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Anionic polysaccharides

A class of substances with hepatoprotective and antiadhesive properties in rat liver preservation

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Abstract In liver preservation, the substitution of the anion Cl^- by lactobionic acid (LB) prevents reperfusion edema and extends the preservation time for human livers. We studied the effect of compounds that are structurally related to lactobionic acid: anionic polycarbohydrates (sulfated anionic polysaccharide, SAP, and pentosan polysulfate, PPS) on liver function and leukocyte-endothelial cell interaction in isolated perfusion and liver transplant models. Rat livers, cold-stored (24 h) in a Cl^--containing control solution, became edematous during 1 h of reperfusion. Substitution of Cl^- by either LB, SAP, or PPS decreased reperfusion edema in a Cl^- concentration-dependent fashion. Reperfusion edema was abolished completely after preservation in 100 mM SAP solution or PPS solution. Also hepatic lactic dehydrogenase (LDH) and aspartate aminotransferase (ASAT) release was lowest after preservation in those solutions. After preservation in LB or anionic polycarbohydrate solutions, portal venous resistance was significantly higher than after preservation in Cl^--containing control solution. Capillary blood flow was 391 ± 83 pl/s and 398 ± 174 pl/s after preservation in SAP solution (SAPs) and PPSs, and 803 ± 117 pl/s and 641 ± 219 pl/s after preservation in LB or Cl^--containing control solution. The number of leukocytes sticking to the vascular wall was lower ($P < 0.05$) after preservation in SAPs or PPSs (109 ± 31 cells/mm^2 and 108 ± 60 cells/mm^2, respectively), when compared with preservation in Cl^--containing control or LB solutions (429 ± 63 cells/mm^2 and 277 ± 59 cells/mm^2). In rat liver preservation, anionic polysaccharides are antiedematous compounds, with a higher potency than LB and additional antiadhesive properties.

Keywords Organ preservation · Liver transplantation · Impermeants · Rat

Introduction

University of Wisconsin (UW) solution has become the standard cold-storage solution for the preservation of human livers [17, 21]. Its superiority to other solutions is based on the addition of cytoprotective substances that prevent reperfusion edema. In organs preserved in solutions other than UW solution, anions, namely Cl^-, are thought to penetrate cell membranes during cold storage [4], thus causing an electrogenically driven influx of Na^+ into cells. Na^+, together with the accumulation of small proteolytic and glycogenolytic solutes, increases the intracellular osmolarity. The resulting osmotic gradient drives the influx of water during reperfusion, thus causing cell swelling and reperfusion edema. The substitution of Cl^- by impermeable anions such as lactobionic acid

(LB), prevents reperfusion edema and therefore became one of the cornerstones of the UW solution [18].

Lactobionic acid is a cyclic carbohydrate with a negatively charged aliphatic side chain and a molecular weight of 358. Due to its size and three-dimensional structure, it does not permeate cell membranes at 4°C. In addition, LB was shown to chelate Ca^{2+} [10] and scavenge oxygen radicals [5].

Anionic polysaccharides are semisynthetic or naturally occurring macromolecules with a basic carbohydrate structure comparable with LB and hydroxyethyl starch, the second key component of the UW solution (Fig. 1). Some anionic polysaccharides were shown to have additional properties of potential advantage for organ preservation: they prevent thrombocyte adherence to activated endothelial cells and microthrombus formation [8, 11].

We here compared the effect of LB and anionic polysaccharides (sulfated anionic polysaccharide, SAP, and pentosan polysulfate, PPS) on liver function, hepatocellular injury, and leukocyte endothelial interaction in a model of rat liver preservation and transplantation.

Materials and methods

Liver procurement

Animal experiments were performed in accordance with the national guidelines for the care and use of laboratory animals and in accordance with the local ethics committee. Male wistar rats (250–320 g; Lewis rats for transplant experiments) had free access to a regular chow and water. After an overnight fast, animals were anesthetised with pentobarbital sodium i.p. (50 mg/kg body weight) and a midline laparotomy was performed. After clamping of the infradiaphragmatic aorta, each liver ($n=6$) was flushed through the portal vein over a period of 5 min with 10 ml of 4°C cold test solution. Livers were surface-cooled with cold test solution, removed, and stored at 4°C for 24 h in all experiments.

Test solutions

Iso-osmolar extracellular-type preservation solutions [19] were used, with 100 mM NaCl serving as control solution (osmolarity adjusted to 300 mosmol with 90 mM mannitol). In the test solutions, Cl^- was gradually substituted by LB, SAP, or PPS (for structure, see Fig. 1) and osmolarity was brought to 300 mosmol with mannitol (Table 1). To prevent interference with the tested impermeants, other protectants were omitted from the solution.

