Etiology of Rising Perfusion Pressure in Isolated Organ Perfusion

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ORGAN perfusion has been a tool of physiological research for over a century. As early as 1911, Alexis Carell described the preservation of human and animal tissues for implantation by hypothermic storage, some with evidence of viability as well as structural integrity. Investigators in this field, however, have experienced difficulty with rising perfusion pressure, tissue edema, and weight gain, with decreased flow and impaired or no function on reimplantation of the organ. A method of extracorporeal perfusion of the kidney, using filtrated plasma, a pulsatile pump, and membrane oxygenator has been described, and a large experience with successful canine preservations has been reported from this laboratory.[6] A unique feature of our method has been the high flow rate, without a rise in perfusion pressure and the absence of tissue edema. Initially, the results obtained were largely empirical, and the exact reason for the avoidance of a rise in perfusion pressure was not known. Recently, a systematic analysis of each aspect was undertaken, and the experience obtained from 120 long-term animal and seven human renal perfusions forms the basis of this report.

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The role of platelet and blood cell aggregates, if whole blood is used, was well established [8, 9, 31] and led to the initial selection of plasma as the perfusate. If the plasma was diluted in a ratio of 1:3 of electrolyte solution, and the osmolarity was maintained between 300 and 340 milliosmoles, edema of the kidney was minimal and perfusion pressure rose only slightly over a 24-hour period of perfusion. However, on reimplantation of these organs, function was greatly impaired and none of the animals survived autotransplantation with immediate contralateral nephrectomy. When undiluted plasma was used, there was a recurrence of the rising perfusion pressure, severe edema, and tissue destruction. This was ameliorated, but not eliminated, by the use of a pulsatile pump and a membrane oxygenator.* Under these circumstances, conventional microscopic studies showed no evidence of thrombi. However, when frozen sections were taken of the perfused kidney, fat stains revealed multiple small emboli in the renal arterioles, and fat droplets in the tubules and intratubular cells (Figs. 1 and 2). It appeared obvious that the rising perfusion pressure was due to blockage of the vessels by lipid components liberated into the perfusate by denaturation. We therefore performed preliminary denaturation of the lipoproteins by freezing and quick thawing. This was done by storing the plasma at minus 20° C. for 12 to 24 hours, followed

* Medical Monitors, 10801—120th Avenue, Kirkland, Washington.

TABLE 1. *Total Lipids Content in Plasma and Perfusate*

Sample	Lipid mg./100 ml.	Phospholipid mg./100 ml.	Neutral mg./100 ml.	Lysolecithin mg./100 ml.	Sphingomyelin mg./100 ml.	Lecithin mg./100 ml.	Phosphatidyl-ethanolamine mg./100 ml.
Plasma	285–330	200–250	36–47	8–11	9–12	157–187	3.3–4.7
Perfusate	197–215	144–167	32–42	5.6–7.7	6.1–8.4	118–143	2.5–3.6
% loss (average)	33%	30%	11%	35%	31%	24%	23%

by rapid thawing in water at 60 to 70° C. Particular care was taken not to warm the plasma to a temperature higher than 38° C. With thawing, a flocculation appeared in the plasma, which could be removed by serial filtration through micropore filters ** with pore diameters of 1.2, 0.45, and 0.22 mμ.

The residue on the filter paper was then analyzed by thin layer chromatography. It consisted primarily of phospholipids, namely lecithin, sphingomyelin, and lysolecithin. Analysis was done by the following method:

Filter paper containing residues from various filtrations was cut into small pieces and extracted in 100 ml. of 2:1 chloroform: methanol at 37° C. for 1 hour. Plasma and perfusate were extracted with the same solvents except that 30 times the volume was used. Extracts were then filtered through a Whatman #43 filter paper, and washed according to the procedure of Folch, Lees and Sloane-Stanley.[14] Total lipids were separated into phospholipids and neutral lipids by silicic acid column chromatography. The neutral lipids were eluted with 40 ml. of redistilled chloroform, the phospholipids were collected with 40 ml. of 5:4 chloroform:methanol, followed by 20 ml. of methanol. The total amount of phospholipids and neutral lipids was analyzed for phosphorus content, according to the spectophotometric method of Bartlett.[3] The phospholipids were separated by thin layer chromatography, using an 18 cm. length of silicic acid-gell precoated plate. To detect the components of the phospholipids, the plate was sprayed with 2-7 dicloroflurescein, and bands of lysolecithin, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were separated. Comparison of total lipid contents of the plasma and perfusate showed that the perfusate contained 30–35% less phospholipids, which were primarily lecithin, sphingomyelin, and lysolecithin. The loss of neutral lipids was less than 10% (Table 1).

