PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 46-51 (1977)

Kinetics of the Release of Zinc and Some Enzymes from Canine Kidney during Isolated Perfusion[1] (39872)

DAVID A. OGDEN, CHARLES F. ZUKOSKI, CLARENCE R. CAZEE, AND MILOS CHVAPIL[2]

The Departments of Medicine and Surgery, College of Medicine, University of Arizona, Tucson, Arizona 85724 and The Veterans Administration Hospital, Tucson, Arizona

There is need for a sensitive, reliable index for determining the progression of ischemic damage to isolated kidneys that are subjected to long-term perfusion. Several methods, consisting primarily of measuring certain enzymes released from the injured tissue (1, 2), have been used. Such changes appear to reflect the organ's integrity. Yet, despite having a normal output of LDH,[3] many kidneys transplanted from human cadavers do not function, and a significant number of these fail immediately after transplantation. Moreover, after 24 hr of preservation, Abouna *et al.* (3) found no significant difference in perfusate LDH and SGOT levels of functioning and nonfunctioning kidneys.

We speculated that tissue hypoxia develops during long-term perfusion of kidneys. To test this hypothesis we measured the distribution pattern of LDS isoenzymes. This reflects the oxygen supply to any given tissue (4). For tissues in which the metabolism is largely aerobic, LDH-1 and LDH-2 predominate. During ischemia, however, the accumulation of lactate results in an increase of LDH-4 and LDH-5. In both perfused and intact tissue, the levels of certain membrane-linked mitochondrial enzymes reflect the extent of tissue injury. For example: Isocitric dehydrogenase (ICDH), proved a useful indicator of myocardial damage or acute liver injury (5, 6). In liver, β-glucuronidase accumulation would be expected to reflect disintegration of lysosomes, although the presence of this enzyme in microsomes has also been reported (7). Determination of total LDH has been shown by several authors to be a sensitive index of kidney integrity (1, 2, 8-10).

In addition to correlating the effect of kidney hypoxia with the activity of some enzyme markers of tissue integrity, we studied the possible effect of this type of tissue injury on the leak of certain cations, especially zinc.

It has been shown that, due to an acute injury to a tissue, zinc leaks out of the damaged cell into the blood. This seems to be the case with acute myocardial infarction (11, 12) and with CCl_4-induced acute liver injury (13). Our study aims to learn the dynamics of changes in zinc in the perfusate during 24-hr perfusion of the isolated dog kidney and to correlate the kinetics of zinc release with kidney injury, as ascertained by assaying the above-mentioned enzymes and isoenzymes.

Materials and methods. Surgical procedures. Twelve female (12- to 17-kg) dogs were used in these studies. The kidneys were removed from the left flank during pentobarbital anesthesia using sterile technique. Half an hour before removal of the kidney, diuresis was induced with 1 liter of 5% dextrose in water and 40 mg of furosemide given intravenously. The excised kidney was flushed by gravity flow at 6° with 200 ml of 5% dextrose in Ringer's lactate solution, containing 2 ml of 1% lidocaine hydrochloride and 10,000 units of heparin. The kidney was then transferred to the perfusion apparatus (Belzer Model L1 400, Life-Med. Corp.). Great care was taken to prevent the

[1] Supported in part by N.I.H. Grants ES 00790 and AM AI 16489 and by the U.S. Veterans Administration Grant 85723.

[2] Send reprint requests to Milos Chvapil, M.D., Ph.D., Department of Surgery, Arizona Health Sciences Center, University of Arizona, Tucson, Ariz. 85724.

[3] Abbreviations used: LDH, lactic dehydrogenase; SGOT, serum glutamicoxalacetic transaminase; SGPT, serum glutamic pyruvic transaminase; CCL_4, carbon tetrachloride; ICDH, isocitric dehydrogenase; CPK, creatine phosphokinase.

