Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Dopamine as additive to cold preservation solution improves postischemic integrity of the liver

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Keywords
dopamine, liver, preservation, viability.

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Received: 7 December 2009
Revision requested: 10 January 2010
Accepted: 4 February 2010
Published online: 4 March 2010

doi:10.1111/j.1432-2277.2010.01068.x

Summary

Dopamine pretreatment has been used to confer protection against cellular injury following hypothermia or anoxia, especially in vascular endothelial cells. Ischemia/reperfusion-associated tissue alterations still represent a major drawback in liver transplantation. The present study was aimed to investigate the effect of dopamine as an *ex vivo* adjunct, added to the cold storage solution, on cold preservation of the liver. Rat livers were excised 30 min after cardiac arrest, flushed with preservation solution and cold stored for 18 h. Dopamine (10, 50 or 100 μM) was added to the preservation solution in other livers. Organ viability was evaluated by 120 min of warm reperfusion *in vitro* ($n = 6$, resp.). Dopamine induced a dose related up to fourfold (at 50 μM) reduction in parenchymal (ALT, LDH) and mitochondrial (GLDH) enzyme release and significantly reduced histologic signs of tissue injury. Bile production and tissue ATP was doubled by dopamine. On the molecular level, dopamine enhanced postischemic phosphorylation of protein kinase A and p42/44 MAP kinase. Inhibition of cAMP-PKA pathway by simultaneous application of RP-cAMPs had no effect on P42/44 phosphorylation, or functional recovery of dopamine-treated grafts. Dopamine supplementation of the flush-out solution appears as a simple way for *ex vivo* augmentation of liver viability during preservation, not mediated via the catecholamine-cAMP signal cascade.

Introduction

Despite efforts to enlarge the donor pool, there is still a major gap between the requests for organs and the availability of donor organs.

The number of patients, waiting for an abdominal organ graft continues to increase and far exceeds the availability of donor grafts [1–3].

Thus, any improvement in the preservation of grafts, which were procured from donors with compromised circulation or even non-heart-beating donors (NHBD) represent a valuable source to enlarge the total number of viable donor organs available for transplantation.

Approaches to maintain structural and functional integrity of the graft between organ retrieval, transportation and final implantation into the recipient include amelioration of flushing and preservation solutions for static cold storage as well as oxygenated machine perfusion preservation.

Technical alternatives for improving the period of *ex vivo* storage of the graft were raised with the intention of achieving aerobiosis of the tissue. Continuous oxygenated perfusion at normothermia has been shown to improve the outcome of porcine liver grafts [4,5]. Transient or continuous aeration of the cold stored liver by gaseous oxygen persufflation during hypothermic storage may be a less complicated alternative that has produced good results in various experimental models [6,7].

Increasing attention has also been given to pharmacologic interventions aiming to improve the fate of marginal organs during and after cold ischemic preservation. Applications of e.g. lidocaine [8] or isoproterenol [9] were effective in protecting the cold stored liver.

Catecholamines in general seem to promote cellular protection against cold preservation injury [10], and dopamine could be disclosed as the one of the most effective among them [10,11].

Accordingly, pre-treatment of kidney organ donors with dopamine [12] was associated with an improved immediate grafts function after transplantation and significantly inhibited tubulitis in human renal allografts subjected to prolonged cold preservation [13].

The aim of the present study was aimed to investigate, whether application of dopamine would also be effective in liver preservation and if applied only to the isolated graft after organ retrieval.

Materials and methods

All experiments were performed in accordance with the federal law regarding the protection of animals. The principles of laboratory animal care (NIH Publication No. 85–23, revised 1985) were followed.

Male Wistar rats, weighing between 250 and 300 g, were anesthetized by intramuscular injection of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg). The abdomen was opened by midline incision, the liver skeletonized and freed from all ligamentous attachments. Cardiac arrest was induced by phrenotomy and the abdomen temporarily covered with plastic foil. Thirty minutes later, the portal vein was cannulated, the livers were excised, rinsed via the portal vein with 40 ml of HTK preservation solution at 4 °C and then cold stored overnight for 18 h at 4 °C.

