The effect of Lazaroid U-74389G on extended liver resection with ischemia in dogs

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Background. *In liver surgery, total clamping of the portal triad (Pringle's procedure) is commonly used, and it sometimes causes liver failure. This study was designed to evaluate the effect of Lazaroid U-74389G (LAZ-G), which inhibits iron-dependent lipid peroxidation, on ischemia-reperfusion injury during liver resection in dogs.*

Methods. *The experiment animals were divided into 2 groups. The control group was subjected to 60 minutes of warm ischemia by partial inflow occlusion. The LAZ-G–treated group received LAZ-G before ischemia and then underwent liver ischemia. After reperfusion, the nonischemic lobes were resected, and the remnant liver function was evaluated.*

Results. *The LAZ-G–treated group showed a significantly improved animal survival rate. Biochemical analysis and morphologic evaluation by electron microscopy suggest that LAZ-G pretreatment protects both hepatic parenchymal cells and sinusoidal endothelial cells from ischemia-reperfusion injury. Expression of IL-1β messenger RNA in hepatic venous blood was measured by a reverse transcriptase-polymerase chain reaction; it was shown to be inhibited in the LAZ-G–treated group after reperfusion. This suggests that LAZ-G decreases the activation of proinflammatory cytokine expression.*

Conclusions. *Lazaroid U-74389G ameliorates ischemia-reperfusion injury caused by Pringle's procedure during extensive liver resection. This agent may therefore be clinically applicable for extended liver surgery involving vascular isolation. (Surgery 1999:126:908-17.)*

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HEPATIC FAILURE INDUCED BY ischemia and reperfusion injury frequently occurs at major hepatectomy. The mechanism of hepatic disorders as the result of ischemia-reperfusion (I/R) injury has not been fully investigated. It is therefore crucial to reveal the mechanism of reperfusion injury and reduce the incidence of such injuries. Lazaroids are a group of new synthetic 21-aminosteroids that inhibit iron-dependent lipid peroxidation without exerting glucocorticoid and mineral corticoid effects.[1] These 21-aminosteroids have been shown to suppress cytokine production,[2] adhesion molecule expression,[3] and neutrophil activation and infiltration.[4] Initially, lazaroid compounds were studied in traumatic, ischemic, and hemorrhagic injury of the central nervous system in animal models.[5-6] The effects of lazaroids have been confirmed in studies of other animal organs.[7-14] Lazaroids are effective in enhancing the liver's resistance to damage as the result of cold I/R injury.[7] Lazaroids potentially therefore improve chances of clinical liver preservation.[9,12] Studies of animal intestinal transplantation models have demonstrated that the addition of lazaroids to the University of Wisconsin solution enhances the resistance of grafts to cold I/R injury and also improves early graft function after surgery.[13] Donor lazaroid pretreatment improves the viability of livers harvested from non–heart-beating donors.[14] In this study, we examined whether Lazaroid compounds can prevent liver injury at the time of extended hepatectomy with 60 minutes of ischemia.

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MATERIAL AND METHODS

Animals. Fourteen adult mongrel dogs of both sexes, weighing 12 to 20 kg, were used in this experimental study. The animals received a standard commercial diet and were allowed free access to food and water until 12 hours before the operation. All animals received humane care in compli-

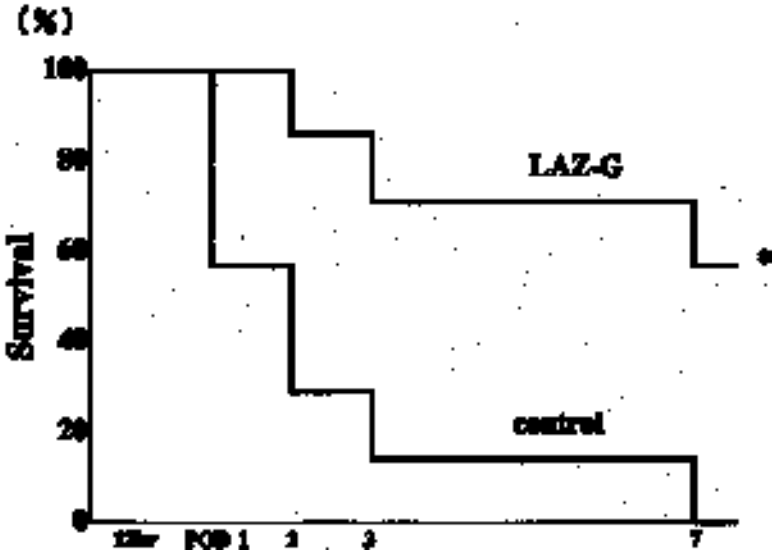

Fig 1. Animal survival course after reperfusion. The LAZ-G–treated group was significantly superior to the control group. **P* < .05 versus control group.

