG CCT CGA AA</td>
<td>CGC GCA AAT TAC CCA CTC CCG A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP7A1</td>
</tr>
<tr>
<td>CYP7A1</td>
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<td>AGG CAT ACA TCC CTT CCG TGA</td>
<td>TGC AAA ACC TCC AAT CTG TCA TGA GAC CTC C</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>BSEP</td>
</tr>
<tr>
<td>BSEP</td>
<td>CCA AGC TGC CAA GGA TGC TA</td>
<td>CCT TCT CCA ACA AGG GTG TCA</td>
<td>CAT TAT GGC CCT GCC GCA GCA</td>
</tr>
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Results

Perfusate Levels of Metformin and Metabolic Parameters during Hepatectomy

To confirm that oral gavage with metformin led to adequate levels, serum metformin levels were measured during the hepatectomy. In the groups in which metformin was administered via oral gavage as preconditioning agent, relatively high metformin serum levels were achieved, while no metformin was detected in rats to whom only saline was administered via oral gavage (Figure 3A). Metformin is an oral antihyperglycemic agent and therefore can lower elevated blood glucose levels. Glucose levels (reference value in rat 4.5 mmol/L) during the hepatectomy were comparable between the group with metformin treatment before the hepatectomy and without metformin treatment (Figure 3B). In addition, also insulin levels showed no significant differences between the metformin-pretreated animals and untreated animals (Figure 3C).

Metformin Concentrations during Normothermic Machine Perfusion

Levels of metformin during 3 hours of NMP are shown in Figure 4. In all groups in which metformin was added as extra ingredient to the perfusion solution, a drop in the concentration was noticed in the first 30 minutes of perfusion, indicating that a part of metformin remains intracellular. Thereafter, levels of metformin in the perfusion solution remained stable. Remarkably, in the group of rats that only received metformin via oral gavage before the hepatectomy and without metformin in the perfusion solution, a low level of metformin was observed in the perfusate during the entire period of NMP.
Figure 3. Serum levels of metformin, glucose, and insulin during hepatectomy. Panel A: Serum levels of metformin during hepatectomy. Panel B: Serum glucose levels during hepatectomy (rat reference glucose levels 4.5 mmol/L). Panel C: Serum insulin levels during hepatectomy. Data are represented as medians with IQR (error bars).
Figure 4. Metformin concentrations during 3 hours of NMP. Data are represented as medians with IQR (error bars).
**Preconditioning with Metformin Improved ATP Content, Markers for Hepatobiliary Function, and Lowered Levels of Glucose and Lactate during NMP**

Hepatic ATP concentrations after 3 hours of NMP are provided in Figure 5A. Interestingly, the group with metformin preconditioning had a significantly higher ATP production after 3 hours of NMP compared to the reference group (no pretreatment with metformin and normal perfusion solution without metformin). There were no significant differences in hepatic ATP contents between all other groups and the reference group.

Bile production, an important marker for hepatocyte function, is presented in Figure 5B. The group with metformin preconditioning displayed a significantly higher bile production during the entire period of NMP compared to the reference group. In addition, after the first 60 minutes of perfusion, the group with a high concentration of metformin added to the perfusion solution (300 mg/L) displayed a rapid decline in bile production compared to the reference group. Furthermore, no significant differences in bile production were noticed between the group with a low concentration of metformin (30 mg/L) in the perfusion solution and the reference group.

Concentrations of the total biliary bilirubin after 3 hours of NMP, another marker for hepatocyte function, are presented in Figure 5C. The concentrations of total biliary bilirubin were significantly higher after metformin preconditioning compared to the reference group. Moreover, in the group with a high concentration of metformin added to the perfusion solution (300 mg/L), bilirubin concentration was significantly lower compared to the reference group after 3 hours of NMP.

