Artificial
Organs

Hypothermic Machine Perfusion Versus Cold Storage in the Rescuing of Livers from Non-Heart-Beating Donor Rats

*Matías E. Carnevale, *Cecilia L. Balaban, *Edgardo E. Guibert, †Hebe Bottai, and *Joaquin V. Rodriguez

*Binational Center (Argentina–Italy) for Research in Clinical and Applied Cryobiology (CAIC) and †Department of Statistics, National University of Rosario, Rosario, Argentina

Abstract: The aim of this work was to compare the efficiency of cold storage (CS) and hypothermic machine perfusion (HMP) methods of preserving grafts excised from non-heart-beating donors that had suffered 45 minutes of warm ischemia. We developed a new solution for HMP to use in liver transplantation, based on BES, gluconate, and polyethylene glycol (BGP-HMP solution). After 24 h of HMP or CS, livers were reperfused at 37°C with Krebs–Henseleit solution with added dextran. For both procedures, portal pressure and flow were measured and the intrahepatic resistance (IR) was calculated. The pH oscillations and enzyme activities (LDH, AST, and ALT) were evaluated for the perfusion buffer during normothermic reperfusion. O_2 consumption of the liver, glycogen production, and bile flow were also measured during the normothermic reperfusion period. Portal flow and IR showed statistical differences ($P < 0.05$) between the two groups ($n = 5$). HMP with BGP-HMP solution resulted in higher values of portal flow and lower IR than CS with HTK solution. Enzyme release after 90 min of reperfusion did not show statistical differences between groups. With regard to bile flow and O_2 consumption, livers preserved by both processes were able to produce bile, but livers preserved with HMP were able to take up more O_2 than livers preserved by CS. **Key Words:** Hypothermic machine perfusion—Liver—Cold storage—Non-heart-beating donors.

Hepatic transplantation has become the only safe solution to various irreversible liver problems. Currently, there are two approaches to preserving transplantable organs, static and perfused storage: both static cold storage (CS) and hypothermic machine perfusion (HMP) are clinically approved for kidneys, but only CS is approved for other organs. HMP is in different stages of preclinical and early clinical studies (1).

Brain-dead donors are currently used in liver transplantation. However, a growing gap between demand for organs and their availability from brain-dead donors has led to a reexamination of organs from non-heart-beating donors (NHBDs) to expand the pool of organs suitable for transplant. These organs may have decreased survival after being transplanted because they have suffered a period of warm ischemia (2). For the above reasons, it is necessary to develop new strategies for the rescuing of these organs, and preservation conditions should be improved (3,4). There are experimental as well as clinical reports indicating that HMP is more appropriate than cold static preservation for rescuing livers from marginal donors (1,5–7). Consequently, this method could be considered as an alternative for overcoming the present shortage of donors by expanding the existing donor pool and possibly lengthening the storage time (8,9).

HMP is a dynamic technique where a continuous flow of preservation solution perfuses the organ and maintains a residual metabolism, largely dependent on energy generation. This, in mammalian systems, is

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Address correspondence and reprint requests to Lic. Matías E. Carnevale or Dr. Joaquin V. Rodriguez, Centro Binacional (Argentina–Italia) de Investigaciones en Criobiología Clínica y Aplicada (CAIC), Universidad Nacional de Rosario. Avda. Arijón 28 bis, Rosario S2011BXN, Argentina. E-mail: matias_carnevale@hotmail.com (M.E.C.); jrodrig@fbioyf.unr.edu.ar (J.V.R.)

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synonymous with a need for oxygen supply for aerobic metabolism delivered by vascular perfusion (10). In addition, the HMP system has the potential to predict graft function and allows the application of pharmacological measures in the hypothermically perfused organs. However, there still are doubts as to which is the best perfusion preservation solution to use. These solutions, designed to counteract the effects of hypothermia, are made up of different components, but all contain three essential constituents: adequate electrolytes (high or low Na^+/K^+ ratio), an impermeant compound to prevent cell swelling, and a buffer to prevent acidosis (10). In our laboratory, we have developed an HMP solution based on *N*,*N*-bis-2-hydroxyethyl-2-aminoethanesulfonic acid (BES), gluconate, and polyethylene glycol (BGP-HMP); this solution was previously assayed in an in vitro model (11,12), and its efficiency was compared versus the classic solution developed by Bretschneider (13).