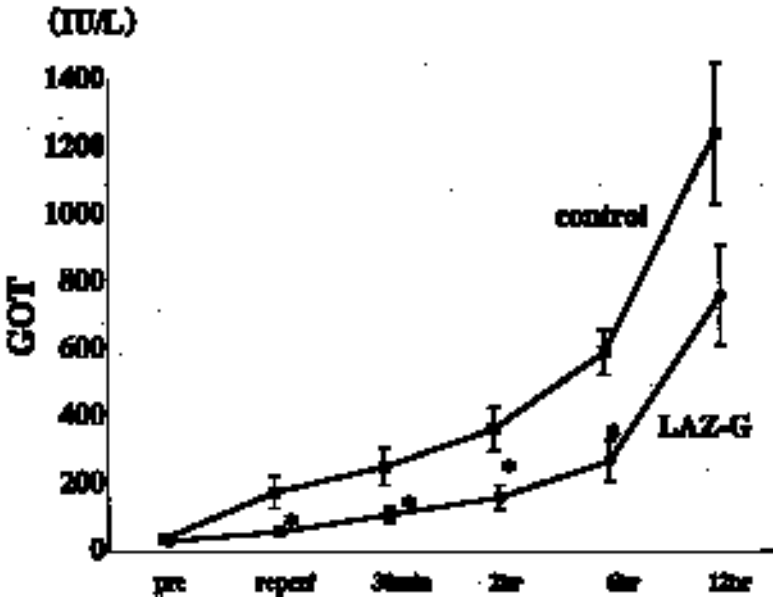

Fig 2. Serum GOT concentrations. Data are expressed as the means ± SEM. **P* < .05 versus control group.

ance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication No 85-23, revised 1985).

Operative procedures. After the administration of ketamine hydrochloride (10 mg/kg intramuscular injection), the animals were anesthetized with pentobarbital sodium (10 mg/kg) and pancuronium bromide (0.2 mg/kg). The animals were then intubated and connected to a volume-cycled ventilator (MD800TM; Senko Med Co Ltd, Tokyo, Japan) at a tidal volume of 20 mL/kg and a rate of 15 breaths/minute. Positive end-expiratory pressure was controlled at 5.0 cm H_2O. Muscular relaxation was obtained with additional pancuronium bromide (0.1 mg/kg).

The operation was performed under sterile conditions. A catheter was inserted into the right femoral artery to monitor the animal's heart rate, arrhythmia, and arterial blood pressure during surgery. Another catheter was inserted through the right femoral vein into the right hepatic vein to collect blood samples. This catheter was also used to infuse a lactated Ringer's solution at a rate of 10 mL/kg/h.

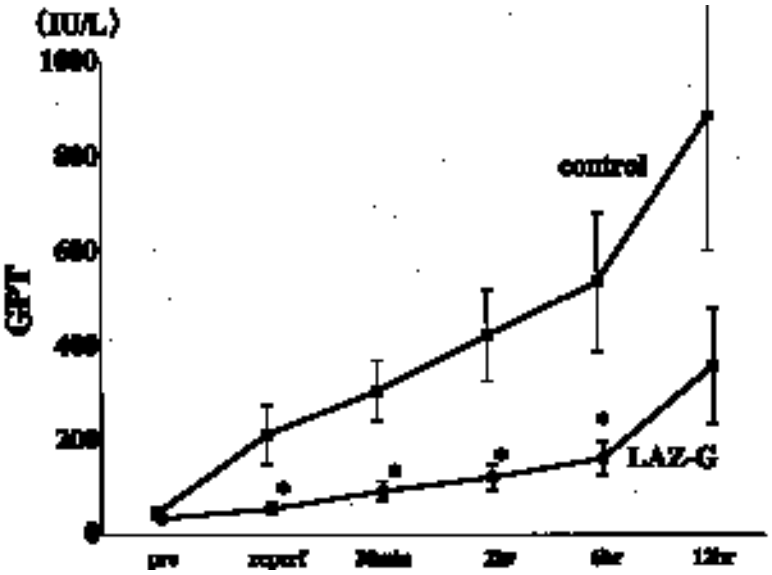

Fig 3. Serum GPT concentrations. Data are expressed as means ± SEM. *$P < .05$ versus control group.

The abdomen was explored through a median incision. The liver was isolated by dissecting the ligaments and all peritoneal attachments of the liver. Both portal branches and hepatic arteries were isolated. The right portal pedicle was clamped for 60 minutes; the left portal branch was patent to avoid portal congestion. After reperfusion, the left portal pedicle was ligated, followed by resection of approximately 75% of the liver (including the right central, quadrate, left central, left lateral, and papillary lobes). Liver specimens were taken after 1 hour of reperfusion, and the abdominal wall was closed.