ISSN 0037-9727

introduction of air into the kidney during the flushing process or the perfusion period. The time between excising the kidney and starting the perfusion was less than 10 min. After 24 h of perfusion, the kidney was removed from the apparatus and transplanted into the neck of the donor (2). The animal's other kidney was then excised. The time between terminating the perfusion and retransplanting the kidney was less than 30 min in all instances. Blood samples were obtained daily from the operated dogs for the next 7 days and creatinine level was measured by standard procedure.

Reimplanted kidneys that caused an increase in serum creatinine above 2.5 mg% were considered malfunctioning and constitute Group B (four kidneys), while the others (eight kidneys) form Group A.

Preparation of the perfusate. Sterile canine plasma was prepared by collecting femoral artery blood from anesthetized dogs into acid citrate–dextrose (ACD) solution (3:1, v/v). The cellular elements of this mixture were separated by centrifugation. Further additions to the plasma were made as suggested by Belzer *et al.* (14). Cryoprecipitated dog plasma was added to the perfusion apparatus using sterile technique. The temperature was kept at 6° when no organ was in the apparatus. The pressure in the transducer line was kept at 60 mm Hg or less once the kidney was placed in the line. The oxygen flow of 4 liters/min was added to the standard Belzer apparatus air pump line. The pH of the plasma in the arterial reservoir was kept at 7.0 to 7.4 by adjusting the concentration of carbon dioxide in the oxygenator. Perfusion pressure was continuously recorded; perfusate flow rate, temperature, pH, pO_2, and pCO_2 were measured. The perfusate was sampled just before organ perfusion was initiated and at 0.5, 1, 2, 4, 8, 10, 12, 16, and 24 hr of perfusion.

Biochemical analysis. Perfusate specimens were stored at 4° until all samples from each experiment were available for analysis. They were not stored longer than 24 hr because longer subjection to cold adversely affected electrophoretic separation of the isoenzymes of LDH.

The LDH activity in the perfusate was assayed by a standard method (15). LDH isoenzymes were separated by electrophoresis using thin-layer agarose gel (Pol-E-Film R_x, Pfizer Diagnostics Division, Charles Pfizer and Co., Inc.). A 1-μl sample was placed in the well in the gel. The gel film was then subjected to electrophoresis at 90 V for 45 min in a barbital buffer adjusted to pH 8.6. The gel film was removed from the cell, placed on a flat surface, and the ends were gently blotted. Three milliliters of Dade's Tetraform R_x substrate stain solution was poured at the anode end of the gel and was spread evenly toward the cathode end in a single smooth motion using a piece of glass tubing. The gel film was then incubated on a water-saturated piece of filter paper at 38° in the dark for 30 min. It was then fixed and cleared in a covered stain dish using a 10% acetic acid in methanol solution for 10 min. Exposure to light was minimized during incubation and fixing. The gel film was subsequently rinsed in deionized water for 15 min, and the cleared film was dried in an oven at 72° until completely dry. The film was cut longitudinally into strips and scanned by a Gelman Digiscreen Scanner-R densitometer at 550 mm to quantitate the isoenzyme fractions.

The activity of β-glucuronidase was assayed according to Fishman (16), and that of OCDH was measured by the uv method given in Sigma Technical Bulletin 150. Serum creatine was measured using an autoanalyzer.

Metal analysis. In aliquots of perfusate, the content of Zn, Mg, and Ca was determined in an atomic absorption spectrometer.

Statistical analysis. This was performed by computing linear regression, correlation coefficients, and by Student's t test. The variability in figures or tables is given as the mean ± SEM.

Results. A total of eight dog kidneys perfused for a period of 24 hr was not rejected within 7 days after reimplantation and is therefore classified as functioning. The results of the functioning kidneys (Group A) are reported first.

