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Hypothermic preservation of rat liver microorgans (LMOs) in bes-gluconate solution. Protective effects of polyethyleneglycol (PEG) on total water content and functional viability

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ABSTRACT

We have reported of an alternative solution to preserve hepatocytes that have three key components: gluconate, sucrose and an aminosulfonic acid (BGS solution). In order to extend the use of this solution to organs as the liver, we evaluate the effect of the addition of PEG of 8, 20 and 35 kDa to BG Solution on the total water content and functional viability of rat liver microorgans (LMOs). LMOs were preserved (48 h 0 ºC) in the following solutions: ViaSpan®; BGS; BG plus 4% PEG 8000 (BG8); BG plus 4% PEG 20.000 (BG20) and BG plus 4% PEG 35.000 (BG35). LDH Release and Total Water Content showed a marked increase in LMOs preserved in BGS. This indicates that, in the absence of PEG, the tissue showed important cell membrane integrity deterioration and was incapable of regulating cell volume. After the preservation period, all groups were reoxygenated (120 min, 37 ºC, KHR) and Total Water Content, Glycogen Content and Oxygen Consumption were determined. After 120 min LMOs preserved in BG35 showed values of Oxygen Consumption similar to controls. On the other hand, LMOs preserved in BG8, BG20 and ViaSpan® showed oxygen consumption rates and glycogen content significantly smaller than controls. In conclusion, BG35 was the most effective preservation solution to protect LMOs against cold preservation injury due to ischemia and reoxygenation. It is a good alternative to ViaSpan® because of its higher buffer capacity, its best indexes of respiration activity and for being considerably less expensive.

Key words. **Cold storage. Preservation solution. ViaSpan® solution.**

INTRODUCTION

One goal in transplantation research is the development of better preservation solutions. There are two different approaches to attain this goal: the modification of the composition of currently available solutions or the development of novel ones. Both strategies require the assessment of tissue viability to analyze and compare the various cold storage solutions. A model commonly used is orthotopic transplantation in dog, pig or rat.[1,2] However, this model is complex, expensive, and labor intense, and do not allow a rapid testing of the myriad of variables that could improve organ preservation. To overcome these shortcomings, simplified models have been developed including isolated or cultured cells, and isolated perfused organs. Isolated cells constitute a simpler model that allows carry out a large number of experiments in a relatively short period of time.[3,4] We have already reported the development of an alternative solution to store functional hepatocytes. This novel preservation solution (BGS) showed the same efficacy as the modified University of Wisconsin solution to protect rat hepatocytes against cold preservation injury and it is considerably less expensive.[5] We have developed BGS solution including three key components: gluconate, which is a non saccharide impermeant that acts as an extracellular oncotic agent,[6] sucrose which gives additional osmotic support,[7] and BES, an aminosulfonic acid, which provides an increased buffer capacity.[8]

Liver microorgans (LMOs) are intact pieces of tissue that maintain a relatively normal architecture, including cell to cell contact and cell to cell

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communication, and can be used as an alternative of whole-organ models.[9] Their usefulness in organ preservation studies has been demonstrated previously; they could be a reliable tool to asses the efficacy of novel preservation solutions developed to include non-heart-beating, older, and marginal donors into the donor pool.[10] A major problem in organs hypothermic preservation is tissue edema. The main cause of cellular swelling under preservation conditions is the inhibitory effect of hypothermia on the activity of the Na^+-K^+ ATPase. Another cause may be the lack of ATP due to effects of hypothermia on energy metabolism.[11] The cellular swelling mechanism seems to be similar in hypothermically preserved organs and LMOs.[12] Colloids are essential to prevent or minimize interstitial edema. Hydroxyethyl starch, included on ViaSpan® solution, has been the focus of several studies and the subject of many debates over the past years.[13] Hydroxyethyl starch can trigger blood red cell aggregation, thereby promoting stasis in hepatic sinusoids that results in incomplete washout of donor graft before transplantation.[14] Since then, other colloids have emerged, including polyethylene glycol (PEG), which has physical and chemical properties of particular interest in organ preservation. New preservation solutions containing PEG, such as IGL-1 (Institute Georges Lopez preservation solution), Cardiosol (a modified cardioplegic solution) and SCOT 15 (Solution de Conservation des Organes et des Tissus), have been shown to efficiently protect organs from ischemia/reperfusion injury.[15,16] PEGs of different molecular weights are available. However, it is not already known which is the optimal for organ preservation.[17]

In order to adapt the BGS composition and make it suitable for the preservation of hole organs, as for example the liver, we have evaluated the effect that the addition of PEG of different molecular weights (MW) (8, 20 and 35 kDa) to this solution has on the total water content and functional viability of LMOs. Also, we have studied the efficacy of these preservation solutions performing viability, functional and histological studies of LMOs both during cold storage and reoxygenation.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats weighing 250-300 g were used in all experiments. The rats were allowed access to standard laboratory diet and water ad libitum prior to the experiments, and received care in compliance with international regulations. The National Council Committee from Argentine approved animal protocols.