Experimental design. The animals were divided into 2 groups; the control group was subjected to warm ischemia with only a Lazaroid U-74389G (LAZ-G; The Upjohn Co, Kalamazoo, Mich) citrate buffer vehicle administered by inflow occlusion as described earlier (n = 7). The LAZ-G–treated group was subjected to warm ischemia with LAZ-G treatment (n = 7). LAZ-G was dissolved in a citrate buffer vehicle (pH 3.0) at a concentration of 1.5 mg/mL. The dissolved LAZ-G is administered to dogs intravenously at a dose of 10 mg/kg through a catheter placed into the right hepatic vein 15 minutes before the hepatic inflow was clamped.

Sample collection and assessments. Blood samples were collected from the right hepatic vein before ischemia, immediately after the initiation of reperfusion, 30 minutes, and 2, 6, and 12 hours after reperfusion and centrifuged. The supernatant was stored at –80°C until biochemical analysis. Liver enzyme levels, including glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LDH), purine nucleoside phosphorylase (PNP) activities and hyaluronic acid (HA) levels were measured at 37°C. GOT, GPT, and LDH activities were determined with an autoanalyzer (JCA-RS1200; Nihon Densi Co Ltd, Tokyo, Japan) with an ultraviolet rate assay. PNP activities were measured by photospectrometer (Hitachi Model 100-30; Hitachi Co Ltd, Tokyo, Japan) with a modified method developed by Kurashige et al.[15] Serum concentrations of HA were measured with HA-binding protein-coated polystyrene beads.[16] Serum levels of lipid peroxide were measured with the method developed by Ohishi et al.[17]

Measurement of IL-1β mRNA. Samples of hepatic venous blood were obtained for examination of expression levels of IL-1β messenger mRNA immediately after and at 1, 3, and 6 hours after reperfusion in each group. The expression levels of IL-1β mRNA were measured by a reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the blood with a TRIZOL LS reagent with a modified method described by Chomozynski and Sacchi.[18] All RNA samples had an A_{260}/A_{280} ratio of more than 1.6. One microgram of total RNA was dissolved in 10.5 μL distilled water and then heated at 95°C for 5 minutes. After quick cooling in an ice bath, 1 μL of 10 mmol/L deoxyribonucleotide triphosphates, 2 μL of 0.009 OD/mL random primer, and 4 μL of 5× reverse transcriptase reaction buffer (0.25 mol/L Tris-HCl [pH 8.3], 0.37 mol/L KCl, 15 mmol/L $MgCl_2$) were added. The mixture was treated with 200 U of Moloney murine leukemia virus reverse transcriptase at 25°C for 10 minutes and at 37°C for 1.5 hours. After the reaction, the reaction mixture was heated at 95°C for 5 minutes and cooled

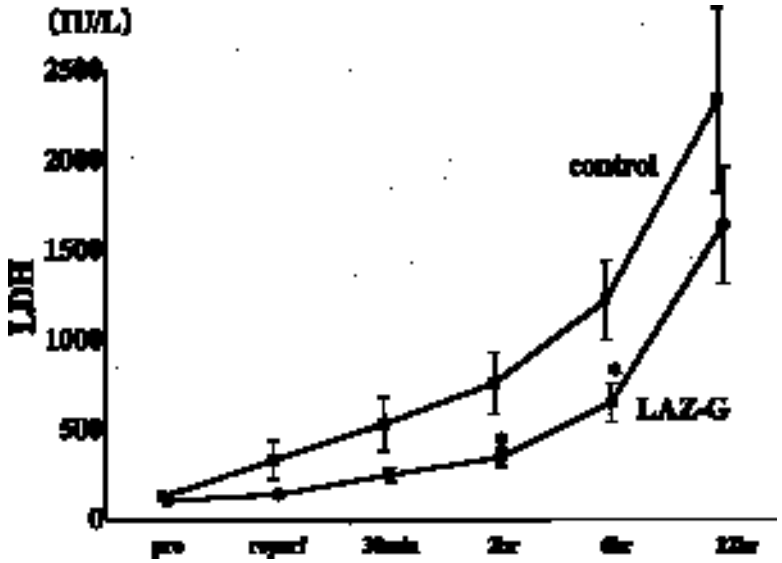

Fig 4. Serum LDH concentrations. Data are expressed as the means ± SEM. *$P < .05$ versus control group.

