The Role of Cell Swelling in Ischemic Renal Damage and the Protective Effect of Hypertonic Solute

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ABSTRACT The failure of blood flow to return to the kidney following a transient period of ischemia has long been recognized. The cause of this "no-reflow" has been investigated in the rat after a transient period of total obstruction of the renal arteries. The vascular pattern of the kidneys as visualized with silicone rubber injection shows a diffuse patchy ischemia throughout the kidney, which persists after release of the obstructed renal artery. Electron microscopic studies of ischemic kidneys showed that all cellular elements were swollen and limiting the available vascular space. Functional studies revealed an increase in plasma urea nitrogen and creatinine after 1 hr or longer ischemic periods. The ischemia, cell swelling, "no-reflow," and subsequent renal dysfunction occurring after obstruction to the renal arteries were corrected by the administration of hypertonic mannitol, but were unaffected by an equivalent expansion of the extracellular fluid volume either with isotonic saline or isotonic mannitol, showing that the osmotic effect was primary. The hypothesis is presented that ischemic swelling of cells may occlude small blood vessels so that recirculation does not resume even after the initial cause of the ischemia is no longer present; solutes which do not penetrate cell membranes are able to shrink swollen cells, increase the available vascular space and thus permit reflow of blood to the ischemic organ.

INTRODUCTION

The failure of circulation to return to a tissue after a transient period of vascular obstruction has been known for many years to occur in several organs, and has been termed, "no-reflow." Recently Ames and his associates (1–3) have demonstrated that swelling of cells in the brain during short periods of anoxia may obstruct small blood vessels resulting in "no-reflow." Such "no-reflow" has also been described in the kidneys following shock and in association with acute tubular necrosis (4–10).

It is well established that regulation of cell volume is dependent on a supply of metabolic energy (11–13). This was first observed by Krebs, Stern, Eggleston, and Hems (14) who noted swelling of isolated tissues when these were made hypoxic. The current understanding of the process of cell volume regulation has been reviewed (15), and a possible role that disturbances in the regulatory process may play in disease states, has been recently pointed out (16). Briefly, it is thought that the primarily extracellular position of sodium ions osmotically balances the intracellular oncotic pressure resulting from the content of proteins and other nondiffusible macromolecules within cells. The extracellular position of sodium, however, is not the result of impermeability of plasma membranes to sodium ions but rather the consequence of a continuous active extrusion of sodium from cell interior to exterior as rapidly as sodium diffuses "down hill" into the cell with its low intracellular concentration and electronegativity from its high concentration in the extracellular fluids. It is the active extrusion of sodium which requires energy to preserve a steady state of sodium distribution between intracellular and extracellular fluids in order to stablize cell volume. When the supply of metabolic energy becomes insufficient to sustain the usual rate of extrusion of sodium from the cell, then sodium continues to enter cells passively; a gain in intracellular sodium and chloride occurs. The resulting increase in solutes within the cell draws water osmotically from the extracellular compartment into the cell with swelling of cells. Ischemia with hypoxia is one such cause of interference with the

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availability of metabolic energy supplies which causes cell swelling (13). If the supply of energy is reestablished before death of the cells transpires, the process is reversible and cell volume returns to its normal state. Obviously, additions to the extracellular fluid of solutes, such as mannitol, which do not penetrate cell membranes readily, will shrink cells osmotically or prevent their swelling during ischemia.

Evidence will be presented: (*a*) that in an experimental animal, temporary obstruction to the renal artery will result in "no-reflow"; (*b*) that there is a persistent patchy ischemia throughout the kidney during "no-reflow"; (*c*) that the ischemia is associated with swelling of cells; (*d*) that the ischemia, cell swelling, "no-reflow", and subsequent renal dysfunction can be prevented or reduced by infusion of hyperosmotic solutes such as mannitol, but not by an equivalent expansion of the extracellular fluid volume with isotonic saline. The evidence seems to indicate that ischemic damage to the kidneys may be self-sustaining and augmenting through a failure of cell volume regulation.

METHODS

Male Sprague-Dawley rats weighing between 280 and 400 g were used in all experiments. The rats were anesthetized with sodium pentobarbital (40 mg/kg); then the jugular vein was catheterized. The kidneys were exposed through an abdominal midline incision. After hemostasis and dissection of perirenal fat from the kidney to interrupt possible collaterals, heparin was administered (4.2 mg/kg). The renal arteries were then clamped for periods of 60–180 min.

