Lazaroids have been reported to attenuate preservation and reperfusion injury. In this study, we examined whether lazaroids can improve the outcome after 48-hr canine liver preservation and transplantation. Adult female beagle dogs were randomized into 4 dosage groups (5 animals each). Lazaroid U-74389G was intravenously administered at a dose of 0 mg/kg, 6 mg/kg, 10 mg/kg, or 15 mg/kg to donors 30 min before harvesting and also to recipients 30 min before revascularization. Control animals (0 mg/kg) were given the lazaroid vehicle. The liver grafts were orthotopically transplanted after 48 hr of hypothermic preservation in UW solution. Lazaroid treatment significantly improved outcome after transplantation. Five-day animal survival increased from 0% in the control to 60% in the 6 mg/kg group, 100% in the 10 mg/kg group, and 80% in the 15 mg/kg group. Lazaroid protected the hepatocytes from damage during preservation, and enhanced energy charge and hepatic blood flow after reperfusion. Histological alterations were significantly less severe in the lazaroid-treated groups. The area of necrotic hepatocytes decreased from 43.7±17.7 in the control to 13.5±3.0 in the lazaroid 10 mg/kg group. These results indicate that lazaroid U-74389G has potential for improvement of clinical liver preservation.

Lazaroids are a group of new synthetic 21-aminosteroids, which inhibit iron-dependent lipid peroxidation without glucocorticoid and mineralocorticoid actions (1). In addition to potent antioxidant properties, lazaroids have been shown to suppress cytokine production (2), adhesion molecule expression (3), and neutrophile activation and infiltration (4). From these unique biological features, lazaroids were first reported to ameliorate ischemia and reperfusion injury of the central nervous system (5-7). Later the effects of lazaroids were confirmed in other tissues and organs in small and large animal experiments (8-15).

In this study, we examined whether lazaroids can improve outcome after 48-hr liver preservation and orthotopic transplantation in beagle dogs. Lazaroid U-74389G was selected from the lazaroid compounds and tested at three different doses. Animals receiving vehicle alone were used as the control.

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MATERIALS AND METHODS

Animals. Adult female beagle dogs, weighing 9 kg to 13 kg, were used as liver donors and recipients. After overnight fasting, the animals were anesthetized with thiopental-sodium (25 mg/kg) for induction, and maintained with fluorane, nitrous oxide, and oxygen by positive pressure mechanical ventilation. Heart rate, arrythmia, arterial blood pressure, central venous pressure, and esophageal temperature were monitored during surgery. Blood gas and electrolytes were measured frequently and corrected if necessary.

Operative procedures. Hepatic homograft procurement and orthotopic liver transplantation were performed using our standard laboratory method (16, 17). In brief, the hepatic ligaments, the distal common bile duct, the portal vein, and the hepatic artery were dissected, and the liver was flushed with 1.5 L cold University of Wisconsin (UW) solution (ViaSpan, Du Pont Merck Pharmaceutical Company, Wilmington, DE) via catheters inserted into the splenic vein (1 L) and the inferior abdominal aorta (0.5 L). The biliary tract was irrigated via cholecystostomy with 100 ml of normal saline. After removal, the graft was placed in a sterile plastic bag containing 0.5 L of UW solution, and kept at 4°C for 48 hr (until transplantation). The recipient liver was dissected and removed with the use of venovenous bypass during the anhepatic phase. The graft was revascularized by end-to-end anastomoses of the suprahepatic vena cava, the infrahepatic vena cava, the portal trunk, and the abdominal aorta. Prior to completion of the infrahepatic vena cava anastomosis, the graft was perfused with 250 ml of cold lactated Ringer’s solution through the portal vein to remove air and UW solution in the graft. The first 50 ml of effluent from the flushing procedure was collected from the infrahepatic vena cava and stored at -70°C for later biochemical analysis. After obtaining hemostasis, the transplant was completed by cholecystoduodenostomy for biliary reconstruction.

Electrolyte solution (Plasmalyte, 1.5-2.0 L) and blood (limited to 1500 ml) collected from donor dogs were transfused to maintain recipient hemodynamics. Cephalosporin 1 g was given to animals intraoperatively and continued daily for 5 days. Animals were allowed to eat and drink from the following morning. Oral cyclosporine 20 mg/kg was administered daily for postoperative immunosuppression until the surviving animals were sacrificed at 14 days.

