Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Science Letters:

A novel way of liver preservation improves rat liver viability upon reperfusion*

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Received Aug. 29, 2006; revision accepted Mar. 5, 2007

Abstract: Background/aim: Currently, the liver is cold-preserved at 0~4 °C for experimental and clinical purposes. Here, we investigated whether milder hypothermia during the initial phase of the preservation period was beneficial for liver viability upon reperfusion. Methods: In the first set of experiments, rat livers were preserved either conventionally in clinically used histidine-trypthopan-ketoglutarate (HTK) solution (Group A: 45 min and Group B: 24 h) or by slow cooling HTK solution (from 13 °C to 3 °C) during the initial 45 min of preservation (Group C: 24 h). In the second set of experiments, additional groups of livers were evaluated: Group BB—preservation according to Group B and Group CC—preservation according to Group C. Further, some livers were preserved at 13 °C for 24 h. Livers were then reperfused using a blood-free perfusion model. Results: Bile production was approximately 2-fold greater in Group C compared to Group B. Alanine transaminase (ALT) and aspartate transaminase (AST) release into perfusate were 2~3-fold higher in Group B compared to Group C. No significant differences were found in ALT and AST release between Group C and Group A. Livers in Group CC compared to Group BB exhibited significantly lower portal resistance, greater oxygen consumption and bromosulfophthalein excretion into bile and lower lactate dehydrogenase (LDH) release into perfusate. Histological evaluation of tissue sections in Group BB showed parenchymal dystrophy of hepatocytes, while dystrophy of hepatocytes was absent in Group CC. Livers preserved at 13 °C for 24 h exhibited severe ischemic injury. Conclusion: These results suggest that the conventional way of liver preservation is not suitable at least for rat livers and that slow cooling of HTK solution during the initial phase of cold storage can improve liver viability during reperfusion.

Key words: Rat, Hepatic graft, Cold ischemia, Liver protection, Histidine-trypthopan-ketoglutarate solution (HTK)
doi:10.1631/jzus.2007.B0289 **Document code:** A **CLC number:** R322.4[+]7

INTRODUCTION

A major factor determining morbidity and mortality after liver transplantation therapy is preservation injury of hepatic grafts (Lemasters and Thurman, 1997; Kukan and Haddad, 2001). Prevention of liver preservation injury may decrease the health care burden of liver transplant recipients as well as the need for retransplantation and thus increase the availability of donor livers for patients on waiting lists (Lemasters and Thurman, 1997; Kukan and Haddad, 2001).

At present, during their procurement, human livers are washed out with cold preservation solution (4 °C) and then immediately submerged into plastic bags containing preservation solution (4 °C). The plastic bag is then placed into iced slush, where the liver is preserved at temperature of 0~4 °C. It is supposed that this procedure is advantageous for the liver, since it allows a rapid decrease of its temperature and minimizes organ metabolism (Hertl *et al*., 1996). However, Drews *et al*.(1998) showed that in rat livers their rapid cooling by iced water during procurement led to a marked deterioration of microperfusion in peripheral and subcapsular regions during cold reperfusion. This interesting work (Drews

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*Project supported by the Ministry of Health of the Slovak Republic under the project of Modulation of Heat Transfer in Isolated Liver (No. 2005/32-SZU-10) and the VEGA Grant (No. 1/1158/04), Slovakia

et al., 1998) remained neglected by scientists working in the area of liver preservation. Furthermore, we found that in our previous experiments surface liver temperature after standard washing out of rat livers with University of Wisconsin preservation solution (4 °C) was approximately 19 °C (Lutterová *et al*., 2001). Thus, if one takes into consideration the difference between the temperature of liver surface and the preservation solution, it is not clear whether the current technique of liver preservation is appropriate. Indeed, we found very recently that preservation of rat livers in physiologic saline at the initial temperature of 10 °C was better than preservation at the initial temperature of 4 °C (Kebis and Kukan, 2006).

Therefore, this study was designed to test the hypothesis that the initial hypothermia close to liver surface temperature as well as slow cooling of histidine-trypthopan-ketoglutarate (HTK) solution (which is in clinical practice for human liver preservation) during the initial phase of cold storage is beneficial for liver viability during reperfusion.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 280~320 g, obtained from Velaz Co. (Prague, Czech Republic) were used. The animals were housed in an air-conditioned room at 22 °C and fed Velaz rat chow and water *ad libitum* up to the time of the experiments. The study was approved by the local animal welfare committee.