Isolated perfused livers

After cold storage, a catheter was placed in the portal vein and in the common bile duct. Livers were reperfused through the portal vein in a closed system with Krebs Henseleit buffer (gassed with O_2/CO_2, 37°C), at a constant portal venous pressure (PVP) of 12 cm H_2O as described previously [19].

Portal flow (PVF) and bile production was measured over a period of 1 h and portal venous resistance was calculated as PVR = PVP/PVF. Tissue edema was measured as total tissue water (TTW in grams of H_2O per gram of dry weight, DW) after cold storage and after reperfusion. After 5 min and 60 min, samples were taken from the perfusate and ASAT and LDH were measured with an automated Hitachi 911 analyzer (Hitachi, Zurich, Switzerland). In a separate set of experiments, samples were taken from the perfusate and electrolytes were measured in the perfusate. The hepatic uptake of electrolytes during reperfusion was calculated and is given in millimoles per gram DW.

Intravital video microscopy

Rat livers were cold-stored (at 4°C, 24 h) in the respective solution. After storage, livers were rinsed with 5 ml Ringer's lactate solution

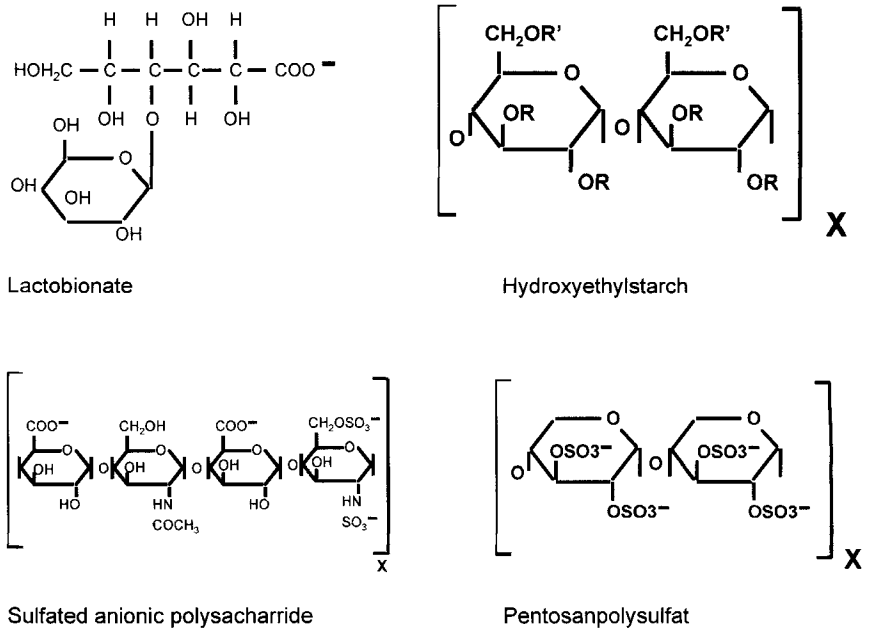

Fig. 1 Structure of lactobionate, hydroxyethylstarch, sulfated anionic polysacharride (SAP) and pentosan polysulfate (PPS). SAP and PPS are zwitter molecules to lactobionate and hydroxyethylstarch, being polycarbohydrates and having anionic side groups. Sodium salts of both substances were used

Table 1 Composition of sodium lactobionate (Na^+-*LB*), sulfated anionic polysaccharide (*SAP*) and petosan polysulfate (*PPS*) based preservation solutions. Osmolarity was adjusted to ~300 mosmol with mannitol (90 mM), and pH was adjusted to 7.35–7.45 with NaOH

	Control	LB			SAP			PPS		
		100	66	33	100	66	33	100	66	33
Na^+-LB (mM)	0	100	66	33	0	0	0	0	0	0
Na^+-SAP (mM Na^+)	0	0	0	0	100	66	33	0	0	0
Na^+-PPS (mM Na^+)	0	0	0	0	0	0	0	100	66	33
NaCl (mM)	100	0	33	66	0	33	66	0	33	66
KH_2PO_4 (mM)	10	10	10	10	10	10	10	10	10	10

and transplanted orthotopically using the technique described by Kamada. Portal vein clamping time was between 12 and 14 min for all transplants and the same between the experimental groups. Intravital video microscopy was performed 30 min after reperfusion and after placing the left liver lobe on a custom-made stage. To avoid exsiccation, the exposed lobe was moistened with normal saline and covered with a cellophane foil.