Before the last filtration through the 0.22 mμ. micropore filter, the following substances were added to the perfusate per liter of ACD collected plasma. Dextrose—50%, 5 ml.; Insulin—80 units; Hydrocortisone—100 mg.; Penicillin—200,000 units; Magnesium Sulfate—8 mEq.; and Phenolsulfonphthalein—12 mg.

Perfusion of the kidney with this filtered plasma completely eliminated the rising perfusion pressure. Fat stains showed that the previously seen lipid particles were completely eliminated. After 72 hours of perfusion, kidney function was proven by reimplantation with immediate contralateral nephrectomy. The kidneys appeared normal and urine production usually occurred within 5 minutes after release of the vascular clamps. Postoperatively, all animals produced copious amounts of urine. Blood urea nitrogen rise was noted in all animals, especially in the 72-hour group, but all returned to normal within 2 weeks in the 24-hour group and within 5 weeks in the 72-hour group.[6]

** Millipore Corporation, Bedford, Massachusetts 01730.

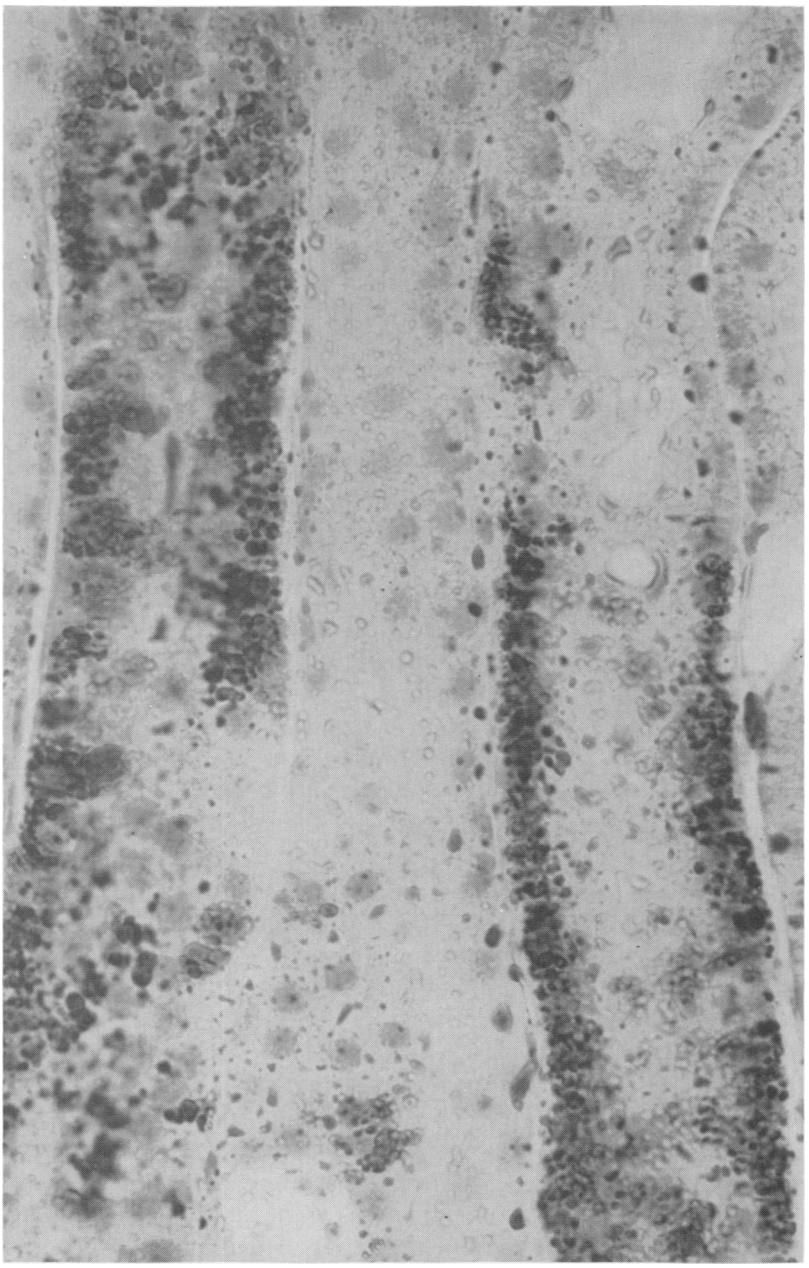

FIG. 1. Fat droplets in tubular and intratubular cells. (Oil red O H. P.)

Animals in both the 24 and 72-hour groups were followed for periods beyond 6 months, and studies at that time revealed normal renal function and renal architecture with no evidence of hypertension.[4]

Subsequently, our first human cadaver kidney preservation was done, and survival was proven by reimplantation after a period of 17 hours preservation outside the body.[7] However, the period of total anoxia was still considerable, as the kidney was obtained in one hospital, and required transportation to the preservation unit in other hospital. The perfusion unit was then modified into a transportable unit, with its own power supply, so that it could be taken

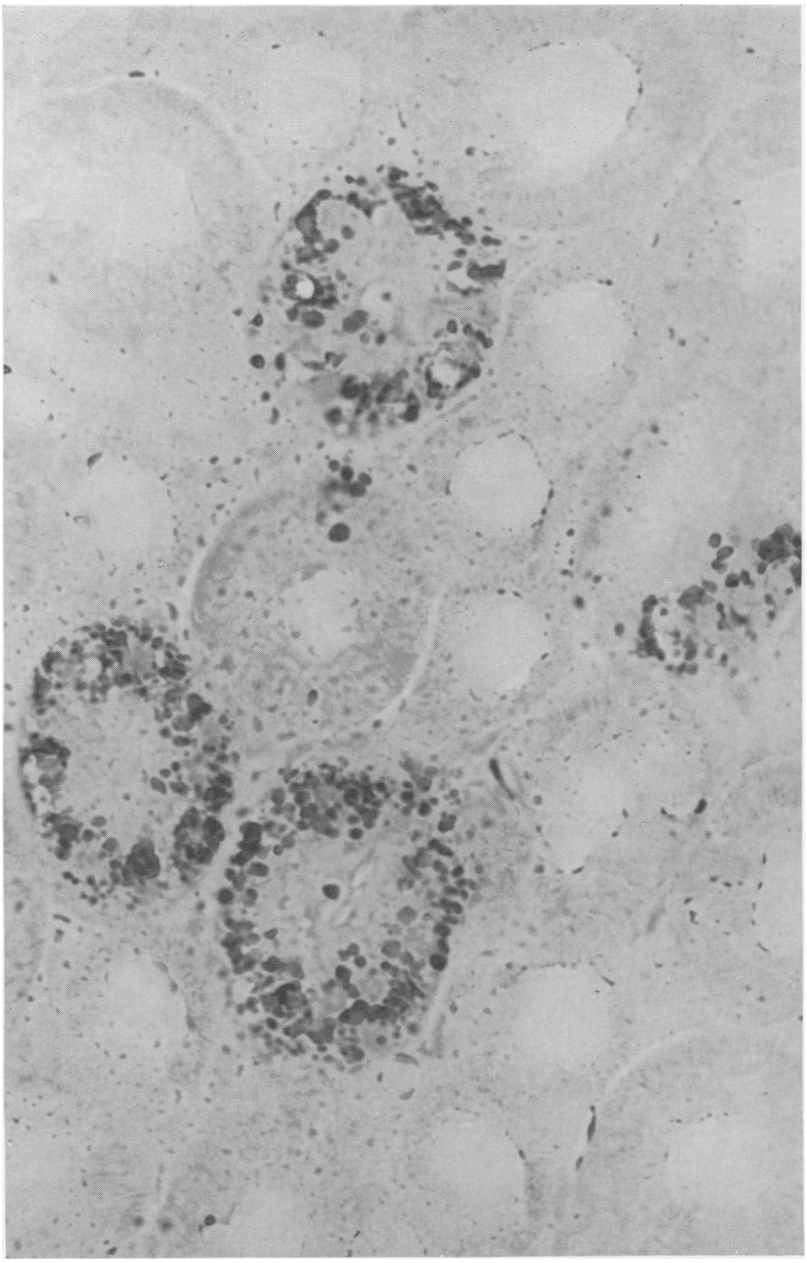

FIG. 2. Fat droplets in tubular and intratubular cells. (Oil red O H. P.)

