




The Novel *N,N*-bis-2-Hydroxyethyl-2-Aminoethanesulfonic Acid–Gluconate–Polyethylene Glycol–Hypothermic Machine Perfusion Solution Improves Static Cold Storage and Reduces Ischemia/Reperfusion Injury in Rat Liver Transplant

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Organ transplantation is the treatment of choice against terminal and irreversible organ failure. Optimal preservation of the graft is crucial to counteract cold ischemia effects. As we developed an *N,N*-bis-2-hydroxyethyl-2-aminoethanesulfonic acid–gluconate–polyethylene glycol (BGP)–based solution (hypothermic machine perfusion [HMP]), we aimed to analyze the use of this solution on static cold storage (SCS) of rat livers for transplantation as compared with the histidine tryptophan ketoglutarate (HTK) preservation solution. Livers procured from adult male Sprague Dawley rats were preserved with BGP-HMP or HTK solutions. Liver total water content and metabolites were measured during the SCS at 0°C for 24 hours. The function and viability of the preserved rat livers were first assessed *ex vivo* after rewarming (90 minutes at 37°C) and *in vivo* using the experimental model of reduced-size heterotopic liver transplantation. After SCS, the water and glycogen content in both groups remained unchanged as well as the tissue glutathione concentration. In the *ex vivo* studies, livers preserved with the BGP-HMP solution were hemodynamically more efficient and the O₂ consumption rate was higher than in livers from the HTK group. Bile production and glycogen content after 90 minutes of normothermic reperfusion was diminished in both groups compared with the control group. Cellular integrity of the BGP-HMP group was better, and the histological damage was reversible. In the *in vivo* model, HTK-preserved livers showed a greater degree of histological injury and higher apoptosis compared with the BGP-HMP group. In conclusion, our results suggest a better role of the BGP-HMP solution compared with HTK in preventing ischemia/reperfusion injury in the rat liver model.

Liver Transplantation 25 1375–1386 2019 AASLD.

Received January 29, 2019; accepted May 16, 2019.

Solid organ transplantation has gained clinical importance in the treatment of terminal and irreversible

organ failure, saving lives and improving life quality and life expectancy of transplant recipients worldwide.⁽¹⁻⁴⁾ The success of transplantation depends on multiple variables, but one key is the adequate preservation of the organ that is to be engrafted.

For liver transplantation, static cold storage (SCS) has become the main method for storage, whereas

Abbreviations: ALT, alanine aminotransferase; BGP, *N,N*-bis-2-hydroxyethyl-2-aminoethanesulfonic acid–gluconate–polyethylene glycol; H & E, hematoxylin-eosin; HMP, hypothermic machine

hypothermic machine perfusion (HMP) or normothermic machine perfusion still awaits being used in the clinical practice.^(5,6)

SCS depends on the effects of cooling, necessary to reduce the tissue oxygen demand and cellular metabolism upon the onset of ischemic damage.⁽⁵⁾ The composition of the preservation solution facilitates the control of biochemical changes developed during the cold storage and ischemic period and maintains organ viability.⁽⁷⁾ Solutions designed to counteract the effects of hypothermia are made up of different components, but they all contain 4 essential constituents:

1. Adequate extracellular or intracellular electrolyte concentration (high or low Na⁺/K⁺ ratio).
2. A buffer to prevent acidosis.^(8,9)
3. An impermeant compound to prevent cell swelling.
4. A colloid to minimize the interstitial edema due to the diffusion of the preservation solution into the interstitium.⁽¹⁰⁾

The gold standard solution used worldwide was the University of Wisconsin (UW; Viaspan, USA) solution developed in the late 1980s.⁽⁵⁾ Since then, other solutions have been introduced in the market, such as the histidine tryptophan ketoglutarate (HTK) solution, developed by Bretschneider,⁽¹¹⁾ and Celsior, which

combines compounds from UW and HTK and was initially used mainly for intrathoracic organs.⁽¹²⁾ The discontinuation of the UW solution favors the need for continuing to search for a solution that will not only improve preservation but could also increase the time span for the organ to be safely engrafted.

We developed an HMP solution based on *N,N*-bis-2-hydroxyethyl-2-aminoethanesulfonic acid–gluconate–polyethylene glycol (BGP). This solution was previously studied storing rats' livers by HMP, assayed in the *in vitro* model of isolated perfused rat livers (IPRLs),^(10,13) and its efficiency was compared with the HTK solution.⁽¹⁴⁾ IPRL offers different advantages in resembling *in vivo* conditions and standardized experimental setups,⁽¹⁵⁾ but nevertheless, it fails to fully translate all of the clinically relevant parameters. Thus, to further expand our understanding of this novel preservation solution, in this work, we studied the efficacy of the BGP-HMP solution in SCS as compared with HTK solution, using not only the *ex vivo* system but also a reduced-size heterotopic rat liver transplant model to stress the new solution, as an alternative for organ preservation.

Materials and Methods

ANIMALS

Male Sprague Dawley rats from the animal facility of the Faculty of Medical Sciences of the National University of Rosario (Rosario, Argentina), 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 they 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 the HTK solution was described previously.⁽¹¹⁾ The BGP-HMP is an extracellular-like preservation solution⁽¹⁰⁾ that was prepared in our laboratory, as previously described.⁽¹⁴⁾ The HTK solution and the BGP-HMP solution were saturated with 600 mL/minute air flow at room temperature.

