Fructose 1-6 Bisphosphate Versus University of Wisconsin Solution for Rat Liver Preservation: Does FBP Prevent Early Mitochondrial Injury?

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ABSTRACT

Background. Fructose 1,6-biphosphate (FBP) has been shown to exert therapeutic effects in models of ischemia-reperfusion in organs other than the liver. This study compared FBP and University of Wisconsin (UW) solution during cold storage and reperfusion, among mitochondria of adult male Wistar rat livers.

Methods. Adult male Wistar rats were assigned to two groups according to the preservation solution used; UW or FBP Aspartate transaminase (AST), alanine transferase (ALT); and lactic dehydrogenase (LDH) were measured in samples of the storage solution obtained at 2, 4 and 6 hours of preservation. After 6 hours of cold storage, we reperfused the liver, taking blood samples to measure AST, ALT, LDH, and throbarbituric acid reactive substances (TBARS). Hepatic fragments were processed for histologic analysis; for determinations of TBARS, catalase, and nitric oxide as well as for mitochondrial evaluation by infrared spectroscopy.

Results. During cold preservation, levels of AST and LDH in the storage solution were lower among the FBP group, but after reperfusion, serum levels of AST, ALT, and LDH were higher in this group, as was catalase activity. TBARS and nitric oxide were comparable between the groups. In the UW group there was a higher amide I/amide II ratio than in the FBP group, suggesting an abnormal protein structure of the mitochondrial membrane. No signs of preservation injury were observed in any liver biopsy, but sinusoidal congestion was present in livers preserved with FBP.

Conclusion. FBP showed a protective effect for preservation during cold storage seeming to protect the mitochondrial membrane although it did not prevent reperfusion injury.

Orthotopic liver transplantation (OLT) is the standard treatment for acute and chronic end-stage hepatic diseases.[1] Optimal allograft preservation is essential to reduce post-ischemic organ dysfunction.[2,3] At present, preservation is preferably achieved using University of Wisconsin (UW) solution.[4] Although it provides good preservation, it is not an ideal solution. Primary non-function after OLT continues to occur and may be related to preservation injury.[5,6]

Fructose 1-6 bisphosphate (FBP), a high-energy glycolytic intermediate,[7] has been shown to exert substantial therapeutic effects in a variety of clinical conditions, eg, septic shock, and in models of ischemia-reperfusion involving organs other than the liver.[8–12] Moresco et al[13] showed that FBP may exert a protective effect on rat liver preservation during and after cold storage. Considering that

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restoration of oxygenated blood flow is much more deleterious than the ischemia in the pathophysiology of preservation injury,[14–16] it may be more important to study the role of FBP in this scenario. Ischemia-reperfusion (I/R) injury has mitochondria as major target with secondary generation of reactive oxygen species (ROS) by this organelle. ROS interact with membrane and cytoplasmic compounds inducing a pronounced injury. Usually, this kind lesion is assessed as the balance between oxidant and antioxidant substances. Some structural changes are ROS-induced; infrared spectroscopy, is able to detect changes in the mitochondrial permeability membrane pore complex as shown by Ricchelli et al.[17] To our knowledge, FBP has not been studied yet, especially by mitochondrial spectroscopy, cold for effects on hepatic (I/R) injuries.

MATERIALS AND METHODS

Animals

Adult male Wistar rat weighing 300 to 450 g were used as donors and recipients respectively. The rats were randomly assigned to two experimental groups. 1) UW solution (n = 5; Viaspan, Bristol-Myers-Squibb) FBP (n = 5; 10 mmol/L in saline) Donors and recipients were anesthetized with inhaled isoflurane (1.5% Isoflurano, Abbot, North Chicago, USA).

Experimental Procedure

Surgical procedures were performed according to reperfusion model[18] Which included cold storage, warm ischemia, and reperfusion. Samples from the cold storage solution obtained at 2, 4 and 6 hours during cold preservation were measured for aspartate transferase (AST), alanine transferase (ALT), and lactic dehydrogenase (LDH) using a kinetic assay (Liquiform kit, Roche Diagnostic). After reperfusion, blood samples were obtained from the suprahepatic vena cava of the reperfused liver to measure AST, ALT, LDH, and thiobarbituric acid reactive substances (TBARS). Afterwards, the reperfused liver was rinsed with 10 mL of saline solution via the portal vein, and fragments were processed for histologic analysis; measurements of TBARS (colorimetric reaction with thiobarbituric acid), catalase (Chance method, 1979)[15] and nitric oxide derivatives (NO) as well as subjected to mitochondrial infrared spectroscopy.