Microscopy was performed using an Axioplan microscope (Zeiss, Oberkochen, Germany) [16], equipped with a 100-W mercury lamp and a fluorescent filter set for epiillumination. FITC-dextran (M_r 150,000; 0.1 ml of 5% solution in 0.9% NaCl/100 g body weight) and rhodamine 6G (0.04 ml of a 0.05% solution in 0.9% NaCl/100 g body weight) was injected i.v. to enable visualization of the microcirculation and quantification of the leukocyte-endothelium interaction. Regions of interest were recorded by a CCD video camera (CF 8/1 FMC; KAPPA Messtechnik, Gleichen, Germany) and an S-VHS video-cassette recorder (AG 7355; Panasonic, Osaka, Japan) for off-line analysis.

Microcirculatory analysis

Liver microcirculation was assessed as sinusoidal perfusion in more than 60 randomly selected vascular segments of each animal [20]. Venous-like 100-μm vessel segments with a straight course and a diameter of less than 35 μm were recorded for 30 s for the evaluation of leukocyte-endothelium interactions and for 60 s for hemodynamic measurements. Internal diameter (ID), in micrometers, and red blood cell velocity (v_{RBC}), in micrometers per second, were measured using a digital video image shearing monitor. The number of sticking leukocytes (N_S) is expressed as the number of white blood cells (WBC) firmly adhering to the inner surface of the investigated 100-μm vessel segment: $N_S = 10^6$ WBC/($\pi \times$ ID $\times$ 100) cells per millimeter squared. Rolling leukocytes (WBC_R) were defined as cells moving at two-fifths of the velocity of erythrocytes in the center of the vessel or as leukocytes adherent for 0.2–20 s. Rolling count (N_R) is given as a percentage: $N_R = 100 \times WBC_R$/WBC. Wall shear rate (γ) was derived from Poiseuille's law for Newtonian fluids: $\gamma = (v_{RBC}/ID) \times 8$ (liters per second). Intravital microscopy blood flow (IVM-BF) was calculated as follows: IVM-BF = $v_{RBC} \times ID^2 \times \pi/4{,}000$ (picoliters per second).

Chemicals

SAP (certoparin-natrium) was purchased from Novartis (Basel, Switzerland) and PPS (SP54) from Bene (Munich, Germany). All other chemicals were purchased from Sigma Chemie, Buchs, Switzerland and were of analytical grade.

Statistical analysis

Results are given as mean ± SD. Statistical differences between means were calculated by ANOVA, with Bonferroni correction for multiple comparisons. Regression analysis was performed by SIGMAPLOT and INSTAT. $P < 0.05$ was considered statistically significant.

Results

Isolated perfused livers

To validate our isolated perfused liver (IPL) model, livers were perfused without cold storage. After flush-out and immediate reperfusion, rat livers ($n = 4$) had a TTW of 2.67 ± 0.42 g H_2O/g DW and a portal venous flow of 16.2 ± 1.8 ml/g DW. As expected, those livers did not become edematous and, after 1 h of reperfusion, TTW was 2.80 ± 0.35 g H_2O/g DW and portal venous flow was 12.9 ± 3.5 ml/g DW ($P = 0.14$ vs immediate flow). During 1 h of reperfusion, those livers produced 49 ± 39 μl bile/g DW.

Livers preserved in control solution containing 100 mM Cl^-, did swell significantly during 1 h of reperfusion (to a TTW of 3.85 ± 0.39 g H_2O/g DW; Fig. 2). A stepwise substitution of Cl^- with LB decreased reperfusion edema in a concentration-dependent manner, and substitution of Cl^- by SAP abolished reperfusion edema completely (Fig. 2).

For LB-preserved livers, but not for SAP-preserved livers, a reversed linear association between reperfusion edema and hepatic uptake of Na^+ and Cl^- was found (Fig. 3).

Hepatic release of LDH and ASAT was lowest after preservation in PPS solution (PPSs) or SAP solution (SAPs), but 30 to 40 times higher after preservation in the Cl^--containing control solution (Table 2).

Bile production was highest after preservation in LB solutions. Due to the high interindividual variance, however, the difference was not significant to LB- or PPS-preserved livers.

Because bile production in choleretic-free perfusion models is a function of electrolyte uptake [9] and impermeable anions prevent ion transport [4], we measured the hepatic uptake of electrolytes from the perfusate (Table 3).