In the *ex vivo* liver perfusion model (IPRL), the composition of the perfusate was 2% dextran in Krebs–Henseleit bicarbonate (KHB) buffer with 118 mM of NaCl, 4.8 mM of KCl, 25 mM of NaHCO₃, 1.2 mM

perfusion; HTK, histidine tryptophan ketoglutarate; IPRL, isolated perfused rat liver; IR, intrahepatic resistance; IRI, ischemia/reperfusion injury; KHB, Krebs–Henseleit bicarbonate; LDH, lactate dehydrogenase; LPT, long preservation time; NADH, reduced nicotinamide adenine dinucleotide; NHBD, non-beat-beating donor; SCS, static cold storage; SD, standard deviation; SPT, short preservation time; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UW, University of Wisconsin.

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This study was supported by a grant of the international relations of the Regione FVG (Friuli, Venezia, Giulia) given to the Italian Liver Foundation and by PICT (Proyectos de Investigación Científica y Tecnológica) 2012–2530 from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina.

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DOI 10.1002/lt.25573

Potential conflict of interest: Nothing to report.

of KH_2PO_4 , 1.2 mM of MgSO_4 , 1.5 mM of CaCl_2 , 2 UI/mL of heparin, and 5 mM of glucose.

STUDY DESIGN

The study was developed in the following steps. The aim of the first step was to characterize the effect of the 2 preservation solutions (HTK and BGP-HMP) on (1) the variations of total water content and metabolites (glutathione and glycogen content) in the liver during the cold storage period (24 hours at 0°C) and (2) the function and viability of rat livers after 24 hours of SCS followed by perfusion in an ex vivo system (IPRL) during 90 minutes at 37°C. In the second step, we aimed to assess the effect of both preservation solutions (HTK and BGP-HMP) on the hepatic function and viability in an experimental model of reduced-size heterotopic liver transplantation in rats.

STEP 1: SCS AND REPERFUSION IN THE EX VIVO SYSTEM

Surgery

Rats were anesthetized with chloral hydrate (Parafarm, Buenos Aires, Argentina; 500 mg/kg body weight, intraperitoneally), and their livers were prepared according to our reported standard technique.⁽¹⁶⁾

Step 1a: Total Water and Metabolite Content in the Liver After Cold Storage

There were 2 experimental groups. In group A (HTK), livers were obtained and immediately flushed with 20 mL of cold HTK ($n = 3$). A tissue sample was taken ($t_{0\text{-HTK}}$ sample), and then they were stored for 24 hours in HTK solution at 0°C. In group B (BGP-HMP), the livers were flushed with BGP-HMP, a tissue sample was taken ($t_{0\text{-BGP-HMP}}$ sample), and then they were stored for 24 hours in BGP-HMP solution at 0°C ($n = 3$). After the SCS period, tissue samples were obtained ($t_{24\text{ hours}}$ samples). In the samples $t_{0\text{ hours}}$ and $t_{24\text{ hours}}$, the total content of water, glutathione, and glycogen concentrations was determined.

Step 1b: Liver Cold Storage and Reperfusion in the Ex Vivo System

There were 3 experimental groups. In group 1 (controls), livers were perfused immediately after the procurement on the IPRL circuit ($n = 5$). In group 2 (HTK), livers were stored for 24 hours in HTK

solution at 0°C ($n = 5$), and in group 3 (BGP-HMP), livers were stored for 24 hours in BGP-HMP solution at 0°C ($n = 5$). After 24 hours of SCS, livers from groups 2 and 3 were reperfused in the IPRL at 37°C with KHB and a 2% dextran buffer.⁽¹⁷⁾

Isolated Perfused Rat Liver

Livers were perfused at a constant pressure of 10.3 mm Hg using a recirculating system.⁽¹⁶⁾ The reperfusion buffer solution was constantly oxygenated through a thin-wall silicone tubing curled in a ventilated carbogen (5% CO_2 /95% O_2) chamber, yielding a perfusate with a partial pressure of oxygen >450 mm Hg. This buffer was subsequently passed through a heated water bath at 37°C and a bubble trap prior to entering the liver. Portal venous pressure was measured during perfusion by means of a water column connected to the portal inflow line. Hepatic effluent was collected through a steel catheter placed firmly into the suprahepatic inferior vena cava and drained to the reservoir.⁽¹⁷⁾

In all studied groups, the livers were then perfused for 90 minutes at constant pressure; the portal flow rate was measured at 15-minute time intervals for up to 90 minutes; and the intrahepatic resistance (IR) was calculated. Perfusate pH oscillations and lactate dehydrogenase (LDH) activity in the perfusate were evaluated during normothermic reperfusion. A 300- μM taurocholate solution was infused into the perfusion medium at 0.2 mL/minute throughout the experiment to sustain bile production. The bile was collected in preweighed tubes for 90 minutes, and the bile flow was estimated gravimetrically. The ability of the liver to extract oxygen was also measured as a function of perfusate flow. O_2 consumption was determined at 30-minute intervals from samples obtained at the liver inflow and outflow perfusates using a YSI model 5300 biological oxygen monitor (YSI, Yellow Springs, OH), equipped with a Clark-type sensor (YSI 5331 oxygen probe, YSI). At the end of perfusion, the livers were collected, weighed, and cut into small samples (4-mm thickness) for histological studies. Also, a biopsy was frozen and stored at -0°C for glycogen content determinations.