Preparation of Mitochondria

Mitochondria from the liver of Wistar rats were prepared as previously described,[19,20] but with some modifications; diced livers (10 to 15 g) were washed three times with 20 mL of a solution containing 250 mmol/L sucrose, 1 mmol/L ethyleneglycoltetracetic acid (EGTA), 10 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7,4) before being macerated in a Potter Homogenizer and centrifuged at 2500 rpm for 5 minutes. The supernate was removed. It was centrifuged at 10,500 rpm for 5 minutes and the pellet suspended in a solution containing 250 mmol/L sucrose, 0.3 mmol/L EGTA, 10 mmol/L HEPES (pH 7.4) before centrifugation at 6000 rpm for 15 minutes. The final pellet was suspended in 1 mL of a solution containing 250 mmol/L sucrose and 10 mmol/L HEPES (pH 7,4). The protein concentration was measured using the biuret method.[21]

Assessment of Mitochondrial Infrared Spectroscopy

Mitochondrial membrane proteins, amide I and II, were analyzed by spectroscopy.[17,22–25] The spectra related to amide I show low frequency; 1615 and 1700 cm^{-1} and a C=O bond; whereas those related to amide II had a frequency of 1500 and 1600 cm^{-1}.[22–24,26] All of these procedures were performed within 4 hours after liver extraction to ensure mitochondrial viability. To compare results, mitochondria were also isolated from five decapitated control rats.

Histologic Analysis

Liver biopsy specimens were fixed in 10% buffered formalin before embedding in paraffin, sectioning, and staining with hematoxylin-eosin. Histologic assessment of the liver was performed by one pathologist who was blinded to the group assignments.

Data Analysis

Statistics were calculated using SPSS 10.0 for Windows. Results are expressed as mean values ± SD or medians. The statistical analysis of enzyme, TBARS, catalase and NO concernations was performed using the Mann-Whitney test. Fisher exact test was used categorical variables. The level of statistical significance was defined as $P < .05$.

Ethical Guidelines and Hazardous Procedures

All experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (revised 1985). The protocol was approved by the local Research Ethics Committee, complying with all local regulations for research involving experimental animals.

RESULTS

Body weight was similar in both groups of donor rats: UW group, 400 ± 39 g versus FBP group, 394 ± 28 g. After 2, 4, or 6 hours of preservation, AST release into the storage solution was significantly higher in the UW group ($P = .008$; Fig 1). After 4 or 6 hours of preservation, LDH measurements in the storage solution were lower in the FBP than the UW group ($P = .03$ and $P = .02$, respectively, Fig 1). However, neither the LDH level at 2 hours after storage, nor the ALT level at 2, 4, or 6 hours after preservation showed significant differences ($P = .09$; $P = .06$; $P = .2$; $P = .7$, respectively; Fig1).

The warm ischemia time was similar between the groups: UW, 57 minutes, versus FBP group, 58 minutes ($P = .7$). On the one hand, serum levels of AST, ALT, and LDH after reperfusion with FBP were significantly higher than those with UW ($P = .02$; Fig 2). Also, the catalase level was higher in the FBP group ($P = .03$, Table1). TBARS (liver tissue and post-reperfusion serum) and No values were comparable between the two groups (Fig 2, Table1).