Preparation of LMOs

LMOs were prepared using "free-hand" techniques, by cutting the liver into thin blocks. They were cut using a microtome blade attached to a plastic handle (designed in our laboratory), to facilitate handling. We worked at 0 °C (on ice) to reduce tissue deterioration, and over a paper filter to prevent the slippage of the blocks that could prevent the precise cutting of the sheets. After slicing, LMOs were placed in different solutions. Fresh LMOs (controls) were placed in Krebs-Henseleit Reoxygenation media (KHR) its composition was: 114 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.5 mM $CaCl_2$, 10 mM hepes, 25 mM glucose, 1 mM allopurinol, 3 mM glycine; pH = 7.40, 310 mOsm/kg water. LMOs that underwent the preservation process were placed in the different preservation solutions, whose compositions are detailed below.

Before starting with the preservation experiments, and in order to validate the recently described cutting technique, LMOs thickness was determined, and then they were incubated (37 °C, KHR, 120 min) to measure the Total Water Content (TWC), Extra and Intracellular Water Space, and K^+ and Na^+ content. LMOs architectural integrity was assessed by histological analysis.

LMOs preservation

Once cut, LMOs were placed in the different solutions. Approximately 50 LMOs were stored for up to 48 h at 0 ºC in a glass Boeco bottle containing 50 mL of the following preservation solutions:

a) ViaSpan® (UW Bristol-Myers-Squibb, Bruxelles, Belgium).
b) BGS.
c) BG plus 4% PEG 8 kDa (BG8).
d) BG plus 4% PEG 20 kDa (BG20).
e) BG plus 4% PEG 35 kDa (BG35) (Table 1).

48 h cold storage were chosen because in preliminary experiments (data not shown) the viability evaluated by Lactate Dehydrogenase (LDH) Release was only moderately altered by 24 h of cold ischemia, but a clear cut difference after 48 h was seen.

A sample was removed daily from all the preserved groups, in order to estimate the time course changes in viability (see Viability parameters and functional assays).

LMOs reoxygenation

After 48 h of cold storage, LMOs were washed thoroughly with a rinse solution previously described by our group[18] to remove residual preservation solution that could interfere during the subsequent reoxygenation. This step was carried out at 37°C during 120 min in a Dubnoff metabolic shaker in KHR under carbogen atmosphere (95%O_2:5% CO_2) in 6-wells culture plates. Two LMOs with 5 mL of KHR were placed in each well.

Experimental procedures

First, the BGS composition was modified to adapt it to LMOs preservation. The main modifications involved the addition of PEG of different MW (8, 20 and 35 kDa), the remotion of sucrose, and the decrease on BES concentration (from 70 mM to 50 mM). As it was already stated, one of the key components of BGS solution is the buffer agent BES, a suitable buffering agent to avoid preservation-related changes in the intracellular pH.[8] So the aim of part I of this paper was the determination of the buffering capacity of the developed preservation solutions, and the evolution of pH of these solutions during the cold storage of LMOs.

Part II was meant to evaluate the effects that the changes introduced to BGS, especially the PEG addition, have on the total water content, and the viability and functionality of LMOs. During the preservation period, we have evaluated LDH Release and Total Water Content. To distinguish between tissue responses displayed during the cooling step, and those caused by cold storage but exposed during the reoxygenation process, we have also assessed LDH Release, Total Water Content, Glycogen Content, Oxygen Consumption and Histological Characteristics of the preserved LMOs during reoxygenation. Freshly cut LMOs were used as controls.

- ***Determination of the Buffering Capacity of the preservation solutions:*** The buffering capacity of a preservation solution is defined as the concentration of H^+ needed to produce a decline of 1 pH unit from the initial pH of the solution.[19] Each tested solution was titrated with 0.1 M HCl under continuous stirring and the pH was continuously measured at 0 ºC and plotted out.
- ***Time course evolution of preservation solution pH during cold storage of LMOs.*** The preservation solutions pH changes were measured at 0 ºC using a pH-meter (JENCO, MODEL 6071) after 24 and 48 h of cold storage.

Validation of the cutting technique: assessment of LMOs quality

- ***Thickness.*** LMOs thickness was determined using a gauge DIGIMESS® (Pocket Thickness Gage, 0.1 mm-10 mm). Sections were placed between two coverslips and the thickness was measured; the thickness of the two coverslips was previously measured and substracted to obtain the LMO thickness.
- ***TWC.*** Tissue water content was determined by a dessication method (12 h in a hot oven at 105 °C). TWC was expressed as mL water/g dry tissue and performed in quadruplicated for each experiment to produce a mean value.
- ***Extra and Intracellular Water Space.*** LMOs were incubated (in triplicate) for 120 minutes in KHR containing the extracellular volume marker inulin (4 mg/mL). After 60 and 120 min of incubation the sections were dried, weighed and homogenized to determine the inulin space. The tissue and medium inulin concentration were determined using Roe assay.[20] The volumes of extra and intracellular compartments were obtained from the distribution volume of the extracellular tracer inulin. Extracellular Water Space was expressed as mL extracellular water/mL total water and Intracellular Water Space as mL intracellular water/ mL total water. They were performed in quadruplicated for each experiment to produce a mean value.
- ***Tissue K^+ and Na^+ content.*** Each dry tissue was dissolved in HNO_3 (1mM) overnight to determinate Na^+ and K^+ levels by flame photometry. Na^+ and K^+ levels were expressed as µEq/ g dry tissue.