The control group ($n = 6$) consisted of conventional cold preservation of the grafts without any additive.

In other groups ($n = 6$, resp.) the preservation solution was supplemented with varying concentrations of dopamine (10, 50 or 100 μmol/l) prior to organ retrieval.

To delineate the mechanistic contribution of dopamine-induced activation of the cAMP-PKA pathway, additional experiments were performed including the combined application of dopamine (50 μmol/l) and the protein kinase A (PKA) inhibitor RP-cAMPs (50 μmol/l).

Viability of all livers was evaluated after 18 h of preservation upon reperfusion *in vitro* according to previously described techniques [14] in a recirculating system for 120 min at 37 °C with 250 ml of oxygenated (95% O_2–5%CO_2; $pO_2 > 500$ mmHg) Krebs-Henseleit buffer, including 3 mg/100 ml of bovine serum albumin (BSA; Fraction V, Sigma Chem, Munich, Germany), at a constant flow of 3 ml/g/min. This set-up has been shown to allow for sensitive and adequate approximation tissue integrity and detection of structural changes in rat livers after hypothermic preservation [14].

To simulate the period of slow rewarming of the organ during surgical implantation *in vivo* [15], all livers were exposed to room temperature on a petri dish for 20 min prior to reperfusion.

Perfusate enzyme activities of alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and the intramitochondrial glutamate dehydrogenase (GLDH) were assessed photometrically using commercialized standard kits (Fa. Roche, Mannheim, FRG).

Metabolic activity of the livers was approximated by the calculation of hepatic oxygen utilization. Perfusate samples were taken at the portal inflow and from the venous effluent and the respective contents of O_2 were measured immediately in a pH-blood gas analyzer (ABL 500 acid-base laboratory; Radiometer, Copenhagen, Denmark). Oxygen uptake of the livers was calculated from the differences between portal and venous sites and expressed as μmol/g liver per min according to transhepatic flow and liver mass.

Tissue extraction and assay of high-energy phosphates

Tissue specimen for assessment of energetic status after reperfusion was taken with precooled steel tongs, immersed in liquid nitrogen and stored at −80 °C until later analysis.

Tissue specimens for assessment of energetic status were immersed in liquid nitrogen and stored at −80 °C until later analysis. Specimens were weighed and preserved for at least 5 days in a vacuum freezer (−45 °C, −0.001 atm). After tissue water was evaporated during freeze-drying, the samples were weighed again to determine their total dry weights. The samples were then homogenized and deproteinized in the cold with 0.33 M $HClO_4$ by means of an Ultra-Turrax (Jahnke & Kunkel KG, Staufen im Breisgau, Germany). After centrifugation and neutralization of the protein-free supernatant, the contents of ATP in the tissue were determined from the extract by standard enzymatic tests with hexokinase and glucose-6-phosphate dehydrogenase reactions [16]. The results were corrected for the respective dry weight to wet weight ratios of the tissue samples and expressed as micromoles per gram of dry weight.

To approximate the impact of reactive oxygen species on tissue integrity, tissue concentrations of lipid peroxides were measured at the end of reperfusion after homogenization in 0.9% KCl by fluorimetric detection, as described elsewhere [17].

Analyses of PKA and p42/44 phosphorylation

Frozen tissue was homogenized on ice with 30 volume of hypotonic lysis buffer, designed to prevent degradation of the phosphorylation sites during preparation (20 mM Tris–HCl, pH 7.5, 50 mM NaF, 50 mM Na_3VO_4, 20 mM

nitrophenyl phosphate, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X 100, 5 mM freshly added benzamidine). The suspension was left on ice for 30 min and then centrifuged at 4 °C for 15 min at 15 000 *g*. Aliquots from the clear supernatant were diluted to 2 mg protein/ml. The whole cell extracts were then used for Western blot analysis as described previously [17]. In brief, equal amounts of protein (20 μg) were suspended in Laemmli buffer and loaded on SDS-PAGE. After gel electrophoresis, proteins were blotted onto nitrocellulose membrane (0.2 μm) and homogeneous transfer was confirmed by staining with Ponceau S. Membranes were then blocked for 1 h at room temperature with 5% nonfat dry milk in TBST and subsequently incubated overnight at 4 °C with primary antibody recognizing only the phosphorylated form of rat p42/44 or PKA (Cell Signaling; Frankfurt, Germany).