Total hepatic electrolyte uptake during reperfusion correlated inversely with the Cl^- concentration in the

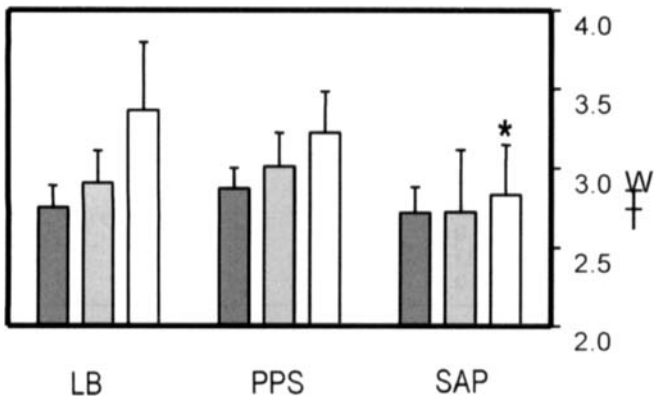

Fig. 2 Total tissue water (TTW) in rat livers cold-stored in 33 mM (*white bars*), 66 mM (*gray bars*), and 100 mM (*black bars*) of either lactobionate (*LB*), SAP solution or PPS solution 60 min after reperfusion in an isolated perfused liver system (TTW after preservation in Cl^--containing control solution 3.85 ± 0.39 g H_2O/g dry weight; $*P < 0.05$)

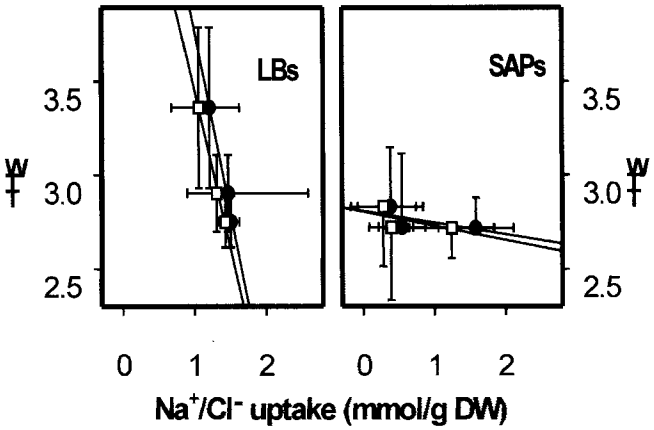

Fig. 3 Correlation between total tissue water (TTW; after 60 min of reperfusion) and hepatic uptake of Na^+ (*empty squares*) or Cl^- (*filled circles*) after cold storage in 33 mM, 66 mM, and 100 mM (*from left to right*) of either LBs or SAPs (correlation coefficient between Na^+ and TTW, $r^2 = 0.99$; Cl^- and TTW, $r^2 = 0.99$ in LB-preserved livers; Na^+ and TTW, $r^2 = 0.39$; Cl^- and TTW, $r^2 = 0.38$ in SAP-preserved livers). Note that there is a negative correlation between the Na^+ or Cl^- uptake and TTW

preservation solution, and the lowest electrolyte uptake was found after preservation in 33 mM SAPs.

Interestingly, bile production (or portal venous resistance) did not correlate with the development of tissue edema. This was true when correlation was analyzed for all groups together or for the different preservation solutions (data not shown).

Intravital microscopy

After preservation in LB or anionic polycarbohydrate solutions (APs), portal venous resistance was significantly higher than after preservation in Cl^--containing control solution; and hepatic capillary blood flow was 391 ± 83 pl/s and 398 ± 174 pl/s after preservation in SAPs and PPSs, and 803 ± 117 pl/s and 641 ± 219 pl/s after preservation in LB or control solution (Table 4) (Fig. 4).

The number of leukocytes sticking to the vascular wall was significantly lower after preservation in SAPs or PPSs (109 ± 31 cells/mm^2, 108 ± 60 cells/mm^2), when compared with preservation in Cl^--containing control or LB solutions (429 ± 63 cells/mm^2, 277 ± 59 cells/mm^2) (Table 5). The percentage of leukocytes rolling along the sinusoidal endothelium, however, was not different between the four preservation groups.

Discussion

Since the introduction of the UW solution, many investigators consider liver preservation a problem solved. However, with improved immunological control, preservation-associated liver dysfunction has become a major cause for early graft loss [1, 14]. The need to further improve liver preservation, not only for marginal grafts, must therefore not be neglected.

