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ORIGINAL ARTICLE

Grzegorz Nowak
Ulf G. Norén
Annika Wernerson
Hanns-Ulrich Marschall
Lennart Möller
Bo-Göran Ericzon

Enteral donor pre-treatment with ursodeoxycholic acid protects the liver against ischaemia-reperfusion injury in rats

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G. Nowak (✉) · B.-G. Ericzon
Department of Transplantation Surgery,
Karolinska Institute,
Huddinge University Hospital B56,
141 86 Stockholm, Sweden
E-mail: grzegorz.nowak@cfss.ki.se
Tel.: +46-8-58587706
Fax: +46-8-7743191

U. G. Norén · L. Möller
Unit for Analytical Toxicology,
Department of Biosciences,
Karolinska Institute,
Huddinge University Hospital,
Stockholm, Sweden

A. Wernerson
Department of Pathology,
Karolinska Institute,
Huddinge University Hospital,
Stockholm, Sweden

H.-U. Marschall
Department of Medicine,
Karolinska Institute,
Huddinge University Hospital,
Stockholm, Sweden

Abstract Liver donor pre-treatment with ursodeoxycholic acid (UDCA) may protect against injury during transplantation. In the present study we evaluated whether enteral administration of UDCA has an effect on bile flow and protects the liver from injury related to transplantation. Wistar rats were used in liver perfusion (LP) and transplantation (LTx) models. Rats were enterally administered UDCA (800 mg/kg) 3 h before cold perfusion. In LP, bile flow and bile acid composition were analysed. In LTx, serum ALT and liver histology were analysed. LP showed biliary UDCA enrichment up to 36 ± 13% in pre-treated rats, causing higher bile flow ($P = 0.026$) compared with control rats. LTx showed lower ALT and TUNEL positive hepatocytes in the UDCA group ($P < 0.02$ and $P < 0.05$). In conclusion, augmented bile salt-dependent bile flow is preserved in the liver after cold storage. Enteral donor pre-treatment with UDCA protects the liver against ischaemia-reperfusion injury.

Keywords Ursodeoxycholic acid · Ischaemia-reperfusion injury · Liver perfusion · Liver transplantation

Introduction

Liver transplantation is the treatment of choice in patients with end-stage liver disease and some inborn errors of metabolism. Beside the recipient's condition, liver graft function is mainly the result of a number of different factors arising before, during and after transplantation [1, 2]. Ischaemia-reperfusion injury is one of the main causes of graft non-function and a determinant of successful liver transplantation. This injury could be reduced by minimising storage time, but probably also by donor pre-treatment. Such strategy is aimed at increasing organ resistance to injuries during preservation and transplantation [3].

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid accounting for up to 3% of the bile acid pool in humans [4], improves symptoms, biochemistry, and probably clinical outcome in cholestatic liver diseases, such as primary biliary cirrhosis, primary sclerosing cholangitis and cholestasis of pregnancy, through mechanisms that have not yet been fully elucidated [5]. In animal experiments, parenteral administration of UDCA shortly before cold perfusion of the donor liver has been shown to have a protective effect on hepatocytes and cholangiocytes [6, 7, 8]. This protective effect of UDCA could be related to the inhibition of apoptosis [9, 10], an event often observed during ischaemia-reperfusion injury [11, 12].

The aims of the present study in rat liver perfusion and liver transplantation models were to evaluate whether enteral administration of a high dose of UDCA before liver procurement (1) has an effect on bile flow in the liver after cold preservation, and (2) protects the donor liver from injury occurring during transplantation.

Material and methods

Adult male Wistar rats (B&K, Sollentuna, Sweden; weighing 250-300 g) were used in vitro liver perfusion (LP; 28 outbred rats) and transplantation (LTx with arterialisation; 24 inbred rats) models. In the study group, the rats ($n=11$ in LP; $n=6$ donors in LTx) were administered UDCA (800 mg/kg b.w. in 3 ml of water, Ursofalk, Falk Pharma, Germany) by gavage 3 h before the livers were harvested. Control rats ($n=17$ in PM; $n=6$ donors in LTx) were administered 3 ml of water only. The high dose of UDCA used in the present experiment corresponds to a dose of 20 mg/kg b.w. in humans. This is calculated from differences between human and rat bile acid pool size and turnover [13]. The total bile acid pool size is 3 g in humans and 40 mg in a 200 g rat with bile acid turnover rates of 500 and 12 mg/day, respectively. This corresponds to 43 mg/kg b.w. and 7 mg/kg b.w. per day for bile pool size and turnover in humans, compared with 200 mg/kg b.w. and 60 mg/kg b.w. per day, respectively, in the rat.