Experimental groups. Lazaroid U-74389G was supplied by The Upjohn Co. (Kalamazoo, MI) and dissolved in a citrate buffer vehicle (pH 3.0) at a concentration of 1.5 mg/ml. The dissolved lazaroid was administered intravenously to donors 30 min before harvesting and to recipients 30 min before graft revascularization. Animals were randomized into four groups: lazaroid U-74389G at 0 mg/kg (Laz [0]), 6 mg/kg (Laz [6]), 10 mg/kg (Laz [10]), or 15 mg/kg (Laz [15]). The 6 mg/kg dose was the dose that The Upjohn Company recommended. Control animals (Laz [0]) were given the same amount of vehicle as the Laz 10 group.

Assessments. Liver enzyme levels, including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and lactate dehydrogenase (LDH), from the effluent of the final flushing solution and from postoperatively collected blood samples were measured using a Technicon RA500 autoanalyzer (Bayer, Tarrytown, NY).
Wedge biopsy samples of liver tissues were collected before graft procurement, at the end of the 48-hr preservation period, and 1 hr after graft reperfusion. The biopsy specimens were halved: one section was immediately frozen in liquid nitrogen for biochemical analysis and the other was fixed in buffered formalin for histopathologic study.

Liver tissues taken for biochemical analysis were analyzed for protein concentration, adenine nucleotides, purine catabolites, malondialdehyde, and myeloperoxidase. Protein concentration of the homogenate was measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) method described by Bradford et al. (18). Adenine nucleotides (AN) and purine catabolites (PC) were measured using a Waters HPLC system (19) (Waters Chromatography Division/Millipore Corp., Milford, MA; Model 510 pumps, Model 484 absorbance module, and Model 717 WISP system). Concentrations of AN and PC were monitored at 254 nm (Waters 484, Tunable Absorbance Detector). Energy charge (EC) was calculated using the equation \( \text{EC} = \frac{ATP + 1/2 ADP}{ATP + ADP + AMP} \) (20). Malondialdehyde (MDA) concentration was estimated by thiobarbituric acid reaction by fluorospectrophotometry (21) (excitation wavelength 515 nm; emission wavelength 535 nm; Shimadzu fluorospectrophotometer, Model RF5000U, Shimadzu Corp., Kyoto, Japan). Myeloperoxidase (MPO) activity assay was measured using the fluorospectrophotometric method of Kravzic et al. (22). One unit of MPO activity was defined as the concentration that caused a 1.0 change in optical density at 460 nm for 1 min at 22°C.

Liver sections taken for histopathology were stained by hematoxylin and eosin and examined by a single pathologist without knowing the groups and the timing of tissue sampling. In addition, the area of necrotic hepatocytes was quantified by a morphometric method using an eyepiece with 135 intersections. Morphometry was performed on five randomly selected portal fields per section at a magnification of \( 400 \times \).

Measurement of hepatic blood flow was performed before liver harvesting and one hr after graft reperfusion using an ultrasonic Doppler flowmeter (Transonic T201D, Transonic Systems Inc., Ithaca, NY) and a laser Doppler flowmeter (Transonic ALF21). Flow values for both machines were expressed as ml/min/100 g liver tissue.

**Results**

**Clinical observations.** Intravenous administration of U-74389G or vehicle alone caused no important hemodynamic changes in either donors or recipients. There was mild but transient acidosis after infusion in all animals. Cold ischemia time (49.2±1.2 hr), warm ischemia time (53±13 min), and graft weight loss during cold storage (15.3±6.8%), were not statistically significantly different in the experimental groups.

**Survival.** Lazaroid U-74389G significantly improved animal survival and reduced the incidence of graft failure during the early postoperative period (Fig. 1). While all animals in the Laz 0 group died of graft failure, only 2 animals in the Laz 6 group and no animals in the Laz 10 or Laz 15 group died of graft failure one day after transplantation. Animals dying of graft failure had pulmonary edema and various amounts of serosanguinous ascites at autopsy. One Laz 15 animal died of intussusception on postoperative day 3. Five-day animal survival was 0% in the Laz 0 group, 60% in the Laz 6 group, 100% in the Laz 10 group, and 80% in the Laz 15 group. However, after 5 days only 4 animals (1 in the Laz 10 and 3 in the Laz 15 group) survived for 14 days. The remaining 8 animals were lost to vascular thrombosis (n=3), cholangitis (n=2), intussusception (n=1), peritonitis (n=1), or bleeding duodenal ulcer (n=1). The difference in animal survival in the treated groups was not statistically significant.