One of the cornerstones of UW solution is the prevention of reperfusion edema. Reperfusion edema is one of the first steps in the multifaceted sequence of reperfusion injury and is thought to be preventable by substituting Cl^- in the preservation solution with imper-

Table 2 Bile production (*BP*; in microliters per gram per hour) and lactic dehydrogenase (*LDH*) and aspartate aminotransferase (*ASAT*; in units per gram dry weight) release into perfusate during reperfusion of rat livers cold-stored in a lactobionate, sulfated anionic polysaccharide (*SAPs*), or pentosan polysulfate (*PPSs*) solution ($n = 6$ each)

	NaCl	LB			SAP			PPS		
		100	66	33	100	66	33	100	66	33
LDH	266 ± 219*	9 ± 2	32 ± 22**	58 ± 56**	7 ± 1	8 ± 4	6 ± 3	3 ± 1	5 ± 2	7 ± 3
ASAT	74 ± 16*	8.3 ± 2.8	16.4 ± 7.2**	19.2 ± 4.8**	2.6 ± 3.4	3.4 ± 2.5	2.6 ± 1.7	5.4 ± 1.6	4.2 ± 0.8	3.8 ± 0.8
BP	13 ± 9	22.5 ± 16	29 ± 26	16 ± 12	16 ± 11	9 ± 9	3 ± 3	7 ± 4	5 ± 3	4 ± 2

$*P < 0.05$ vs all;
$**P < 0.03$ vs SAP and PPS

Table 3 Electrolyte uptake (millimoles per gram dry weight, micromoles per gram dry weight for K^+) from perfusate by rat livers during 60 min of reperfusion after storage in LB or SAP solution ($n=6$ each)

	Control	LB			SAP		
		100	66	33	100	66	33
Na^+	1.94±1.47	1.51±0.12	1.47±1.12	1.21±0.42	1.58±0.53	0.54±0.33	0.38±0.46*
Cl^-	1.86±1.46	1.43±0.09	1.31±0.41	1.06±0.38	1.24±0.60	0.39±0.31	0.28±0.46*
K^+	107±76	75±18	61±21	54±15	63±40	22±17*	18±15*

*$P<0.01$ vs control

Table 4 Capillary blood flow (picoliters per second) and number of leukocytes rolling along or sticking (cells per millimeter squared) onto venules of rat livers preserved for 24 h in chloride or impermeable anion-based preservation solutions ($n=3$–4 each group; *OLT* immediate orthotope liver transplantation without cold ischemia)

	Native liver	OLT	NaCl	Lactobionate	SAP	PPS
Flow (pl/s)	376±34	212±18	641±219	803±117	391±83*	398±174*
Shear rate	290±29	230±44	287±43	306±26	219±24	224±84
Sticker	106±25	301±117	429±63	277±59	109±31*	108±60*
Roller	10.2±3.1	13.2±4.5	26.7±9.2	24.6±4.2	26.3±9.6	22.4±14.5

*$P<0.01$ vs control

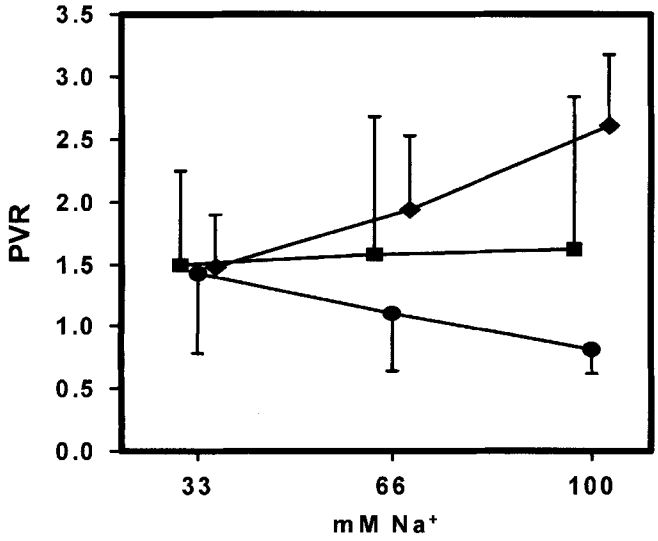

Fig. 4 Portal venous resistance (in centimeters of H_2O × minutes per milliliter × grams wet weight) as a function of impermeable concentration in rat livers cold-stored in a LBs (*filled circles*), SAPs (*filled squares*) or PPS (*filled diamonds*)