into the operating room where the donor kidney was removed, and transported if necessary. The anoxia time was thus decreased to a minimum and consisted only of the warm anoxia time between the death of the donor and removal of the kidney and a short period of cold anoxia time required for reimplantation of the organ.

Figures 3 and 4 show a photograph and diagrammatic explanation of the portable perfusion unit,* which, at the present time, is used for storage of human cadaver kidneys.

* Built by Mr. Chester Truman, Research and Development Laboratory, University of California San Francisco Medical Center.

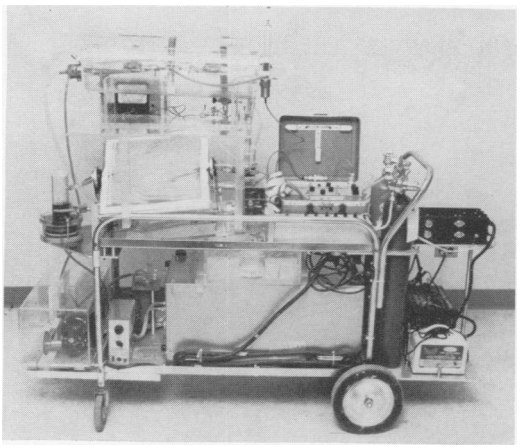

FIG. 3. Photograph of transportable renal preservation unit.

We have now stored seven human cadaver kidneys, and six of these have functioned after periods of perfusion ranging from 4 to 17 hours. One kidney, which was perfused for 4 hours, was lost because of a technical error. This transplant was done in an unprepared uremic recipient, and although the kidney produced urine after reimplantation, postoperative bleeding and compression by hematoma made it necessary to remove the kidney on the fifth postoperative day. However, the other kidney from the same cadaver donor was successfully transplanted into another recipient after having been on perfusion for more than 8 hours.

Perfusion has permitted transplantation of cadaver kidneys under circumstances when it would not have been possible, as in bilateral Wilms' tumor.[11]

The perfusate for the human perfusions was identical to that used in the animal experiments, except that the plasma was obtained from the blood bank as frozen plasma, AB+. Filtration of the plasma and the addition of the previously mentioned substances was done at the time of notification of a potential donor.

Discussion

The occurrence of fat emboli in extracorporeal circulation has been recognized for many years. Owens *et al.*[26, 27] observed widespread fat embolization in animals perfused with a film oxygenator, but reported no pathologic findings when the membrane lung was inserted into this same extracorporeal circuit. They concluded that fat molecules may be separated from lipoproteins at the blood-gas interphase and then coalesced to form lipid aggregates large enough to cause histologically-recognizable emboli. Lee *et al.*[20] reported the formation of chylomicron aggregates and free fat globules up to 30 μ in size, following prolonged recirculation of plasma in bubble, film, and disc oxygenators. Wright *et al.*[32] confirmed the observation that fat globulemia occurred in all experiments in which blood or plasma was recirculated through a screen oxygenator. In their studies, the phenomenon could be eliminated by the interposition of a Teflon® membrane, between the blood and gas interphase. However, although the occurrence of fat emboli appeared to be diminished when a membrane oxygenator was used, fat emboli were still noticed in our experiments after prolonged perfusion.

