1126

INHIBITION OF FREE RADICAL GENERATION AND IMPROVED SURVIVAL BY PROTECTION OF THE HEPATIC MICROVASCULAR ENDOTHELIUM BY TARGETED ERYTHROCYTES IN ORTHOTOPIC RAT LIVER TRANSPLANTATION[1]

PRAKASH N. RAO,[2] THOMAS R. WALSH, LEONARD MAKOWKA, TAO LIU, ANTHONY J. DEMETRIS,[3] RANDY S. RUBIN, JAMES T. SNYDER, HANS J. MISCHINGER, AND THOMAS E. STARZL

The Transplant Laboratories, Departments of Surgery, and Pathology, University Health Center of Pittsburgh, University of Pittsburgh; and the Veterans Administration Medical Center, Pittsburgh, Pennsylvania

The capacity of specifically targeted erythrocytes to inhibit free radical–mediated injury to the endothelial cell after cold preservation, and improve liver function was studied in two experimental models: An isolated perfused rat liver (IPRL) system and syngeneic orthotopic rat liver transplantation. In the IPRL model, livers were preserved in University of Wisconsin solution for 24 h at 4°C. At the end of the preservation period, livers were flushed with lactated Ringer's (control), immunoerythrocytes (IES), or blank intact erythrocytes prior to warm reperfusion for 2 h using an assanguinous Krebs-Henseleit buffer. Production of superoxide (O_2^-) anion during warm reperfusion in the IES-treated liver was reduced by 65% as compared with controls ($P<0.001$) and by 74% ($P<0.001$) when compared with blank erythrocyte–treated livers. Endothelial cell preservation, as assessed by levels of purine nucleoside phosphorylase (PNP), was much better in the IES-treated group ($P<0.001$) when compared with untreated livers.

Hepatocellular preservation was markedly improved in the IES-treated livers.

In the syngeneic liver transplantation model, livers were preserved in UW solution for 24 h at 4°C. Prior to implantation, livers were flushed with 5 ml of cold lactated Ringer's or immunoerythrocytes. Survival after three weeks was 60% in the IES-treated group and 30% in the untreated group. Survival in the IES-treated group was not significantly different from a control (no preservation) group.

IES-treated livers in both models demonstrated better endothelial cell integrity and ultimate liver function. IES treatment therefore appears to protect the hepatic microvascular endothelial cell from reperfusion injury and could prove to be an easy reproducible method of donor organ preparation after cold preservation.

Liver transplantation is now the therapy of choice for end-stage liver disease (*1*). Reperfusion injury following preservation is caused by toxic oxygen radicals (free radicals) localized at the microvascular endothelial cell (*2, 3*) and has deleterious biochemical and immunological consequences for the entire organ (*4, 5*). Free radical scavengers have been used in the past with mixed success (*6, 7*), unless they are delivered intracellularly, such as by liposomes (*8, 9*). Through the use of liposomes— or, for more specific targeting—immunoliposomes,

[2] Address correspondence to: Dr. Prakash N. Rao, Ph.D., Transplant Laboratories, University of Pittsburgh, 8200 Blood Bank Building, 203 DeSoto Street, Pittsburgh, PA 15261.

[3] Department of Pathology, University of Pittsburgh.

(10) an increase in intracellular concentration of antioxidants can be achieved with subsequent inhibition of free radical generation.[4] Intact red blood cells can prevent damage to the endothelial cell by hydrogen peroxide (H_2O_2) (11, 12), presumably due to their high levels of endogenous antioxidants (13). Targeting of these erythrocytes should in theory be able to inhibit intracellular superoxide generation following reperfusion after cold preservation. In our experiments specific targeting was accomplished by the conjugation of antibodies to factor VIII (endothelial cell–specific antigens) to the surface of freshly washed erythrocytes (14) (immunoerythrocytes [IES].[*] We tested the capacity of these IES to protect the endothelial cell in two experimental models: isolated perfused rat liver (IPRL) and orthotopic liver transplantation.