Therefore, the aim of this work was to compare the efficiency of CS and HMP methods in preserving grafts excised from NHBDs that have suffered 45 min of warm ischemia.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250–300 g were used in all experiments. The rats had access to a standard diet and water ad libitum prior to the experiment and received care in compliance with international regulations. The ethical committee of the School of Biochemistry and Pharmaceutical Sciences, National University of Rosario, approved the animal protocols.

Solutions

The composition of Bretschneider solution (Bretschneider's histidine–tryptophan–ketoglutarate [HTK], Franz Kohler Chemie GmbH, Bensheim, Germany) has already been described (13). The BGP-HMP solution (12) was prepared in our laboratory; it had the following composition: 100 mM sodium gluconate, 7 mM potassium gluconate, 20 mM sucrose, 30 mM BES, 2.5 mM KH_2PO_4, and 0.03 mM polyethylene glycol (35 kDa), 5 mM $MgSO_4$, 3 mM glutathione, 5 mM adenosine, and 15 mM glycine, together with 0.25 mg/mL streptomycin and 10 U/mL penicillin G. Osmolarity was 297 ± 4 mOsm/kg H_2O; pH was 7.40; Na^+ and K^+ concentrations were 120 ± 2 and 10 ± 1 mEq/L, respectively. Medicinal synthetic air was bubbled through the HTK solution at the selected temperatures at a gas flow of 600 mL/min for 20 min before use. In contrast, the BGP-HMP solution was saturated with room air.

The hypothermic perfusion system

A photograph of the recirculating perfusion system is shown in Fig. 1. It consisted of a reservoir that contained the perfusion solution, a bubbler to deliver air, a sample port, a Masterflex peristaltic pump (Cole Parmer Instrument Co., Chicago, IL, USA), a flowmeter, a nylon filter, and a bubble trap that connected the inflow fluid with a three-way stopcock used to communicate between the liver inflow and the hydrostatic manometer. The liver was set up