Liver procurement

The rats were given ketamine/xylazine [(100/10) mg/kg] intraperitoneally to induce anesthesia before surgery and the liver was prepared as described (Kukan, 1999). Briefly, the bile duct was cannulated with polyethylene PE 50 tubing and 250 IU of heparin was injected intravenously. To ensure that global liver function was comparable between the groups, bile production was measured for 5 min during liver procurement. The portal vein was then cannulated with PE 240 tubing and the hepatic artery was ligated. The liver was flushed out in situ through the portal vein with 30 ml of cold (4 °C) HTK solution (Dr. Franz Köhler Chemie GmbH, Alsbach-Hähnlein, Germany) at constant pressure of 12 cmH_2O. Then, as rapidly as possible, the livers were excised and transferred to the preservation/perfusion chamber made from plexiglass containing 70 ml of HTK solution (Kebis and Zvrškovec, 2002).

Liver preservation

In the first set of experiments, rat livers were preserved either conventionally in HTK solution [Group A: 45 min (n=6) and Group B: 24 h (n=6)] or by slow cooling HTK solution (from 13 °C to 3 °C) during the initial 45 min of preservation [Group C: 24 h (n=6)]. In the second set of experiments, additional groups of livers were evaluated: Group BB (n=5)—preservation according to Group B and Group CC (n=5)—preservation according to Group C). Finally, some livers were preserved at 13 °C for 24 h (n=3).

Liver perfusion

The livers were perfused through the portal vein from a height of 12 cm in a recirculating perfusion system at 37 °C (Kukan, 1999). As a perfusion medium, Krebs-Henseleit bicarbonate buffer (KHB) (200 ml, pH=7.4) containing glucose (10 mmol/L) and saturated with 95% oxygen and 5% carbon dioxide (inflow P_{O_2} =400~500 mmHg) was used. In the first set of experiments, the following parameters were assessed: portal flow, bile production, alanine transaminase (ALT) and aspartate transaminase (AST) release into perfusate. In the second set of experiments, we measured portal resistance, oxygen consumption, bromosulfophthalein (BSP) excretion, (BSP was added into the reservoir at 30 min of reperfusion as a 6 μmol bolus), and lactate dehydrogenase (LDH) release into perfusate.

Temperature measurements

We measured the temperature of HTK solution inputing rat livers, surface liver temperature, and HTK solution in the preservation/perfusion chamber (for the first 60 min of preservation) by a thermometer (HI 8751 Minitherm, Hanna Instruments, Kahl am Rhein, Germany).

Histological analysis by hematoxylin and eosin (HE) staining

After each perfusion of the second set of experiments, the middle part of the great left lateral lobe

(lobus sinister lateralis) was fixed in 10% formalin. Fixed samples were cut and stained by HE. Pathologist (Ján Jakubovský) performed morphological evaluation of liver tissues. Morphological evaluation was carried out on HE-stained sections at original magnification×100.

Assays

The activities of perfusate ALT, AST, and LDH were measured with standard reagent kits, which were purchased from Pliva-Lachema Diagnostica (Brno, Czech Republic). BSP concentrations were measured in 0.1 mol/L NaOH spectrophotometrically at 580 nm after appropriate dilution of bile samples. Oxygen consumption was assessed by 248 pH/Blood Gas Analyzer (Ciba Corning Diagnostics Limited, UK).

Treatment of data and statistics

Oxygen consumption was calculated from the equation:

$$O_2 \text{ consumption}=(C_{in}-C_{out}) \text{ ml/(min·g liver)},$$

where C_{in} and C_{out} are oxygen perfusate levels at input and output.

Percentage change in liver weight during the reperfusion period was calculated as follows:

$$(\Delta\text{liver weight/initial liver weight})\times 100\%.$$

Values are given as mean±*SEM*. The data from three groups of livers were analyzed by ANOVA followed by Bonferroni correction test for multiple comparisons. Statistical evaluation between two groups was performed using Student's *t* test. A difference was considered significant when $P<0.05$.

RESULTS

The first set of experiments

1. Animal weight, liver weight and bile production prior to preservation

There were no significant differences in animal weight (g) between Group A, Group B, and Group C (Group A: 309±6; Group B: 306±3; Group C: 302±2). Similarly, no significant differences were found in liver weight (g) (Group A: 10.8±0.2; Group B: 10.7±0.1; Group C: 10.6±0.1). Bile production [μl/(min·g liver)] in vivo, as a sign of global hepatic function, was similar prior to cold preservation (Group A: 1.95±0.1; Group B: 2.2±0.2; Group C: 2.19±0.2).

2. The temperature of the preservation solution and of liver surface after liver procurement

There were no significant differences in the temperature (°C) of HTK solution inputing livers between the groups (Group A: 4.0±0.3; Group B: 4.5±0.1; Group C: 4.8±0.3). Likewise, surface liver temperatures (°C) after washing out of the liver were similar between the groups (Group A: 16.8±0.3; Group B: 16.8±0.9; Group C: 17.7±0.3).