MATERIALS AND METHODS

Preparation of erythrocytes. Immunoerythrocytes were prepared by conjugating factor VIII antibodies, (ICN, Immunobiologicals No. 68–112) using chromium chloride as a conjugating agent (15). This method resulted in an average specific binding of 15%, as measured by ^{125}I protein-A. Blank erythrocytes were processed in a similar mannner, except for the conjugation of antibodies to their surface.

IPRL. Livers harvested from male Lewis rats (250–300 g) were preserved for 24 h in University of Wisconsin lactobionate solution at 4°C (16). At the end of the preservation period, the livers were flushed in a retrograde fashion through the infrahepatic IVC with 5 ml of cold lactated Ringer's (control n=12), IES solution (hematocrit 35–40%, n=10), or intact nonimmune erythrocytes (n=12). Warm reperfusion was carried out for 2 h at 37°C with an asanguinous Krebs-Henseleit buffer containing 2% bovine serum albumin.

OLTX. Sygeneic transplants were carried out using male Lewis rats as donors (200–250 g) and recipients (250–300 g). Livers were preserved in UW solution for 24 h at 4°C. Immediately prior to implantation, livers were flushed in a retrograde fashion (17) with 5 ml of cold lactated Ringer's (n=10) or IES solution (n=10). Orthotopic liver transplantation was carried out according to the method of Miyata (18). Three-week survival in these two groups was compared with that of a control group where transplants were carried out immediately after harvest.

Assays. Endothelial cell preservation was assessed by measuring effluent levels of purine nucleoside phosphorylase (PNP) (19). Free radical generation was analyzed by measuring levels of superoxide anion (20). Hepatocyte preservation was assessed by measuring transaminase levels (16) using a Technicon RA 500 autoanalyzer (Technicon Instruments, Tarrytown, NY).

Histology. Analysis of tissue by light microscopy was carried out by a staff pathologist in a blinded fashion.

Statistical analysis. Data are expressed as means ± SD. The statistical significance of difference between group means was analyzed by the Student's *t* test. The alpha level has been adjusted for multiple comparisons using Bonferroni's adjustment (21). The alpha level per comparison was $P<0.01$.

RESULTS

IPRL: superoxide generation. Reperfusion following preservation resulted in a burst of superoxide generation (Fig. 1) in all the three groups. Levels of superoxide anion at the end of 30 min of reperfusion were 0.67±0.31, 1.06±0.59, and 0.91± 0.47 nmol cytochrome c reduced/ml in untreated, IES-treated,

[*] Abbreviations: IES, immunoerythrocytes; IPRL, isolated perfused rat liver; PNP, purine nucleoside phosphorylase.

and blank erythrocyte-treated livers, respectively. As reperfusion continued, superoxide levels in untreated and blank erythrocyte-treated livers steadily increased to 1.32±0.39 and 1.75± 0.33 nmol cytochrome c reduced/ml, respectively. These levels were significantly higher ($P<0.001$ for both) than those observed in IES-treated livers (0.47±0.24 nmol cyto. c red./ml).

PNP levels. A steady rise in PNP levels was observed in all three groups, indicating increasing leakage of the cytosolic enzyme. The rate of leakage was most marked in control untreated livers. The PNP value at the end of 2 h of reperfusion in these livers was 182.17±79.34 mu/ml, which was significantly higher ($P<0.001$) than that observed in IES-treated livers of 84.80±26.03 milliunits/ml. The amount of PNP produced by the blank erythrocyte treated livers was 113.0±29.09 milliunits/ml, which was significantly lower than ($P<0.01$) that observed in untreated livers. PNP values at the end of reperfusion were not statistically different in the two erythrocyte-treated groups (Fig. 2).