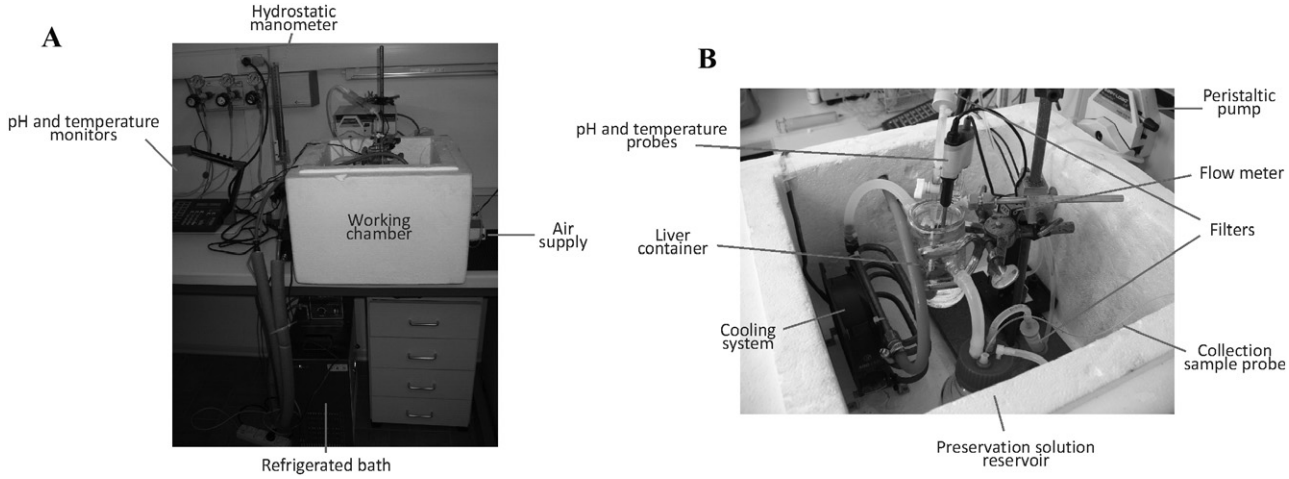

FIG. 1. Recirculating perfusion system is shown. (A) This equipment ensured perfusion at a constant pressure with an air-saturated BGP-HMP solution, while temperature and pH were constantly monitored. Flow rate and hydrostatic perfusion pressure were followed during the experiment. (B) Peristaltic pump, flow meter, filters, pH and temperature probes, liver container, cooling system, preservation solution reservoir, and collection sample probe.

floating in a thermostatic chamber that allowed the fluid exiting from the liver to reach the reservoir. The perfusion portal pressure was measured with a hydrostatic manometer. A thermocouple and a pH glass electrode were also inserted into the thermostatic chamber to measure the fluid temperature and pH during device operation. They were all assembled in a chamber with a thermostat, the temperature of which could be set to 5.0 ± 0.5 or 10.0 ± 0.5°C.

NHBD rat model, HMP, and cold storage

Male Wistar rats were anesthetized by intraperitoneal injection of chloral hydrate (Parafarm, Buenos Aires, Argentina) (500 mg/kg). The abdomen was opened by midline incision and the liver freed from all ligamentous attachments. In brief, the bile duct was cannulated with a PE-50 catheter (Intramedic, Parsippany, NJ, USA) and the portal vein was cannulated with a 14G catheter (Abbocath, Abbott Park, IL, USA), but perfusion was delayed until later. Cardiac arrest was induced by intravenous injection of concentrated potassium chloride solution (2 M) (14) and 150 U of heparin into the femoral vein. Forty-five minutes later, perfusion with Krebs–Henseleit buffer was started, and the suprahepatic inferior vena cava was cannulated with steel tubing (internal diameter 3 mm). Finally, the liver was removed and either (i) connected to a recirculating perfusion system maintained at 5°C and perfused with 250 mL of BGP-HMP solution or (ii) flushed with 20 mL of cold Bretschneider solution (HTK) and then stored (0–4°C) in the same solution for 24 h for CS studies. HMP was performed for 24 h at a constant pressure of 40 mm H_2O (equivalent to 25% of the normothermic portal pressure) and a flow of 0.23 mL/min/g liver. The perfusion solution was air-equilibrated during HMP. After 24 h of HMP or CS, livers were reperfused at 37°C with Krebs–Henseleit buffer with 4% dextran added (15). For both procedures, portal pressure and flow were measured and the intrahepatic resistance (IR) was calculated. Perfusate pH oscillations and the activities of enzymes (lactate dehydrogenase [LDH], aspartate transaminase [AST], and alanine transaminase [ALT]) in perfusate were evaluated during normothermic reperfusion. Bile was collected in preweighed tubes every 15 min, and bile flow was estimated gravimetrically, assuming the bile density to be equal to water, and expressed as μL/min/g liver.

The ability of the liver to extract oxygen was also measured as a function of perfusate flow. Oxygen consumption was determined at 30-min intervals from samples of liver inflow and outflow perfusates using a YSI Model 5300 biological oxygen monitor (YSI, Yellow Springs, OH, USA) equipped with a Clark-type sensor (YSI 5331 oxygen probe). At the end of perfusion, the livers were collected, weighed, and cut into small blocks (4 mm in thickness) for histological studies. The liver blocks were fixed in 10% formaldehyde in phosphate buffered saline (pH = 7.40) and processed for paraffin embedding. Slices 5 μm in thickness were cut, stained with hematoxylin and eosin, and analyzed with bright field microscopy. Also, a biopsy specimen was frozen and stored at −20°C for glycogen content determination.