Viability parameters and functional assays

- ***LDH Release.*** The LDH activity was measured in the incubation medium and in the tissue as described previously.[21] Results were expressed as

the percentage of the total enzyme activity released to the incubation medium.

- ***Glycogen Content.*** The glycogen content of LMOs was determined using the Carr and Neff's assay.[22] Two LMOs were removed from the incubation medium and were stored at -20 ºC until the colorimetric determination was made. The glycogen content was expressed as mg glycogen/g tissue.
- ***Oxygen Consumption.*** Fresh (controls) and cold stored LMOs were incubated in KHR medium at 37 ºC in a Dubnoff metabolic bath under carbogen atmosphere with continuous shaking for 120 min. At different times two LMOs were added to a thermostatized oxygen electrode chamber designed in our laboratory. The chamber contains 10.5 mL of respiration medium (Krebs-Henseleit media with 10 mM hepes and 2 mM pyruvate, pH = 7.40 at 36 ºC). The oxygen content of this respiration medium equilibrated with air was determined according to Robinson[23] and found to be 0.524 ± 0.024 µmol/mL at 36 ºC and at a barometric pressure of 760 mmHg, n = 10. Oxygen consumption was measured using a Clark-type oxygen electrode (YSI 5300, Yellow Spring, OH, USA). The endogenous respiration rate was assessed after a stabilization period of 2 min. The rate of oxygen uptake was recorded and calculated over a 5 min period. Results were expressed as µmol O_2/min/g tissue.

Histology

The efficacy of the different preservation solutions in conserving LMOs morphology after cold preservation and normothermic reoxygenation was evaluated. Samples of all the experimental groups were fixed in 10 % formaldehyde in PBS (pH = 7.40) and histologically processed for paraffin embedding. Slices of 5 µm thick were cut and stained with hematoxilin & eosin. Afterward, they were analyzed with a light field microscopy (Olympus Co, LTD. Model U-MDOB, 20X objectives, equipped with a digital camera Olympus model D-360 Zoom-3.2 megapixels of resolution) taking into account hepatocyte cords integrity, presence of vacuoles, blebs or necrotic focus, sinusoidal endothelial cells shape, and the general morphological aspect of the hepatic lobules.

Statistical analysis

Statistical differences between values were assessed by analysis of variance (ANOVA) followed by Scheffe's multiple range test. A *p* value less than 0.05 was considered statistically significant.

RESULTS

Cutting technique: Viability parameters to asses LMOs quality

Although LMOs were manually cut, thin and homogeneous cuts were obtained. The LMOs average thickness was 436 ± 18 µm, n = 50.

Table 1 shows TWC, Extra and Intracellular Water Space, and Na^+ and K^+ content (37 ºC-120 min-KHR) of LMOs freshly cutted, n = 20.

The TWC value (mL water/g dry tissue) at the beginning of the incubation was 3.74 ± 0.32 and no significant differences were observed after 60 and 120 min (3.72 ± 0.32 and 3.69 ± 0.22, respectively).

The Extra and Intracellular Water Spaces did not change after 120 min of incubation. Extracellular water values (mL extracellular water/mL total water) were: 0.37 ± 0.03 after 60 min and 0.43 ± 0.02 after 120 min; whereas the values obtained for the Intracellular Water Space (mL extracellular water/mL total water) were 0.62 ± 0.03 after 60 min and 0.57 ± 0.02 at 120 min.

The LMOs also maintained the intracellular Na^+ and K^+ contents after 120 min of reoxygenation. The tissue K^+ content values were 88 ± 22 and 64 ± 23 µEq K^+/g dry tissue, and the Na^+ levels were 572 ± 94 and 637 ± 89 µEq Na^+/g dry tissue, after 60 and 120 min.

Buffering capacity of the preservation solutions and extracellular pH evolution of LMOs cold stored in these solutions up to 48 h

Potentiometric titration curves were generated for each solution by adding small amounts of HCl 0.1 N to the solution of interest. As illustrated in Figure 1A, BG8, BG20 and BG35 showed a substantially greater buffering capacity than ViaSpan®. The buffering capacity expressed as mEq H^+/L/pH unit obtained for BG8 was 37.2 ± 1.2; 38.5 ± 1.3 for BG20; 36.1 ± 0.8 for BG35 and 11.3 ± 0.9 for ViaSpan®. No differences were observed between BG8, BG20 and BG35.

Figure 1B shows the time course evolution of the preservation solution pH from LMOs cold stored in BG8, BG20, BG35 and ViaSpan®. No significant differences on the pH were found after 48 h of cold storage in BG8, BG20 and BG35. However, pH of the

Table 1. Composition of the preservation solutions ViaSpan®, BGS, BG8, BG20 and BG35.