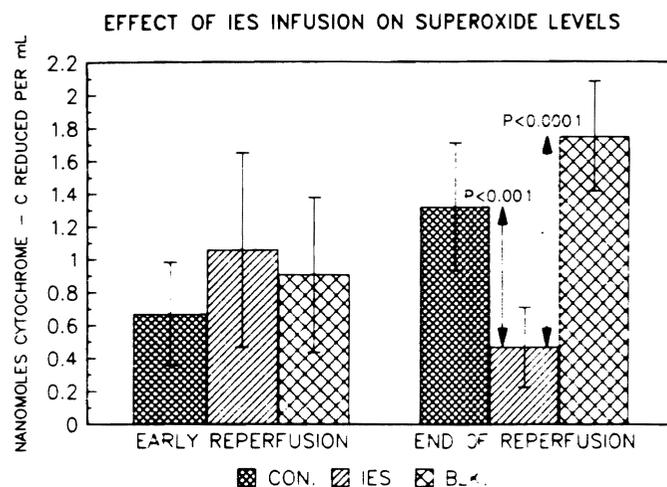EFFECT OF IES INFUSION ON SUPEROXIDE LEVELS

FIGURE 1. Reperfusion following preservation results in a burst of superoxide generation in all the three groups. As reperfusion progresses, superoxide generation is significantly reduced in the IES-treated livers when compared with the control untreated ($P<0.001$) or blank erythrocyte-treated livers ($P<0.0001$).
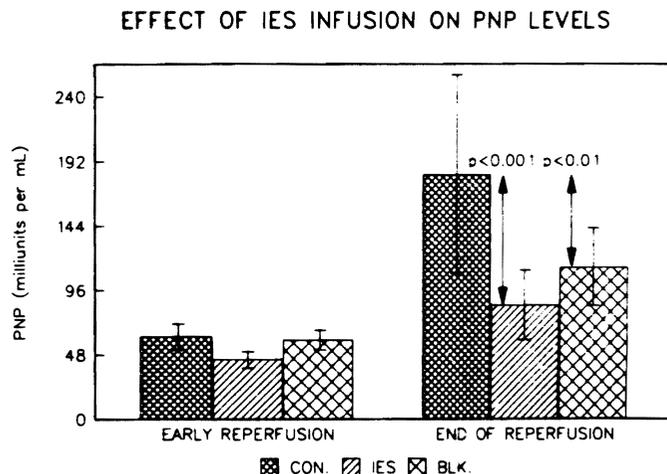
EFFECT OF IES INFUSION ON PNP LEVELS

FIGURE 2. Reperfusion following cold preservation was associated with a rise in PNP levels in all the three groups. Endothelial cell function was best preserved in the IES-treated livers where PNP levels were significantly lower ($P<0.001$) than those in control untreated livers.

SGOT and SGPT. In the untreated livers reperfusion was associated with a significant leakage of both transaminases into the effluent. SGOT and SGPT values at the end of 2 h were, respectively, 1056.42±650.45 and 1082.58±773.08 IU/ml. These values were significantly higher than those observed in IES-treated livers: 319.7±146.05 ($P<0.005$) and 319.6±253.12 ($P<0.01$) IU/ml. Transaminase levels in IES-treated livers were similar to those observed in blank erythrocyte–treated livers; 485.42±381.70 and 507.17±499.91 IU/ml, respectively (Figs. 3 and 4).

Bile production. Mean rates of bile production at the end of 30 min in untreated, IES-treated, and blank erythrocyte–treated livers were, respectively, 0.56±0.19, 0.51±0.19, and 0.37±0.16 ml/min/g of tissue. As the perfusion progressed, bile production decreased 42% in the control (untreated) ($P<0.01$) and 33% in the blank erythrocyte–treated livers ($P<0.0001$), as compared with a 4% decline at the end of the reperfusion in IES-treated livers (Fig. 5).

OLTX. Eleven of fourteen animals (78.57%) survived 3 weeks

after OLTX in the control, nonpreserved group. This dropped significantly to 30% (3 of 10) ($P<0.001$) for the rats that received livers not treated after 24 h of cold preservation. Treatment of livers with IES prior to reperfusion resulted in a increase of the survival to 60% (6 of 10), which was higher than the untreated UW preserved group and similar to the survival obtained in fresh nonpreserved livers (Fig. 6).

Histology. IES treated livers in both experiments appeared normal on gross examination and morphologically on light microscopy (Fig. 7).