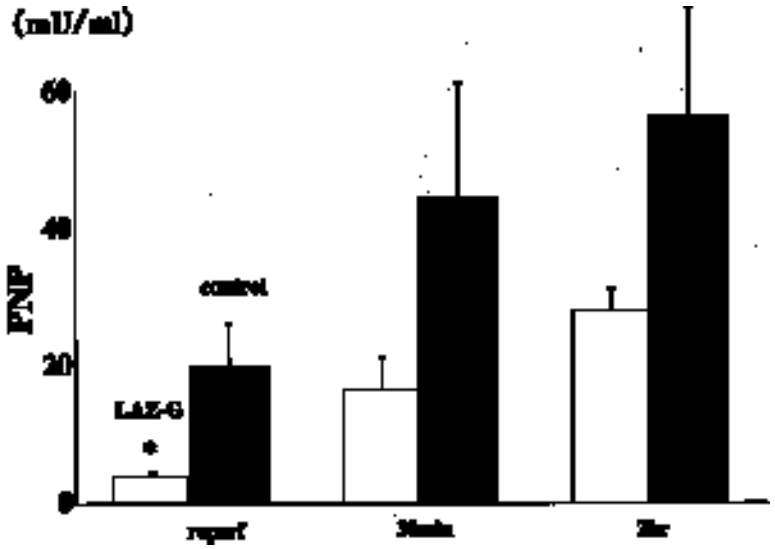

Fig 5. Serum PNP concentrations. Data are expressed as means ± SEM. *$P < .05$ versus control group.

quickly in an ice bath. Eighty microliters of distilled water were added to the mixture.

To obtain IL-1b and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) complementary DNA fragments by PCR, we designed oligonucleotides primers from mouse, rat, and human cDNA sequences and synthesized 4 oligonucleotides with an Applied Biosystems DNA synthesizer (model 392; Applied Biosystems, Inc, Foster City, Calif): 5,-TGAGCTGAAAGCTCTCCACCTC-3, (bases 419-440 of the rat IL-1b coding region; sense); 5,-TTGA GAGGTGCTGATGTACCAG-3, (bases 726-705 of the rat IL-1b coding region; antisense); 5,-GAACGGGAAGCTCACTGGCATGGC-3, (bases 666-689 of the rat G3PDH coding region; sense); 5,-TGAGGTCCACCACCCTGTTGCTG-3, (bases 976-954 of the rat G3PDH coding region; antisense).

PCR was performed on 20 μL of reverse-transcribed reaction mixture,[19] 8 μL of 1.25 mmol/L deoxyribonucleotide triphosphates, 50 pmol of each oligonucleotide primer, 5 μL of 10 × PCR buffer (100 mmol/L Tris-HCl [pH 8.3], 500 mmol/L KCl, 15 mmol/L $MgCl_2$) and 1.25 U of Taq polymerase in a 50 μL reaction volume with a DNA thermal cycler (model PJ 2000; Applied Biosystems, Inc). The amplified IL-1β and G3PDH cDNA fragments were obtained from 27 and 19 PCR cycles, respectively; each cycle consisted of a

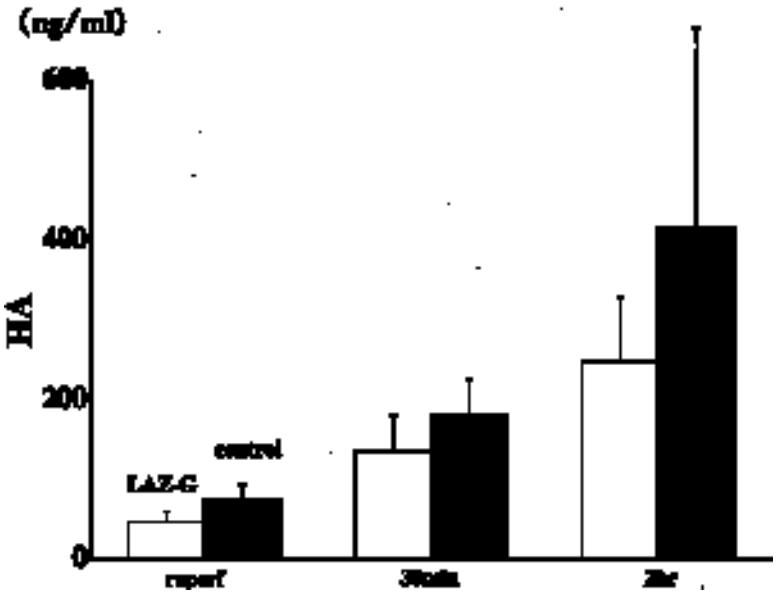

Fig 6. Serum HA concentrations. Data are expressed as the means ± SEM.

denaturing at 94°C for 1 minute and an annealing at 60°C for 2 minutes. Aliquots of the reaction mixture were electrophoresed on a 2% agarose gel. For quantitative assessment, fluorescence intensities were measured by a Fluor Imager (Molecular Dynamics, Sunnyvale, Calif).