Liver glycogen content after reperfusion

The technique of preservation and the composition of the preservation solution might contribute in different ways to restore energy during reperfusion. Liver glycogen was determined in biopsy specimens taken after perfusion. Glycogen content was calculated from the amount of glucose released by treatment of homogenized tissue with α-amyloglucosidase following the determination of free glucose content (16). Glycogen content was expressed as mg glycogen/g liver tissue.

Histology

Hematoxylin and eosin staining was used to assess the morphological integrity of the parenchyma. We analyzed the morphology of the sinusoid by measuring sinusoidal dilatation and the amount of cell damage. Two operators, working independently but concurrently, examined at least 15 microscopic fields from three different biopsies by light microscopy. The fields were chosen randomly within the liver parenchyma from each experimental group, and the images were captured with a Canon PowerShot A620 digital camera (Canon, Tokyo, Japan). Images were analyzed with ImageJ software (NIH, Bethesda, MD, USA) using the point-counting method, with grids that contained 80 points placed over the images (17). Every point was analyzed, and the field under the point was evaluated for the occurrence of sinusoidal dilatation, vacuolization, or sinusoidal endothelial cell detachment, each expressed as a percentage.

Calculations

IR, LDH, AST, ALT, and oxygen consumption were calculated using the following formulas:

1. IR (mm Hg/mL/min/g liver) = portal pressure (10.3 mm Hg)/portal flow (mL/min/g liver);
2. LDH (U/L/g liver) = [LDH]/liver weight;
3. AST (U/L/g liver) = [AST]/liver weight;
4. ALT (U/L/g liver) = [AST]/liver weight;

5. Oxygen consumption (μmol O_2/min/g liver) = (C_{in}–C_{out})/portal flow (mL/min/g liver) where C_{in} and C_{out} are the oxygen concentrations in the inflow and outflow, respectively. Oxygen solubility in water = oxygen concentration (μmol O_2/mL)/ partial pressure of oxygen (kPa). Oxygen solubility in water at 37°C°= 0.01056 μmol O_2/mL/kPa (15).

Statistical analysis

Data are presented as mean ± SD. Statistical significance of the differences between values was assessed by ANOVA. Student's *t*-test was applied for glycogen data analysis. For the morphometric analysis, an ANOVA was performed. A P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Perfusion flow and IR

Figure 2 shows the evolution of perfusion flow and IR over 90 min of normothermic reperfusion. As indicated in Fig. 2a, perfusion flow was constant, and there were statistical differences between the portal flow of HMP livers and that of CS livers at all the analyzed times during the experiment ($P < 0.05$). IR was calculated as a function of perfusion flow at a constant perfusion pressure of 10.3 mm Hg. Figure 2b shows the evolution of IR during the perfusion period; statistical differences were found between the HMP and CS groups only at 75 and 90 minutes. These data suggest that HMP has the mechanical advantage of maintaining a healthy microcirculation.

In the design of an optimal HMP apparatus, biomechanical parameters, such as perfusion flow, pressure, and shear stress, are critical. One of the criticisms of HMP has been that it produces a time-dependent increase in vascular resistance and may reduce tissue perfusion (18,19). In our study, both portal flow and intrahepatic resistance remained constant throughout the period of preservation and reperfusion.

Organs donated from NHBDs are particularly prone to form microthrombi and erythrocyte aggregations within the vascular system, impeding successful flush-out upon harvest (20). The main benefit of HMP preservation may hence relate to better progressive microcirculatory equilibration of the graft tissue with the preservation fluid. Constant perfusion gradually compensates for uneven perfusion of the tissue during initial flush-out and eliminates regions of reduced protection resulting from an incomplete equilibration with the preservation solution.