Regarding the mitochondrial evaluation, the transmittance absorption infrared spectra are shown in Fig 3. The UW group had a higher amide I/amide II ratio than the FBP or control group (Fig 4). Mild hepatocyte vacuolization was observed in 80% and 60% of the UW and FBP groups, respectively ($P = .4$). Steatosis was observed in 40% of the UW and 60% of the FBP group ($P = .5$). No signs of

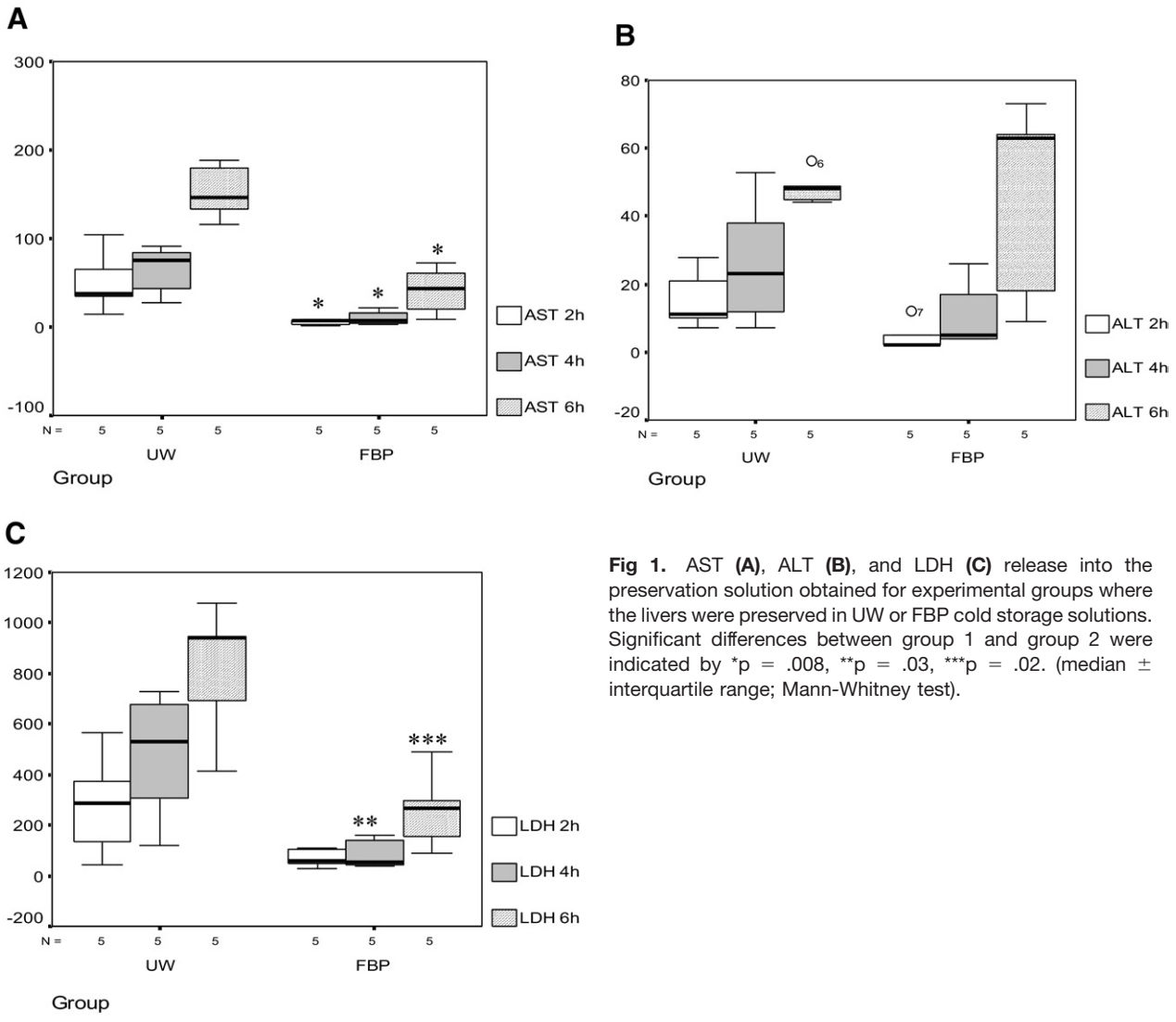

Fig 1. AST **(A)**, ALT **(B)**, and LDH **(C)** release into the preservation solution obtained for experimental groups where the livers were preserved in UW or FBP cold storage solutions. Significant differences between group 1 and group 2 were indicated by *p = .008, **p = .03, ***p = .02. (median ± interquartile range; Mann-Whitney test).

preservation injury were detected in any organ. However, sinusoidal congestion was observed in all livers preserved with FBP.