3. The temperature of the preservation solution during the initial phase of cold storage

Fig.1 shows temperature of HTK solution during the first 60 min of preservation. It can be seen that there were significant differences in the temperature of HTK solution between Group C and Group A or Group B during the first 35 min of preservation. After 45 min of preservation temperatures were similar.

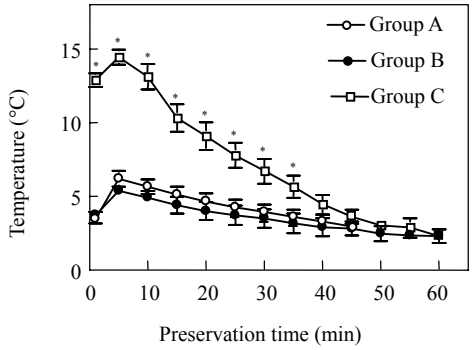


Fig.1 The temperature of HTK solution (°C) during the first 60 min of preservation of rat livers. After procurement livers were preserved in HTK solution for 45 min (Group A) and 24 h (Group B) by a conventional way of preservation. HTK solution was slowly cooled from 13 °C to 3 °C during the initial 45 min of preservation (Group C: 24-h preservation). Data represent mean±*SEM* (6 livers per group). *Significantly different (Group C vs Group A or Group B) at $P<0.05$

4. Effects of the initial phase of cold storage on liver viability during reperfusion in Group A, Group B, and Group C

Viability parameters of isolated reperfused rat livers are shown in Figs.2a~2d. Portal flow was comparable between Group A and Group C. Although

nonsignificant, but better flow was recorded in Group C compared to Group B (Fig.2a).

Bile production, as a sign of global hepatic function, was severely suppressed in 24-h preserved livers. Slow cooling of HTK solution at the initial phase of cold preservation (Group C) improved significantly bile production after 30 min reperfusion compared to Group B (Fig.2b).

ALT and AST release into perfusate, as measures of hepatocellular membrane integrity, were nonsignificantly different between the groups at 5 min of reperfusion, but they were 2~3 times higher in Group B compared to Group A at 60 min of reperfusion (Figs.2c and 2d). Release of ALT and AST into perfusate of Group C after 60 min of reperfusion was comparable to control livers (Group A).

The second set of experiments

1. Effects of the initial phase of cold storage on liver viability during reperfusion in Group BB and in Group CC

To prove that slow cooling of the preservation solution is indeed better than the conventional way of preservation, we performed further experiments and measured other viability parameters of livers during reperfusion (Note: livers in Group BB were preserved according to Group B and livers in Group CC were preserved according to Group C). Portal resistance during reperfusion of Group BB and Group CC is shown in Table 1. It can be seen that after 30 min of reperfusion portal resistance was significantly lower in Group CC compared to Group BB.

Likewise, oxygen consumption was greater after 30 min of reperfusion in Group CC compared to Group BB (Table 2).

Bile production was nonsignificantly better in Group CC compared to Group BB during the first 30 min of reperfusion (Table 3). However, BSP-induced bile flow was significantly greater in Group CC compared to Group BB. Similarly, BSP excretion into bile was significantly greater in Group CC compared to Group BB (Table 3).