Distribution pattern of LDH isoenzymes. We first tested the hypothesis that, during isolated perfusion of the canine kidney, hypoxia, which should be reflected in the

change of LDH isoenzyme pattern, develops in the tissue. One representative experiment out of three similar studies is presented in Fig. 1. The data on LDH isoenzymes are given in percentage of total LDH activity at various time intervals of the perfusion.

The initial value of LDH isoenzymes refers to the analysis of perfusate of a kidney removed from the dog and inserted, within 10 min, into the Belzer apparatus. LDH-1 constitutes 17% of the total and LDH-2 14.2% of the total. Thus, approximately 31% of LDH isoenzymes at the beginning of the perfusion belong to the "aerobic" variety. After 24 hr of perfusion these fractions represented only 9% of the total activity. Isoenzymes LDH-4 and LDH-5, representing the "anaerobic" fractions, increased from the original 23 and 14% of the total activity to 36 and 34%, respectively. Thus a striking increase occurred in the plasma anaerobic forms of LDH isoenzymes during 24 hr of perfusion. As reported elsewhere (17), the most prominent isoenzyme in dog plasma at the start of the perfusion is LDH-3, which represents almost 30% of the total LDH activity. At the end of 24-hr perfusion it comprised only 20% of the total LDH activity.

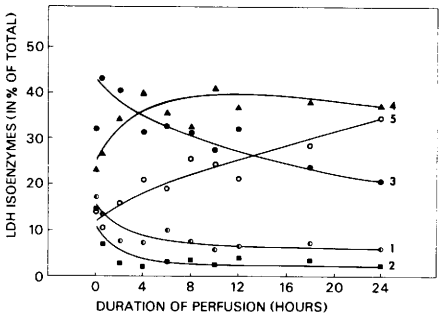

FIG. 1. Changes in LDH isoenzymes in the plasma during the isolated perfusion of canine kidney. The data for individual LDH isoenzymes 1 to 5 are presented as percentage of total LDH activity at a certain time period and refer to perfusion of kidney from Group A.

The shift in LDH isoenzymes started rapidly; changes were evident within 30 min of perfusion and quite pronounced within 2 hr.

Our data refer to the perfusion characteristics of the eight kidneys that were classified as functioning. The kidneys were maintained at stable pressure, pO_2 and pCO_2. The pH of the perfusate increased from 7.29 to 7.31 during the 24 hr of perfusion and the flow rate decreased from 60 to 28 ml/min.

Activity of some enzymes and zinc content. We next analyzed enzyme and zinc content in the kidney perfusate in the course of 24-hr perfusion (Table I). As shown in Fig. 2, comparing the satisfactory and nonsatisfactory kidney perfusions, a significant increase in LDH activity appears in malfunctioning kidneys after 4 hr of perfusion, while the first significantly different increase in zinc content is evident at 12 hr of perfusion.

A significant positive correlation exists among every parameter shown in Table I and the duration of perfusion. Because cryoprecipitated, filtered plasma was used in all experiments, the initial values of zinc are lower by approximately 30% than the values of intact fresh plasma. This reflects pri-

TABLE I. SUMMARY OF THE ACTIVITIES OF SOME ENZYMES AND ZINC CONTENT IN THE PLASMA DURING THE ISOLATED PERFUSION OF CANINE KIDNEY.[a]

Time (hr)	Zinc (μg/100 ml)	LDH (U/ml)	ICDH (U/ml)	β-Glucuronidase (ΔOD-100 ml)
0	65.0 ± 3.3	76.8 ± 4.94	35.0 ± 18.0	7.5 ± 0.42
0.5	73.7 ± 4.1	113.3 ± 9.50	70.0 ± 10.0	7.9 ± 0.37
2	83.0 ± 7.2	135.0 ± 12.6	107.0 ± 16.7	8.3 ± 0.51
4	85.4 ± 11.6	178.8 ± 15.6	145.0 ± 35.0	8.4 ± 0.46
6	92.5 ± 13.1	206.4 ± 22.7	180.0 ± 25.1	8.6 ± 0.41
8	94.8 ± 19.8	236.2 ± 30.7	250.0 ± 20.0	9.2 ± 0.34
10	107.7 ± 23.1	247.6 ± 15.7	282.0 ± 35.0	9.8 ± 0.38
12	93.4 ± 14.6	305.0 ± 31.7	310.0 ± 38.5	9.4 ± 0.25
18	120.8 ± 17.8	411.2 ± 27.2	452.5 ± 46.0	10.0 ± 0.33
24	150.8 ± 19.7	507.6 ± 56.5	513.0 ± 49.0	10.2 ± 0.38