After the ischemic period, reflow of blood to the kidney was permitted for 10 min, 30 min, or 24 hr. A silicone rubber suspension (Microfil, Canton Biomedical Products, Boulder, Colo.) was injected via the mesenteric artery after ligating the aorta in two places around a catheter below the renal arteries and also above the mesenteric artery. An incision in the vena cava permitted drainage of excess silicone from the kidney vasculature for the 3–4 min period of injection which proceeded at pressures no higher than 130 mm Hg as measured with the aortic catheter. A complete cast of the vasculature was obtained by dehydrating the kidneys in a graded ethanol series and subsequently clearing with methylsalicylate. Another group of rats was kept alive for 24 hr after release of the clamps in order to examine subsequent renal function. To avoid dehydration, the rats were force-fed an isotonic glucose solution (4 ml/100 g) 8–10 hr after the surgical procedure.

In additional experiments a possible role for the red blood cells in the "no-reflow" phenomenon was examined. All blood was washed out of the left kidney by perfusing this organ with warmed saline after it had been surgically isolated temporarily from the circulation by appropriate clamping of aorta and inferior vena cava. As soon as the color of the perfusate indicated clearing of blood from the kidney, the left renal pedicle was clamped to provide the period of renal ischemia. At the end of the ischemic period, the silicone rubber was injected immediately or 10 min after reestablishing blood flow to the kidney.

Solutions were administered by intravenous injection through the jugular vein during the last 15–30 min of vascular obstruction. The solutions used and the amounts administered were: isotonic saline, 3.2 ml/100 g; isotonic mannitol, 2.8 ml/100 g; hypertonic mannitol 25% (calculated to yield 1400 mOsm/kg of water), 1 ml/100 g; and hypertonic sodium sulfate 0.7 M (calculated from the osmotic coefficient to yield 1400 mOsm/kg of water), 1.3 ml/100 g. The dose of each solution was chosen to provide an equivalent expansion (or slightly greater expansion in the case of saline and sodium sulfate) of extracellular fluid volume. In the case of hypertonic mannitol plasma osmolality was found to be 360 ± 10 mOsm/kg water ($n = 22$), as measured 5 min after completion of the injection; following sodium sulfate the corresponding value was 347 ± 2.7 mOsm/kg water ($n = 7$). Initial osmolality was found to be 304 ± 4 mOsm/kg water ($n = 7$).

Tissue for electron microscopy was removed surgically and quickly cut into 2 mm cubes at 4°C under a 1% glutaraldehyde solution (phosphate-buffered, pH 7.4). The trimmed pieces of kidney were fixed for a minimum of 90 min at 4°C in the solution above. After further fixation with osmium tetroxide the tissues were dehydrated in a graded ethanol series and embedded in an epon-araldite mixture. Sections were cut on a Reichert OmU2 ultramicrotome (American Optical Corp., Buffalo, N. Y.), stained with uranyl acetate and lead citrate, and examined in a Philips EM-200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) For quantitative purposes all micrographs were taken at the same instrumental magnification and enlarged identically.

In order to provide direct information on the assumed swelling and shrinking of renal cells during these studies, measurements were made of the mean endothelial cell thickness and the circularity of renal interstitial cells as visualized in electron micrographs of tissues obtained from the subcortical zones of the respective kidneys.

The mean thickness of capillary endothelium was determined by planimetry. Measurements were made on the micrographs of the areas enclosed by the basement membrane and by the luminal surface of each capillary. From these measurements the mean radii of the two concentric circles were obtained. The mean endothelial thickness for each capillary was taken as the difference between these two radii. All capillaries with mean outer radii of 10 μ or less were included in these measurements provided that nuclei of endothelial cells were not visible in the plane of the section.

Another index of cell swelling was obtained by measurement of the circularity of profiles of interstitial cells in electron micrographs. The perimeter and area of each cell was measured using the cell membrane as the limiting boundary in each case. The degree of circularity of each interstitial cell was calculated as the per cent, that the actual area of the cell constituted, of the maximum area which could be enclosed by the measured cell perimeter.

Per cent circularity $= [4\pi A(100)]/[(P)^2]$ where A is the measured area of each interstitial cell and P is its measured perimeter. Since this index refers to the cross-sectional area of cells whereas the volume of the cell is concerned in the process of swelling, this index understates changes occurring in the latter. Tissue and photomicrographs for these histological measurements were selected randomly.