**Liver enzyme release.** Liver enzymes (GOT, GPT, LDH) released into the effluent after final flushing were significantly lower in the Laz 10 group than the other three groups (Fig. 2). Similarly, LDH blood levels 1 hr after reperfusion were lower in animals receiving higher doses of U-74389G, but the difference was not significant. Liver enzymes of the surviving animals did not return to normal 14 days after transplantation.

**Biochemistry.** At higher doses (Laz 10 and Laz 15), lazaroid U-74389G retarded the degradation of adenine nucleotides to purine catabolites during preservation, and enhanced energy resynthesis after graft revascularization (Table 1). Cold storage induced a universal decline in adenine nucleotides and a corresponding increase in purine catabolites in all groups. The tissue concentration of total adenine nucleotides was significantly higher in the Laz 10 group than the Laz 0 group, and the tissue concentration of total purine catabolites was
significantly lower in the Laz 15 group than the Laz 0 group. At 1 hr after reperfusion the energy charge of Laz 10 livers returned to normal levels, while the energy charge of the other 3 groups was significantly below normal. Although lazaroid U-74389G has been known to inhibit lipid peroxidation, MDA levels at the end of preservation and at 1 hr after reperfusion showed no significant difference among the groups. Levels increased equally during preservation and declined to below normal after reperfusion. Although MPO levels were undetectable after the neutrophils were washed from the liver by the preservation solution, they rose significantly, and they were higher than normal after reperfusion. Neutrophil accumulation in livers given vehicle alone was double that of the Laz 15 group animals.

**Hepatic blood flow.** Upon portal unclamping, heterogeneous reperfusion and the occurrence of outflow block were always seen in livers treated by vehicle alone. Lazaroid U-74389G ameliorated these disturbances and improved hepatic blood flow, particularly at the higher doses (Fig. 3). Portal blood flow, hepatic arterial blood flow, and hepatic tissue blood flow of normal livers were measured at 186 ± 80 ml/min/100 g liver, 53 ± 24 ml/min/100 g liver, and 13 ± 2 ml/min/100 g liver, respectively. At 1 hr after reperfusion, these three estimates in Laz 0 and Laz 6 livers decreased to one-third of the normal values, whereas both hepatic arterial blood flow and hepatic tissue flow in Laz 10 and Laz 15 groups were significantly higher than those of the other two groups.

**Histopathology.** No morphological difference could be detected between the groups prior to revascularization. After reperfusion, livers from the animals receiving no treatment (Laz 0 group) showed severe structural abnormalities, such as diffuse hepatocyte necrosis, disarray of hepatocyte cords, disaggregation of hepatocytes, and hepatic congestion (Fig. 4A). In contrast, the treated livers, particularly from the Laz 10 group animals, had rather well-preserved hepatic architecture with small foci of the necrotic area and/or single-cell necrosis (Fig. 4B). The necrotic hepatocyte area was measured at 43.7 ± 17.7% in the Laz 0 group, 16.8 ± 6.3% in the Laz 6 group, 13.5 ± 3.0% in the Laz 10 group, and 31.5 ± 5.7% in the Laz 15 group (Fig. 5). The necrotic area of Laz 6 and Laz 10 was significantly less than the other two groups.

**DISCUSSION**

This study demonstrated that lazaroid U-74389G significantly improved the outcome of beagle dogs after 48-hr liver preservation.
Liver damage from ischemia and reperfusion has largely been explained by the generation of superoxide radicals (30, 31). Superoxide anions and hydrogen peroxides are produced by the xanthine oxidase enzyme system via the conversion of hypoxanthine to xanthine. Highly toxic hydroxyl radicals are generated in the presence of ferrous iron (Fe **+) via the Haber-Weiss reaction or Fenton reduction. Other superoxide anion species are produced by the NADPH-dependent oxidase system. These superoxide radicals interact with lipids, proteins, nucleic acids, and cell membranes, and cause lipid peroxidation in hepatocytes, endothelial cells, Kupffer cells, and infiltrating inflammatory cells. Lipid peroxides, in turn, impair cellular and subcellular function, and often lead to cell death. Lipid peroxides also stimulate cytokine production, mediate arachidonic acid metabolism, induce expression of adhesion molecules, and activate leukocyte adherence and infiltration. Although cells are equipped with defensive endogenous antioxidants, supplemental exogenous antioxidants are needed to protect cells from increased free radical generation (32).