Assessment of Hepatic Function and Cell Injury

LIVER GLYCOGEN CONTENT AFTER COLD STORAGE (1A) AND AFTER REPERFUSION (1B)

Liver glycogen was determined in biopsy specimens taken after SCS (1a experiments) and after SCS

followed by reperfusion (1b experiments). Glycogen content was calculated from the amount of glucose released by treatment of homogenized tissue with α -amylglucosidase following the determination of the free glucose content.⁽¹⁸⁾

TISSUE WATER CONTENT

Water content of liver samples was gravimetrically determined as the difference between the wet and dry weight of liver specimens. Tissue samples were dried in an oven (48 hours, 120°C), performed in triplicate to produce a mean value, and given as a percentage of wet weight.

GLUTATHIONE DETERMINATION

Reduced glutathione content of liver samples was carried out in homogenates prepared in cold 5% trichloroacetic acid in cold 0.01N HCl and measured as described by Ellman.⁽¹⁹⁾

LIVER ENZYMES RELEASED DURING REPERFUSION

LDH concentration was measured in 1 mL of perfusate samples by following the rate at which reduced nicotinamide adenine dinucleotide (NADH) was converted to NAD⁺ at 340 nm in the presence of pyruvate.⁽²⁰⁾

HISTOLOGY

Hematoxylin-eosin (H & E) staining followed by light microscopy observation was used to assess the morphological integrity of the parenchyma. There were 2 operators, working independently but concurrently, who examined more than 50 microscopic images ($\times 400$ magnification) of different biopsies from each one of the experimental groups. Fields were chosen randomly within the liver parenchyma from each experimental group, and the images were captured by a digital camera. Histopathological observations were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD) using a scoring method consisting of 80-point grids superimposed over the images.⁽²¹⁾ Every point was analyzed, and the field under each point was evaluated for sinusoidal dilatation, vacuolated cytoplasm, and sinusoidal endothelial cell injury, each expressed as a percentage.⁽²²⁾

STEP 2: SCS AND REPERFUSION IN THE IN VIVO MODEL

Surgery and Experimental Design

Heterotopic auxiliary and reduced (30% of the total volume) rat liver transplantation with portal vein arterialization was performed in adult, male Sprague Dawley rats using a described technique.⁽²³⁾ The engraftment was done without performing the hepatic artery anastomosis. Macroscopy appearance, color, and consistency of the engrafted livers after reperfusion was identical to the native livers (Fig. 1).^(24,25)

Groups

A total of 32 rats were used. Of these, 5 presented internal bleeding at the portal vein anastomoses and were not considered, and 3 extra rats were included to obtain baseline data. Then, 12 recipients were randomly divided into 2 groups with 6 animals each, considering short preservation time (SPT) 1 ± 0.5 hours and long preservation time (LPT) 24 ± 1 hour:

1. Livers in the HTK group were transplanted after a SPT (HTK-SPT; $n = 3$) or transplanted after a LPT (HTK-LPT; $n = 3$).
2. BGP-HMP group livers were transplanted after a SPT (BGP-HMP SPT; $n = 3$) or transplanted after a LPT (BGP-HMP LPT; $n = 3$).

All recipients were killed 24 hours after reperfusion for the assessment of ischemia/reperfusion injury (IRI) pathological changes.

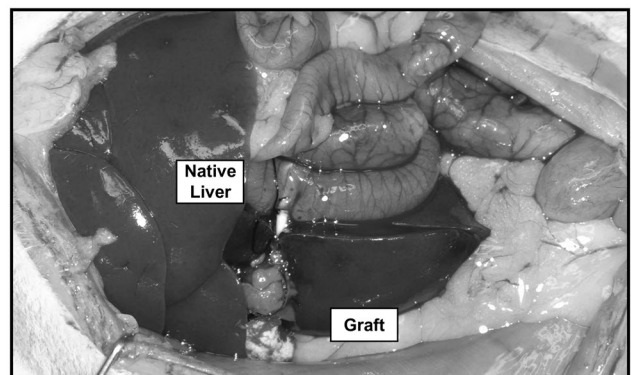


FIG. 1. Anatomic situation after heterotopic liver transplantation immediately after removing the right kidney.

Evaluation of Graft Damage and Cell Injury in the Living Model

Blood samples were collected from the inferior vena cava 24 hours after reperfusion in each group. Alanine aminotransferase (ALT) levels were tested using a commercial kit. Biopsies of liver grafts were also collected at 24 hours after surgery. They were fixed with 10% formalin solution, embedded with paraffin, stained with H & E, and observed under light microscopy. Quantification of liver damage was determined using a grading system originally reported by Camargo et al.⁽²⁶⁾ and modified by Abu-Amara et al.⁽²⁷⁾ Seven parameters were considered for each analyzed sample:

1. Hemorrhage.
2. Neutrophil infiltration.
3. Cell necrosis.
4. Eosinophilic changes of hepatocytes.
5. Discohesive hepatocytes.
6. Cytoplasmic vacuolization.
7. Sinusoidal dilatation.

Each parameter was scored (0, normal; 1, mild change; 2, moderate change; and 3, severe change). Each sample received a general score resulting from adding each evaluated parameter.

An immunohistochemical study to evaluate apoptosis was performed in each group by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique using the In-Situ Cell Death TMR (Roche, Basel, Switzerland) following the manufacturer's instructions. Counting of apoptotic cells was performed considering the number of apoptotic cells in 10 fields per sample (including 5 photographs per sample) using a FV1000 confocal microscope (Olympus, Tokyo, Japan).⁽²⁸⁾

STATISTICAL ANALYSIS

All quantitative data are expressed as mean \pm standard deviation (SD). Statistical significance of the differences between values was assessed by *t* test, chi-square test, or analysis of variance. A *P* value <0.05 was considered statistically significant. Analyses were performed with SPSS, version 20 (IBM, Armonk, NY).