	ViaSpan®	BGS	BG 8	BG20	BG35
Impermeants (mM)					
Lactobionate	100				
Gluconate		100	100	100	100
Raffinose	30				
Sucrose		40			
Buffers (mM)					
KH_2PO_4	25	2.5	2.5	2.5	2.5
BES		70	50	50	50
Substrates (mM)					
Allopurinol	1	1	1	1	1
Glutathione	3	3	3	3	3
Adenosine	5	5	5	5	5
Glycine		15	15	15	15
Colloids (g/L)					
HES	50				
PEG 8 kDa			40	40	40
PEG 20 kDa			40	40	40
PEG 35 kDa			40	40	40
pH 7.40	7.40	7.40	7.40	7.40	
Osm (mOsm/kgwater)	320 ± 4	326 ± 5	331 ± 3	335 ± 6	339 ± 4

To adapt the BGS solution to LMOs preservation, its composition was modified. The sucrose was replaced by PEG and the concentration of the buffer agent BES was diminished. The different solutions only vary in the MW of the PEG they contain. All the solutions were bubbled with 100% N_2 for 45 min at 0 ºC before use. **BES:** [N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid]. **HES:** Hydroxyethyl starch. **PEG:** Polyethyleneglycol. Dexamethasone 16 mg/L, insulin 40 UI/L and penicillin G 200.000 UI/L were added to Viaspan® before use. Streptomycin 250 mg/mL and penicillin G 10.000 UI/L were added to BGS, BG8, BG20 and BG35 before use.

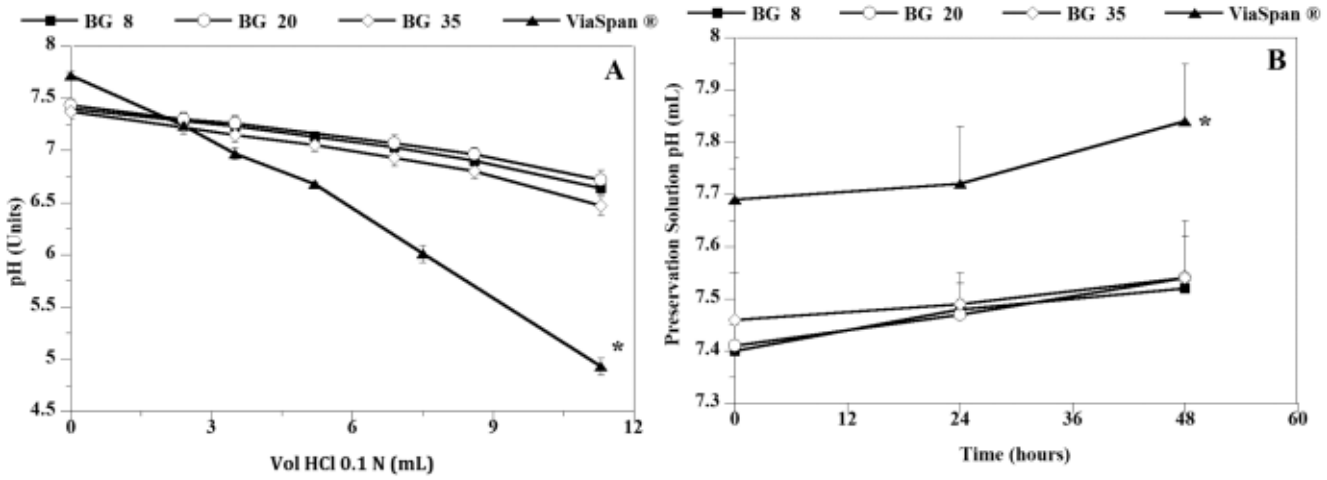

Figure 1. A. *Determination of the buffering capacity of BG8, BG20, BG35 and ViaSpan® solutions. Each tested solution was titrated with 0.1 M HCl, and the pH was measured at 0 ºC sequentially and plotted out. Data are expressed as mean ± SD for 4 determinations. * different from BG8, BG20 and BG35, p < 0.05.* ***B.*** *Evolution of preservation solution pH of LMOs cold stored in BG8, BG20, BG35 and ViaSpan® solutions during 48 h at 0 ºC. Each point is expressed as mean ± SD for 3 LMOs preparations.* * *different from pH = 7.40 and from BG8, BG20 and BG35, p < 0.05.*

LMOs preserved in ViaSpan® showed a statistical increase ($p < 0.05$) after 48 h of preservation (from 7.68 ± 0.03 at time 0 until 7.87 ± 0.04 at 48 h, n = 3). This value was also different to that observed in LMOs stored in BG8 (7.55 ± 0.04); BG20 (7.51 ± 0.04) and BG35 (7.39 ± 0.07) ($p < 0.05$, n = 5).

LMOs viability during the cold preservation period

An increase in LDH Release during cold storage for all the evaluated solutions was observed (Figure 2A). However, the LDH Release for LMOs preserved