LP livers were stored in cold University of Wisconsin (UW) solution (ViaSpan, DuPont, the Netherlands) for 24 h prior to in vitro perfusion. Livers in the LTx groups were transplanted after 8 h of cold preservation in UW solution. Principles of laboratory animal care were followed, and the experiments were carried out with the approval of the local ethics committee at the Karolinska Institute.

Perfusion model

In the present study a previously described rat liver perfusion model was used [14]. During the liver harvesting operation the common bile duct was cannulated (PTFE-tubing, 0.6 mm outer diameter), and an inflow tube (Venflon, 16 G, Becton Dickinson) was ligated into the portal vein. Cold UW solution (20 ml) was flushed into the portal vein immediately after liver removal. The isolated livers were submerged in UW solution and stored for 24 h at 4°C. After cold storage the liver was connected to the perfusion system for 4 h. The perfusion medium consisted of Krebs Ringer bicarbonate solution, pH 7.4, 3% bovine serum albumin, 10 mM alanine, 5.5 mM glucose, heparin (2000 IU in 70 ml) and 23% washed bovine erythrocytes (Statens Veterinärmedicinska Anstalt, SVA Håtunaholm, Sweden). The perfusion medium was pumped (Dosing pump FE 211, B. Braun, Erlangen, Germany) into an oxygenator and saturated with 5% CO_2 in O_2. The whole apparatus was kept at 37°C by circulating water and the perfusion medium entered the liver at a constant pressure of 15 cm H_2O. The first 30 min of perfusion was used for calibration.

Bile flow was monitored by bile sampling in 1.5 ml tubes. Bile samples were analysed for total bile acids using a 3-alpha-hydroxysteroid dehydrogenase assay [15]. To determine the lactate metabolising capacity (i.e. the liver function), a bolus dose of lactate was added to the perfusion medium. Perfusate samples were denatured in 8% perchloric acid at 30 min intervals. Residual lactate in the supernatant was measured using an enzymatic kit (Sigma 826-B, Sigma, St. Louis, MO, USA) following the manufacturer's protocol. Lactate is oxidised, by lactate dehydrogenase using nicotinamide adenine dinucleotide (NAD) as cofactor, to pyruvic acid, which is trapped with hydrazine to force the reaction to completion. NADH is measured spectrophotometrically at 340 nm. The experiments were terminated after 4 h due to apparently decreasing liver viability.

Liver transplantation

A rat liver transplantation model with re-arterialisation using the recipient right renal artery was used [16]. Surgery was performed under isoflurane anaesthesia (Fluovac Unit, IMS, Cheshire, UK). An inbred rat strain (syngeneic combination) was used in order to eliminate immunological response and injury, and to avoid the need of immunosuppression. After laparotomy in the donor, all the ligamentous attachments were divided and the liver was mobilised. Arterial supply from the coeliac axis up to the hepatic artery was presented. The common bile duct was transected, cannulated with a 5 mm long polyethylene tube (outer diameter 0.9 mm, Portex, Hythe, UK) and secured with 6-0 silk (Ethicon). The animals were injected with 50 units of heparin through the penile vein and the donor liver was perfused via the portal vein with 10 ml cold UW solution. After procurement with subsequent portal

vein and infrahepatic vena cava cuff preparation in an iced bath with UW solution (outer diameter of the cuff in portal vein 2.08 mm; outer diameter of the cuff in infrahepatic vena cava 2.8 mm; Portex), the livers were stored in UW solution at 4°C for 8 h.

During the recipient operation, the central venous catheter was placed in the jugular vein (Venflon 20 G, Becton Dickinson). After laparotomy, the recipient's liver and right kidney were excised. The right renal artery was prepared for graft arterialisation with a cuff (outer diameter 0.9 mm, Portex). The donor liver was placed orthotopically. The suprahepatic vena cava was anastomosed end-to-end with a running Prolene 7-0 suture (Johnson and Johnson). The continuity of the infrahepatic vena cava and the portal vein were established by means of the cuff technique [17]. The hepatic arterial circulation was re-established under the microscope by anastomosis of the donor coeliac axis to the recipient's right renal artery on the prepared cuff. Bile ducts were connected onto the tube. Recipient blood samples were collected 24 h after transplantation. The animals were killed 36 h after transplantation when liver tissue was harvested for histopathological examination.