Nearly all of the lipid components in blood plasma are combined with proteins, and the soluble lipoproteins are responsible for the transport of lipids in blood.[16] There are at least three major groups of lipoproteins present in the plasma of mammals. These are the high density lipoproteins, the low density lipoproteins, and the chylomicrons. Certain factors effect the stability of lipoproteins in plasma, mostly in the low density group.[18] Lipoproteins are readily damaged by conditions that are usually hazardous to plasma proteins, such as extremes of pH, and, in some cases, ionic strength, heat, freezing, the presence of ethanol (except at low temperature), and exposure to interphases such as gas-water or air-water. All of these agents tend to disrupt the complex, consisting of an aggregate of mixed lipids stabilized and limited by a specific peptide chain. This

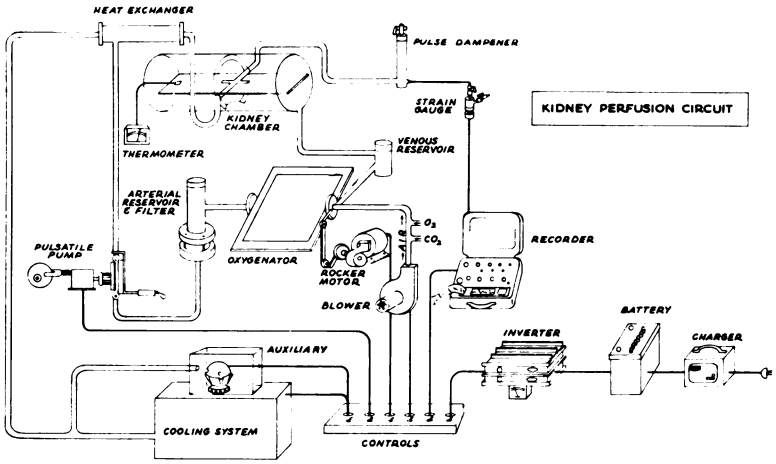

FIG. 4. Diagram of transportable renal preservation unit.

leads to aggregation of the lipids into larger particles, which greatly increases the turbidity of the solution. By deliberate preliminary denaturation of the lipoproteins and subsequent filtration through micropore filters, we were able to remove the lipid aggregates, thus obtaining a perfectly clear plasma solution (Fig. 5). Our studies have shown that only 30–35% of the lipid components were removed (Table 1). This probably consisted primarily of the low density lipoprotein group because the high density lipoproteins have a higher proportion of peptide to lipid molecule linkage and thus have a greater stability to temperature variations. It has been shown by Oncley[25] that the low density lipoproteins make up 40% of the total phospholipids in plasma. With preliminary denaturation and subsequent filtration, we were able to remove 75–90% of the phospholipids, which we believe were primarily in the low density group, thus eliminating the problem of lipid aggregates. Even in our 72-hour perfusions, no fat emboli could be found after this preliminary filtration. It is our opinion that the gentleness of the perfusion and the presence of a membrane oxygenator prevented further denaturation of the more stable residual lipoproteins.

Cort *et al.*,[10] studying kidney cortex slices in *in vitro* experiments, suggested that hypothermia alone produces cellular swelling. This was confirmed by Enerson[12, 13] in studies on rat diaphragms. Enerson also showed that the total tissue gain was only 5% between normothermic conditions and hypothermia at 7° C. He then showed that although hypothermia alone produces a minimal increase in cellular volume, the combination of hypoxia and hypothermia produces a much greater and more significant cell swelling. Figure 6 graphically demonstrates the vicious cycle which occurs in isolated organ perfusion when micro-emboli occlude the capillary circulation, resulting finally in cellular swelling and tissue destruction. Tissue edema and increase in organ weight of more than 5%, after perfusion, is indicative of tissue destruction and results in cell death. Tissue edema was not observed in our experiments, and for this reason, long-term observation in our animal experiments revealed no permanent damage.

The choice of a pulsatile flow instead of nonpulsatile flow was based on the studies of several investigators,[23, 24] which, in general, revealed a significant increase in perfusion pressure and vascular resistance in response to nonpulsatile flow. Only one dog out of a group of four survived after his kidney was maintained on hypothermic extracorporeal perfusion for 24 hours, with nonpulsatile flow. Initial function, after reimplantation, was much worse as compared