[a] Data, presented as arithmetic mean ± SEM, are based on the analysis of eight Group A kidney perfusion experiments. For details see "Methods."

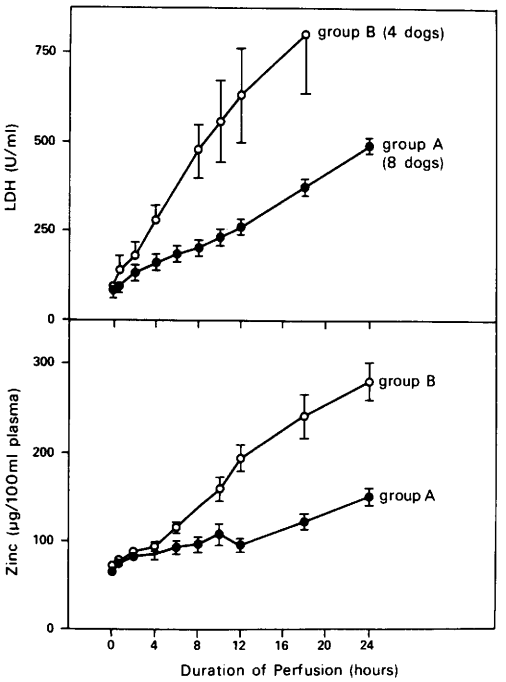

FIG. 2. LDH activity and zinc content in the perfusate during the isolated perfusion of functioning and malfunctioning canine kidneys. This figure gives the statistics based on eight successful perfusion experiments (Group A) and four perfusions of kidneys which did not function after reimplantation (Group B). The data are presented as mean ± SEM

marily the loss of α_2-macroglobulins, which, along with albumin, transport zinc. It has been shown previously that the enzyme activity in a perfusate indicates the integrity of cells of the perfused organ. Therefore, we could conclude that during perfusion of the kidney there is a progressive leakage of some enzymes and zinc from the cells.

The correlation coefficient of zinc and LDH activity changes during perfusion was $r = 0.91$ ($P < 0.001$). This indicates a close relationship between continuous increase in zinc plasma content and leak of either enzyme from injured kidney cells. As both LDH and ICDH are zinc-dependent enzymes, as are most dehydrogenases (18), this may explain some increase in plasma zinc. Still, the magnitude of the rise of zinc concentration is too high to be explained solely by this mechanism.

The increase in plasma zinc level was not paralleled by Ca and Mg content in the perfusate (data not shown), both cations remaining almost unchanged during kidney perfusion.

Strikingly different results were noted in testing the perfusate of the four kidneys that did not remain viable after reimplantation. Due to the complex and involved procedure for analyzing LDH isoenzymes, we did not analyze the distribution patterns in the four nonfunctioning kidneys. Our results are confined to enzyme activity and zinc content of the perfusate of these kidneys.

LDH activity and zinc content. While the data presented in Table I show a gradual increase in enzyme activity and zinc content of the perfusate of the functioning kidneys, these parameters increased abruptly between 4 and 8 hr in the kidneys that were later rejected (Fig. 2). Furthermore, the final values of LDH activity and zinc content after 24 hr of perfusion were twice as high as the values of the Group A kidneys. The only abnormal physiological parameter that might explain the elevated LDH activity and zinc content is the rise in pH above 7.4.