Bile production

Bile production was impaired in the non-heart-beating donor rat model, as shown in Fig. 3. Bile flow was diminished in the HMP group with respect to the CS group ($P < 0.05$). Additionally, the mean bile flow in the HMP group did not change over the 90 min of

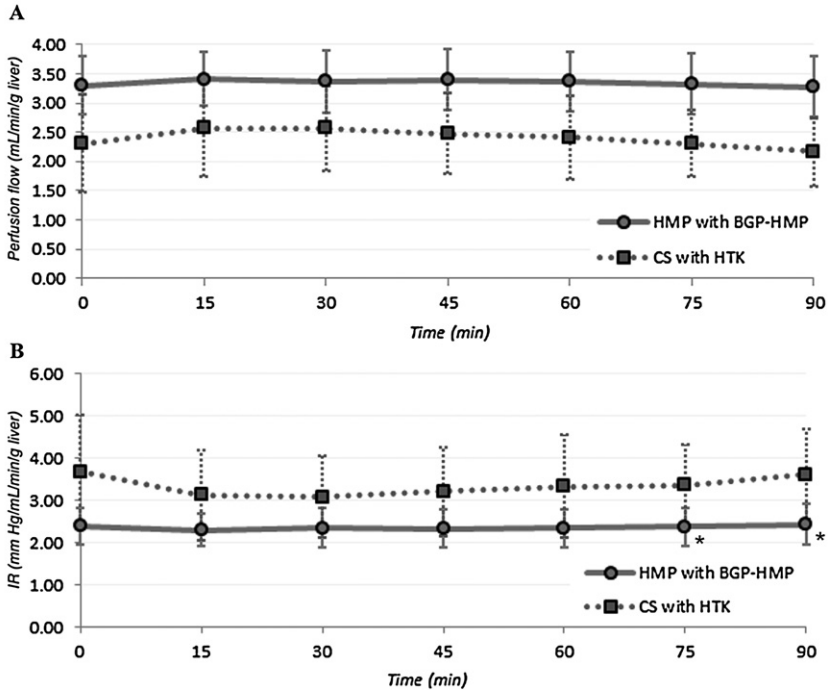

FIG. 2. (a) Perfusion flow and (b) intrahepatic resistance over 90 min of reperfusion. Livers were perfused for 90 min at constant pressure, and the intrahepatic resistance was calculated and expressed as mm Hg/mL/min/g liver in five separate experiments in each analyzed group. *$P <$ 0.05 compared with CS.

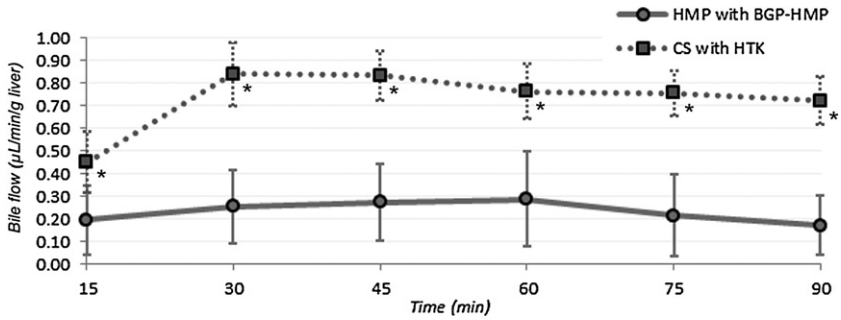

FIG. 3. Bile flow. The bile was collected in preweighed tubes every 15 min, and the bile flow was estimated gravimetrically and expressed as µL/min/g liver in five separate experiments in each analyzed group. $^*P < 0.05$ compared with HMP group.

perfusion. This phenomenon could be due to liver washout of the bile salts over the 24 h of HMP. Another interesting aspect to consider at this point is blood supply. In contrast to hepatocytes, which receive a dual blood supply, the bile duct has a single blood supply from the hepatic artery and is more vulnerable to ischemic damage. In our work, we only perfused the liver via the portal vein, ignoring the hepatic artery due to the necessary complicated handling during surgery; thus, the biliary tract probably did not receive enough oxygen. Similarly, we should further examine our preservation solution, because elevated preservation solution viscosity may increase biliary damage (21).

Oxygen consumption rate

Figure 4 shows how HMP and CS affected metabolic activity of livers in terms of O_2 consumption. HMP livers showed statistically significantly higher rates of consumption with respect to CS livers. Efficiency in O_2 consumption is essential to maintain the energy state of the cells. Rapid depletion of ATP under anaerobic metabolism may cause cellular swelling and acidosis. This leads to the degradation of ATP to adenosine, causing the accumulation of toxic substances and the formation of lactic acid, resulting in cell death (22–24).