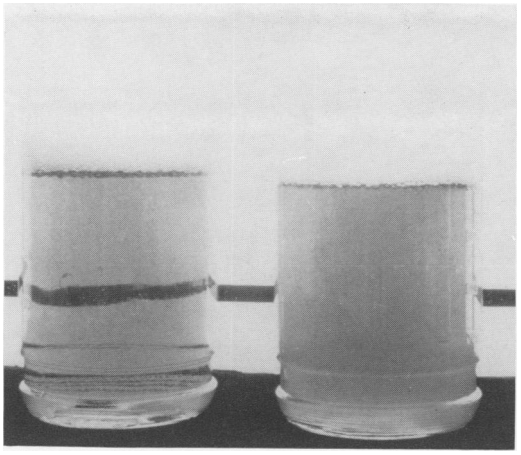

FIG. 5. Appearance of plasma before (right) and after (left) filtration. Note marked turbidity of unfiltered plasma (right).

to the group of dogs in which the kidney was perfused with pulsatile flow over 24 hours. In addition, this dog's blood urea nitrogen returned to normal much later than in the pulsatile group. Interestingly enough, this dog is now 1 year postoperative and has normal renal function, suggesting that no permanent damage was produced. We believe that pulsatile flow produces better cortical perfusion and that it is essential for preservation over extended periods of time.

Although the pH is maintained at physiologic or slightly alkaline conditions (7.40 to 7.55), we have noted that the organ will still survive even if the pH is more alkaline under these hypothermic conditions. In the animal experiments, occasional kidneys have survived after 24 hours, with pH's in the range of 7.8 to 7.9. In our experience with human kidneys, however, we have kept the pH as close as possible to 7.5, which is easily controlled by adding small amounts of CO_2 to the air blowing through the membrane oxygenator.

In our early experiments we used 100% oxygen in the membrane oxygenator and found that the oxygen consumption by the renal tissues was extremely small.[5] We noticed that when air was used in the membrane oxygenator, enough oxygen was available as the oxygen content and the pO_2 of the venous affluent appeared to be well above safe values. If the temperature of the kidney was raised, further drop of the venous pO_2 was observed, thus proving that the organ was able to extract more oxygen if needed. The value of high oxygen tension in organ preservation has been established by many investigators,[1, 21] however, it is still uncertain if this function is due to more of a metabolic inhibition than to direct improved oxygenation of the tissues. In our experiments, when high flow rates were maintained, oxygenation appeared to be adequate, even if the pO_2 was maintained within normal limits. It appeared to us that the metabolic inhibition for prolonged periods of time might actually be damaging to tissues, and for this reason we have deliberately kept the pO_2 in the normal range of around 150 mm. Hg measured at 37° C.

The additives to the perfusate (insulin, cortisone, etc.) were chosen on an entirely empirical basis and further studies are required to show their necessity. It has been suggested that calcium acts as a membrane stabilizer.[17] Because of the use of citrate in our perfusate, no calcium is available to the tissues, and the substitution of magnesium might counteract the absence of calcium.[2] In addition, elevations of serum magnesium appear to be characteristic of hibernation. Magnesium values during deep hibernation in the hedgehog were 170% higher than those before the onset of sleep.[29] However, insulin also appears to be required. Suomalainen[30] showed that the subcutaneous injection of magnesium solutions in hedgehogs, and subsequent refrigeration of the animals produced a condition of magnesium anesthesia different from natural hibernation. If subcutaneous injections of insulin and magnesium were given before transfer to an icebox, however, the animals then went into a cold-blooded state which closely resembled natural hibernation. In addition, the ad-

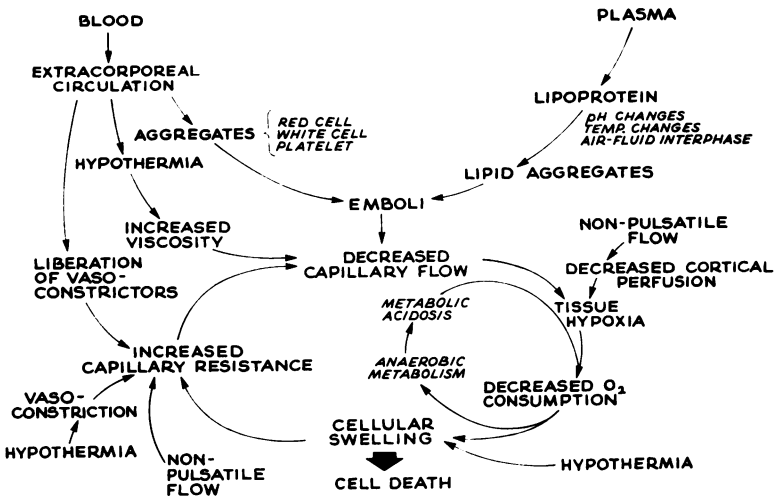

FIG. 6. Etiology of cellular swelling during isolated organ perfusion.