Creatinine levels in functioning and nonfunctioning kidneys. The creatinine levels of the eight functioning kidneys (Group A) did not rise above the normal limit of 2.5 mg%. In the four nonfunctioning kidneys (Group B), the malfunction was accompanied by a sharp rise in plasma creatinine. During the first 24 hr after reimplantation the creatinine level rose from 1.9 to 6.1 mg%. It fell to 5.8 mg% by 48 hr but assays were not carried further because of the declining health of the dogs and rejection of the reimplanted kidneys.

Discussion. A crucial problem in organ transplantation is the viability of the organ to be grafted. What is the structural-functional integrity of the donor organ? The development of ischemia in the transplanted organ is a major limitation to success of the transplant. For this reason several techniques have been suggested to test the viability of the tissue. Most depend upon the determination of LDH activity in the perfusate (1, 2). Determination of LDH activity in the serum seems to be a satisfactory index of the function of kidneys during isolated perfusion (2, 19). Belzer *et al.* (1) concluded that LDH determinations 1 and 2 hr

after the onset of perfusion may provide a satisfactory method for choosing kidneys appropriate for transplantation. Our data on LDH support Belzer's findings and add new information about the physiological meaning of the increased activity of LDH.

As stated by Belzer *et al.* (1), four enzymes (SGOT, SGPT, LDH, and CPK) increased with duration of ischemia during 24 hr of continuous perfusion. LDH measurement appeared to be the most sensitive of the enzymes tested.

Our study extends Belzer's conclusions (1) in several ways. With successful kidney perfusions, we found that the initial LDH activity in perfusate is low (below 100 U/ml) and does not rise above 500 U/ml after 24 hr of perfusion. The level of LDH of kidneys that failed was initially comparable to successful kidneys. After 24 hr, however, it reached an activity above 1000 U/ml. Hence, the activity of LDH in plasma shortly after starting the perfusion (1–2 hr) is not necessarily of prognostic value in determining eventual damage to the organ after more-prolonged perfusion. Furthermore, comparison of functioning and malfunctioning kidneys showed that the earliest observed difference in LDH activity in perfusate was found after 4 hr of perfusion.

Statistical analysis of multiple intercorrelations showed the closest relation existing between LDH and ICDH ($r = 0.89$, $P < 0.01$), LDH and Zn ($r = 0.91$, $P < 0.01$). The determination of β-glucuronidase was the least-sensitive method for assaying the viability of perfused kidneys. The correlation coefficient between LDH and β-glucuronidase varied, as evident from the variation coefficient of the average of seven independent experiments where $r = 0.616 \pm 0.275$ (44%). Still, the correlation coefficient is statistically significant.

Of great interest are the findings regarding a rapid shift in LDH isoenzymes in the plasma perfusate. Our results on the LDH isoenzyme pattern in plasma perfusate point to two basic problems. First, there is a continuous and marked change in the content of M and H subunits. An increase in M subunits, reflected mostly in an increase in LDH-4 and LDH-5, indicates a prevalence of anaerobic metabolism, since LDH-5 is relatively insensitive to increased concentrations of O_2 (17). Second, there is an almost immediate response in the LDH isoenzyme pattern, reflecting a rapidly changing microenvironment within the kidney tissue.

Based on our results, we conceived of the following biochemical events occurring in perfused dog kidneys. A healthy kidney is able to function under these conditions for a certain time (which in our experimental arrangement was approximately 12 hr) without evidence of a marked biochemical or metabolic lesion. During this period mitochondrial structures, being more susceptible to ischemic injury, show early signs of distortion reflected by an elevation of ICDH in the perfusate. At this time, we observed insignificant changes in β-glucuronidase. The later elevation of activity of this enzyme, coupled with the early increase in ICDH activity, suggests that the sequence of injurious events starts in mitochondria and later involves lysosomes with discharge of their enzymes or disruption of vacuoles. This later stage is documented by significantly more LDH, ICDH, and zinc appearing in the perfusate.