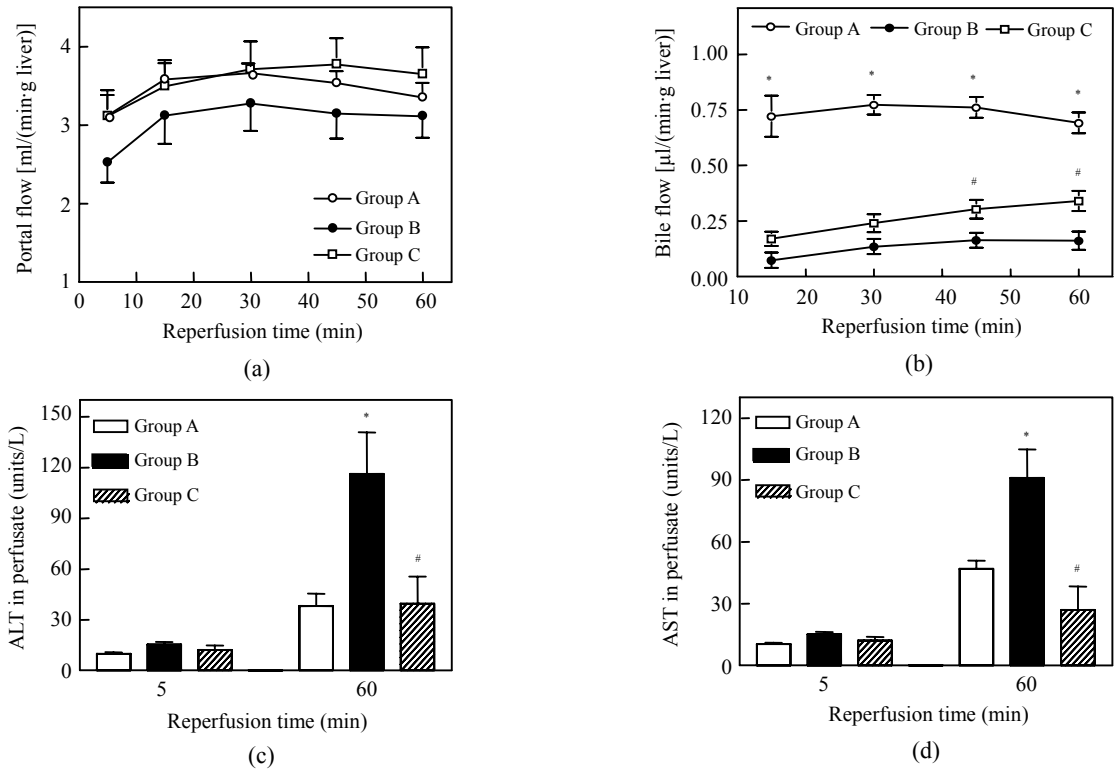


Fig.2 Viability parameters of isolated perfused rat livers after preservation. (a) Portal flow; (b) Bile flow; (c) ALT release into perfusate; (d) AST release into perfusate

Livers were perfused after standard cold preservation for 45 min (Group A), after standard cold preservation for 24 h (Group B), or following slow cooling of the preservation solution from 13 °C to 3 °C during the initial 45 min of preservation (Group C). Data represent mean±*SEM* (6 livers per group) and were expressed per gram liver weight. Significantly different at $P<0.05$: *Group B vs Group A or Group C, #Group C vs Group B

Table 1 Portal resistance during reperfusion [mmHg/(ml·min)]

Reperfusion time (min)	Group BB	Group CC
5	0.516±0.058	0.518±0.029
15	0.460±0.046	0.464±0.025
30	0.438±0.031	0.406±0.015
45	0.450±0.026	0.384±0.007*
60	0.450±0.026	0.374±0.008*

Data represent mean±*SEM* (5 livers per group); *Significantly different at $P<0.05$

Table 2 Oxygen consumption during reperfusion [μmol/(min·g liver)]

Reperfusion time (min)	Group BB	Group CC
5	0.734±0.076	0.760±0.072
15	0.826±0.104	0.882±0.081
30	0.854±0.106	1.056±0.067
45	0.800±0.070	1.088±0.058*
60	0.842±0.073	1.108±0.058*

Data represent mean±*SEM* (5 livers per group); *Significantly different at $P<0.05$

Table 3 Effects of the way of preservation on bile flow and BSP excretion

Treatment groups	[a]Bile flow [μl/(min·g liver)]	[b]BSP-induced bile flow [μl/(min·g liver)]	BSP excretion [μmol/(30 min·liver)]
Group BB	0.30±0.06	0.43±0.04	3.63±0.52
Group CC	0.42±0.04	0.62±0.05*	4.62±0.56*

[a]Bile flow during the first 30 min of reperfusion; [b]Bile flow after addition of BSP into the reservoir during the last 30 min of reperfusion. Data represent mean±*SEM* (5 livers per group); *Significantly different at $P<0.05$

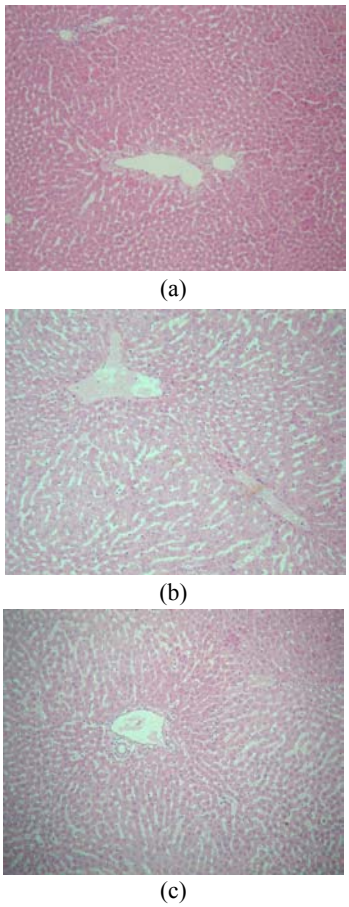
LDH release into perfusate, as a measure of hepatocellular membrane integrity, was (240±52) units/L in Group BB (after 60 min of reperfusion), while in Group CC LDH release was only (116±25) units/L.