Lazaroids are a novel form of antioxidant that inhibits iron-dependent lipid peroxidation, and they were developed in studies of the effect of large doses of methylprednisolone on central nervous ischemia. Compared with a large dose of methylprednisolone, Lazaroids were reported to be 10,000 times more potent in inhibiting lipid peroxidation (1). Although the mechanism of action is still unclear, attenuation of ischemia and reperfusion injury by Lazaroid was found in the central nervous system of rats (3, 6) and dogs (7), and later in patients (33). The protective effect was subsequently
confirmed in the heart (8, 9), lung (10), kidney (14, 15), and intestine (13) after ischemia (warm and cold) and reperfu-
sion. Recent reports have shown that lazaroid compounds 
maintain endothelial cell viability in a dose-dependent man-
ner (11, 12). Pretreatment of the donor with lazaroid reduced 
enzyme release and phospholipase A2 release after 
24-hr preservation in an ex vivo isolated pig liver perfusion 
model (12). They also showed that lazaroid pretreatment of 
rats receiving 24-hr-preserved livers allowed survival to in-
crease from 30% without treatment to 90% with treatment. 
These results, except for changes in MDA, are consistent 
with the findings obtained in our study. Cosenza et al. found 
that postperfusion MDA increased in only vehicle-treated 
livers, while our study showed that MDA increased in all 
experimental groups during preservation and decreased to 
below normal after reperfusion. Ferguson et al. (34) reported 
MDA changes in rat livers that were similar to our results. A 
more specific and sensitive analytical method is required to 
eluicate these differences, since the measurement of MDA 
to estimate lipid peroxidation is rather crude.

In conclusion, lazaroids are effective for prolonging the 
preservation period of hepatic homografts, with a potential 
for clinical use. However, before embarking on a clinical trial, 
several issues need to be considered. First, despite successful 
48-hr canine liver preservation, the agent appears 
ful for improving graft viability within the current 
preservation time frame (35). This last question is currently 
under investigation in multicenter trials with central nerv-
ous injuries.