DISCUSSION

The present study compared FBP and UW solutions. The rat liver reperfusion model included cold storage, ischemia, and reperfusion to simulate OLT. The model was tested in a pilot study and shown to be reproducible.

During the pilot study, no more than 15 minutes of reperfusion were possible without hemodynamic derangements. Most published studies have evaluated hepatocellular injury after 30 minutes of reperfusion insult,[27–29] however, recent publications have shown that early markers, especially those of oxidative stress, estimate graft damage after a hepatic rinse[30] or as early as 15 minutes after reperfusion.[31] Moreover, considering that the I/R syndrome is defined as a 30% decease in mean arterial pressure, which usually occurs within the first 5 minutes of graft reperfusion and lasts for at least 1 minutes,[32] the chosen period seemed adequate, and was therefore adopted as the standard time.

At 2, 4, and 6 hours of preservation, AST and LDH release into the storage solution was lower among the FBP group. These results suggested that FBP was better protected the liver during cold ischemia than the UW solution. The mechanisms responsible for these effects are still uncertain, and this study was not designed to examine them. FBP interacts with biomembranes, modifying ion permeability and maintaining it viability.[5] In addition, it preserves intracellular adenosine triphosphate levels by activating the glycolytic pathway[33,34] and exerts a chelating action of Ca^{2+}, thereby modulating intracellular Ca^{2+} homeostasis.[35]

Furthermore, after reperfusion, serum levels of AST, ALT,and LDH were higher in the FBP than the UW group, Suggesting the FBP was not able to prevent hepatocellular reperfusion injury. Oxidative stress (OS) reflects an imbal-