Concentrations of biliary bicarbonate, a marker for cholangiocyte function, were measured after 1.5 hours of NMP (Figure 5D). The biliary bicarbonate concentration...
was significantly lower in the group of livers with a high concentration of metformin added to the perfusion solution (300 mg/L) compared to the reference group. In addition, a significantly higher concentration of bicarbonate was measured in the bile samples after 1.5 hours in the group with metformin preconditioning versus the reference group. Biliary pH levels after 1.5 hours of NMP are shown in Figure 5E. Biliary pH was not significantly different between the study groups.

The perfusate levels of lactate and glucose during 3 hours of NMP are presented in Figure 6A and B. The group with metformin added to the perfusion fluid in a high concentration (300 mg/L) showed a significantly steeper increase in lactate and glucose concentrations almost over time compared to the reference group. Furthermore, in the group with metformin preconditioning, lactate and glucose levels were significantly lower than the reference group from 60 minutes onwards.

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

Concentrations of total bile salts after 1.5 hours of NMP are presented in figure 7A. The group with metformin preconditioning displayed a significantly lower concentration of total bile salts after 1.5 hours of NMP compared to the reference group. Between the groups with metformin in the perfusion solution (high and low concentration) and the reference group no significant differences were found. Remarkably, the concentration of total bile salts in the group with metformin added to the perfusion solution in a high concentration (300 mg/L) was almost comparable to the concentration of total bile salts in the group with preconditioning. However, the total bile production in the group with metformin in a high concentration added to the perfusate was almost 4 times lower (Figure 5B).

Relative mRNA expression of CYP7A1 and BSEP immediately measured after SCS (baseline groups; rats pretreated with metformin or only saline) and after 3 hours of NMP are presented in Figure 7A and 7B. Metformin pretreatment significantly decreased mRNA levels of CYP7A1 and BSEP compared to the groups with saline feeding as pretreatment. In addition, after 3 hours of NMP, mRNA levels of CYP7A1 and BSEP were significantly lower in the group with metformin preconditioning compared to the reference group.
Figure 6. Lactate and glucose during 3 hours of NMP. Panel 6A: Lactate concentrations during 3 hours of NMP. Panel 6B: Glucose concentrations during 3 hours of NMP. Almost during the entire period of NMP, levels of lactate and glucose were significantly higher in the group with metformin added to the perfusion solution in the high concentration (300 mg/L) compared to the reference group (*p<0.05). Furthermore, in the group with metformin preconditioning, lactate and glucose levels were significantly lower after the first hour of NMP in comparison with the reference group (**p<0.05). Data are represented as medians.
Pre- and Reconditioning with Metformin Did Not Lower Preservation Injury during NMP

Markers for hepatocellular injury, AST, ALT, and LDH measured in the perfusate after 3 hours of NMP showed no significant difference between the groups with metformin pre- or reconditioning and reference group (Figure 8A-C). In addition, levels of TBARS, a marker for oxidative stress, were not significantly different between groups after 3 hours of NMP (Figure 8D).

Markers for cholangiocyte injury, LDH and gamma-GT, measured in bile after 3 hours of NMP are provided in Figure 8E and F. The concentrations of biliary LDH and gamma-GT were also not significantly different between the groups with metformin pre- or reconditioning and the reference group.

Figure 8G presents caspase-3 activity, a marker for apoptosis, immediately measured after SCS (baseline groups; rats pretreated with metformin or only saline) and after 3 hours of NMP. Interestingly, caspase-3 activity was significantly lower in the group with metformin pretreatment compared to the group with only saline pretreatment. However, after 3 hours of NMP no significant differences were found in caspase-3 activity between the groups with metformin pre- or reconditioning and the reference group.