Analyses on small volumes of plasma were performed as follows: plasma urea nitrogen by the diacetyl monoxime

FIGURE 1 Vascular pattern of hemisections of kidneys visualized by silicone rubber injected into renal artery. At the left is shown the vascular pattern of a normal kidney while middle and right kidneys show the vascular pattern after 60 min of obstruction of the renal artery and of 10–30 min of reflow of blood to the kidneys, respectively, after release of the arterial obstruction. The blood vessels are filled with white silicone rubber and the dark areas represent portions of the kidney which were ischemic. The patchy ischemia in the kidneys is evident. × 2.

method (17) using an autoanalyzer; plasma creatinine by the Folin Wu method adapted for microdetermination (Beckman Ultramicro Analytical System, model 150, Beckman Instruments, Inc., Fullerton, Calif.); plasma osmolality, using an Advanced Instruments Osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.).

RESULTS

To examine the vascular pattern in the kidney following a period of obstruction to the renal artery, silicone rubber was injected intra-arterially 10–30 min after release of arterial obstruction. Fig. 1 compares the vascular pattern in the normal control kidney with that seen 10 and 30 min, respectively, after 60 min of total renal ischemia. In the normal kidney the zones are clearly identifiable as cortical, subcortical, outer, and inner medullary zones. A diffuse patchy ischemia affecting all zones of the kidney is seen after release of the temporary obstruction of the renal artery even though return of circulation had been allowed for 10 or 30 min before injection of the silicone rubber. The effect of infusion of hypertonic mannitol sufficient to produce a mean increase of plasma osmolality from control values of 304 ± 4 ($n = 7$) to 360 ± 10 ($n = 22$) mOsm/kg water is shown in Fig. 2 for an experiment in which the duration of ischemia was 60 min.

Since the infusion of hypertonic mannitol will expand the extracellular fluid volume, a volume of saline was infused equal to or greater than the expansion of extracellular fluid volume calculated to have accompanied the mannitol. As Fig. 2 shows, the infusion of isotonic saline failed to improve the vascular pattern, suggesting that mannitol exerts its effect osmotically rather than by expansion of extracelular fluid volume. Fig. 3 shows in greater detail the subcortical zone of the same kidneys

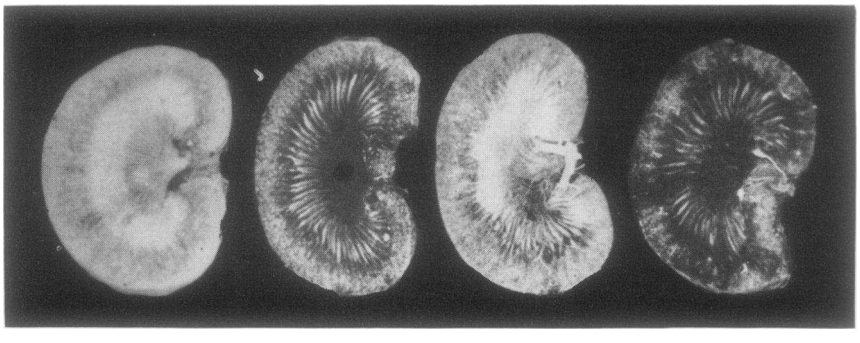

FIGURE 2 Vascular pattern of hemisection of normal kidney at the left and of kidneys after 60 min of obstruction of the renal artery, 60 min of obstruction of the renal artery plus hyperosmotic mannitol, and 60 min of arterial obstruction plus a volume of saline calculated to expand extracellular fluid volume to a greater degree than did the hypertonic mannitol. The silicone rubber was injected 10 min after release of the renal artery. × 1.5.