Results

EFFECTS OF SCS ON LIVER TOTAL WATER CONTENT AND METABOLITES

Table 1 shows the time-related changes of water, glutathione, and glycogen content of the livers preserved with BGP-HMP and HTK solutions. The percentage of water and glycogen content in both groups remained unchanged after the preservation period. On the contrary, tissue glutathione concentration was not affected by the 24 hours of SCS in the HTK group but was significantly increased in the BGP-HMP-preserved livers ($P < 0.05$).

EX VIVO LIVER HEMODYNAMICS AND FUNCTION AFTER SCS AND REPERFUSION

Figure 2 shows the evolution of perfusion flow and IR over 90 minutes of normothermic reperfusion. The perfusion flow was constant; perfusion flow was not different between BGP-HMP and the control group (Fig. 2A). The IR, calculated as a function of perfusion flow at a constant perfusion pressure of 10.3 mm Hg (Fig. 2B), was significantly lower in the BGP-HMP group at 45, 60, 75, and 90 minutes of reperfusion.

TABLE 1. Effect of Cold Storage Time on the Tissue Water, Glutathione, and Glycogen Content

Preservation Solution	Time of Hours at 0°C			Time of 24 Hours at 0°C		
	Liver Total Water Content (%)	Glutathione ($\mu\text{mol/g}$ of liver)	Glycogen (mg/g of liver)	Liver Total Water Content (%)	Glutathione ($\mu\text{mol/g}$ of liver)	Glycogen (mg/g of liver)
BGP-HMP (n = 3)	69.19 \pm 0.47	6.36 \pm 0.46	18.23 \pm 2.61	70.11 \pm 0.40	7.29 \pm 0.17*	17.17 \pm 2.19
HTK (n = 3)	69.46 \pm 1.25	5.89 \pm 0.05	17.43 \pm 4.74	71.18 \pm 0.84	5.49 \pm 0.89	12.82 \pm 3.59

NOTE: Data are expressed as mean \pm SD.

*Different from HTK at 24 hours. Statistical significance was defined as $P < 0.05$.