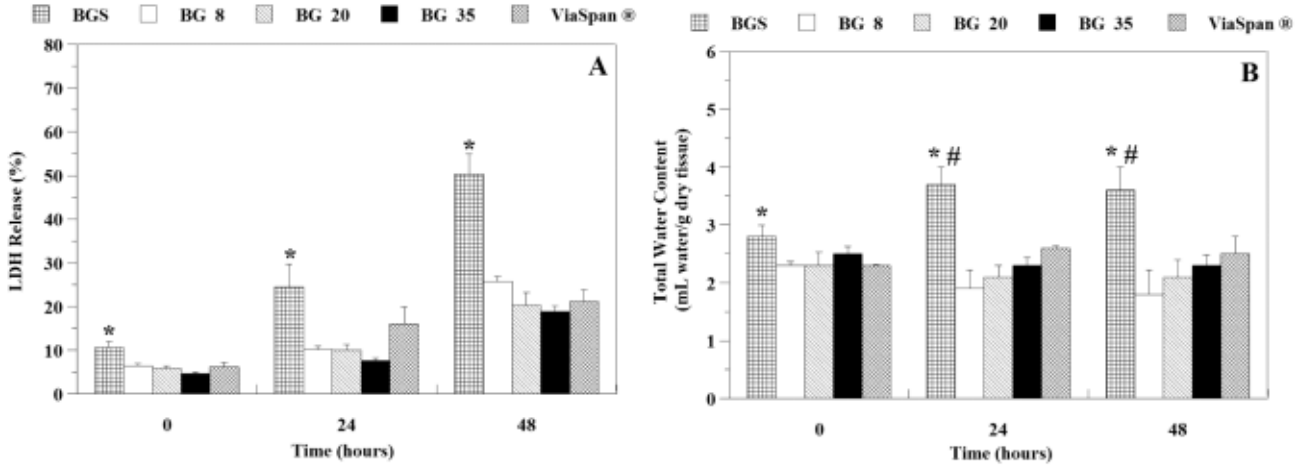

Figure 2. A. *Time course LDH Release of LMOs preserved in BGS, BG8, BG20 BG35 and ViaSpan® solutions during cold preservation (48 h at 0 °C). Data are expressed as mean ± SD for 4 HMOs preparations. * different from all the other groups, p <0.05.* ***B.*** *Time course of Water Content of LMOs preserved in BGS, BG8, BG20, BG35 and ViaSpan® solutions. Data are expressed as mean ± SD for 4 LMOs preparations. * Different from all the other groups. # different from the initial preservation time (t = 0), p < 0.05.*

in BGS was significantly higher than the values obtained for all the others groups. After 48 h, LDH Release was 50.3 ± 3.7 % for LMOs preserved in BGS solution whereas for LMOs preserved in the other solutions this parameter was statistically minor ($p < 0.05$, n = 5): 25.7% ± 2.6 for BG8, 20.2% ± 3.1 for BG20, 18.9% ± 2.9 for BG35 and 21.2% ± 1.7 for ViaSpan®.

Figure 2B shows the TWC evolution for LMOs during the cold storage in the different preservation solutions. For LMOs preserved in BGS, the TWC was significantly higher ($p < 0.05$, n = 5) than for LMOs preserved in the others solutions. After 48 h, the BGS group contained 3.6 ± 0.4 mL water/g dry tissue, against 1.8 ± 0.4 for BG8, 2.1 ± 0.3 for BG20, 2.3 ± 0.2 for BG35 and 2.5 ± 0.3 for ViaSpan®. In addition, only for LMOs cold stored in BGS solution we found a significant increase in TWC with an increased preservation time (from 2.8 ± 0.4 at t = 0 until 3.6 ± 0.6 after 48 h). Due to these results, we decided not to evaluate this group during the reoxygenation period.

LMOs viability and function during the reoxygenation period

After 120 min of rewarming, no statistically significant differences were found neither in LDH Release (Figure 3A) nor in TWC (Figure 3B) between fresh LMOs (controls) and LMOs preserved in BG8, BG20, BG35 and ViaSpan®. After 120 min LDH Release (%) was: 30.4 ± 6.8 for control, 30.2 ± 4.9 for BG8, 29.4 ± 6.5 for BG20, 23.4 ± 3.4 for BG35 and 35.4 ± 5.5 for ViaSpan® (n = 5). Figure 3C shows the TWC of controls and LMOs cold preserved in the different solutions. At the beginning of the rewarming, controls showed a TWC significantly higher than the LMOs preserved in the different solutions analyzed (3.72 ± 0.32 for control, 3.00 ± 0.08 for BG8, 2.73 ± 0.09 for BG20, 2.90 ± 0.18 for BG35 and 3.10 ± 0.37 for ViaSpan®, n = 5, $p < 0.05$). However, after 60 and 120 min no significant differences were observed, indicating that all the LMOs from the preserved groups can regulate the TWC. Figure 3C shows the Glycogen Content and the Oxygen Consumption during the reoxygenation step. At the beginning (time 0), only LMOs preserved in ViaSpan® showed a glycogen content significantly smaller than the rest of the studied group ($p < 0.05$, n = 5). The values, expressed as mg glycogen/g tissue were: 27.50 ± 1.40 for controls, 21.70 ± 1.70 for BG8, 27.70 ± 5.60 for BG20, 25.60 ± 2.60 for BG35 and 13.70 ± 2.00 for ViaSpan®. A significant reduction of the glycogen content as the reoxygenation time increase was observed for all the analyzed groups ($p < 0.05$, n = 5). However, only LMOs from BG20 and BG35 groups showed a glycogen content similar to controls (18.83 ± 4.70 for controls; 18.04 ± 1.45 for BG20 and 19.51 ± 2.45 for BG35, 9.95 ± 1.70 for BG8 and 10.50 ± 1.40 for ViaSpan®). It is important to note that while LMOs preserved in Viaspan® showed smaller glycogen contents than the others groups at the beginning of the reoxygena-