Proteins were eventually visualized on X-ray film via chemiluminescence after exposure to horseradish peroxidase-conjugated secondary antibody (Phototope® New England Biolabs Inc., Schwalbach/Taunus, Germany). Subsequently, blots were stripped and were probed with an antibody recognizing total (phosphorylated and unphosphorylated) rat p42/44, or PKA (Cell Signaling).

Quantification of protein content was performed based on the ratios of individual signals of phosphorylated versus total protein, determined densitometrically using U N-SCAN-IT gel v 6.1 (Silk Scientific Corporation, Orem, UT, USA).

Histology

Liver tissue was collected at the conclusion of the experiments, cut into small blocks (3 mm thickness) and fixed by immersion in 4% buffered formalin overnight at 4 °C. The next day, the blocks were embedded in paraffin and cut into 2-μm sections using a microtome. Hematoxylin & Eosin staining was used to judge the morphological integrity of the parenchyma. Sections were examined and the extent of necrosis graded semiquantitatively from 1 (no necrosis) to 4 (severe necrosis with disintegration of hepatic cords) as described elsewhere [18], in a blinded fashion by two independent investigators.

Statistics

All values were expressed as mean ± SEM. After proving the assumption of normality and equal variance across groups, differences between treated and untreated groups were tested by analysis of variance (ANOVA) followed by Dunnett's test unless otherwise indicated. Statistical significance was set at $P < 0.05$.

Results

Effects of dopamine dosage on postischemic liver integrity

Enzyme releases of ALT and LDH during reperfusion were taken as general parameters of hepatocellular injury of the liver.

Perfusate activities of ALT were significantly reduced to up to ¼ by supplementing the preservation solution with dopamine (Fig. 1). This effect was dose-dependent; among the doses under investigation, best protection was achieved at a concentration of 50 μM.

A similar pattern was also found with respect to LDH; the maximal perfusate activities of were 977 ± 115; 613 ± 112*; 489 ± 103* and 678 ± 119* U/l in controls and dopamine supplemented (10. 50 and 100 μM) groups, respectively (*$P < 0.05$ vs. control).

The dose–effect relationship of dopamine-related tissue protection was closely corroborated by functional recovery of hepatic bile production upon reperfusion (Fig. 2). Among the three concentrations tested, maximal effects were obtained with 50 μM dopamine while 100 μM was less effective. Thus, 50 μM was chosen for further investigations.

Histopathologic changes

Architecture of liver structures was quite well preserved in all groups. Notwithstandingly, areas of hepatocyte

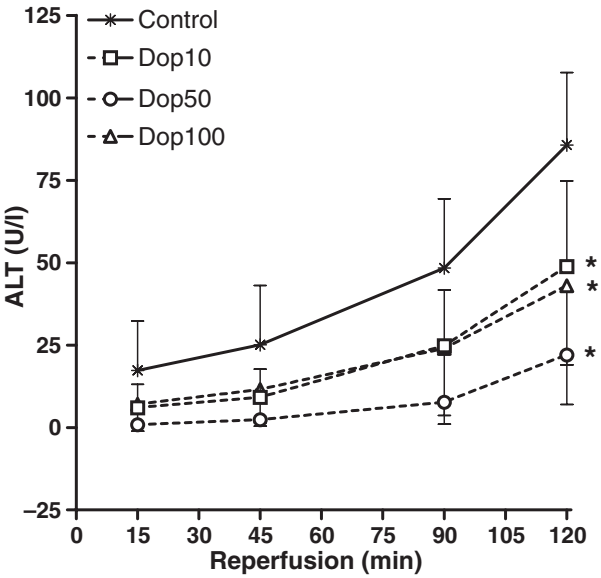

Figure 1 Hepatic loss of alanine aminotransferase (ALT) during normothermic reperfusion after 18 h of cold storage with or without (control) supplementation of the preservation solution with 10, 50 or 100 μM dopamine (Dop). Values are given as mean ± SD; $n = 6$ per group, *$P < 0.05$ vs. control.