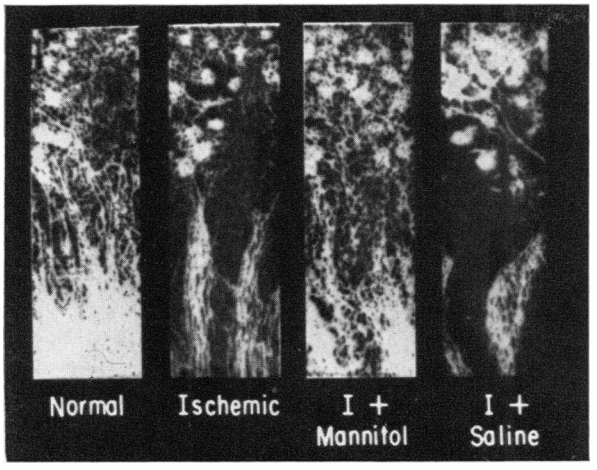

FIGURE 3 Magnification of the vascular pattern in the subcortical zone of the kidneys shown in Fig. 2. × 20.

illustrated in Fig. 2 since the subcortical zone characteristically shows marked ischemia.

Fig. 4 shows that the more abnormal vascular pattern following 120 min of renal ischemia can likewise be markedly improved with hypertonic mannitol. The number of similar experiments, the treatment and duration of ischemia are shown in Table I in which the figures are the number of kidneys examined in each experimental group. The effects of hypertonic mannitol were similar to those shown in Fig. 2 and Fig. 4 in all instances except after 180 min of ischemia in which case the improvement in the vascular pattern with mannitol was inconstant. In all instances isotonic saline failed to improve the vascular pattern.

In order to examine further the role of osmolality in the action of mannitol on the renal vasculature two additional kinds of experiments were performed: (*a*) Mannitol in a concentration isotonic with rat plasma and in a total volume equal to the expansion of extracellular fluids calculated to have accompanied the infusion of the hypertonic solution, was administered to seven animals; (*b*) Sodium sulfate in hypertonic solution was administered in a manner similar to that for the hypertonic mannitol in eight animals to determine whether a solute other than mannitol can produce the same improvement in the vascular pattern after renal ischemia. With isotonic mannitol the vascular pattern was variable. In one of seven experiments the vascular pattern was distinctly improved while in the remainder there was either slight or no improvement after 60 min of ischemia. Hypertonic sodium sulfate in eight animals produced definite improvement in the vascular pattern. Although in some experiments improvement was as good as that expected with hypertonic mannitol, the degree of improvement was not so great in all instances.

TABLE I

Number of Kidneys Examined in Each Experimental Group

Treatment	Duration of ischemia, min				
	0	60	90	120	180
None	20	18	8	34	14
Hypertonic mannitol		30	6	14	12
Isotonic saline		12			
Isotonic mannitol		14			
Hypertonic sodium sulfate		16			

Examination of renal tissue by both light and electron microscopy suggested that all cellular elements in the ischemic kidney were swollen and that such swelling

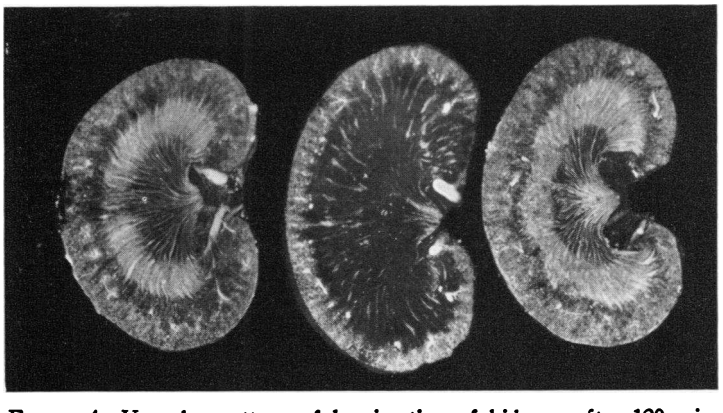

FIGURE 4 Vascular pattern of hemisection of kidneys after 120 min of obstruction of the renal arteries with and without infusion of hypertonic mannitol. On the left is a normal kidney. The middle and right kidneys were injected with silicone rubber after 120 min of ischemia, but mannitol was administered in the case of the kidney on the right. × 2.