ministration of magnesium facilitates experimental hypothermia.[19, 28]

Penicillin was added because of its nontoxic properties and with good surgical aseptic technic, infection has never been a problem in the animal or human preservation experiments. Steroids were added because of their theoretical advantage as tissue stabilizers.[22] Dextrose was used on the basis of the excellent work reported by Folkman *et al.*[15] We continue to add this to our perfusate, although even after 72 hours of perfusion we have been unable to show a definite utilization of glucose. Probably the organ is at such a low temperature that glucose is barely used. The elimination of glucose, or perhaps the substitution of fructose, is presently under investigation. Phenosulfonphthalein is used as a pH indicator, and is of great value as a rough estimate of pH during perfusion.

Although it would be a practical advantage to substitute a stable synthetic solution instead of plasma for the perfusion fluid, we believe that homologous plasma is necessary for maintenance of good organ viability. Our experiments with plasma substitutes have been unrewarding. Although perfusion appeared to be adequate, viability of the organ was always greatly impaired after reimplantation and immediate contralateral nephrectomy, even after perfusion periods of only 24 hours.

In the animal experiments, we have limited the period of perfusion to 72 hours. We feel that this is adequate for tissue typing and preparation of the recipient, and in human renal preservation cases, usually no more than 12 hours are required. Further work is in progress to improve the preservation over this time period of 72 hours, rather than to prolong the total period of preservation.

Summary

Fat emboli appear to be the principal causative agent for rising perfusion pressure and tissue edema in isolated organ perfusion in which whole blood or plasma is used. Such aggregates produce tissue hypoxia which sets up a vicious cycle, resulting in tissue edema and cell death. Preliminary denaturation of the lipoproteins and removal of the aggregates by microfiltration has resulted in elimination of rising perfusion pressure. A transportable perfusion circuit, which utilizes a membrane oxygenator, pulsatile pump, and hypothermia has been used successfully in human cadaver transplants as well as in animal experiments.