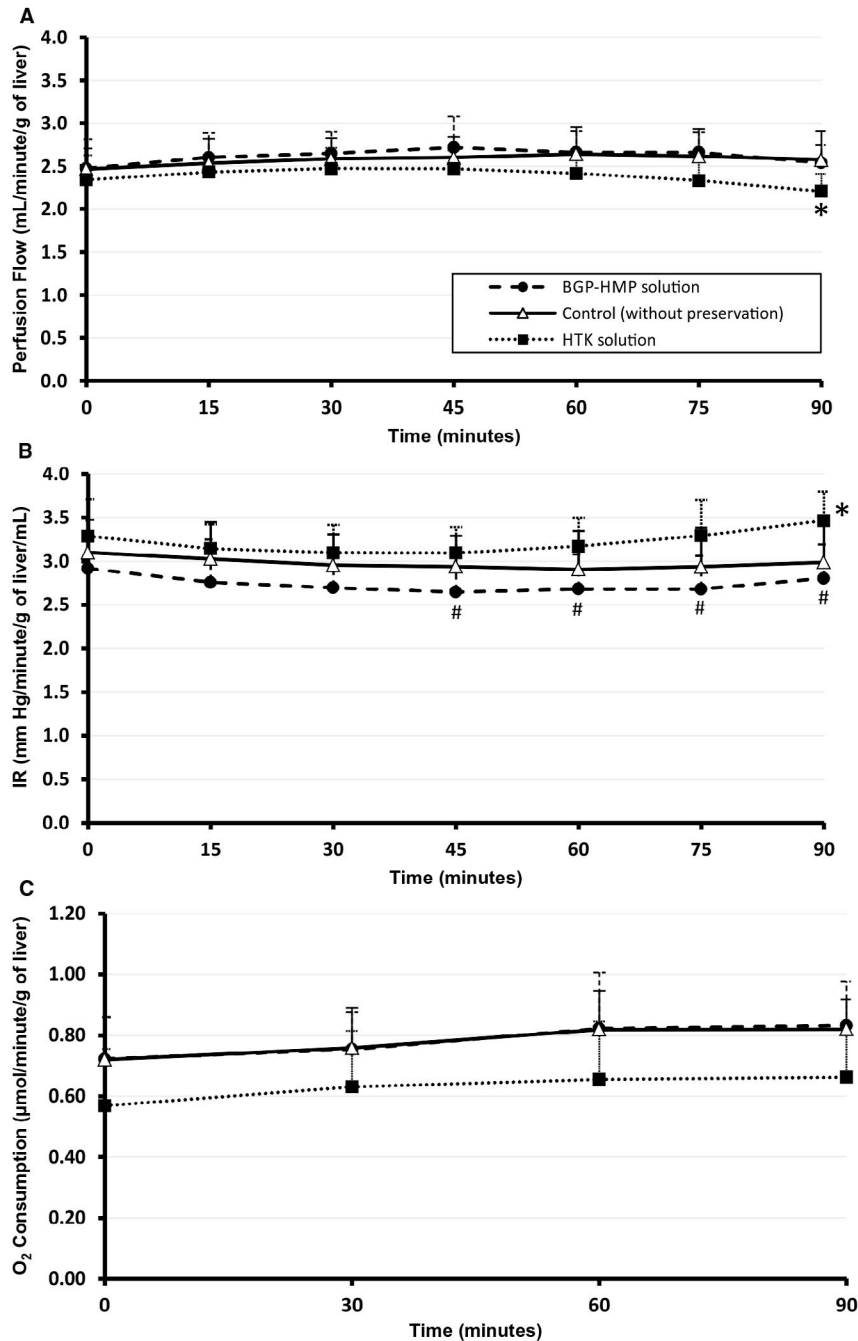


FIG. 2. (A) Perfusion flow, (B) IR, and (C) O₂ consumption during 90 minutes of reperfusion. Livers were perfused at constant pressure. The IR was calculated and expressed as mm Hg/minute/g of liver/mL. O₂ delivery and consumption was determined at 30-minute intervals from samples of liver inflow and outflow perfusate and are expressed as μmol/minute/g of liver. Results are expressed as the mean ± SD (n = 5); P < 0.05. *HTK is different from the control. #HTK is different from BGP-HMP.

Figure 2C shows the time course of O₂ consumption. Although not statistically significant, the HTK-preserved livers showed a reduced rate of consumption compared with the control and BGP-HMP groups,

which was consistent with a better perfusion flow rate and a lower IR of the BGP-HMP group.

Figure 3A shows that the bile production was equally impaired in the preserved groups compared

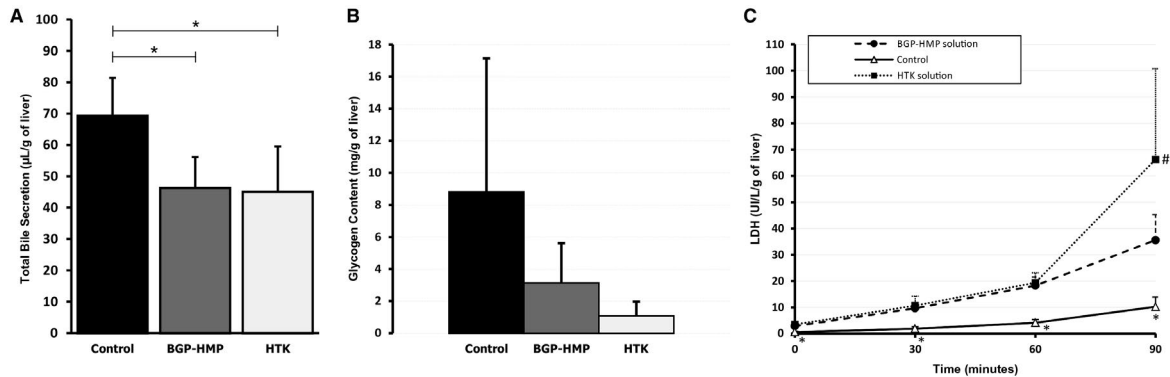


FIG. 3. (A) Bile flow production. The bile was collected in preweighed tubes for 90 minutes, and the bile flow was estimated gravimetrically and expressed as $\mu\text{L/g}$ of liver. (B) Glycogen content. Liver glycogen was determined in biopsy specimens taken after reperfusion, and it was expressed as mg/g liver tissue. (C) Time course of changes in the release of LDH to the perfusate during the reperfusion after the cold storage period (IU/L/g of liver). Results are expressed as the mean \pm SD ($n = 5$); $P < 0.05$. *BGP-HMP and HTK are different from the control. #HTK is different from BGP-HMP.

with the control group. The hepatic glycogen content was severely affected at the end of preservation time, but the effect was significantly more in the HTK-preserved livers (Fig. 3B).

The accumulation of LDH in the perfusate indicating the extent of cell injury was increased in the treated groups compared with the control during reperfusion (Fig. 3C). At 90 minutes, the LDH activity in the HTK group was significantly higher than in the BGP-HMP group (66.26 ± 34.5 versus 35.55 ± 9.75 IU/L/g of liver weight, respectively; $P < 0.05$).

HISTOLOGICAL ASSESSMENT

Figure 4 shows representative histologies of livers of control (Fig. 4A), BGP-HMP (Fig. 4B), and HTK groups (Fig. 4C). The experimental groups presented a rather conserved liver histology with organized hepatocyte plates; only isolated necrotic/apoptotic areas and well-structured portal triads were observed. The morphometric analysis showed that sinusoidal dilatation was significantly more frequent ($P < 0.001$) in the treated groups than in the control group, with no difference between the treated groups. Endothelial injury was also more evident in the treated livers ($P < 0.001$ versus controls), but it differed between the 2 solutions, being almost twice as high in the BGP-HMP group. Sinusoidal endothelial cells were all rounded, and most of them appeared protruding in the sinusoidal lumen. Vacuolization was seen around central veins with portal areas without vacuoles. Both groups differed from the control, but the vacuolization observed in the

HTK-preserved livers was more elevated than that observed in the BGP-HMP-preserved group (Fig. 4).

EVALUATION OF GRAFT DAMAGE AND CELL INJURY OBSERVED AFTER THE HETEROTOPIC AUXILIARY AND REDUCED (30% OF THE TOTAL VOLUME) RAT LIVER TRANSPLANTATION

Plasma ALT levels in each group increased in a time-dependent manner with LPT compared with baseline data and SPT (Fig. 5A). There were no significant differences between both evaluated solutions in a SPT and LPT after 24 hours of graft reperfusion.