DISCUSSION

Reperfusion following cold preservation results in significant injury to the microvascular endothelial cell, as evidenced by an increasing leakage of purine nucleoside phosphorylase. This is in keeping with the finding of Caldwell Kenkell et al. (*22*), and

EFFECT OF IES INFUSION ON BILE PRODUCTION

FIGURE 5. During early reperfusion, bile production is similar in control (untreated), IES-treated, and blank erythrocyte–treated livers. As reperfusion progresses bile production is significantly reduced in the untreated livers ($P<0.01$) and blank erythrocyte–treated group ($P<0.0001$) as compared with the IES-treated group.
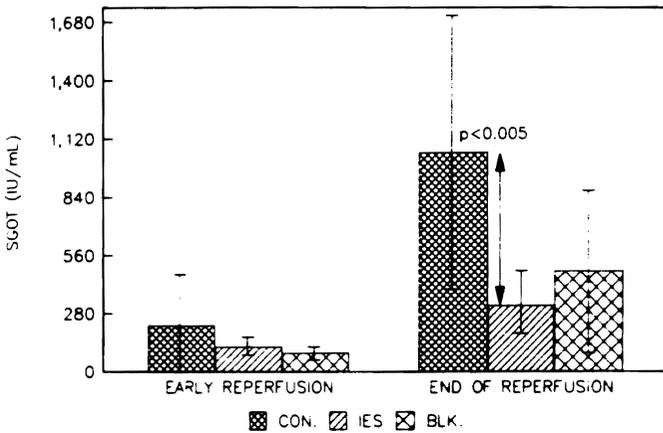
EFFECT OF IES INFUSION ON SGOT LEVELS

FIGURE 3. In the untreated livers reperfusion results in significant leakage of SGOT into the effluent. IES treatment results in protection of the liver, as evidenced by significantly lower levels ($P<0.005$) in this group.
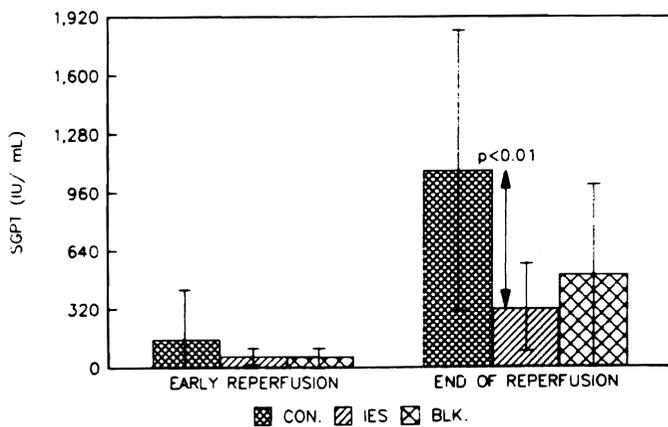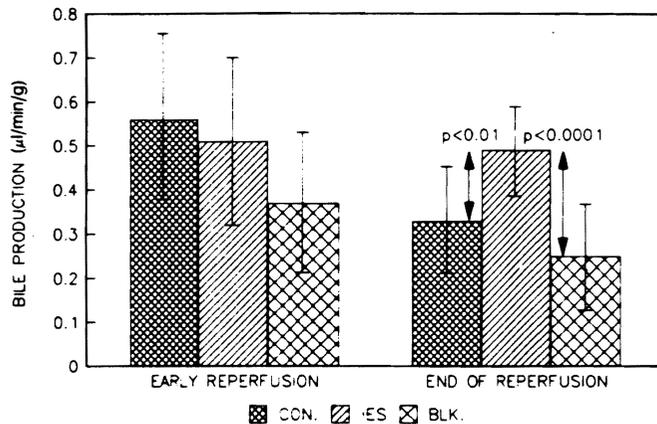
EFFECT OF IES INFUSION ON SGPT LEVELS

FIGURE 4. Control untreated livers demonstrated increasing hepatocyte injury, as reflected by high levels of SGPT in the effluent. These values were significantly higher ($P<0.001$) than those observed in the effluent of IES treated livers.