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References

1. Ackermann, J. R. W. and Barnard, C. N.: Successful Storage of Kidneys. Brit. J. Surg., **53**:525, 1966.
2. Adelman, W. J., Jr.: The Effect of External Calcium and Magnesium Depletion on Single Nerve Fibers. J. Gen. Physiol., **39**:753, 1956.
3. Bartlett, G. R.: Phosphorus Assay in Column Chromatography. J. Biol. Chem., **234**:466, 1959.
4. Belzer, F. O. and Ashby, B. S.: Unpublished data.
5. Belzer, F. O. and Ashby, B. S.: Unpublished data.
6. Belzer, F. O., Ashby, B. S. and Dunphy, J. E.: 24-Hour and 72-Hour Preservation of Canine Kidneys. Lancet, September 9, 1967, p. 536.
7. Belzer, F. O., Ashby, B. S., Gulyassy, P. F. and Powell, M.: Successful Seventeen-Hour Preservation and Transplantation of a Human Cadaver Kidney. New Eng. J. Med., **278**:608, 1968.
8. Belzer, F. O., Park, H. Y. and Vetto, R. M.: Factors Influencing Renal Blood Flow During Isolated Perfusion. Surg. Forum, **15**:222, 1964.
9. Born, G. V. R., and Cross, M. J.: Effects of Inorganic Ions and of Plasma Proteins on the Aggregation of Blood Platelets by Adenosine diphosphate. J. Physiol. (London), **170**:397, 1964.
10. Cort, J. H. and Kleinzeller, A.: The Effect of Temperature on the Transport of Sodium and Potassium by Kidney Cortex Slices. J. Physiol. (London), **142**:208, 1958.
11. deLorimier, A. A., Belzer, F. O., Kountz, S. L. and Kushner, J. H.: Simultaneous Bilateral Nephrectomy and Renal Homotransplantation for Bilateral Wilms' Tumor. (in preparation)
12. Enerson, D. M.: Cellular Swelling. I. Hypothermia Graded Hypoxia and the Osmotic Effects of Low Molecular Weight Dextran on Isolated Tissues. Ann. Surg., **163**:169, 1966.
13. Enerson, D. M. and Berman, H. M.: Cellular Swelling. II. Effects of Hypotonicity, Low Molecular Weight Dextran Addition and pH Changes on Oxygen Consumption of Isolated Tissues. Ann. Surg., **163**:537, 1966.
14. Folch, J., Lees, M. and Sloane Stanley, G. H.: A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem., **226**:497, 1957.
15. Folkman, J., Cole, P. and Zimmerman, S.: Tumor Behavior in Isolated Perfused Organs: *In vitro* Growth of Biopsy Material in Rabbit Thyroid and Canine Intestinal Segment. Ann. Surg., **164**:491, 1966.
16. Fredrickson, D. S. and Gordon, R. S., Jr.: Transport of Fatty Acids. Physiol. Rev., **38**: 585, 1958.
17. Frankenhaeuser, B. and Hodgkin, A. L.: The Action of Calcium on the Electrical Properties of Squid Axons. J. Physiol. (London), **137**:218, 1957.
18. Hanahan, D. J.: Lipide Chemistry. New York, Wiley, 1960.
19. Hermann, J. B.: Effects of Certain Drugs on Temperature Regulation, and Changes in Their Toxicity, in Rats Exposed to Cold. J. Pharmacol. Exp. Ther., **72**:130, 1941.
20. Lee, W. H., Jr., Krumhaar, D., Fonkalsrud, E. W., Schjeide, O. A. and Maloney J. V., Jr.: Denaturation of Plasma Proteins as a Cause of Morbidity and Death after Intercardiac Operations. Surgery, **50**:29, 1961.
21. Lillehei, R. C., Manax, W. G., Bloch, J. H., Eyal, Z., Hidalgo, F. and Longerbeam, J. K.: *In Vitro* Preservation of Whole Organs by Hypothermia and Hyperbaric Oxygenation. Cryobiology, **1**:181, 1964.
22. Lotke, P. A.: Lysosome Stabilizing Agents for Hypothermic Kidney Preservation. Nature (London), **212**:512, 1966.
23. Mandelbaum, I. and Burns, W. H.: Pulsatile and Nonpulsatile Blood Flow. JAMA, **191**: 657, 1965.
24. Nakayama, K., Tamiya, T., Yamamoto, K., Izumi, T., Akimoto, S., Hashizume, S., Iimori, T., Odaka, M. and Yazawa, C.: High-Amplitude Pulsatile Pump in Extracorporeal Circulation with Particular Reference to Hemodynamics. Surgery, **54**:798, 1963.
25. Oncley, J. L.: *In* The Lipoproteins: Methods and Clinical Significance, Homberger and Bernfeld, Eds. New York, Karger, 1958, p. 14.
26. Owens, G., Adams, J. E., McElhannon, F. M. and Youngblood, R. W.: Experimental Alterations of Certain Colloidal Properties of Blood During Cardiopulmonary Bypass. J. Appl. Physiol., **14**:947, 1959.
27. Owens, G., Adams, J. E. and Scott, H. W., Jr.: Embolic Fat as a Meansure of Adequacy of Various Oxygenators. J. Appl. Physiol., **15**:999, 1960.
28. Sollmann, T. H.: A Manual of Pharmacology and its Application to Therapeutics and Toxicology, 8th ed. Philadelphia, Saunders, 1957.
29. Suomalainen, P.: Production of Artificial Hibernation. Nature (London), **142**:1157, 1938.
30. Suomalainen, P.: The Magnesium and Calcium Metabolism of Hibernating Hedgehogs and the Production of Artificial Hibernation. Suom. Kemistilehti, **11**:29, 1938.
31. Swank, R. L., Fellman, J. H. and Hissen, W. W.: Aggregation of Blood Cells by 5-Hydroxytryptamine (Serotonin). Circ. Res. **13**:392, 1963.
32. Wright, E. S., Sarkozy, E., Dobell, A. R. C. and Murphy, D. R.: Fat Globulemia in Extracorporeal Circulation. Surgery, **53**:500, 1963.