Liver damage was also scored according to the classification system of Abu-Amara et al.⁽²⁷⁾ The analysis of biopsies obtained after SPT showed a general injury with a range between 6 and 7. In these groups, the most common observation was cytoplasmic vacuolization and a moderate grade of sinusoidal dilatation, with no differences between HTK and BGP-HMP solutions. When the preservation was extended to the extreme of 24 hours (LPT), the BGP-HMP LPT group showed a moderate grade of injuries, and although they displayed a severe grade of cytoplasmic vacuolization and sinusoidal dilatation, there was no evidence of necrosis (Fig. 5C). On the other hand, the HTK-LPT group showed a greater degree of tissue injury ($P < 0.05$ versus BGP-HMP LPT), with severe and confluent coagulative necrosis and hemorrhage into hepatic chords leading to loss

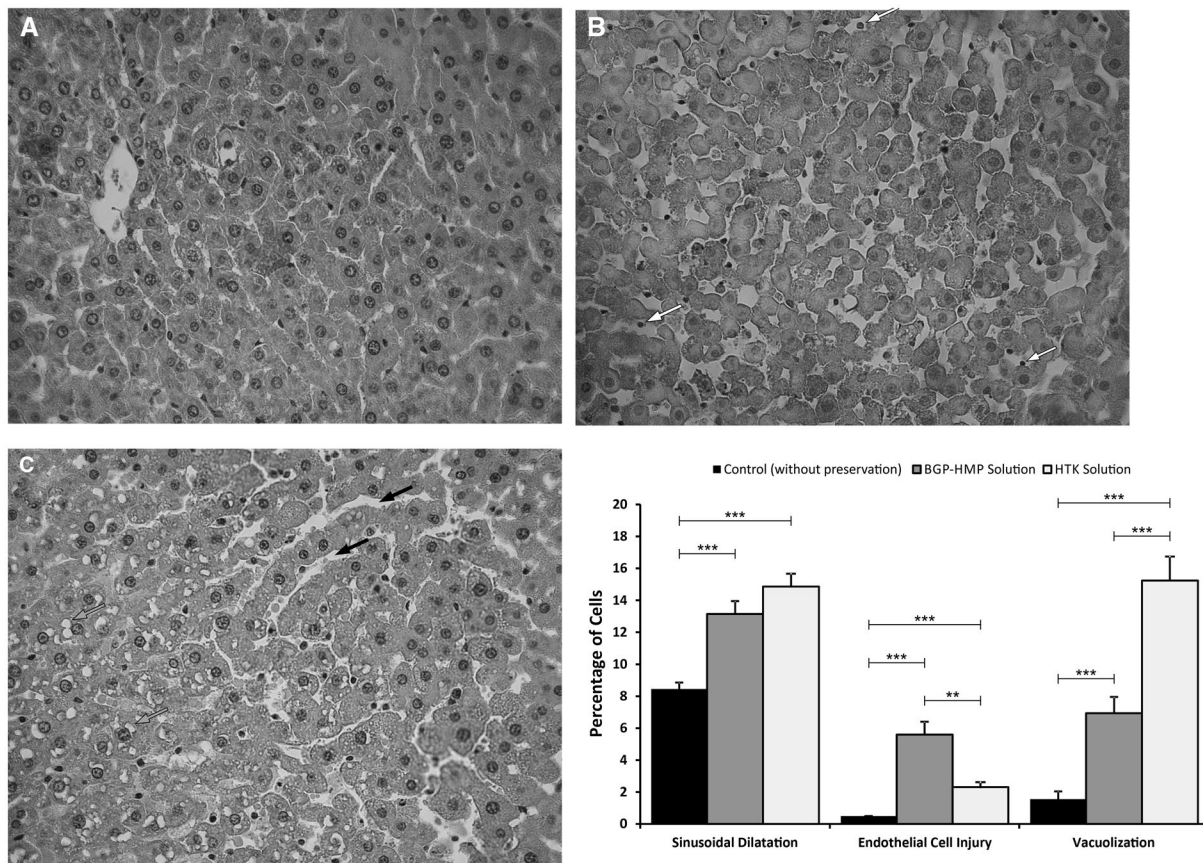


FIG. 4. H & E–stained liver sections after cold preservation under different conditions and 90 minutes of ex vivo reperfusion. Representative photographs ($\times 400$ magnification) from the 3 experimental groups are shown. Histopathological observations, such as sinusoidal dilatation (black arrows), vacuolated cytoplasm (gray arrows), and endothelial cell rounding and retraction (white arrows), were semiquantified by a scoring method. The graph shows the results of morphometric analysis ($n = 5$; mean \pm SD; ** $P < 0.01$, *** $P < 0.001$). Percentage values of each studied injury were calculated from the observed frequencies in each experimental group. (A) Control group, (B) BGP-HMP–preserved group, and (C) HTK–preserved group.

of tissue architecture (Fig. 5D) in an extreme liver transplant model. Also, apoptotic cell evaluation by TUNEL technique was included. Results showed a significant increase in HTK LPT when compared with the BGP-HMP LPT group (Fig. 6).

The immunohistochemical study performed to evaluate apoptosis by TUNEL technique further supports the benefit observed by preserving the liver with BGP-HMP solution both in the SPT and the LPT groups. Figure 6 shows a significant difference in the number of apoptotic hepatocytes between the HTK and BGP-HMP SPT groups ($P < 0.01$) and between the HTK and BGP-HMP LPT groups ($P < 0.01$). On the contrary, no difference was observed when the HTK SPT and LPT groups were

compared; similar observations were found between the BGP-HMP SPT and LPT groups ($P > 0.05$).