LDH, AST, and ALT release during reperfusion

The accumulation of LDH, AST, and ALT enzymes in the perfusate over 90 min of perfusion indicates the level of cell injury. Normothermic ex vivo evaluation after 24 h of CS or HMP revealed that the liver released large amounts of LDH, AST, and ALT during the reperfusion experiments. No statistical differences were found between groups. After 90 min from the beginning of perfusion, the LDH concentration was 25.01 ± 9.31 U/L/g liver in the CS group and 26.74 ± 11.78 U/L/g liver in the HMP group. AST concentration was 4.66 ± 0.67 U/L/g liver in the CS group and 5.07 ± 2.57 U/L/g liver in the HMP group. ALT concentration was 1.98 ± 0.31 U/L/g liver in the CS group and 2.25 ± 1.38 U/L/g liver in the HMP group.

Glycogen content after cold preservation and 90 min of reperfusion

Simple cold storage of livers for transplantation activates glycolysis due to lack of oxygen. Energy derived from glycolysis may be critical for cell survival, and liver cell death may occur once glycolysis is inhibited in the liver due to accumulation of end products or lack of substrate (glycogen) (25). In Fig. 5 it is possible to appreciate the variation in glycogen content after CS and HMP. Paradoxically, glycogen depletion was critical during reperfusion after 24 h of HMP, in contrast with CS ($P < 0.05$). The glycogen depletion was probably due to the continuous hypothermic perfusion with an oxygenated solution (HMP-BGP solution) that did not contain energy-rich substrates such as glucose to replenish the glycogen reserves.

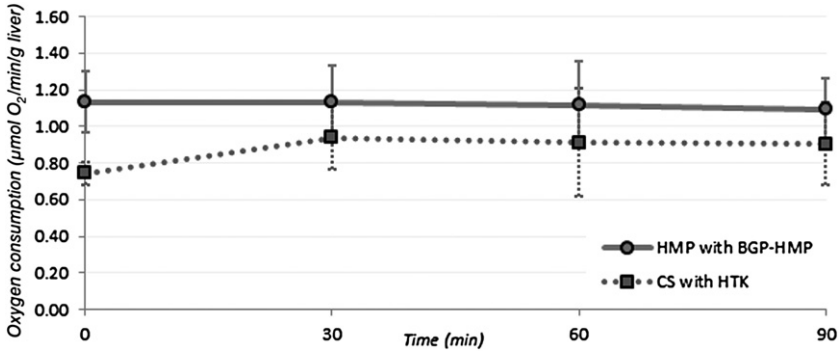

FIG. 4. Oxygen consumption. Oxygen delivery and consumption was determined at 30-min intervals from samples of liver inflow and outflow perfusate and expressed as µmol O_2/min/g liver in five separate experiments in each analyzed group.

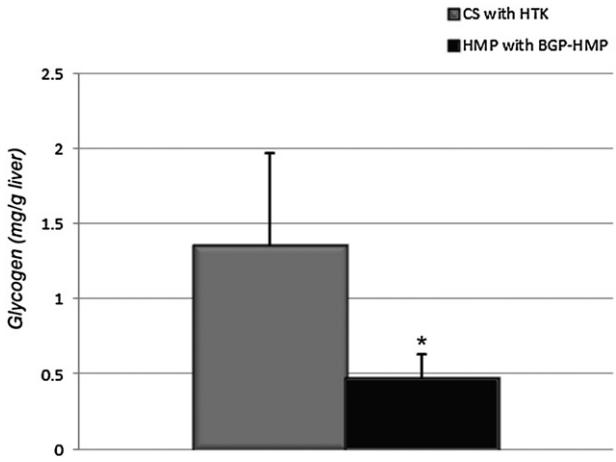

FIG. 5. Glycogen content. Liver glycogen was determined in biopsy specimens taken after reperfusion and expressed as mg glycogen/g liver tissue in five separate experiments in each analyzed group. $^*P < 0.05$.