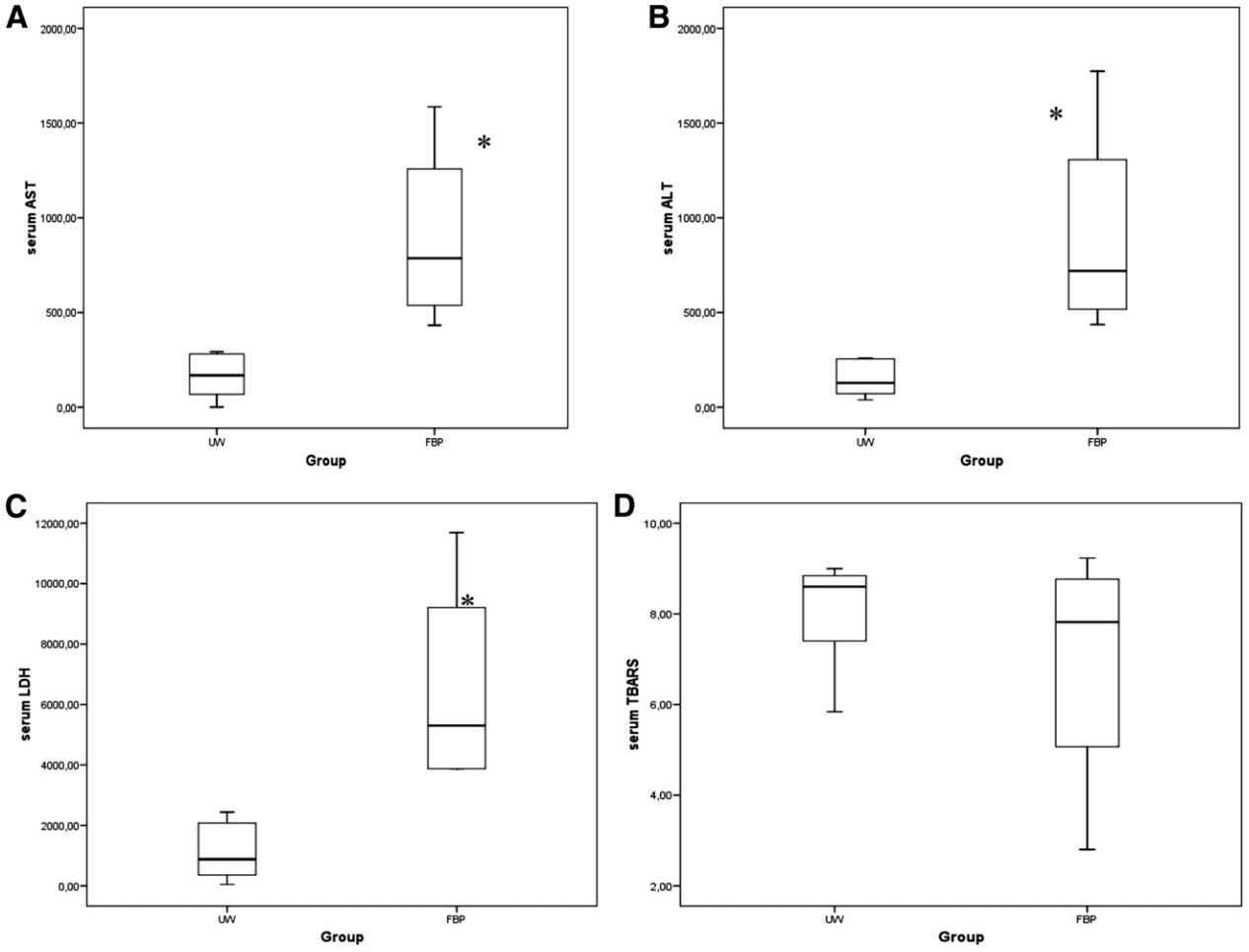

Fig 2. AST **(A)**, ALT **(B)**, LDH **(C)** and TBARS **(D)** levels in the post-reperfusion serum obtained form experimental groups where the livers were preserved in UW or FBP cold storage solutions. Significant differences between group 1 and group 2 were indicated by *$P = .02$ (median ± interquartile range; Mann-Whitney test).

ance between the availability of cellular oxidants and antioxidants. Several experimental studies have shown that reperfusion of the liver after ischemia produces OS due to the overproduction or ROS, which are believed to be a major factor mediating liver damage.[36] Presumably, the most important source of graft damage is the overproduction of ROS during the initial phase of reperfusion, which is mainly mediated by activated Kupffer cells.[37] Their harmful effects are offset by efficient antioxidant mechanisms, including glutathione and catalase, which are essential for cell integrity.[38] In the present study, OS was evaluated by TBARS values in the serum and liver tissue as well as catalase and NO activities post-reperfusion. TBARS in liver tissue and serum as well as NO measurements were comparable between the two groups, suggesting that FBP may exert some protective effects after reperfusion. In this regard, it is interesting that catalase activity in liver tissue was higher among the FBP than the UW group. Possibly, it reflects an FBP additional protective ability.

Table 1. Levels of TBARS, Catalase, and NO Derivaties in Post-reperfusion Liver Tissue

	Group 1 (UW) (n=5)	Group 2 (FBP) (n=5)	
TBARS (nmol/g)	9.8 (7.4–12.7)	11.1 (7,1–14,4)	$P = .6$
Catalase (U/mg/prot)	327 (255–378)	435 (350–497.5)	$P = .03$*
NO derivatives (nmol/mg/prot)	0.06 (0.04–0.12)	0.07 (0.05–0.09)	$P = 1.0$

Values expressed as median ± interquartile range. Mann-Whitney test.
*Significant difference was defined as $P < .05$.

Amide I and amide II are the most important components of the mitochondrial pore; modifications of their ratio can alter membrane permeability. It was remarkable that analysis of mitochondrial damage by infrared spectroscopy yielded a higher amide I/amide II ratio among the UW group. This observation may reflect more pronounced mitochondrial damage in this group. Besides, similar results

were obtained in the control compared to the FBP group. These results agree with those of Sano et al,[33] who demonstrated that FBP protected mitochondrial function. Also, Kim et al investigated I/R in cultured hepatocytes, demonstrating, more apoptosis than necrosis due to increased ATP availability when FBP was used.[39] Once there is necrosis, there is more inflammatory reaction. One author of our group (PEH, unpublished data) assessed a septic model in rats, demonstrating that the amide I/amide II ratio was similar to the UW ratio in our study. One can speculate whether this protection was related to an anti-inflammatory ability of FBP. Although no difference in ROS production was observed between the FBP and UW groups, it seems that the FBP solution was related to a higher antioxidant release and a lower mitochondrial injury than UW. Based on the well-known statement that mitochondria play an essential role in OS being the trigger of the injury, protecting this organelle may prevent th I/R lesion.