Figure 7. Total concentration of bile salts and mRNA expression of CYP7A1 and BSEP. Panel A: Total bile salts production after 1.5 hours of NMP. Levels of total bile salts were significantly lower in the group with metformin preconditioning compared to the reference group (*<0.05). Panel B: Relative mRNA expression of CYP7A1. Panel C: Relative mRNA expression of BSEP. Messenger RNA expression of CYP7A1 and BSEP in baseline biopsies immediately after static cold storage (SCS) showed that metformin pretreatment significantly decreases mRNA levels of CYP7A1 and BSEP compared to the groups with saline feeding as pretreatment. In addition, after 3 hours of NMP, mRNA levels of CYP7A1 and BSEP were significantly lower in the group with metformin preconditioning compared with the reference group. Data are represented as medians with IQR (error bars).
Figure 8. Markers for hepatobiliary injury. Panel A: Perfusate AST levels after 3 hours of NMP. Panel B: Perfusate ALT levels after 3 hours of NMP. Panel C: Perfusate LDH levels after 3 hours of NMP. Panel D: Perfusate TBARS levels after 3 hours of NMP. Panel E: Biliary gamma-GT after 3 hours of NMP. Panel F: Biliary LDH after 3 hours of NMP. Panel G: Caspase-3 activity immediately after static cold storage (SCS) (baseline biopsies) and after 3 hours of NMP. The caspase-3 activity was significantly lower in the group with metformin pretreatment compared to the group with only saline pretreatment in baseline biopsies (*p<0.05). Data are represented as medians with IQR (error bars).
**Metformin Reconditioning Lowered Glycogen Content during NMP**

The group with metformin added to the perfusion solution in a high concentration (300 mg/L) displayed a breakdown of the liver glycogen content during 3 hours of NMP, as indicated by PAS staining (Figure 9A). In contrast, the PAS stains of all other study groups indicated higher glycogen levels in liver tissue (Figure 9B).

**Discussion**

The aim of the current study is to examine whether metformin is able to reduce injury when it is administered before the period of ischemia, as preconditioning agent or as reconditioning agent when it was added as extra additive during NMP. Our study demonstrates that preconditioning with metformin significantly improved ATP content, markers for hepatobiliary function (total bile production, cumulative levels of biliary bilirubin and bicarbonate), and lowered levels of glucose and lactate during 3 hours of NMP. However, metformin preconditioning also lowered total bile salt secretion and expression of the FXR target genes CYP7A1 and BSEP. In addition, metformin preconditioning did not lower markers for hepatobiliary injury such as AST, ALT, LHD, TBARS, biliary gamma-GT, biliary LDH, and caspase-3 activity, a specific marker for apoptosis. With respect to the reconditioning effects of metformin, examined in two different perfusate concentrations (30 and 300 mg/L), our study did not show beneficial effects in improving hepatobiliary function or reducing hepatobiliary injury. In contrast, metformin added to the perfusion solution in a high concentration (300 mg/L) had adverse effects as it significantly increased levels of lactate and glucose, decreased bile production, and reduced glycogen content during 3 hours of NMP.

As described in the introduction, metformin partly blocks complex I of the respiratory chain. Inhibition of complex I during ischemia and in the beginning
of reperfusion can lower I/R injury via two different pathways. Firstly, partial blocking of complex I stimulates AMPK activation, inhibition of opening of the MPTP, a depressed release of cytochrome c and ultimately less apoptosis (8-10). Secondly, partial inhibition of complex I is related with lower production of ROS (11). Some ROS are normally generated during oxidative phosphorylation (12). However, when the oxidative phosphorylation is partly blocked by metformin, less ROS will be produced (11). ROS are an important cause for cellular and in particular mitochondrial injury as ROS are extremely unstable and react with several cellular components (12). In our experiments, we found evidence that preconditioning with metformin significantly improved mitochondrial function, as reflected by higher ATP content and lower levels of lactate in the perfusate during 3 hours of NMP compared to the reference group. In addition, our data also showed that metformin preconditioning lowered the degree of apoptosis. Hepatic caspase-3 activity was significantly lower immediately after SCS in the group with metformin preconditioning compared to the reference group. However, levels of TBARS after 3 hours of NMP, a marker for lipid peroxidation of membranes due to ROS injury, were not significantly different between the group with metformin preconditioning and the reference group.