Histologic studies. After 1 hour of reperfusion, liver specimens were collected and fixed in 10% formalin. The tissue was dehydrated, embedded in paraffin, cut into 3 to 5 μm sections, and mounted. After deparaffinization, the tissue samples were stained with hematoxylin and eosin for the histologic study. Moreover, the ultrastructural analysis was conducted by transmission electron microscope. The tissue for electron microscopic observation was fixed in a 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer solution (pH 7.4). Samples were taken postfixed in 2.0% phosphate-buffered osmium tetroxide. After dehydration in alcohol, they were embedded in Quetol 812 (Nissin EM Co Ltd, Tokyo, Japan). The ultra-thin sections were poststained in uranyl acetate and lead citrate. These specimens were examined in a blind analysis by a single pathologist who was uninformed about the grouping and the timing of tissue sampling.

Statistical analysis. All values are expressed as the means ± SEM for each experimental group. Statistical analysis was performed with the Mann-Whitney *U* test. The animal survival rate was determined with the Kaplan-Meier method, and the log-rank test was used to determine significance. *P* values less than .05 were considered statistically significant.

RESULTS

Intravenous administration of LAZ-G or vehicle alone caused no important hemodynamic changes in any experimental animals.

Clinical course. LAZ-G significantly improved animal survival and reduced the incidence of fatal acute liver failure during the early postoperative period (Fig 1). The 3-day animal survival rate was 14% in the control group and 71% in the LAZ-G–treated group. The 7-day animal survival rate was 0% in the control group, and 57% in the LAZ-G–treated group. Several animals in the control group died of hepatic failure immediately after surgery. In the LAZ-G–treated group, only 2 dogs died of hepatic failure within 3 days after surgery. One dog in the LAZ-G group died within 7 days after surgery because of infection that persisted throughout the experiment. All animals that survived more than 7 days after surgery were killed by an overdose of pentobarbital at 7 days after surgery.

Liver enzyme release. Liver enzymes (GOT, GPT, LDH) released into the serum showed lower levels in the LAZ-G–treated group than in the control group. GOT and GPT concentrations immediately after, 30 minutes, and 2 and 6 hours after reperfusion were significantly lower in the LAZ-G–treated group than in the control group (Figs 2 and 3). Serum levels of LDH 2 and 6 hours after reperfusion were significantly lower in the LAZ-G–treated group than in the control group (Fig 4). Serum levels of PNP and HA were compared in the 2 groups immediately after, 30 minutes, and 2 hours after reperfusion. In the LAZ-G–treated

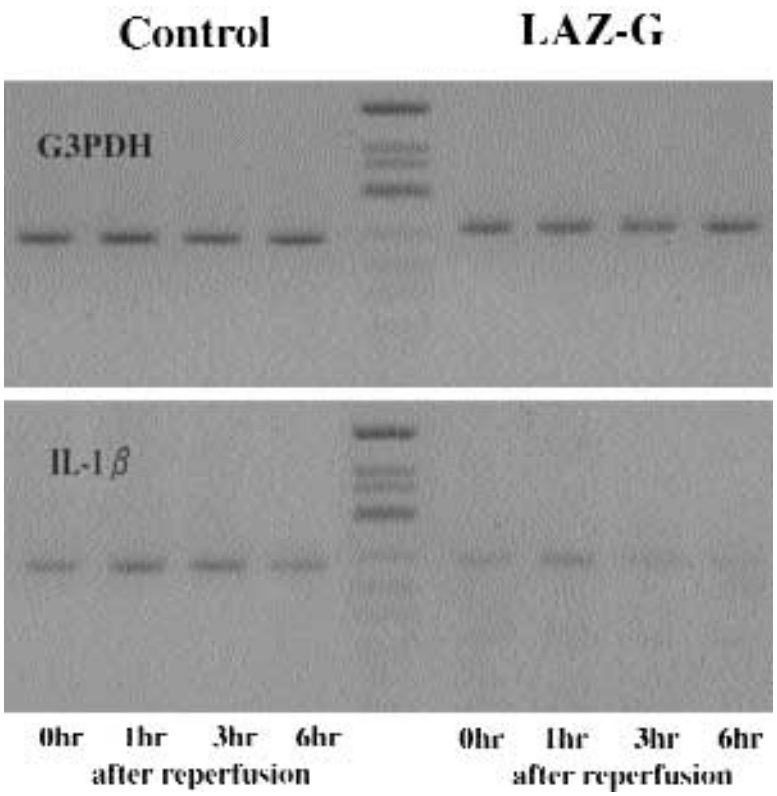

Fig 7. Expression of IL-1ß mRNA in the control group and the LAZ-G–treated group. In the control group, IL-1ß was strongly expressed after reperfusion. Expression of IL-1ß was inhibited in the LAZ-G–treated group compared with that of the control group at each examination point after reperfusion.

group, serum levels of PNP just after reperfusion were significantly lower compared with those of the control group (Fig 5). Serum levels of HA were lower in the LAZ-G–treated group than in the control group, but the difference was not significant (Fig 6). Serum levels of lipid peroxide were compared between the 2 groups, but there were no observable differences.