Histopathological examination

Tissue samples were taken from the same part of the transplanted livers and fixed in 4% phosphate buffered formalin. The specimens were then embedded in paraffin and cut in a microtome (Leica Microsystems AB) into 4–5 μm-thick sections; the sections were stained with haematoxylin-eosin.

Histopathological signs of liver injury were semi-quantified under light microscope using modified Suzuki's classification [18]. Thus, we evaluated the amount of neutrophils, hepatocyte necrosis and fatty changes graded from 0 to 4. According to used classification absence of necrosis, fatty changes and neutrophils was scored as 0, while severe fatty changes, hepatocytes necrosis and neutrophils was given a score of 4, respectively. The same investigator performed all evaluations blinded to the identity of the sample.

DNA fragmentation was detected by terminal deoxyribonucleotidyl-transferase-mediated dUTP digoxigenin nick end labelling (TUNEL) (ApoTag, Oncor, MD), using the manufacturer's protocol. Apoptotic index defined as a ratio between the number of TUNEL positive hepatocytes and the total number of evaluated hepatocytes was compared between the groups. Apoptotic bodies negatively stained by the TUNEL method were not counted. Stained hepatocytes at the edge of the specimen were also excluded. 2500 hepatocytes were examed using random systematic sampling procedure.

Statistical methods

Statistical evaluation was performed using Student's *t*-test for independent variables and the ANOVA test for repeated measures. The Mann–Whitney U-test was used for non-parametric variables. Parametric data are presented as the mean ± standard deviation (SD). Non-parametric data obtained from histopathological examination are presented as the median (columns) and range (whiskers). The differences were considered statistically significant if P value of less than 0.05 was achieved.

Results

Perfusion model

UDCA was found to be enriched in the bile of the study group animals at 30, 90 and 150 min of perfusion (36.9 ± 13; 18.2 ± 14.9 and 7.6 ± 6.1% of total bile salts, respectively). While UDCA decreased during perfusion, the relative amount of cholic acid (CA) increased (19.5 ± 10.8%, 32.6 ± 14.8% and 43.9 ± 13.3 %, respectively). In control animals, CA was the major bile acid (52.3 ± 2.2 %) and UDCA was consistently low (2.2 ± 1.1 %). Throughout perfusion, bile production was higher in the UDCA group ($P = 0.026$) (Fig. 1). There were no differences in lactate levels over time between the groups.

Transplantation model

The groups did not differ with regard to cold ischaemia time (8.1 ± 0.2 h in controls; 8.2 ± 0.2 h in the UDCA group) or anhepatic time (18.1 ± 2.4 min and 17.1 ± 2.2 min, respectively). Lower serum ALT levels were observed 24 h after LTx in the UDCA group (31.8 ± 7.6 IU vs. 47.1 ± 10.5 IU in controls, $P < 0.02$). Regarding histopathological signs of liver injury, only minor changes were observed with no differences between the groups [Table 1]. However, a lower apoptotic index (AI) was found in the UDCA group (median AI 0.01 [range 0.002 - 0.015] in the control group and median AI 0.003 [range 0.002 – 0.004] in the UDCA group; $p < 0.05$) [Figure 2 and 3]. The bile duct epithelium was TUNEL negative in both groups.

Discussion

Despite improvements in organ preservation and liver surgery, the incidence of significant liver injury related to ischaemia re-perfusion is still a problem. Various strategies have been proposed to reduce the injury from