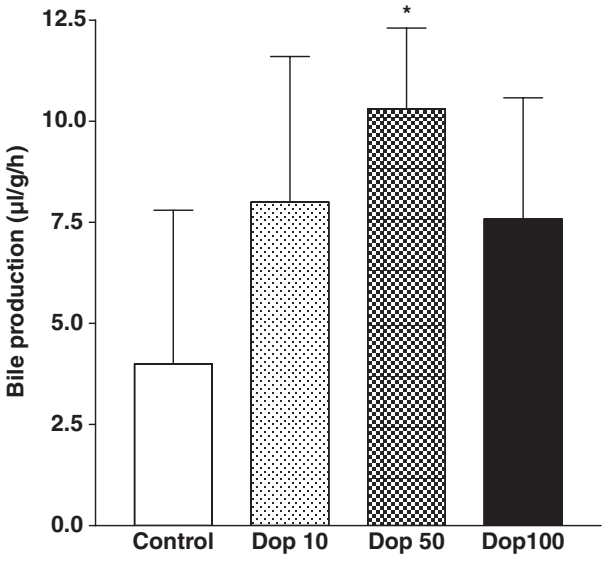

Figure 2 Hepatic bile production during normothermic reperfusion after 18 h of cold storage with or without (control) supplementation of the preservation solution with 10, 50 or 100 µM dopamine (Dop). Values are given as mean ± SD; $n = 6$ per group, $^*P < 0.05$ vs. control.

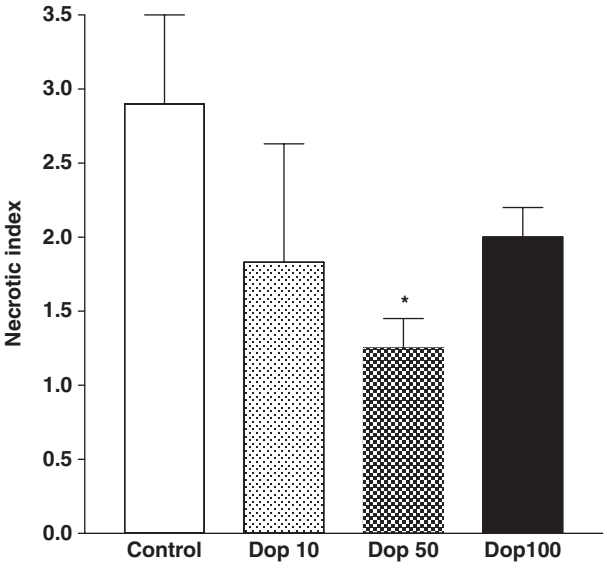

Figure 3 Histologic necrosis score after normothermic reperfusion following reperfusion after 18 h of cold storage with or without (control) supplementation of the preservation solution with 10, 50 or 100 µM dopamine (Dop). Values are given as mean ± SD; $n = 6$ per group, $^*P < 0.05$ vs. control.

necrosis, distributed throughout the parenchyma and cytoplasmic vacuolization, mostly in the pericentral segment of hepatic lobules were found more often in controls than after dopamine treatment. Significant reduction in score values for necrotic tissue injury was seen at a concentation of 50 µM dopamine. (cf. Fig. 3).