Measurement of IL-1β mRNA. In the control group, IL-1β mRNA was strongly expressed after reperfusion. Expression of IL-1β mRNA was inhibited in the LAZ-G–treated group compared with that of the control group at each examination point after reperfusion (Fig 7).

Histopathologic findings. Histopathologically, hepatocellular cyto-aggregation and microvesicular steatosis were observed in the control group. Marked neutrophilic exudation was also seen in the sinusoids of the control group 1 hour after reperfusion (Fig 8, *A*). In the LAZ-G–treated group, neither significant hepatocellular changes nor neutrophilic exudation was observed (Fig 8, *B*). As seen with transmission electron microscopy, Kupffer cells in the control group showed mild hypertrophic change without abundant vacuoles (Fig 9, *A*). In the LAZ-G–treated group, Kupffer cells showed remarkable hypertrophic cellular volume containing various sizes of vacuoles. Cytoplasmic projections and pseudopodia were prominent compared with those observed in the control group (Fig 9, *B*).

DISCUSSION

In liver surgery, it is important to control blood loss during hepatic resection. Minimizing hemorrhage is essential for obtaining a satisfactory clinical outcome after liver resection.[20-21] Total occlusion of the hepatic portal triad, referred to as "Pringle's maneuver," is commonly used for the purpose of minimizing hemorrhage. However, this procedure interrupts blood supply to the liver; therefore when followed by reperfusion, the Pringle's maneuver sometimes causes hepatic injury. Compared with other organs, the liver is especially sensitive to ischemia and reperfusion. Therefore various mechanisms of hepatic injury after ischemia and reperfusion have been investigated. Some investigators have suggested that late-phase reperfusion injury after hepatic no-flow ischemia is mediated mainly by neutrophils.[22-23] The activated neutrophils adhere to the vascular endothelium and subsequently release superoxide anion or other reactive

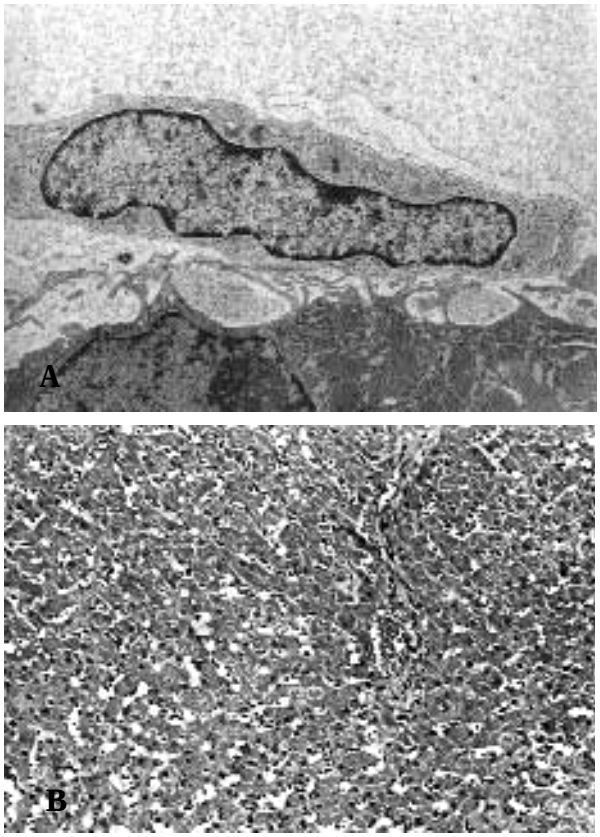

Fig 8. Histopathologic findings of the control group (**A**) and the LAZ-G–treated group (**B**). In the control group, hepatocellular cyto-aggregation and microvesicular steatosis were observed, and remarkable neutrophilic exudation was also seen in the sinusoids. On the other hand, no significant hepatocellular changes and neutrophilic exudation were observed in the LAZ-G–treated group. (Hematoxylin eosin stain; original magnification, ×50.)