2. Histological analysis by HE staining

Fig.3 shows histological findings of HE staining. Compared to the control liver, livers in Group BB (by conventional preservation) and Group CC (by novel way of preservation) showed typical widening of sinusoids and dettachments of endothelial cells. Parenchymal dystrophy of hepatocytes was absent in the control liver and in Group CC, while in Group BB parenchymal dystrophy of hepatocytes was observed (Fig.3). Thus, it can be concluded that the above novel way of preserving hepatocytes is consistent with measurements of viability parameters.

3. Preservation of livers at 13 °C for 24 h

Liver swelling (calculated as percentage change of liver weight during reperfusion) was 44.6%±4.1%, indicating severe ischemic injury. Compared to Group BB, portal resistance was approximately doubled and oxygen consumption halved, bile production during the first 30 min of reperfusion was one tenth that of normal, BSP-induced bile flow was approximately one twentieth that of normal and BSP excretion into bile was negligible. Finally, LDH release into perfusate was (1840±591) units/L.



(a) (b) (c)

Fig.3 HE staining of control liver (a), conventionally preserved liver (b), and liver preserved by a novel way of preservation (c) (original magnification×100)

DISCUSSION

The objective of the present study was to investigate the hypothesis that slow cooling of the preservation solution during the initial phase of cold storage may improve liver viability during reperfusion and thus decrease liver reperfusion injury. We used for these experiments our preservation/perfusion chamber (Kebis and Zvrškovec, 2002) since our previous study showed that preservation of rat livers is more suitable in a preservation chamber than in plastic bags (Kebis *et al*., 2004). In addition, from the technical point of view a controlled decrease of the preservation solution can be achieved more precisely in plexiglass chamber than in plastic bag (Note: in the plastic bag the temperature of the preservation solution may interfere with ice slush surrounding the bag).

The data presented in Figs.2b~2d, Fig.3 and Tables 1~3 strongly suggest that slow cooling is indeed beneficial for liver viability during reperfusion and that the current procedure used in liver preservation is not suitable at least for the rat model. Of all viability parameters studied during liver reperfusion, bile flow is considered to be the most reliable indicator of ischemic and preservation damage (Bowers *et al*., 1987; Kukan and Haddad, 2001). Bile flow correlated with ATP tissue levels after liver transplantation and rapid resumption of bile flow in both rat liver transplantation (Sumimoto *et al*., 1988) and clinical liver transplantation (Lemasters and Thurman, 1997) is considered to be an early sign of a successful graft. Data on improved bile flow and BSP excretion into bile, as an index of the hepatic organic anion transport, indicate that milder initial hypothermia and slow cooling of the preservation solution may improve transplantation prognosis.

Interestingly, Fujita *et al*.(1993) showed that moderate hypothermia of 10 °C to 20 °C was more appropriate for maintaining structure and function of rat livers than temperature at 5 °C when the continuous perfusion of livers was used for preservation. However, for static liver preservation, temperatures close to 0 °C were found to be more appropriate than 4 °C (Hertl *et al*., 1994; Yoshida *et al*., 1999). In line with the latter findings, we observed severe liver injury, when livers were preserved at 13 °C for 24 h.

The question now arises as to whether results presented in this study can be extrapolated to human livers. On one hand, surface-to-volume ratio of the rat liver is much higher than that of the human liver. Therefore, the injury resulting from human hepatic grafts may be much smaller than that resulting from rat liver hepatic grafts. On the other hand, the percentage of apoptotic hepatocytes in human hepatic grafts was the highest (30%) in the subcapsular region of the grafts (Borghi-Scoazec *et al*., 1997), i.e. in the region mainly exposed to the effects of cold environment during the procurement phase. Further, apoptosis of hepatocytes was correlated with postoperative serum levels of AST (Borghi-Scoazec *et al*., 1997).

CONCLUSION

The results of the present study strongly suggest that slow cooling of the preservation solution during the initial phase of cold storage is more appropriate procedure for liver preservation than the currently used method. Whether these results hold true in humans is not clear. Therefore, preclinical significance of this novel way of liver preservation should be studied using larger animal liver e.g., the pig liver in preservation-transplantation experiments.

ACKNOWLEDGEMENT

The authors sincerely thank Mr. Eduard Mališ for his excellent technical assistance.

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