Discussion

The gold standard preservation solutions used in liver transplantation have been the UW and HTK. Recently, the need to have new alternatives has opened the field to new solutions, such as Celsior and Institut Georges Lopez 1, but researchers do not completely agree regarding which one provides better short-term and longterm outcomes.⁽²⁹⁻³⁵⁾ Although there are a significant number of research projects going on, there is still a lack of evidence supporting the development of new

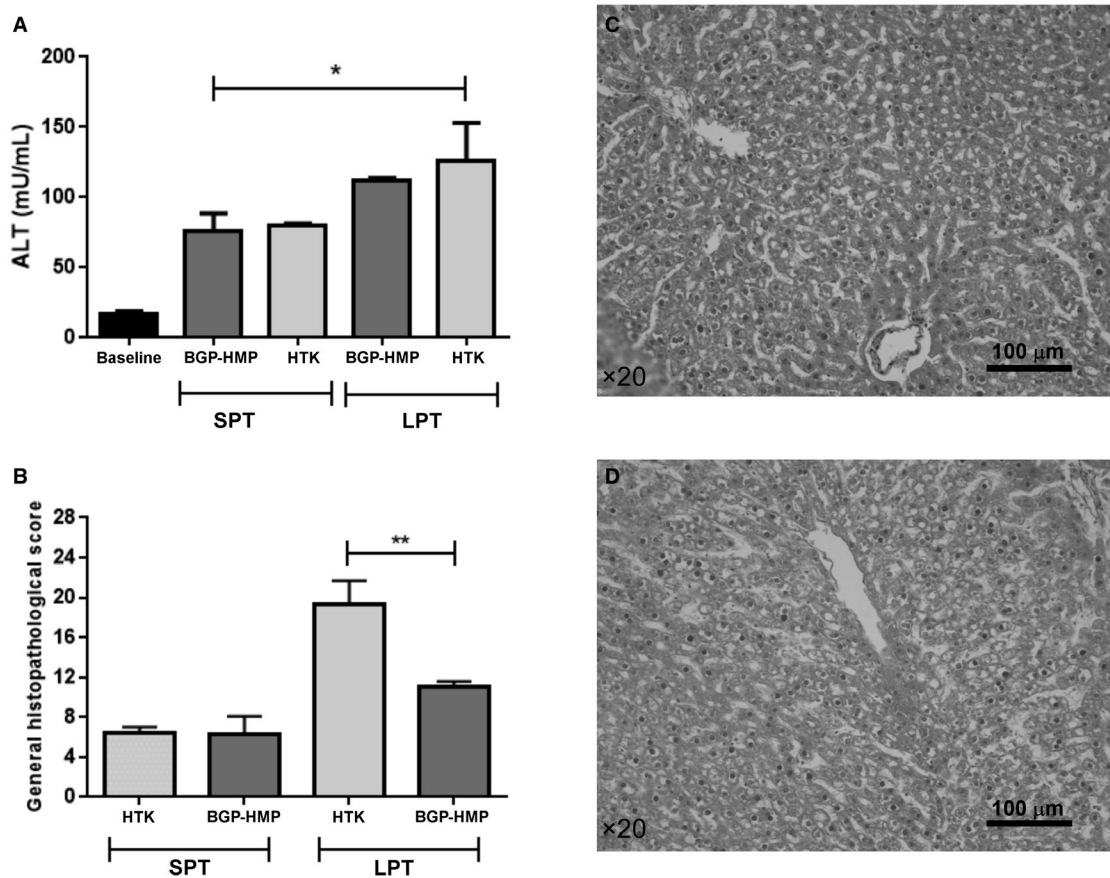


FIG. 5. Cell injury and graft damage in the living model. (A) Plasma liver enzyme levels in nontransplanted rats and recipients with SPT or LPT. No differences were noted between transplanted rats of each group ($*P < 0.05$ all groups versus baseline data). (B) General histopathological score of the transplanted livers. No differences were found between the groups preserved for a short period of time. After LPTs, HTK-LPT showed a much greater degree of injury compared with the BGP-HMP LPT group ($*P < 0.01$). (C) Representative images of BGP-HMP LPT and (D) HTK LPT ($\times 20$ magnification).

alternatives that will not only improve results but also will allow us to extend the required times for the engraftment. This possibility will clearly improve quality of life for the surgical time, result for the recipients, and availability of organs, as well as perhaps the costs.

Here, we aim to develop a new preservation solution capable of being used in all the abdominal organs as well as for static and dynamic preservation techniques. In a previous work, we evaluated our new BGP-HMP solution using HMP to rescue rat livers from non-heart-beating donors (NHBDs) and proved that this solution used only for preservation might lead to a lower degree of injury than the one observed after using HTK for SCS.⁽¹⁴⁾ In the present work, we compared our BGP-HMP solution with HTK by using SCS, IPRL, and heterotopic reduced nonarterialized

auxiliary rat liver transplantation. In order to reduce interstitial edema during the SCS period, we added the colloid osmotic agent polyethylene glycol 35 kDa^(7,36-39) to the BGP-HMP solution, and we observed that the percentage of water in tissues for both groups was still constant during the preservation time; no edema was observed. The glutathione content of livers preserved with HTK solution showed a lower concentration than those preserved with BGP-HMP. This difference might be a result of the addition of 3 mM of glutathione to the BGP-HMP solution.