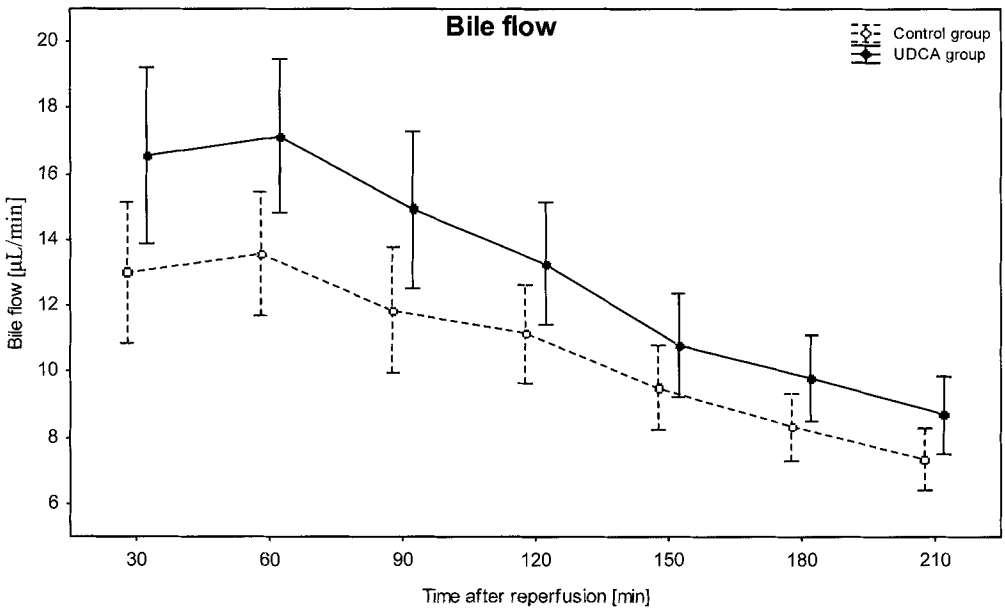

Fig. 1 Bile flow in perfusion model

ischaemia and re-perfusion during liver transplantation [3]. One possible method is to pre-treat the donor with drugs that may increase organ resistance to transplantation-related injuries [19]. Such drugs must be simple to administer and must rapidly reach high concentrations in the transplanted organ. Among the substances investigated for donor pre-treatment in liver transplantation, special attention has been paid to ursodeoxycholic acid (UDCA) and its taurine conjugate (TUDCA).

Table 1 Severity of liver injury at 36hrs after transplantation according to modified Suzuki's classification (mean ± SD)

	Control group	UDCA group
Fatty changes	0 ± 0	0 ± 0
Neutrophils infiltration	0.3 ± 0.5	0.2 ± 0.4
Necrosis	0.3 ± 0.5	0.2 ± 0.4

UDCA is a naturally occurring bile acid present in small amounts in human [5] and rat bile [20]. It has been shown that UDCA improves symptoms and serum liver biochemistry, possibly delays disease progression to severe fibrosis or cirrhosis, and perhaps prolongs post-transplant free survival in primary biliary cirrhosis [5]. Animal experiments have shown that parenteral donor pre-treatment with TUDCA reduces reperfusion injury as reflected by lower serum liver enzyme levels and better preservation of the bile duct, as seen in electron microscope studies [8, 21]. Similar results were obtained when TUDCA was administrated into the portal vein and/or added to the preservation solution [22].

In the present in vitro experiment, the increase in bile flow seen in the UDCA-treated group was similar to that observed in a previous in vivo study [20]. This indicates that bile-salt dependent bile flow is not only preserved after cold ischaemia, lasting as long as 24 h, but can also be increased by oral pre-treatment of the donor with the seemingly non-toxic substance UDCA. This suggests

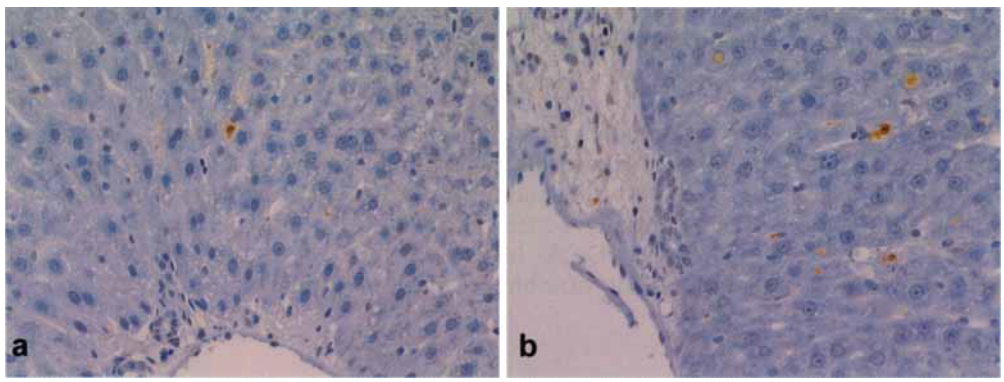

Fig. 2a,b TUNEL positive stained apoptotic changes. **a** In the UDCA group; **b** in the control group