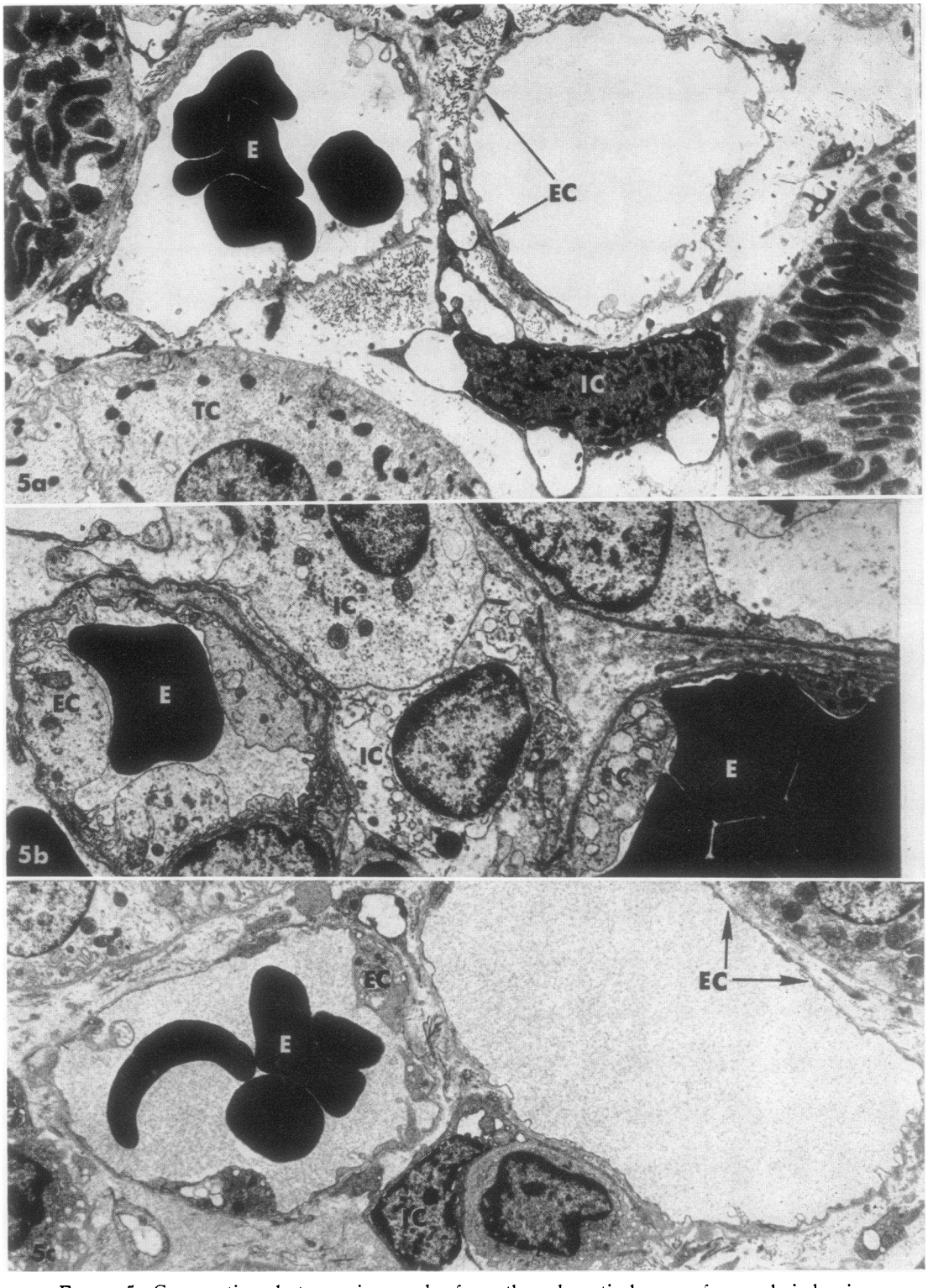

FIGURE 5 Comparative electron micrographs from the sub-cortical zone of normal, ischemic control, and mannitol-treated ischemic kidneys. A, Normal kidney showing patent vessels, flat endothelial cells (EC), and an interstitial cell (IC) with a very irregular profile; erythrocyte (E), tubular cell (TC). B, Ischemic kidney with obviously swollen endothelial cells

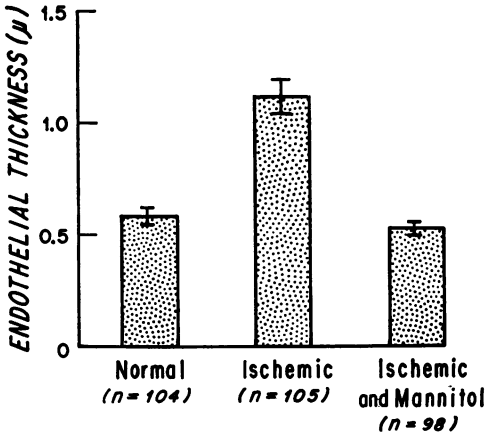

FIGURE 6 Mean endothelial thickness of renal capillaries (radii $<10\,\mu$).