Liver histology

The histological observations were semiquantified using a point-counting method consisting of 80 point grids superposed over the images. Points that were in contact with injured morphology were counted and the result divided by the total number of points on the grid ($n = 50$ for each group). The results are shown in Fig. 6.

Sinusoidal dilatation

Histology showed that the widening of sinusoids after perfusion and reperfusion was more evident for the HMP group than the CS group. The HMP group had a slightly higher grade of sinusoidal dilatation ($P = 0.06$); see Fig. 6a. This suggests the possibility of an inadequate balance of hydrostatic pressure during perfusion despite the presence of polyethylene glycol and low perfusion pressure.

Vacuolization

Neither group showed an elevated incidence of vacuoles in the hepatocyte cytoplasm (a sign of cell damage; data not shown).

Endothelial cell injury

The two groups of preserved livers presented differences in endothelial cell injury levels (Fig. 6b). The CS group showed a slight but statistically significant difference from the HMP group ($P < 0.05$).

CONCLUSION

The livers showed the same membrane integrity with both preservation methods (shown by enzyme release), and both groups were able to maintain an active metabolism. However, livers preserved with hypothermic mechanical perfusion showed better hemodynamic response and enhanced O_2 consumption capacity. Nevertheless, both systems of preservation for livers could be suitable for application in different circumstances to get better use of livers from non-heart-beating donors to increase the pool of available organs. The hypothermic perfusion system

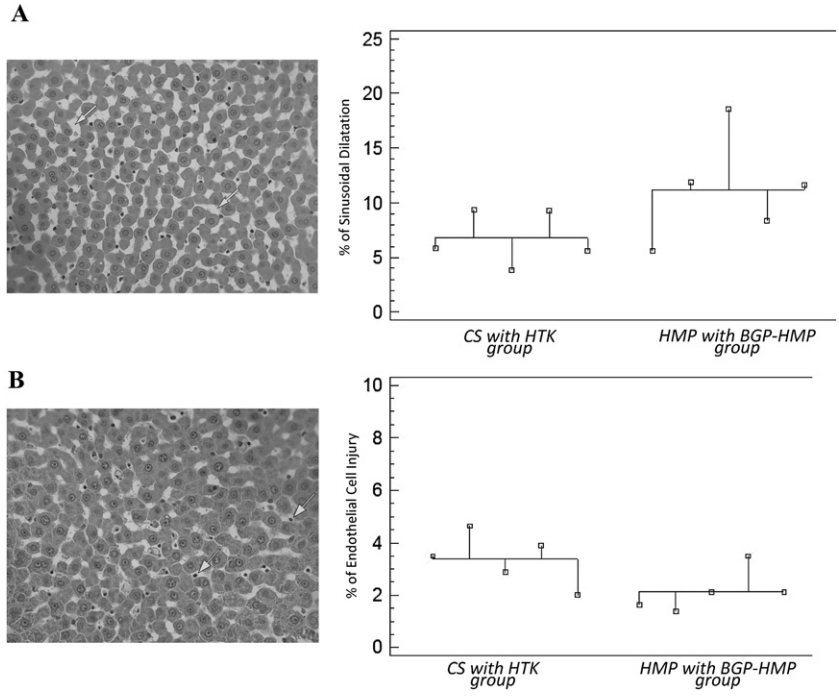

FIG. 6. Hematoxylin/eosin staining. 40× zoom images from H&E-stained biopsies. Arrows show dilatated sinusoids (a) and endothelial cell injury (b). Graphs show the results of morphometric analysis. Percentage values account for frequency of injury event in each microscopic field.

for preserving livers prior to transplantation represents an alternative strategy for diminishing injury due to warm ischemia in NHBDs. Also, the possibility of applying pharmacological measures during this procedure could open up other possibilities, like the addition of bioactive gases (NO, SH_2 or CO) (26). Notwithstanding the fact that normothermic reperfusion is a good in vitro model for evaluating the outcome of preservation, preclinical hepatic transplantation is necessary for better understanding and evaluation of the entire process.

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