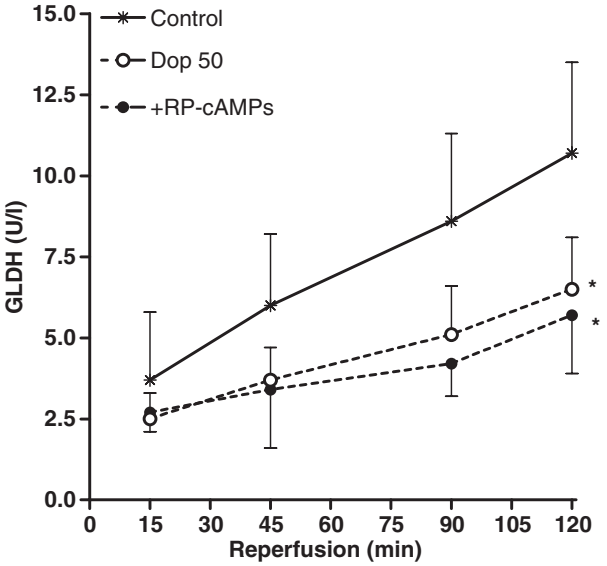

Figure 4 Hepatic loss of intramitochondrial glutamate dehydrogenase (GLDH) during normothermic reperfusion after 18 h of cold storage. Preservation solution was supplemented with 50 µM dopamine (Dop50), with 50 µM dopamine and 50 µM of the protein kinase A inhibitor RP-cAMPs (+RP-cAMPs) or used without additives (control). Values are given as mean ± SD; $n = 6$ per group, $^*P < 0.05$ vs. control.

Dopamine improves mitochondrial function during reperfusion

Following the hepatic liberation of the mitochondrial marker enzyme GLDH (Fig. 4), a marked and significant attenuation of GLDH leakage was obtained by dopamine while untreated livers exhibited an ongoing loss of GLDH, the activity of which rose in the perfusate during the whole observation period. The effect of dopamine was not mediated through catecholamine-induced activation of the cAMP-PKA pathway, because blocking of PKA by RP-cAMPs did not alter the results.

Hepatic oxygen utilization is shown in Fig. 5a: It is seen that dopamine leads to an enhanced oxygen uptake by the reperfused liver, irrespective of additional blockade of PKA signaling. As shown in Fig. 4b, this translates into a significant improvement of hepatic energy metabolism resulting in a more than twofold increase in tissue levels of ATP at the end of the experiment.

The impact of oxygen free radicals on tissue integrity was approximated by the amount of lipid peroxidation in the tissue at the end of reperfusion. It was found that dopamine (50 µM) significantly ($P < 0.05$) reduced hepatic lipid peroxidation from 84.2 ± 11.5 to 35.6 ± 16.4 nmol/mg and this reduction was not influenced by the addition of RP-cAMPs (39.5 ± 5.1 nmol/mg).