As expected histological analysis, did not show any signs of preservation injury in liver biopsy specimens, which was

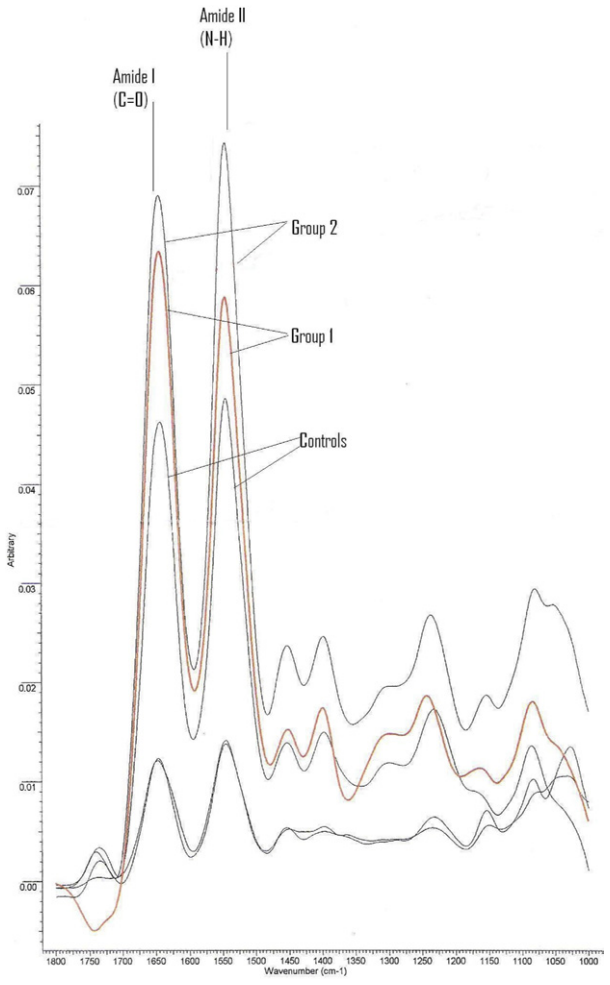

Fig 3. Infrared spectra in the group 1, group 2 and controls (decapitated rats).

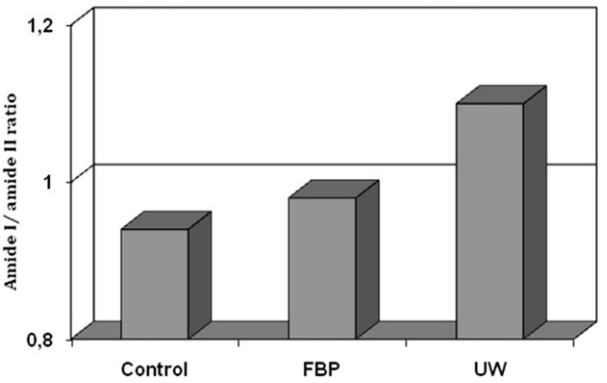

Fig 4. Amide I/amide II ratio in group 1, group 2, and the controls (decapitated rats).

probably related to the short reperfusion time. However all livers preserved with FBP solution showed sinusoidal congestion, suggesting an important injury to the sinusoidal endothelial cells during preservation. Cold ischemia specifically damages SEC. When detached these cells lose their cytoplasmic process and become rounded as result of the changes in the extracellular matrix and the cytoskeleton, sloughing into the sinusoidal lumen.[40] We hypothesized that FBP was not able to prevent the SEC lesion.

In conclusion, in this model, the FBP solution showed protective effects on rat liver preservation during cold storage, but failed to prevent post-reperfusion injury. Moreover, FBP seemed to protect mitochondrial membranes in the reperfusion scenario.

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