With respect to the reconditioning effects of metformin, no significant differences were obtained in ATP content, lactate levels, and caspase-3 enzyme activity compared to the reference group. The observation that reconditioning with metformin has no obvious beneficial effect may have two possible explanations. First, metformin was not present intracellularly in time to inhibit complex I during early reperfusion. It has been shown that closing of the MPTP via complex I inhibition should be performed immediately during reperfusion to lower apoptosis (2,8). Late inhibition of complex I during reperfusion has no effect in the reduction of apoptosis (2,8). Another possible explanation might be that complex I was inhibited too strongly during the entire period of NMP, which may have nullified the potential beneficial effects of metformin during early reperfusion. It is known that toxic inhibition of complex I as occurs in patients with a metformin overdose, suppresses the oxidative phosphorylation with lactic acidosis as a consequence (23). Although in our study reconditioning with metformin in two concentrations (30 and 300 mg/L) did not significantly suppress ATP content, lactate levels in the group with metformin in the high concentration (300 mg/L) were significantly higher compared to the reference group, indicating that also anaerobic glycolysis was necessary for ATP production.

Metformin is widely used for lowering gluconeogenesis in patients with type II diabetes (1,2). In our study, we observed significantly lower glucose levels in the group with metformin preconditioning. Remarkably, this was not the case in the groups with metformin reconditioning. In the group with a high concentration of metformin in the perfusion solution (300 mg/L) considerable increases in
glucose levels were observed. In addition, evaluation of PAS staining revealed a lower glycogen content after 3 hours of NMP, which indicates a higher degree of glycogenolysis in this group. Apparently, the increases in glucose levels were generated from hepatic glycogen stores. A higher rate of glycogenolysis was also shown in earlier studies where rat livers were perfused with high concentrations of metformin (5 mmol/L or 825 mg/L) (33). However, the exact mechanisms how metformin reduces gluconeogenesis but also can stimulate glycogenolysis are not fully known (34).

Another effect of AMPK activation is suppression of FXR transcriptional activity (13). In turn, FXR plays an important role in the control of bile salt homeostasis, inducing BSEP expression and inhibiting CYP7A1 expression (35,36). In our study, in particular in the groups with metformin preconditioning, we observed that metformin reduces the mRNA levels of both CYP7A1 and BSEP. Therefore, 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 our experiment during preconditioning. With preconditioning we obtained circulating metformin levels of 6 mg/L in vivo, while in experiments with cultured hepatocytes concentrations of 165 mg/L were used and FXR suppression was observed (13).

One interesting finding was the relatively higher concentration of total bile salts in the group with metformin added to the perfusion solution in a high concentration (300 mg/L) compared to the group with metformin preconditioning. Levels of total bile salts were almost similar between both groups, while the group with metformin preconditioning produced almost 4 times more bile. Therefore, the question rises if bile in the group with metformin preconditioning was diluted. Bile flow into the bile canaliculi is dependent of the osmotic gradients caused by bile salts (bile salt dependent bile flow) and other solutes (bile salt independent bile flow) (37). 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 (38). In the group with metformin preconditioning significantly more bicarbonate was produced during 3 hours of NMP. As a result, the higher bicarbonate production could play a role in the diluted bile production in the group with metformin preconditioning.

Contrary to expectations, metformin preconditioning or reconditioning was not able to significantly reduce preservation injury in our heart beating donation model. Therefore, it is still interesting to assess in a model with more severe injury at baseline (e.g. in a donation after circulatory death model) whether metformin preconditioning will be able to lower preservation injury and improve hepatobiliary function. However, it should be noticed that levels of total bile salts as well as mRNA expression of CYP7A1 and BSEP are of interest in these future experiments.
Taken together, our results illustrate that preconditioning of rat donor livers with metformin significantly improves hepatobiliary function but does not reduce injury as assessed during NMP viability assessment. Reconditioning with metformin showed no beneficial effects. Further research should be undertaken to investigate whether metformin is able to reduce preservation injury in a model with more severe ischemic injury at baseline.
References