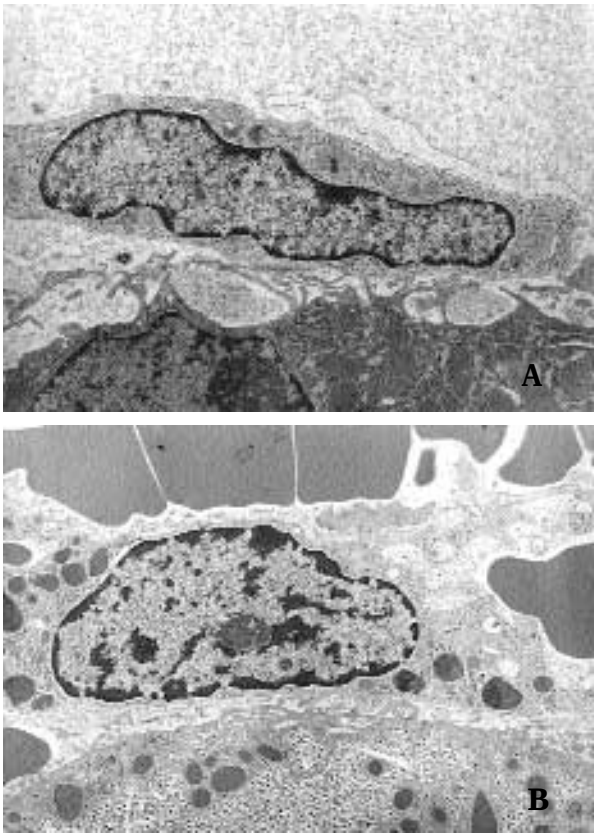

Fig 9. Ultrastructural examination findings by transmission electron microscope of the control group (**A**) and the LAZ-G–treated group (**B**). In the control group, Kupffer cells showed mild hypertrophic change without abundant vacuoles and with a decrease in pseudopodia. In the LAZ-G–treated group, Kupffer cells showed cytoplasmic hypertrophy with various sizes of phagocytic vacuoles. (Original magnification, ×5000.)

oxygen products that increase microvascular permeability.[24] In the presence of ferrous iron, oxygen is converted to superoxide anion, hydroxyl radicals, or both. This process in turn generates lipid peroxide radicals with high levels of reactivity in the cellular plasma membranes.[25-26] Lipid peroxidation of intact membranes and proteolysis of membrane proteins, coupled with inactivation of cytochromal enzymes, results in alterations in cell function, increases in permeability, and potentially results in widespread cytotoxicity. Lipid peroxides also activate leukocyte adherence and infiltration, mediate arachidonic acid metabolism, induce expression of adhesion molecules, and stimulate cytokine production.[27] It is clear that proinflammatory cytokines (eg, TNF-α and IL-1β) play an important role in I/R injury.[28-29] Cosenza et al[7] reported that lipid peroxidation might play a major role in the loss of liver graft viability after prolonged cold I/R injury.

Lazaroids are a group of new synthetic 21-aminosteroids that inhibit iron-dependent lipid peroxidation without exerting glucocorticoid and mineral corticoid effects, and lazaroids are a novel class of potent free-radical scavengers.[1] They have been shown to be effective on reducing organ injury in a variety of settings. The steroid moiety of the lazaroid is able to integrate into cell membranes and the iron-chelating group of lazaroids can scavenge free radicals. The latter group is a more potent class of free-radical scavenger than has previously been available. Killinger et al[12] demonstrated the efficacy of lazaroids on improving endothelial cell viability after hypothermic preservation. Studies of animal kidney, liver, intestine, heart, and lung transplantation models have demonstrated that the treatment with lazaroids improves early graft function after surgery.[7,8,13,30] In the present experimental study, we considered

the possibility that the treatment with LAZ-G before vascular occlusion could potentially ameliorate typical hepatic I/R injury.

We now routinely use an experimental model in which partial ischemia of a canine liver is induced by clamping the portal pedicles of the caudate and right lateral lobes.[31,32] In this model, portal congestion is avoided without performing veno-venous bypass because the left portal vein is patent. After reperfusion, the nonischemic lobes are resected so that only a part of the ischemic liver remains, and its function can thus be evaluated.

Todo et al[9] argued that the most effective dose of LAZ-G was 10 mg/kg in a canine liver preservation and transplantation model. We also determined that LAZ-G was efficient when a dose of 10 mg/kg was administered. Because the mean half-lives of tirilazad mesylate are relatively long (3.7 hours for healthy male volunteers),[33] we administered LAZ-G by bolus infusion of 10 mg/kg before ischemia. As for the method of LAZ-G administration, Todo et al[9] and Cosenza et al[7] used the dissolved lazaroid intravenously, and they reported its effect on the liver. We also used the dissolved LAZ-G intravenously rather than through the portal vein or the hepatic artery.