We conclude that LDH, ICDH, and zinc are sensitive indicators of kidney viability, as suggested by Belzer *et al.* (1, 14). Contrary to Belzer, however, we believe the determinations should be made at the latest possible stage of perfusion. Such determinations are more meaningful than those from early stages of the perfusion because they reveal the functional capacity of the kidney and the existence of hypoxic lesions within the tissue.

Summary. An isolated 24-hr perfusion of 12 dog kidneys in Belzer apparatus was performed, and the activities of LDH, ICDH, and β-glucuronidase, LDH isoenzymes pattern, and zinc content in the perfusion fluid were determined. LDH activity in plasma perfusate rises with time of perfusion at two statistically different rates, slowly up to 12 hr, then faster thereafter. Significant correlation exists between LDH, ICDH, and zinc values during perfusion. β-Glucuronidase activity changed only slightly, mostly after 18 hr of perfusion. The magnitude of LDH, ICDH activity, and zinc content corresponds with the function of the kidney post-

reimplantation and, in satisfactory 24-hr perfusions, should not exceed 500 U/ml and 200 μg% of Zn, respectively. With time of perfusion, there is a continuous shift in LDH isoenzymes to anaerobic forms (LDH-4 and LDH-5), indicating a fast development and a continuous progression of hypoxia in the kidney tissue.

1. Belzer, F. O., Ashby, B. S., and Downe, G. L., Surg. Forum **19,** 205 (1968).
2. Kohn, M., and Ross H., Transplantation **11,** 461 (1971).
3. Abouna, G. M., Garver, F. A., Kogure, H., De-Long, G., and Andres, G. A., Surg. Forum **24,** 318 (1973).
4. Bowers, G. N., Jr., Clin. Chem. **5,** 509 (1959).
5. Cohen, N. N., Potter, H. P., and Bowers, G. N., Ann. Int. Med. **55,** 604 (1961).
6. De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Applemans, F., Biochem. J. **60,** 604, (1955).
7. Grundmann, R., and Pichlmaier, H., Res. Exp. Med. **159,** 298, (1973).
8. Livshits, R. I., Slobodin, V. B., Yakushev, V. S., Bochina, T. V., Popov, G. K., and Potapov, V. S., Biul. Eksp. Biol. I. Med. **74,** 49 (1972).
9. Latner, A. L., Siddiqui, S. A., and Skillen, A. W., Science **154,** 527 (1966).
10. Anderson, G. L., and Bullard, R. W., Proc. Soc. Exp. Biol. Med. **138,** 441 (1971).
11. Handjani, A. M., Smith, J. C., Jr., Herrman, J. B., and Halsted, J. A., Chest **65,** 185 (1974).
12. Lindeman, R. D., Yunice, A. A., Baxter, D. J., Miller, L. R., and Nordquist, J., J. Lab. Clin. Med. **81,** 194 (1973).
13. Chvapil, M., Ryan, J. N., Elias, S. L., and Peng, Y. M., Exp. Mol. Pathol. **19,** 186 (1973).
14. Belzer, F. O., Ashby, B. S., and Dunphy, J. E., Lancet **2,** 536 (1967).
15. Cabaud, P. G., and Wroblewski, F., Amer. J. Clin. Pathol. **30,** 234 (1958).
16. Fishman, W. H., Springer, B., and Brunetti, R., J. Biol. Chem. **173,** 449 (1948).
17. Wilkinson, J. H., "Isoenzymes." E. and F. N. Spon, Ltd., London (1965).
18. Vallee, Bert L., Physiol. Rev. **39,** 443 (1959).
19. Belzer, F. O., *In* "Organ Perfusion and Preservation" (J. C. Norman, ed.), p. 3. Appleton-Century-Crofts, New York (1968).

Received March 23, 1977. P.S.E.B.M. 1977, Vol. 156.