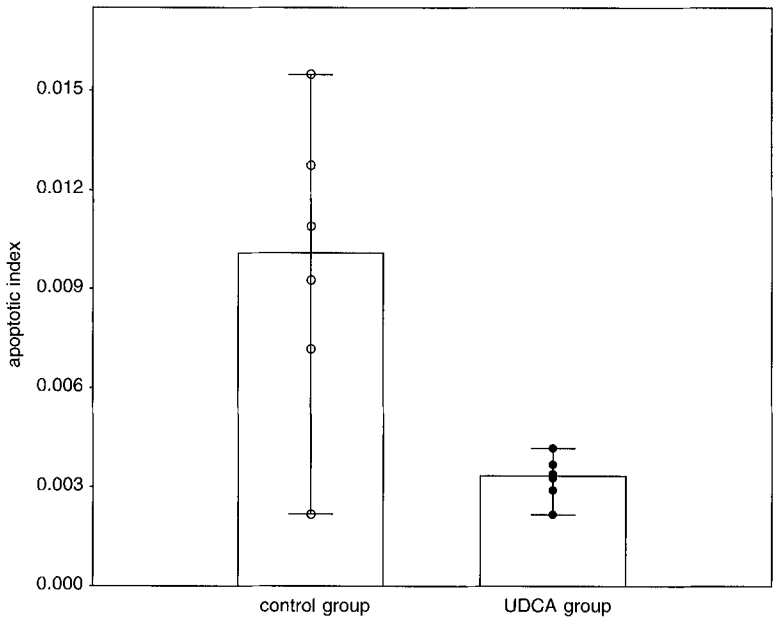

Fig. 3 Apoptotic index in the liver at 36 hrs after transplantation based on TUNEL staining. Columns represent the median, whiskers represent the range. The results for individual animals are marked

improvement of liver function [23]. Washout of bile acids during organ perfusion is probably the reason for the decrease in bile flow over time seen in our perfusion model.

Oral administration of UDCA has the important pharmacological advantage of effective competition with the absorption of more hydrophobic bile acids in the intestine [24]. The enteral administration of UDCA changes the balance between potentially toxic hydrophobic and hydrophilic bile acids in the liver in a positive way, as has been well documented in a number of human studies [5] and in a recent study in rats [20]. In this last study it was found that UDCA became the major biliary bile acid (about 40% of total) at 30 min after intra-duodenal bolus administration, and was almost quantitatively excreted in 60 min after application [20]. Consequently the livers could be harvested from these rats as early as 30-45 min after UDCA administration. In humans with preserved enterohepatic circulation, UDCA peaks in the serum approximately 2-3 h after a single oral dose [25], which makes enteral administration of UDCA via a nasogastric tube suitable for clinical organ donor surgery.

Since the enterohepatic circulation of bile salts is interrupted and liver function deteriorates with time in the perfusion model, a rat liver transplantation model was used to verify the potentially beneficial effect of donor oral pre-treatment with UDCA. Ischaemia during preservation, an independent variable for the outcome of liver transplantation, leads to deterioration in energy metabolism and finally to dysfunction of the hepatocytes [26]. We chose an 8 h period of cold ischaemia in our transplantation model, based on the observation that this duration of ischaemia results in significant injury in rat livers [27]. This injury followed by reperfusion often results in apoptosis [11], leading to initially poor organ function or even dysfunction [12]. Interestingly, apoptosis also plays a role in bile acid related cell toxicity [28], and it may be possible to reduce liver apoptosis by manipulating the composition of the bile acids.

Apoptosis is characterised by a series of well-defined cellular changes, such as altered morphology with shrinkage of the nuclei, chromatin condensation and DNA fragmentation. Our study, based on the TUNEL method, showed a lower number of hepatocytes undergoing apoptosis in transplanted livers from UDCA pre-treated donors. However, the expected reduction in apoptosis in the bile duct epithelium was not observed. Both the reduction in hepatocyte apoptosis and the lower serum levels of ALT observed in the pre-treated group suggest that UDCA had a hepato-protective effect in our transplantation model.

In conclusion, enteral administration of UDCA to rat organ donors enhances bile flow, even after a long period of cold preservation, and reduces liver cell ischaemia/reperfusion injury after transplantation as reflected by the reduction in enzyme levels and the reduced incidence of hepatocyte apoptosis.

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