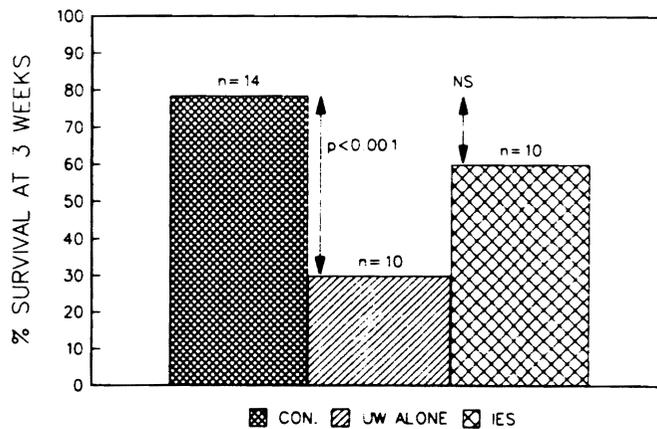EFFECT OF IES INFUSION ON SURVIVAL IN OLTX

FIGURE 6. Eleven of fourteen animals (78.57%) survived 3 weeks after OLTX in the control (nonpreserved) group. This dropped significantly to 30% (3 of 10) ($P<0.001$) for the rats who received livers not treated after 24 h of cold preservation. Treatment of livers with IES prior to reperfusion resulted in an increase of the survival to 60% (6 of 10), which was higher than the untreated UW preserved group and similar to the survival obtained in fresh nonpreserved livers.
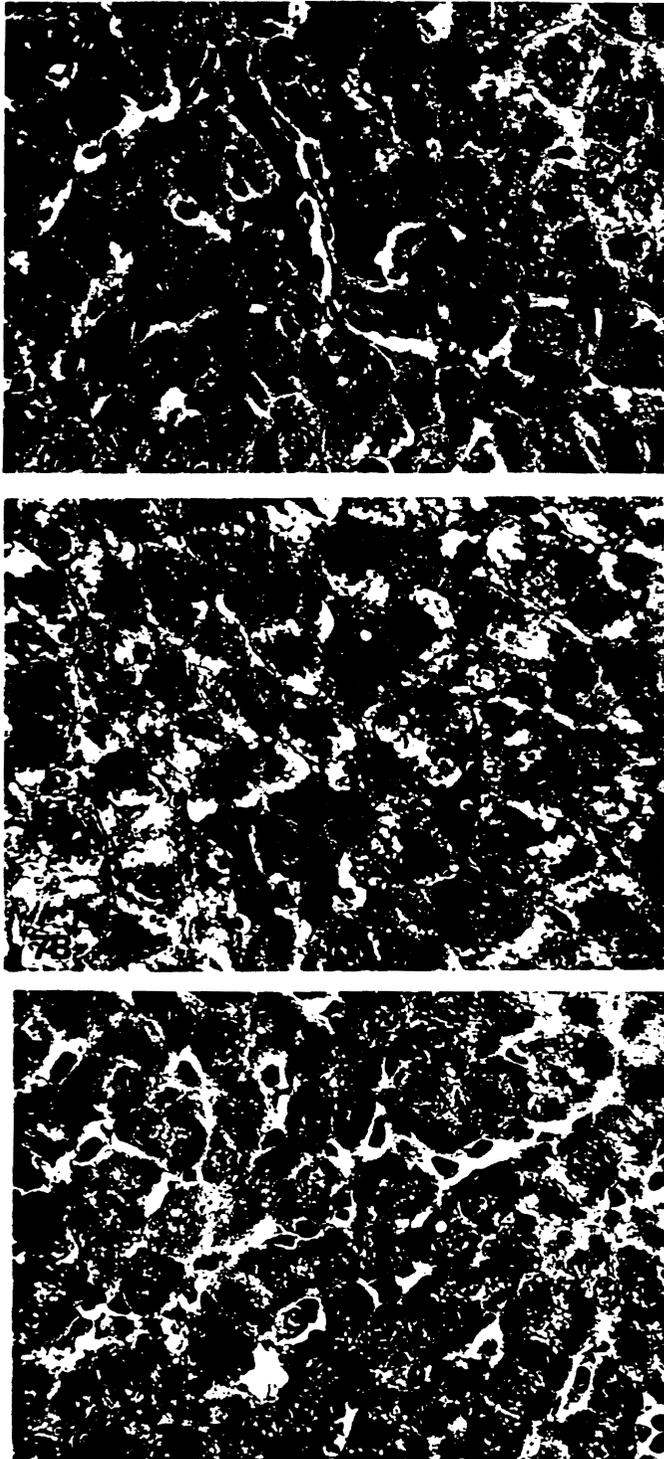
FIGURE 7. (A) Control liver: no preservation. Normal hepatocellular architecture is preserved except for occasional steatosis. Within the microvasculature (sinusoids) multiple normal appearing endothelial and kupffer cell nuclei can be seen. There is a minimal debris within the microvasculature (H & E; original magnification: ×164). (B). Twenty-four-hour-preserved livers (untreated). In contrast to the treated livers there is uniform destruction of hepatocytes and obliteration of the sinusoids. What sinusoids can be found have minimal or no endothelial or kupffer nuclei. Nuclei from perisinusoidal cells are seen free within the lumen of the microvasculature. (H & E; original magnification: ×164). (C) Twenty-four-hour-preserved livers treated with immunoe-rythrocytes. After twenty four hours of cold preservation these livers were loaded with immunoerythrocytes before revascularization. In general hepatocellular architecture is maintained much like