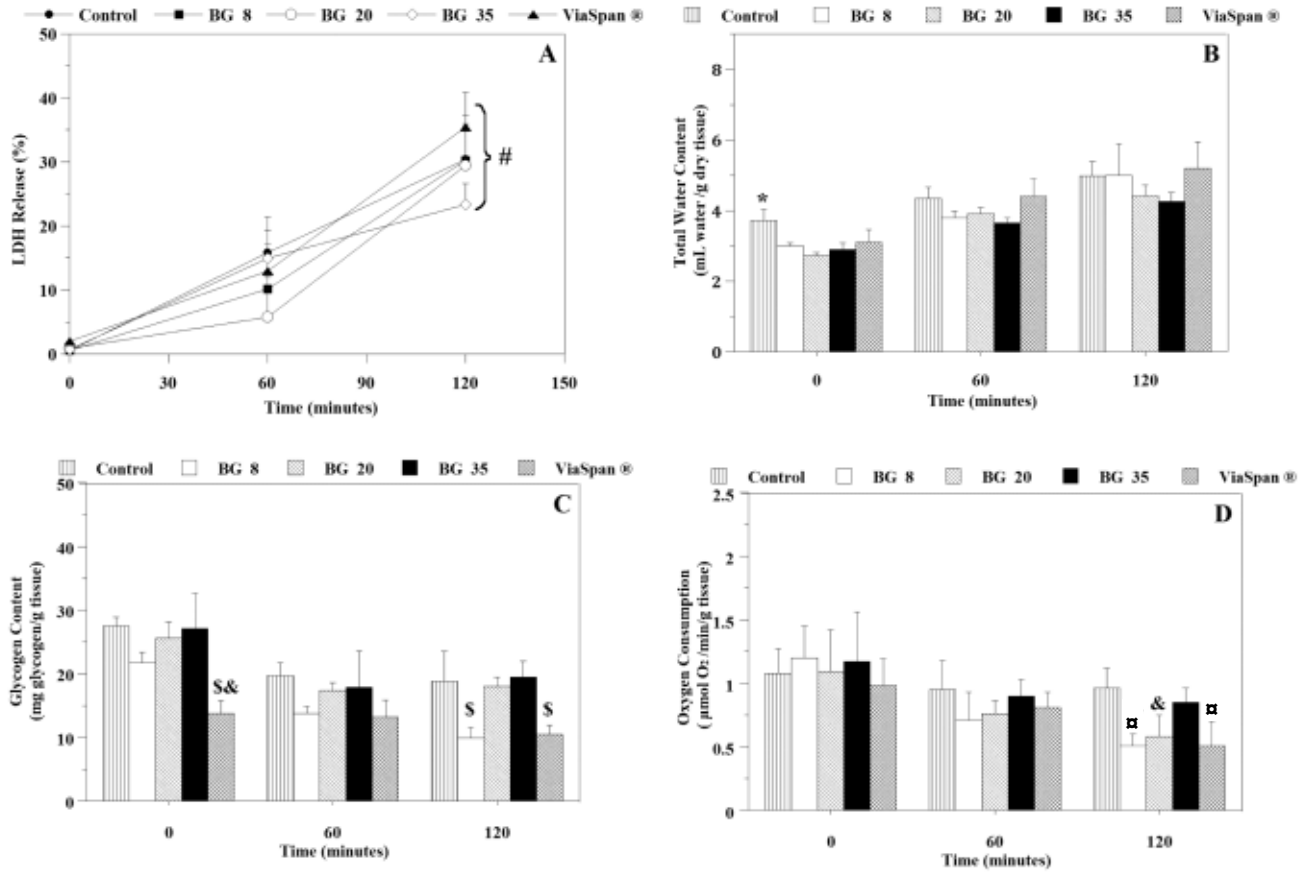

Figure 3. A. *Time course of LDH Release after 120 min of reoxygenation determined in fresh LMOs and cold preserved in BG8, BG20, BG35 and ViaSpan® solutions. Data are expressed as mean ± SD for 5 LMOs preparations. # different from time 0, p < 0.05.* ***B.*** *Time course of Total Water Content during 120 min of reoxygenation determined in fresh LMOs and cold preserved 48 h in BG8, BG20, BG35 and ViaSpan® solutions. Each bar represents the mean ± SD for 5 LMOs preparations. * different from all the other groups, p < 0.05.* ***C.*** *Time course of Cellular Glycogen Content after 120 min of reoxygenation determined in fresh LMOs and cold preserved in BG8, BG20, BG35 and ViaSpan® solutions. Data are expressed as mean ± SD for 5 LMOs preparations. $ different from LMOs preserved in BG20 and BG35 solutions; & different from controls, p < 0.05.* ***D.*** *Time course of Oxygen Consumption in fresh LMOs and cold preserved in BG8, BG20, BG35 and ViaSpan® solutions. Each bar represents the mean ± SD for 5 LMOs preparations. ¤ different of LMOs preserved in BG20, BG35 and controls; & different from controls, p < 0.05.*

tion, the percentage of loss at the end of this step was similar (24 ± 2.1) to those obtained for BG20 (30 ± 1.6), BG35 (26 ± 1.4) and controls (29 ± 2.2). The group that showed the greatest decrease was BG8 with 54± 2.7 of loss at 120 min. Panel D of *figure 3* shows Oxygen Consumption of controls and preserved LMOs during reoxigenation. After 120 min only LMOs preserved in BG35 showed values of Oxygen Consumption (expressed as µmoles O_2/g tissue/min) similar to controls (0.85 ± 0.11 for BG35 and 0.96 ± 0.16 for controls). On the other hand, LMOs preserved in BG8, BG20 and ViaSpan® showed oxygen consumption rates significantly smaller than controls (0.51 ± 0.09 for BG8, 0.58 ± 0.17 for BG20, 0.51 ± 0.19 for ViaSpan®).