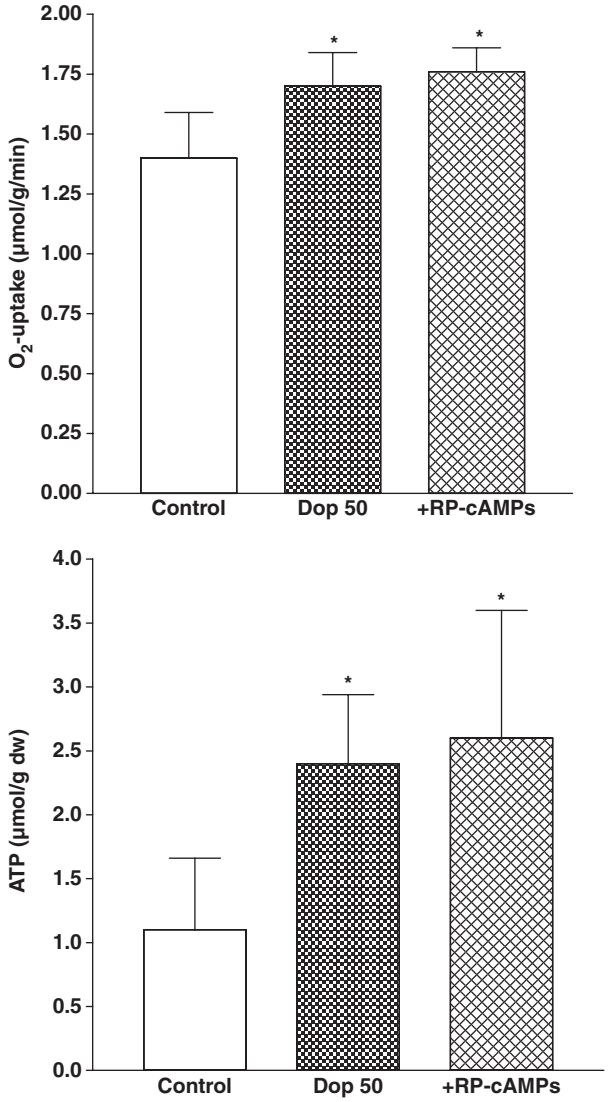

Figure 5 Metabolic recovery (oxygen consumption – upper panel, and tissue concentrations of ATP – lower panel) of the livers upon normothermic reperfusion after 18 h of ischemic preservation. Preservation solution was supplemented with 50 µM dopamine (Dop50), with 50 µM dopamine and 50 µM of the protein kinase A inhibitor RP-cAMPs (+RP-cAMPs) or used without additives (control). Values are given as mean ± SD; $n = 6$ per group, $*P < 0.05$ vs. control.

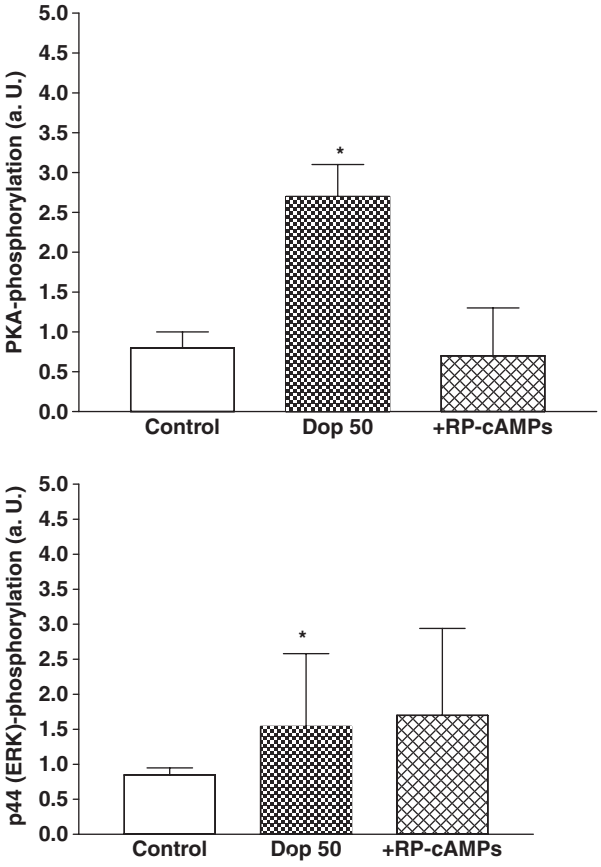

Figure 6 Phosphorylation rate of protein kinase A (upper panel) and p42/44 MAK-kinase (lower panel) of reperfused livers after 18 h of ischemic preservation. Preservation solution was supplemented with 50 µM dopamine (Dop50), with 50 µM dopamine and 50 µM of the protein kinase A inhibitor RP-cAMPs (+RP-cAMPs) or used without additives (control). Values are given as mean ± SD; $n = 4$ per group, $*P < 0.05$ vs. control.

Dopamine activates p42/44 independent of cAMP-PKA signaling

The postischemic activation level of the p42/44 has been investigated by the phosphorylation ratio of the p42/44-MAPkinase (Fig. 6).

While low levels of phosphorylation/activation of p42/44 is seen in the untreated controls, groups receiving dopamine treatment during preservation did show significantly enhanced signs of activation. Inhibition of the catecholamine responsive cAMP-PKA signal pathway by RP-cAMPs (Fig. 6b) did not affect the dopamine-induced activation of p42/44.

Discussion

Although transplantation of grafts, originating from donors with cardio-circulatory compromise, has found its way into clinical practice in several centers, the use of NHBD organs is still impeded by the apparently increased susceptibility of these organs to preservation injury.