REFERENCES

1. Braughler JM, Pregenzer JF, Chase RL, Duncan LA, Jacobsen 
   EJ, McCall UM. Novel 21-amino steroids as potent inhibitors of 
   iron-dependent lipid peroxidation. J Biol Chem 1987; 262: 10, 
   438.
2. Shenkar R, Abraham E. Effects of treatment with the 21-amin-
   osteroid, U7438F, on pulmonary cytokine expression following 
3. Meyer RJ, Juarez RA, Holden WE. 21-aminosteroids protect 
   endothelial cells against injury by neutrophils. Am Rev Respir 
4. Gadaleta D, Verma M, Davis JM. Inhibition of neutrophil leuk-
  ocytes by the 21-aminosteroid, U7438F. J Surg 
5. Hall ED, Paziara KE, Braughler JM. 21-aminosteroid lipid per-
   oxidation inhibitor U74006F protects against cerebral isch-
6. Park CK, Hall ED. Dose-response analysis of the effect of 21-
   aminosteroid tirilazad mesylate (U74006F) upon neurological 
   outcome and ischemic brain damage in permanent focal cere-
7. Perkins WJ, Milde LN, Milde JH, Michenfelder JD. Pretreat-
   ment with U74006F improves neurologic outcome following 
8. Holzgrete HH, Buchanan LV, Gibson JK. Effects of U74006F. a 
   novel inhibitor of lipid peroxidation, in stunned reperfused 
9. Hendry PJ, Anstadt MP, Plunkett MD, Amato MT, Menius JA 
   Jr, Lowe JF. Improved donor myocardial recovery with a new 
   lazaroid lipid antiperoxidant in the isolated canine heart. 
    an additive to University of Wisconsin solution for pulmonary 
    grafts in the rat transplant model. J Thorac Cardiovasc Surg 
    1992; 104: 1333.
11. Killinger WA Jr, Doroff DB, Keagy BA, Johnson G Jr. Improve-
    ment of endothelial cell viability at 4 degrees C by addition of 
    lazaroid U74500A to preservation solutions. Transplantation 
12. Cosenza CA, Cramer DV, Cumneen SA, Tuso PJ, Wang HK, 
    Makowka L. Protective effect of the lazaroid U74006F in cold 
    ischemia—reperfusion injury of the liver. Hepatology 1994; 19: 
    418.
    SM. Improved small intestinal preservation after lazaroid 
    U74389G treatment and cold storage in University of Wiscon-
    Attenuation of renal reperfusion injury in rats by the 21-
15. Shackleton CR, Ettinger SL, Scudamore CH, Toleikis PF, Keown 
    PA. Effect of a 21-aminosteroid, U74006F, on lipid peroxida-
    tion and glomerulotubular function following experimental re-
16. Todo S, Kam I, Lynch S, Starzl TE. Animal research in liver 
    transplantation—with special reference to the dog. Semin 
    Liver Dia 1985; 5: 309.
    3: 253.
18. Bradford MM. A rapid and sensitive method for the quantitation 
    of microgram quantities of protein utilizing the principle of 
    chromatography of nucleotides, nucleosides, and major purine 
    bases and its application to different tissue extracts. Anal 
    Biochem 1985; 144: 258.
    predict primary nonfunction of the human liver homograft? 
    Transplant International 1994; 7: 89.
21. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal 
    tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 
    351.
22. Krawisz JE, Sharon P, Stenson WR. Quantitative assay for 
    acute intestinal inflammation based on myeloperoxidase activ-
    ity. Gastroenterology 1984; 87: 1334.
    Disturbance of microcirculation associated with prolonged 
    preservation of dog livers under UW solution. Transplant Proc 
    of the canine liver by modified simple hypothermic storage 
25. Belzer FO, Southard JH. Principles of solid organ preservation 
    Extended preservation of human liver grafts with UW solu-
    canine liver for 24–48 hours using simple cold storage with UW 
28. Sumimoto R, Lindell SL, Southard JH, Belzer FO. A comparison of 
    histidine-lactobionate and UW solution in 48-hour dog liver
Lazaroid, an inhibitor of iron-mediated lipid peroxidation, has been shown to reduce free radical-mediated injury after ischemia and reperfusion. We thus examined the efficacy of pretreatment with lazaroid (U74500A) in enhancing functional recovery after 24-hr heart preservation. An isolated rabbit heart model perfused with the blood from a support rabbit was used. Before preservation, either U74500A (4 mg/kg, group L; n=6) or solvent (group S; n=7) was given to the donor rabbit. After 24-hr preservation with UW solution at 0°C, all hearts were perfused with cross-circulated blood for 60 min with the Langendorff mode followed by 40 min of the working mode. In group S, ventricular fibrillation (VF) after reperfusion was observed in all hearts, whereas no VF was observed in the U74500A-pretreated group. In group L, the serum creatine phosphokinase; its isozyme, troponin-T; and serum lipid peroxide levels after 10 min of reperfusion were all significantly (P<0.05) lower than those in group S. The Frank-Starling curve (indicating the left atrial pressure-aortic flow relationship) showed a significant left and upward shift in group L compared with that in group S (P<0.0001). The heart pretreated with U74500A showed less ischemia-reperfusion injury, better ventricular function, and a lower lipid peroxide level. We thus conclude that the inhibition of lipid peroxidation with lazaroid appears to offer some potential benefits for long-term heart preservation.

Reactive oxygen species such as the superoxide anion and hydroxyl radical are thought to play an important role in ischemia-reperfusion injury (1-2). Several studies have attempted to reduce myocardial injury using such antioxidants as superoxide dismutase, catalase, and allopurinol (1-6). A failure to neutralize the reactive oxygen species results in the initiation of lipid peroxidation (2), which thus causes cell membrane injury.

U74500A is one of the 21-aminosteroids (lazaroids) without glucocorticoid and mineralcorticoid side effects and strongly inhibits lipid peroxidation by chelating iron (7-8). Although several reports have demonstrated the effect of lazaroids on ischemia-reperfusion injury of the central nerve system (9), kidney (10), liver (11), lung (12-13), and heart (14-17), the cardioprotective effect on prolonged hypothermic cardiac ischemia has not yet been investigated.

In this study, we examined the cardioprotective effect of this agent after 24-hr preservation. An isolated rabbit heart perfused with cross-circulated blood was used for the functional evaluation. We also measured the serum lipid peroxide (LPO)* level to elucidate the effect of this drug.

* Abbreviations: AoF, aortic flow rate; CBF, coronary blood flow rate; CK, creatine phosphokinase; CK-MB, creatine phosphokinase-MB isozyme; LAP, left atrial pressure; LPO, lipid peroxide; MVO2, myocardial oxygen consumption; TnT, troponin-T; VF, ventricular fibrillation; UW, University of Wisconsin.