Histology

LMOs from control and preserved groups (BG 8, BG 20, BG 35 and ViaSpan®, 48 h at 0 °C, followed by normothermic reoxygenation) were analyzed.

Control group showed conserved hepatocyte cords architecture with endothelial cells attached to the perisinusoidal matrix. The shape of these cells was normal and the hepatic lobules preserved their architecture at the end of the reoxygenation period (Figure 4). At the beginning of the reoxygenation time, LMOs preserved in BG 8, BG 20, BG 35 and ViaSpan® showed good hepatocyte cord integrity with a grater calibre compared with controls, and a mixed population of endothelial cells: ones swollen

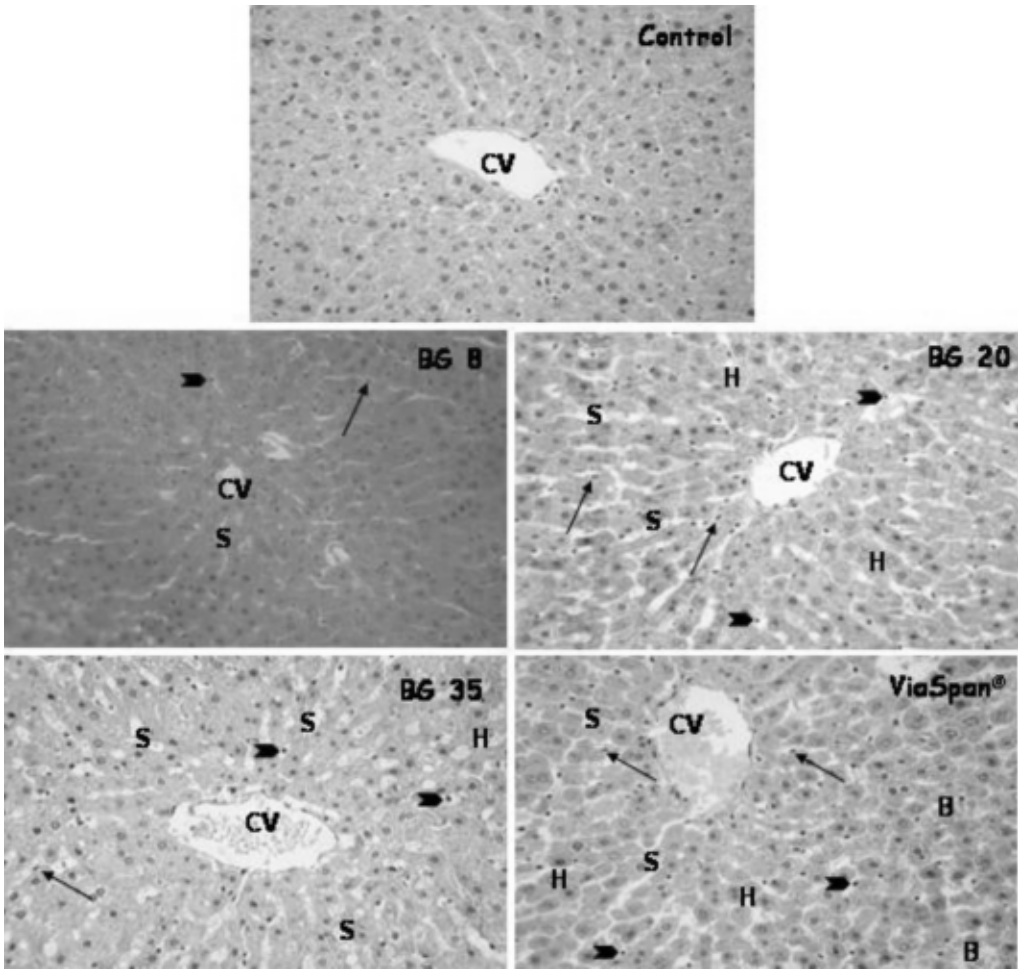

Figure 4. *LMOs morphology. Samples were taken from control and preserved groups at the end (t=120 min) of the normothermic reoxygenation period. Control: normal morphology through the hepatic parenchyma surrounding a central vein (CV). LMOs preserved in BG20, LMOs preserved in BG35 and LMOs preserved in ViaSpan®: all groups presented opened sinusoids (S); swollen hepatocytes (H); rounded (arrow heads) and fusiform (arrows) endothelial cells. ViaSpan® presented blebs (B) and the gratest amount of swollen hepatocytes (H). LMOs preserved in BG8 apparently conserved the architecture of the hepatic lobule; nonetheless, hepatocyte cords and sinusoid were poorly distinguished. Magnifications 20X.*

and attached to the perisinusoidal matrix and others swollen but detached and inside the sinusoidal lumen (data not shown). After 120 min of reoxygenation all the preserved groups showed conserved hepatocyte cord integrity with sinusoides markedly opened. Most of the endothelial cells were swollen and inside the sinusoidal lumen. ViaSpan® solution presented the grater amount of swollen hapatocytes together with some blebs, and BG8 showed an apparently conserve parenchyma. However, hepatocyte cords and sinusoids were not clearly distinguished.