There exists already experimental and clinical evidences in kidneys that dopamine confers protection against cold storage injury [12,19], if administered as a pretreatment to the donor prior to organ harvest. Similarly, preincubation of vascular endothelial cells with dopamine mitigated cellular release of LDH and morphological alterations after hypoxic challenge [10]. By contrast, dopamine was

found ineffective, when given after the ischemic insult [20].

Here we show now, that dopamine also effectively reduces tissue alterations and functional impairments during postischemic reperfusion of the liver.

Interestingly, this was obtained by isolated *ex vivo* application of dopamine during cold preservation.

The use of dopamine after organ retrieval represents an attractive tool circumventing the need of taking measures already in the donor. It is very easily applicable and could be a major contribution to improve organ integrity upon long-term preservation.

Moreover, possible disadvantages of systemic dopamine application in the donor-like pro-inflammatory chemokine induction in the lungs [21] and adverse impacts on heart transplantation outcome [22,23] would be abrogated.

Potential pathways by which dopamine is causative fort he described findings comprise reduction of proteolysis [24] which has actually been shown to compromise in cold stored liver grafts [25,26], as well as protection of vascular endothelial cell integrity through mitigation of oxygen free radical induced cell alterations [10]. Our results on reduced lipid peroxidation after dopamine treatment are in line with the latter.

Dopamine is also known to enhance cellular levels of cAMP [27], which are prone to decline upon anoxic conditions [28]. Pharmacological interventions to increase cellular cAMP have been shown to improve postischemic recovery in the lungs [29], liver [9,30] and gut [31]. Interestingly, dopamine was similarly effective in reducing preservation injury, whether any influence on the cAMP-PKA signal cascade was blocked by RP-cAMPs or not, suggesting a genuine dopamine effect independent of its catecholaminergic property.

Another mechanism that may contribute to tissue protection against ischemia–reperfusion is the ERK–MAPK signal pathway, which actually was found to be activated by dopamine and was not affected by RP-cAMPs in our model. Increased activation of p42/44 is reported to be associated with improved survival in HUVECS after trophic withdrawal [32]. Phosphorylation of p42/44 facilitates the closing of intercellular gaps during rewarming of endothelial cells thus increasing barrier function of the endothelium [33].

To date it is well known, that cold preservation has a prevailing impact on nonparenchymal cells including sinusoidal endothelium [34,35]. Discontinuity in the endothelial lining [34] and alterations in the endothelial cell cytoskeleton [36] are found to be the major culprits of postischemic liver dysfunction.

However, also in isolated hepatocytes, reduced cell survival was observed when ERK activation was inhibited [37], and donor liver adenoviral CT-1 transfer decreased hepatic necrosis in small-for-size liver transplantation, which was mediated in part by activation of ERK signaling pathways [38].

The ability of the liver to restore the tissue pool of high-energy phosphates during postischemic reperfusion has redundantly been shown to be a reliable indicator of graft viability [39,40] and production of bile can be considered as the most important predictor of ultimate graft survival [40]. In line with previous results in isolated endothelial cells, we could demonstrate a marked improvement in whole graft energetic recovery after dopamine treatment. Because of the limitations of the *in vitro* model, it was not possible to investigate ulterior effects of the dopamine treatment, e.g. on *in vivo* microcirculation, blood endothelial interactions and long-term bile duct integrity. Future studies including actual transplantation of the graft are thus emphasized to further substantiate the conclusion that postharvest dopamine treatment by supplementation of the preservation solution represents an easy and effective measure to reduce cold storage-associated liver injury.

Authorship

MK: analyzed data, wrote the paper, participated in the design of the study. JS: performed research, collected data. TM: designed research, wrote the paper.

Acknowledgement

This study was supported by a grant from the German Research Foundation (DFG Mi470/13-1). The authors gratefully acknowledge the valuable technical help of R Lutterbach and P Efferz during the isolated organ experiments and the immunological analyses.

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