Table 5 Recipient survival (in days) after transplantation of rat livers preserved for 24 h in LBs, SAPs, or PPSs (at 100 mM Na^+ concentrations)

	Recipient	Mean
LB	1 day 2 days 1 day	1.3±0.5 days
SAP	2 days 3 days 2 days	2.3±0.5 days
PPS	1 day 4 days 2 days 2 days	2.3±1.1 days

meable anions. Impermeable anions, namely LB, were first used to prevent tissue swelling in ischemia models [13] and subsequently adapted for liver preservation solutions by Southard and Belzer. Lactobionate is thought to remain extracellularly during the cold-storage phase, thus preventing intracellular anion accumulation and osmotic swelling upon reperfusion of the graft [4, 17, 18].

Surprisingly no other impermeants have been studied in liver cold storage. The anionic polysaccharides used in this study were selected for their structural homology with LB and another key component of the UW solution, hydroxethyl starch.

When LB was substituted by SAP or PPS, hepatocellular swelling was prevented more effectively and the equimolar substitution of 100 mM LB by SAP, prevented hepatic reperfusion edema in our experiments completely. That antiedematous effect was associated with a pronounced protection of hepatocytes, as indicated by a lower release of transaminases during reperfusion by one order of magnitude.

Interestingly, when livers were preserved in APs, reperfusion edema was independent from hepatic anion (Cl^-) uptake, and the pronounced antiedematous effect of anionic polysaccharides could be observed despite the presence of Cl^- in the storage solution.

It therefore appears that anion-driven osmotic swelling plays a minor role in the development of hepatic

reperfusion edema, at least in AP-preserved livers, and other edematogenic factors have to be considered.

Several earlier studies have identified leukocyte-induced endothelial disruption and subsequent interstitial fluid accumulation as main factors in reperfusion edema [6, 22], and strategies aiming against leukocyte endothelial interactions with various antiadhesive antibodies have successfully prevented graft swelling in various transplant models [7].

APs in this study were selected not only for their structural similarity to LB, but also for their well-known antiadhesive and antithrombotic properties [8, 11]. When rat livers were preserved in APs, less than half the number of leukocytes stuck to postcapillary venules as compared to LB-preserved livers.

Two further observations from our intravital microscopy studies are noteworthy. The effect of AP added to the storage solutions was perpetuated well into the 1st h of reperfusion and after the preservation solution has been rinsed out of the grafts. Second, the antiadhesive effects of AP solutions were limited to leukocyte sticking but not leukocyte rolling.

Interactions between leukocytes and vascular endothelium are mediated by various proteins, with rolling of leucocytes in microvessels being mediated by selectins, whereas sticking relies on integrins. It appears that APs "blind" circulating leukocytes for the damaged endothelium either by binding to integrins or by inhibiting the rapid incorporation of the integrin receptor from intracellular vesicules into the membrane [15], while the interaction of leukocytic ligands with selectins appears unaffected.

Interestingly, in the isolated perfusion experiments, bile production and reperfusion blood flow were impaired by either impermeable anion when compared with Cl^--containing control solution. In choleretic-free, ILP models, bile production and bile retention were found to be a function of hepatocellular cation transport [9]. Also Na^+ as well as K^+ uptake was significantly lower in livers preserved in anionic polysaccharide solutions, which might explain the lower bile production of livers preserved in solutions containing impermeants.

In addition to the pronounced antiedematous effects, anionic polysaccharide solutions had cyto- or membranoprotective effects, as indicated by the lower LDH and ASAT release from livers preserved in SAPs or PPSs. That effect might at least partially be responsible for the higher PVR in livers preserved in SAPs and PPSs for the following reason: during hepatic ischemia [3], hepatocytes or other parenchymal cells [2] are thought to release adenosine into the perivascular space, where it binds to A_2-adenosine receptors and reduces the vasculotonus [12]. When interstitial adenosine concentrations are increased by inhibiting adenosine deamination, hepatic reperfusion and graft function is improved [20]. Preservation of livers in SAPs and PPSs might not only reduce LDH and ASAT leakage but also decrease the leakage of adenosine or other vasodilators from cold-ischemically damaged rat livers. Therefore, rat livers preserved in APs without vasodilators might maintain a higher vascular tonus, as indicated by the higher PVR in our experiments.

Summarizing our data, the pronounced antiedematous, hepatocellular protective, and antiadhesive effects of anionic polysaccharides during preservation of rat livers seem to warrant further studies in syngeneic and allogeneic [23] liver transplant models, with a future emphasis on preventing low flow after reperfusion [20].

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