Thurman et al. (1988) (*23*), who also demonstrated increasing injury to the microvascular endothelial cell on reperfusion following cold ischemia. Contrary to McKeown's finding (*24*), this injury was manifest on warm reperfusion rather than during cold ischemia. (Fig. 2). Toxic oxygen radicals liberated at reperfusion have been implicated in the injury to the endothelial cell (*25, 26*). In our experiments, warm reperfusion following cold preservation resulted in a significant burst of superoxide generation (Fig. 1) in both the nontreated and treated livers early in reperfusion, followed by a steady decrease in the IES-treated livers. The lag period of superoxide inhibition in the IES-treated livers can be expected, as endocytosis necessary for the delivery of the antioxidants contained within the IES will occur after the liver has warmed to 37°C. Indeed, at low temperature, almost 90% of bound immunoliposomes can be washed away (*10*). As reperfusion progresses, however, a significant inhibition of superoxide generation is achieved in the IES-treated livers. This was not observed in blank erythrocyted-treated livers, which is in keeping with the findings of Tsan and White, who observed that intact erythrocytes could inhibit the generation of H_2O_2 outside the cell but were unable to inhibit the intracellular generation of superoxide. This indicates a role for the intracellular delivery of antioxidants by IES in the amelioration of free radical–induced endothelial cell injury.

Reperfusion injury to the microcirculation eventually results in hepatocellular injury (*19, 24*). This is confirmed by the significantly elevated levels of SGOT and SGPT in the effluent of control untreated livers (Figs. 3 and 4). Protection of the microvascular endothelial cell by immunoerythrocytes appears to protect the hepatocyte as evidenced by significantly lower levels of transaminase leakage in these livers. A limited amount of protection to the hepatocyte was also accorded by treatment with blank erythrocytes. Bile production is considered as an index of ATP generation (*27, 34*)—hence postoperative organ viability. Reperfusion injury to the endothelial cell results in a significant decrease in bile production in control untreated livers. Treatment with immunoerythrocytes appears to inhibit this injury and maintain optimal liver function, as reflected by the maintenance of rate of bile production by these livers.

To test the clinical applicability of the IES we evaluated the protective capacity of the immunoerythrocytes in an orthotopic liver transplantation model. Rats that received livers that were preserved for 24 h in cold UW solution demonstrated a three-week survival rate of 30%. Most of the recipients died early in the posttransplant period of graft nonfunction. Loading the graft with immunoerythrocytes just prior to implantation increased survival two-fold to 60%, which was similar to that observed in fresh nonpreserved livers. Treatment with IES appears to have a role in preserving organ viability following cold preservation in UW solution. Livers appeared normal on gross examination and light microscopy. Immunoerythrocyte treatment did not result in sludging of sinusoids in the perfusion or the transplant experiment, while the untreated preserved livers often swelled considerably.