DISCUSSION

LMOs were used as a valuable tool to adapt the composition of BGS in order to evaluate it application to the preservation of the entire liver. It is well known that LMOs should be thin enough to allow rapid nutrient and gas exchanges. Thick slices (> 500 µm) begin to show ischemic injury in the middle areas, whereas very thin slices (< 200 µm) present a large proportion of damaged cells. Although the LMOs were manually cut, they had an average thickness of 400 µm and viability markers (Table 2) were similar to those obtained using Krumdieck and Brendel-Vitron tissue slicers.[24,25]

According to Belzer and Southard[26] any storage solution should contain among its components:

a) An effective impermeant agent to reduce cell swelling.

Table 2. Validation of the cutting technique: Total Water Content, Extra and Intracellular Water Space and K^+ and Na^+ Levels. LMOs were incubated as described in *Experimental Procedures*. Values are expressed as means ± SD of samples obtained from 20 preparations.

	60 min	120 min
Total water content * (mL water/ g dry tissue **)**	3.72 ± 0.32	3.69 ± 0.22
Extracellular Space (mL extracellular water/mL total water)	0.37 ± 0.03	0.43 ± 0.02
Intracellular Space (mL intracellular water/mL total water)	0.62 ± 0.03	0.57 ± 0.02
K^+ (µEq/ g dry tissue)	88 ± 22	64 ± 23
Na^+ (µEq/g dry tissue)	572 ± 94	637 ± 89

* Total Water Content was also determined at time 0; the value was 3.74 ± 0.32.

b) An ionic balance to minimize loss of intracellular homeostasis.
c) A suitable buffering agent to avoid preservation-related changes in the intracellular pH.

The loss of neutrality during ischemia has an important effect on the structure and function of proteins and enzymes. As was predicted theoretically, ViaSpan® showed a lower buffering capacity than BG8, BG20 and BG35 (Figure 1A) but in our experimental conditions, all the preservation solutions tested could regulated pH during cold storage. However, ViaSpan® maintained the pH at values higher than the physiological pH (Figure 2B).

As impermeant colloid we have used PEG of tree different molecular weights to determine which is the most appropriate for preservation solutions. First, we analyzed the viability and TWC of LMOs during 48 h of cold ischemia in BGS, BG8, BG20, BG35 and ViaSpan®. LDH Release and TWC showed a marked increase in LMOs preserved in BGS. This indicates that, in the absence of an appropriate colloid as PEG, the tissue showed important cell membrane integrity deterioration and was incapable of regulating cell volume. On the other hand, there were no differences in the regulation of TWC between ViaSpan® and BG8, BG20, and BG35, which may indicate that PEG protects against tissue edema in some way not related to its MW.

The actual effect of cold storage on cell function and metabolism could be manifested during the reoxygenation step. At the begging of reoxygenation, all the preserved groups showed a Total Water Content lower than controls. This could be related to the cell volume contraction that occurs during cold storage. This phenomenon is reversed after 120 min because all the experimental groups showed similar TWC.

We determined the glycogen remaining in the liver cells cytoplasm as an index of the energetic resources that could became available for tissue through glycogenolysis.[27] At the beginning of reoxygenation, LMOs preserved in ViaSpan® showed the lowest glycogen content. This phenomenon could be due to a greater utilization of glycogen as an energy substrate during cold storage.

Cellular oxygen consumption was determined during reoxygenation to allow expression of any latent damage that could have occurred during cold preservation. The effects of cold ischemia on mitochondrial function are especially important, since it is essential for cell viability.[28] The mitochondrial function was assayed by measuring the respiratory activity of LMOs. Only the BG35 group showed a respiration rate similar to fresh LMOs.

The histological analysis showed that all the preserved group were able to maintain the hepatic parenchyma architecture after cold preservation/ normothermic reoxygenation. Viability and functional results showed that BG8 presented lower glycogen content and oxygen consumption than BG20 and BG35. On the other hand, as it was already mentioned, the only group that showed a respiration rate similar to controls was BG35. This higher basal respiration rate could suggest that the tissue stored in BG35 has a higher content of cell substrates and is in best conditions to face the reoxygenation step. Southard, *et al*.[29] suggested that fatty acids (or other agents) generated during cold storage contribute to mitochondrial damage. We therefore

speculate that PEG of 35 KDa acts by decreasing the generation of oxygen free radicals during cold ischemia and avoiding the formation of harmful fatty acids that could damage the mitochondria. Further studies needs to be done to establish the mechanism of this protective effect exerted by PEG 35000.

Taking into account all these data together, it was demonstrated that BG35 was the most effective preservation solution to protect LMOs against cold preservation injury due to ischemia and reoxygenation. It is a good alternative to ViaSpan® because of its higher buffer capacity, its best indexes of respiration activity and for being considerably less expensive. Before performing any clinical application of this novel solution for liver preservation other animal sources (pig, rabbit), as well as the presence of apoptotic cells and gene expression regulation should be tested.

ABBREVIATIONS

- **LMOs:** Liver microorgans.
- **PEG:** Polyetyleneglycol.
- **KHR:** Krebs Henseleit Reoxygenation.
- **LDH:** Lactate Dehydrogenase.
- **TWC:** Total Water Content.

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