	Δ	P
Ischemic	—Normal	<0.001
Ischemic and mannitol	—Normal	>0.05
Ischemic	—Ischemic and mannitol	<0.001

was largely reversed or prevented by hypertonic mannitol (Fig. 5). To quantitate the impression of cell swelling, measurements were made from electron micrographs of the mean endothelial thickness and of the circumference and area of interstitial cells, as described in Methods. Fig. 6 shows the mean endothelial thickness of control, ischemic, and mannitol-treated kidneys, Fig. 7 shows the relative circularity of interstitial cells in the same experimental conditions. It is evident that swelling of cells occurs as measured by these indices and that hypertonic mannitol returns these cells to their normal measurements.

Microscopic examination of sections from the medulla of ischemic kidneys frequently showed the lumen of the vasa recta to be filled with packed red blood cells in spite of the fact that the animals were anticoagulated with heparin. To assess the possible role of this phenomenon on the "no-reflow" in 14 experiments all trapped blood cells were removed from the kidneys immediately after clamping the arterial supply to that kidney. During the subsequent 60 min of ischemia the kidney was free of blood cells. When the silicone rubber was injected at the end of the ischemic period and without permitting blood to reenter the kidney, the vascular pattern was the same as in the nonischemic control kidney (seven experiments). However, if a 10 min period

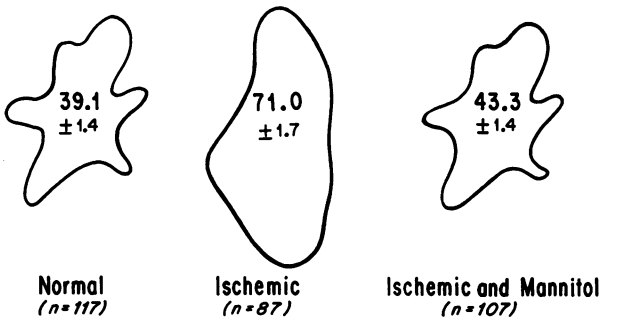

FIGURE 7 Mean per cent circularity of renal interstitial cells.

	Δ	P
Ischemic	—Normal	<0.001
Ischemic and mannitol	—Normal	<0.01
Ischemic	—Ischemic and mannitol	<0.001

of reflow of blood into the ischemic kidney was permitted before the injection of the silicone rubber then the characteristic patchy vascular ischemia was again seen (seven experiments). These results are similar to those recently reported by Summers and Jamison (18).

The effect of transient renal ischemia on subsequent renal function and urine production has been reported in studies by others (18–21). In this study the plasma urea nitrogen was measured 24 hr after relief of ischemia in mannitol-treated and control animals. Fig. 8 shows that the plasma urea nitrogen rose following ischemia but in each instance was significantly lower in the mannitol-treated animals. Fig. 9 shows that mannitol diminished the rise of serum creatinine 24 hr after ischemia to levels significantly lower than control ischemic, or saline-treated animals. Thus infusion of hyperosmotic mannitol did not prevent but did diminish the disturbed function which follows a period of renal ischemia.

DISCUSSION

Although "no-reflow" has been observed experimentally in the kidney after a variety of procedures which reduce perfusion of that organ, the present study is limited to the effects of transient total obstruction of the renal artery. The results in this experimental situation indicate that "no-reflow" is associated with swelling of renal cells in the kidney and that "no-reflow" and cell swelling can be reversed by hypertonic mannitol infused into the animal at the end of the period of renal ischemia.

(EC), and interstitial cells (IC). The smaller vessel (to the left) suggests strongly the role of endothelial swelling in restricting blood flow, while larger vessels (a portion of one is seen at the right) are filled with sludged blood. C, Mannitol-treated ischemic kidney. It is evident here that the endothelial cells (EC) are flattened approximately as in the normal. This field suggests the average results obtained in these preparations. The larger vessel, now free of trapped blood, exhibits greater endothelial flattening than the small one. The interstitial cells (IC) appear considerably less swollen than in the nontreated ischemic preparation. All figures are at a magnification of × 5250.