The vascular endothelium is a primary target for rejection reactions (*35, 36*), and T lymphocytes play a major role in cell mediated allograft rejection (*37*). It has been demonstrated

in the nonpreserved group. In particular though the sinusoids and perisinusoidal cells have normal appearing architecture with nuclei intact and with minimal if any debris within the lumen of the microvasculature (H & E; original magnification: ×164).

that antioxidant therapy can inhibit the proliferation of T lymphocytes, probably through an inhibition of interleukin-2 receptor expression (38, 40). Our experiments demonstrate that pretreatment of rat livers with immunoerythrocytes targeted to the microvascular endothelial cell can prevent oxidant-induced reperfusion injury—and, therefore, potentially T lymphocytes proliferation. In the clinical situation donor organ pretreatment with immunoerythrocytes could prove to be an easy, recipient-specific method to protect the vascular endothelium from post-ischemic reperfusion injury.

REFERENCES

1. Maddrey WC. Van Thiel DH. Liver transplantation: an overview. Hepatology 1988; 8: 948.
2. McCord JM. Oxygen derived free radicals in post-ischemic tissue injury. N Engl J Med 1985; 312: 159.
3. Southard JH, Marsh DC, McAnulty, JF, Belzer FO. The importance of oxygen-derived free radical injury to organ preservation and transplantation. Transplant Proc 1987; 19: 1380.
4. Galumbeck MA, Sanfilippo FP, Hagen PO, Seaber AV, Urbaniak JR. Inhibition of vessel allograft rejection by endothelial removal. Ann Surg 1987; 206: 757.
5. Forbes RDC, Guttmann RD, Gomersall M, Hibberd J. A controlled serial ultrastructural tracer study of first set cardiac allograft rejection in the rat. Am J Pathol 1983; 3: 184.
6. Crapo JD, Delong DM, Stostrom K, Hasler GR, Drew RT. The failure of aerosolized superoxide dismutase to modify pulmonary oxygen toxicity. Am Rev Resp Dis 1977; 115: 1027.
7. Pyatak PS, Abuchoski A, Davis FA. Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulating life and antiinflammatory activity. Res Commun Chem Pathol Pharmacol 1980; 29: 113.
8. Tsan MF, Davis EH, Del Vecchio PJ, Rosano CL. Enhancement of intracellular glutathione protects endothelial cells against oxidant damage. Biochem Biophys Res Commun 1985; 127: 270.
9. Freeman BA, Turrens JF, Mirza Z, Crapo JD, Young SL. Modulation of oxidant lung injury by using liposome entrapped superoxide dismutase and catalase. Fed Proc 1985; 44: 2591.
10. Huang A, Kennel SJ, Huang L. Interaction of immunoliposomes with target cells. J Biol Chem 1983; 258: 14034.
11. Van Asbeck BS, Hoidal J, Vercelloti AM, Schwartz BA, Moldow EF, Jacob HS. Protection against lethal hyperoxia by tracheal insufflation of erythrocytes: role of red cell glutathione. Science 1985; 227: 756.
12. Tsan MF, White JE. Red blood cells protect endothelial cells against H_2O_2 mediated but not hyperoxia induced damage. Proc Soc Exp Biol Med 1988; 188: 323.
13. Beutler E. Red cell metabolism: a manual of biochemical methods. New York: Grune & Stratton, 1984.
14. vd Kwast TH, Stel HV, Cristen E, Bertina RM, Veerman ECI. Localization of factor VIII procoagulant antigen: an immunohistological survey of the human body using monoclonal antibodies. Blood 1986; 67: 222.
15. Muzykantov VR, Sakharov DV, Domogatsky SP, Goncharov NV, Danilov SM. Directed targeting of immunoerythrocytes provides local protection of endothelial cells from damage by hydrogen peroxide. Am J Pathol 1987; 128: 276.
16. Ontell SJ, Makowka LM, Ove P, Starzl TE. Improved hepatic function in the 24 hour preserved rat liver with UW lactobionate solution and SRI 63-441. Gastroenterology 1988; 95: 1617.
17. Liu T, Walsh TR, Nalesnik M, Makowka LM, Starzl TE. Improved preservation of the rat liver for orthotopic liver transplantation using UW-Lactobionate and retrograde reflushing. Surgery (in press).
18. Miyata M, Fisher JH, Fuhs M, Isselhard W, Kasai Y. A simple method for orthotopic liver transplantation in the rat: cuff tech-