Although the content of tissue glycogen of livers from the HTK group showed a reduction after 24 hours of preservation, the same phenomenon was not observed in the BGP-HMP group. This lack of change in glycogen content in the BGP-HMP group during cold ischemia

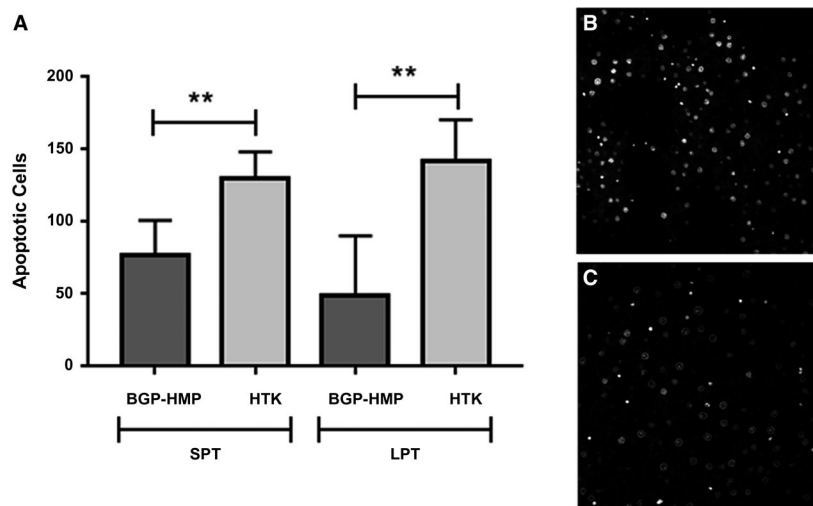


FIG. 6. (A) Bar graph shows apoptotic cell quantification by TUNEL assay in both groups evidencing an increase in the number of apoptotic cells in HTK LPT comparing to BGP LPT (** $P < 0.01$). (B). Representative images of HTK LPT and (C) BGP-HMP LPT group ($\times 20$ magnification) by TUNEL assay.

suggests that the solution was able to guarantee a sufficient reduction of glucose metabolism, including glucose utilization and release by glycogenolysis as well as gluconeogenesis. On the other hand, the content of glycogen in the livers preserved with both solutions and reperfused was significantly reduced compared with the control group. This depletion is probably a response to metabolic demands after reperfusion, which is a surrogate for an increased uptake and consumption of glucose by the cells and is a consequence of the secondary hypoxia suffered by the liver during SCS.⁽⁴⁰⁻⁴²⁾

The composition of the preservation solutions can greatly influence the maintenance of the microvasculature of the organ. At this point, the control of the osmotic and oncotic balance is mandatory.⁽⁴³⁾ Our data show that livers preserved with BGP-HMP tolerate the perfusion flow better and exert a lower vascular resistance, suggesting a clear advantage in maintaining a healthier microcirculation than HTK.

The maintenance and recovery of metabolism after preservation is another important point to analyze. In our previous work, using the BGP-HMP solution with HMP, we found that the production of bile was diminished in relation to the SCS group with HTK in livers obtained from NHBDs.⁽¹⁴⁾ In this work, we confirmed a comparable decrease in the amount of bile secretion/production in livers preserved with both solutions, suggesting that the injury produced at the hepatocyte/canalicular level was similar with both solutions.

Furthermore, the O_2 consumption rate in the BGP-HMP group was identical to that of the control group and better than livers preserved with HTK.

These findings, together with the favorable reduction on IR observed during IPRL with the BGP-HMP solution, suggest that the energy capacity of these livers remains close to intact. Efficiency in O_2 consumption is essential to maintain the energetic state of the cells.

Although our results showed enzymatic release after the IPRL, when histological preparations were analyzed, reversible tissue damage was shown in the BGP-HMP group. This can be considered as a direct effect of cellular injury during SCS with the inevitable and, therefore, expected consequence of the reperfusion injury.

In the same way, after a long SCS time of 24 hours and following of the subsequent transplant chosen during the study design (reduced size and heterotopic), the amount of cytoplasmic enzymes released (ALT) was comparable in preserved livers with our BGP-HMP solution or with the commercial HTK solution.

Moreover, and more important than the biochemical evaluation, the histopathological analysis showed after 24 hours of preservation (longterm preserved livers) that the livers of the BGP-HMP group had a lower degree of tissue injury with absence of irreversible histological damage, characterized by the absence of hepatocyte necrosis seen in the HTK group under conditions of preservation and surgical procedure. These results are reinforced by the data observed by

the TUNEL analysis, showing a significantly lower degree of apoptotic cells in the BGP-HMP SPT and LPT compared with the HTK group. Therefore, BGP-HMP not only reduces the degree of ischemic cell deaths but also the amount of apoptotic cell deaths.

In summary, after testing our new BGP-HMP solution in 2 models—the IPRL in vitro model and the transplant model—the preliminary results support a promising role in preventing IRI compared with one of the clinically used preservation solutions. However, it is necessary to evaluate the solution in larger animal models, with whole-liver transplants and longer survival times as a preliminary step for clinical trials.

Acknowledgments: We thank Enrique L. Portiansky and Carolina Zanuzzi from the Laboratorio de Análisis de Imágenes Patología General, Facultad de Ciencias Veterinarias, Universidad Nacional de la Plata, for their technical support.

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