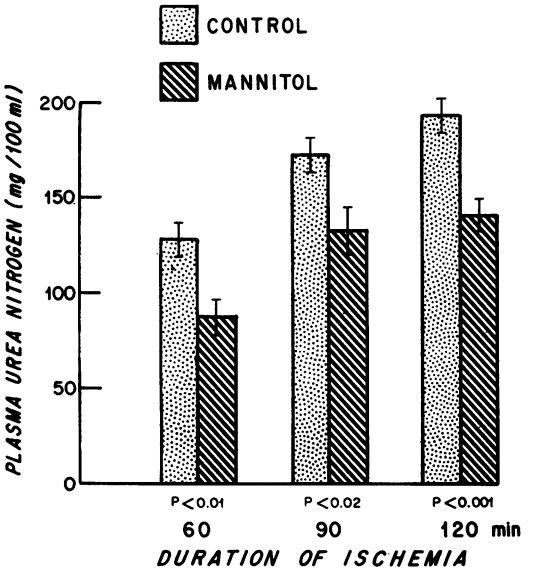

FIGURE 8 The concentration of urea nitrogen in plasma 24 hr after the period of ischemia with and without mannitol nfusion.

Since isotonic saline and isotonic mannitol failed to improve renal circulation following ischemia but hypertonic sodium sulfate as well as hypertonic mannitol were effective, the evidence supports the hypothesis that it is the rise in extracellular osmolarity by solutes which penetrate cell membranes poorly that shrinks the swollen cells osmotically, thus improving the renal circulation.

To compare the effects of various solutes, solutions of equivalent osmolality were infused. However, the reflection coefficient of plasma membranes to these solutes is not known nor has the effect of ischemia on the permeability of renal cell membranes been determined. The fact that hypertonic sodium sulfate was slightly less effective than an equal osmolar solution of mannitol may be due to the slightly greater ease with which sodium sulfate can penetrate plasma membranes of ischemic cells than can mannitol. Also isotonic mannitol seemed to protect the vascular pattern better than did isotonic saline. If plasma membranes are significantly permeable to sodium and other solutes of the extracellular fluids then introduction of a poorly penetrating solute, such as mannitol, even in isotonic concentrations, may exert some effect in preventing cell swelling.

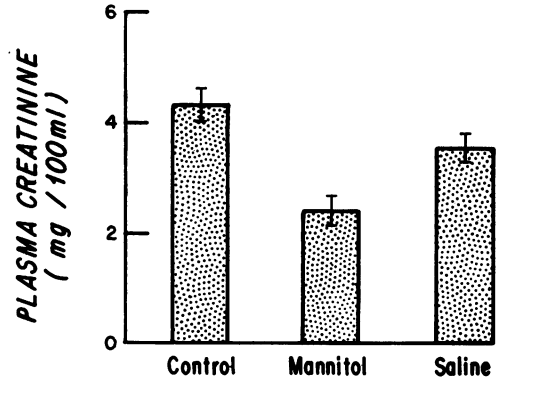

FIGURE 9 Plasma creatinine levels 24 hr after 60 min of ischemia with and without hypertonic mannitol or isotonic saline.

	Δ	P
Control	—Mannitol	<0.001
Control	—Saline	>0.05
Mannitol	—Saline	<0.01

Although measurments were made to document the swelling of endothelial and interstitial cells, microscopy revealed that all cellular elements in the ischemic areas were swollen. The thickness of endothelial cells in small vessels was measured, as changes in the dimensions of these cells must directly affect vascular resistance. The circularity of interstitial cells was determined as an index of swelling of cells which might also affect the size of the lumen of small blood vessels. Although renal tubular cells also appeared distinctly swollen and contributing to compression of small blood vessels, this observation was not documented quantitatively. Comparison of the size of these cells is difficult because of the heterogeneous types involved, and these cells in their normal state exhibit a high degree of circularity.