In this experimental study, serum levels of GOT, GPT, and LDH were measured as markers for damage to hepatic parenchymal cells. Serum levels of PNP and HA were also measured to detect damage to hepatic endothelial cells.[34] PNP, which is released into circulation at the time of hepatic injury, has been reported as a specific marker enzyme for the sinusoidal endothelial cells.[35-36] However, it has been recently reported that PNP is also localized in the liver parenchymal cells. PNP is therefore unlikely to be a specific marker of endothelial cell damage.[37-38] Because it is rapidly absorbed from the blood, HA has also been characterized as an indicator of endothelial cell injury. Increased serum levels of HA indicate impaired HA uptake by hepatic endothelial cells.[39-41] Thus considering the serum levels of both PNP and HA, we have evaluated the damage to hepatic endothelial cells.

The present study suggests that LAZ-G pretreatment significantly improves animal survival after major liver resection with vascular occlusion. After reperfusion, GOT, GPT, and LDH levels were significantly lower in the LAZ-G–treated group as compared with the control group. Serum enzyme activity usually expresses proteolytic degradation and extrusion of enzyme from damaged liver parenchymal cells; a reduction in these levels indicates that LAZ-G pretreatment reduces cell damage in the liver. The protective effects of pretreatment may be related to the pharmacologic characteristics of LAZ-G: namely, the inhibition of tissue lipid peroxidation through cooperative mechanisms such as chemical antioxidant effects and membrane stabilization. After reperfusion, serum levels of PNP were significantly lower in the LAZ-G–treated group as compared with the control group. Thus LAZ-G appears to protect both hepatocytes and sinusoidal endothelial cells from I/R injury. However, serum levels of HA were not shown to be significantly different between groups. It is therefore possible that, as a result of the suppression of damage to parenchymal cells, the sinusoidal endothelial cells may also avoid damage.

To evaluate the morphologic state of Kupffer cells after reperfusion, electron microscopic studies were performed in these 2 experimental groups. Kupffer cells in the LAZ-G–treated group showed remarkable hypertrophic cellular volume containing various sizes of vacuoles, suggesting the rapid recovery of Kupffer cell phagocytic activity. In contrast, the Kupffer cells in the control group appeared emaciated, suggesting poor phagocytic activity. Sankary et al[42] reported that, in an in vivo rat liver transplant model, prolonged donor fasting decreases Kupffer cell phagocytosis and tumor necrosis factor production after revascularization. In the study by Sankary et al,[42] Kupffer cells are thought to be inactivated by means of starvation at ischemia after reperfusion. In the present study, it was assumed that Kupffer cells would be well preserved under LAZ-G treatment; this would render them fully capable of regeneration after tissue damage caused by I/R.

To analyze the mechanism of action of LAZ-G, we examined the expression of cytokine and performed a quantitative analysis of IL-1β mRNA expression in hepatic venous blood using reverse transcriptase-polymerase chain reaction. IL-1β mRNA expression was evidently suppressed in the LAZ-G–treated group after reperfusion; however, we did not analyze the expression of cytokines in hepatic cells. Inside the liver, the typical source of inflammatory cytokine production is thought to be Kupffer cells. Our data suggest that LAZ-G might control the expression of IL-1β in Kupffer cells. Thus LAZ-G may inhibit I/R injury by suppressing cytokine production in this canine model. However, it is not clear that LAZ-G inhibits IL-1β directly or by the inhibition of lipid peroxides because the difference of serum levels of lipid peroxide was not significant between the 2 groups in this experiment. Cosenza et al[7] evaluated the effects of lazaroids by measuring levels of lipid peroxides in the liver. To clarify the effects of LAZ-G on the inhibition of lipid

peroxides, it seems necessary to measure not only lipid peroxide levels in the serum but in the liver tissue as well. Although 21-aminosteroids appear to be potent inhibitors of lipid peroxidation, suppressing proinflammatory cytokines may occupy a important role in inhibiting I/R injury in the present study. Tracey and Cerami[43] suggested that IL-1β and TNF-α were pleiotropic cytokines associated with various inflammatory conditions such as hepatic injury after I/R. To clarify the mechanism of LAZ-G in the inhibition of proinflammatory cytokines, it seems prudent to examine not only IL-1β but also TNF-α. However, the analysis for TNF-α has not yet been performed because we have not found a suitable primer for polymerase chain reaction in dogs.

In conclusion, LAZ-G effectively reduces hepatic dysfunction after major hepatic resection with inflow occlusion. Antilipid peroxidation is a useful approach to the study of major liver resection with warm ischemia. From the present study, we conclude that Lazaroid U-74389G may be clinically applicable in liver surgery such as extended hepatic resection and hepatic transplantation, which is associated with vascular occlusion.

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