19. Rao PN, Walsh TR, Makowka LM, Rubin R, Snyder JT, Starzl TE. Purine nucleoside phosphorylase (PNP) a new marker for ischemia reperfusion oxidative liver injury. Hepatology 1988; 11: 193.
20. McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244: 6049.
21. Milliken GA, Johnson DE. Analysis of messy data; vol 1. Designed experiments. Belmont, CA: Wadsworth, 1981.
22. Caldwell-Kenkel J, Thurman RG, LeMasters JJ. Liver preservation for transplantation: early loss of nonparenchymal cell viability. Transplantation 1988; 45: 834.
23. Thurman RG, Marzi I, Seitz G, Thies J, LeMasters JJ, Zimmerman F. Hepatic reperfusion injury following orthotopic liver transplantation in the rat. Transplantation 1988; 46: 502.
24. McKeown CMB, Edwards V, Phillips MJ, Harvey PRC, Petrunka CN, Strasberg SM. The critical injury in cold preservation of liver allografts in the rat is sinusoidal lining cell damage. Transplantation 1988; 46: 178.
25. Parks DA, Bulkley GB, Granger DN. Role of oxygen free radicals in shock, ischemia and organ preservation. Surgery 1983; 94: 428.
26. Warren JS, Ward PA. Review: Oxidative injury to the vascular endothelium. Am J Med Sci 1980; 292: 97.
27. Abouna GM. Pig liver perfusion with human blood: the effect of preparing and flushing the liver with various balanced solutions on its subsequent viability and function. Br J Surg 1968; 55: 761.
28. Turner MD, Alican P. Successful 20-hour storage of the canine liver by continuous hypothermic perfusion. Cryobiology 1970; 6: 293.
29. Lee D, Holland RK. Improved performance of the isolated rat liver when perfused with purified bovine serum albumin. Transplantation 1979; 27: 384.
30. Kamada N, Calne RY, Wight DGD, Lines JG. Orthotopic rat liver transplantation after long-term preservation by continuous perfusion with fluorocarbon emulsion. Transplantation 1980; 30: 43.
31. Lee D, Clark DG. Influence of ischemic time on the production of bile by perfused rat liver. Cryobiology 1977; 14:37.
32. Hanks JB, Meyers WC, Wellman CL, et al. The effect of cell-free and erythrocyte-containing perfusions in rat liver. J Surg Res 1980; 29: 149.
33. Eiseman B, Lieman DS, Roffucci F. Heterologous liver perfusion in treatment of hepatic failure. Ann Surg 1965; 162: 329.
34. Kamiike W, Nakahara M, Nakao K, et al. Correlation between cellular ATP level and bile excretion in the rat liver. Transplantation 1985; 39: 50.
35. Demetris AJ, Lasky S, Vantheil DH, Starzl TE, Whiteside T. Induction of DR/IA antigens in human liver allografts. Transplantation 1985; 40: 504.
36. Pober JS, Collins T, Gimbrone MA, Libby P, Reiss CS. Inducible expression of class II major histocompatibility complex antigens and the immunogenecity of vascular endothelium. Transplantation 1986; 41: 141.
37. Pober JS, Gimbrone MA, Collins T, et al. Interactions of T-lymphocytes with human vascular endothelial cells: role of endothelial cells surface antigens. Immunobiology 1984; 168: 483.
38. Pober JS, Gimbrone MA, Cotran RS, et al. Ia expression by vascular endothelium is inducible by activated T cells and by human gamma interferon. J Exp Med 1983; 157: 1339.
39. Chaudhari C, Clark IA, Hunt NH, Cowden WB, Ceredig R. Effect of antioxidants on primary alloantigen induced T cell activation and proliferation. J Immunol 1986; 137: 2646.
40. Chaudhari C, Hunt NH, Clark IA, Ceredig R. Antioxidants inhibit proliferation and cell surface expression of receptors for interleukin 2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and Ionomycin. Cell Immunol 1988; 115: 204.