How does mannitol get into renal vessels which are blocked by prior ischemia? Since not all vessels are totally obstructed, mannitol enters the interstitium of the kidney by crossing the endothelial cells of patent vessels. The endothelial cells of these capillaries are then reduced in volume as are other neighboring cells, releasing the obstruction in the small vessels so that circulation resumes. This sequence of events is supported by the measurements which revealed that endothelial cells of larger arterioles were most flattened by mannitol, interstitial cells next, and endothelium of the smallest vessels least. Thus, comparing the endothelial thickness of the ischemic kidneys with those treated with mannitol, it was observed that after the mannitol there was a decrease of 0.74 $\pm 0.1\ \mu$ ($P < 0.001$) in the mean endothelial thickness of the larger vessels (with radii 5.5–10 μ), whereas in the smaller vessels (with radii $< 5.5\ \mu$) the decrease was only $0.40 \pm 0.1\mu$. The difference between the reduction in mean thickness is itself significant ($P < 0.02$). Furthermore, the larger vessels of ischemic kidneys treated with mannitol had a mean endothelial thickness which was even less than that of normal vessels of the same size (0.45 ± 0.04 and $0.57 \pm 0.04\ \mu$ mean thickness, respectively with P of the difference < 0.05). This reduction in endothelial thickness of about 20% may contribute to the reduced vascular resistance that others have observed after injections of

hypertonic mannitol even into nonischemic tissues (22–27).

Although the studies were done with the experimental animals anticoagulated with heparin, it was not uncommon to see the lumen of small vessels in the vasa recta occluded with tightly packed red blood cells. The role of the red blood cells in the "no-reflow" phenomenon has been recently examined by Summers and Jamison (18) who found that India ink failed to perfuse all vessels in the kidney following a period of temporary total ischemia. However, if the blood cells were washed out of the kidney with saline before the period of ischemia, India ink injected immediately upon release of the arterial obstruction entered all the renal vessels. Red cell ghosts which were rendered rigid by treatment with calcium also prevented the India ink from filling all the renal vessels while ghosts made pliable by treatment with water failed to prevent the India ink from entering all the renal vessels. From this they conclude that packing of red cells even in the heparinized animals does contribute to the "no-reflow" but that there must be some increased resistance of the vessels which causes the red cells and calcium-treated red cell ghosts to obstruct. The cause of the increased vascular resistance, which they postulate, could well be the cell swelling documented in the present study. Similar effects of the blood cells on the vascular pattern of the ischemia kidney were obtained in this study.

There is no reason that the cell swelling which related to "no-reflow" after ischemia in the kidney is limited to this organ. Ames and associates (1–3, 28) indeed have previously clearly demonstrated this phenomenon in the brain following transient ischemia to that organ. Willerson and associates (29) have recently demonstrated that following temporary ligation of the left anterior descending coronary artery, hypertonic mannitol improves the depressed myocardial contractility, lessens elevation of S-T segments over the area of ischemia, and increases total and collateral coronary blood flow into the ischemic zone. The rate at which cell swelling may occur and "no-reflow' develop with ischemia may be inversely related to the metabolic rate of the tissue, but this is conjecture.

Fig. 10 summarizes the sequence of events which are thought to follow transient arterial occlusion. The self-sustaining vicious cycle of cell swelling, further ischemia, and final tissue death is depicted. Mannitol or other poorly penetrating solute can disrupt this cycle by shrinking cells, improving circulation, and relieving ischemia. Hypoxic damage already incurred is obviously not reversed by such treatment but further damage may be prevented.

The therapeutic implications for the use of hypertonic mannitol to disrupt this sequence of events is obvious. Mannitol has been recommended in the management of acute renal failure for several years (30). Its use, however, has been largely empirical. Although sustaining urine flow may help avoid tubular obstruction when proteinuria is present, its major usefulness is probably dependent upon improvement of the circulation by shrinking swollen cells.

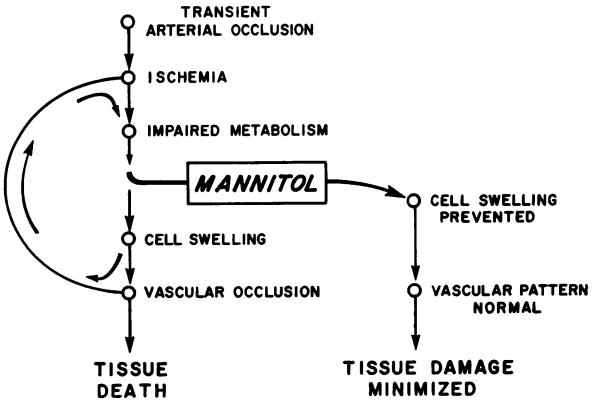

FIGURE 10 The effect of transient arterial occlusion on tissue ischemia and the corrective role of manitol.

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