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Effects of cold ischemia and reperfusion on trapping of erythrocytes in the rat kidney

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Abstract. After reperfusion of kidneys subjected to a period of warm ischemia, the medulla displays a vascular congestion of erythrocytes, especially in the inner stripe of the outer zone, a phenomenon referred to as "trapping." This trapping causes reflow alterations, thus contributing to postperfusion medullary ischemia. The purpose of the present investigation was to study whether trapping also occurs after reperfusion of kidneys following varying periods of *cold* ischemia and to determine if there is any correlation between the degree of cold ischemic injury and the extent of erythrocyte trapping. Rat kidneys stored at +4 °C for 0-30 h were transplanted into recipient animals pretreated with a ^{51}Cr-labelled erythrocyte suspension. Twenty minutes after reperfusion, the grafts were removed and microdissected into cortex, outer and inner stripes of the outer medullary zone, and inner zone, respectively. The radioactivity of these specimens was measured, and the erythrocyte content for each specimen was calculated. The results show a maximal trapping for cold ischemia time (CIT) of about 12-15 h. A linear correlation between the amount of trapping and CIT could be found in all parts of the kidney (except for the cortex) for CIT 0-15 h. The best correlation was found in the part where the trapping was most prominent, i.e., in the inner stripe. After CIT of 15 h or more, no correlation could be found. It is suggested, as described in models of *warm* ischemia, that the obstructions of the capillaries by trapped erythrocytes following reperfusion is of pathophysiological significance for the development of post-transplant acute renal failure. Furthermore, the strong correlation between CIT and the extent of erythrocyte trapping, particularly in the inner stripe, indicates that measurement of erythrocyte trapping after reperfusion could be a sensitive indicator of the degree of cold ischemic damage.

Key words: Organ preservation - Erythrocyte trapping - Experimental kidney transplantation.

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The outcome of renal transplantation has greatly improved since the introduction of cyclosporine A for immunosuppression. However, graft failure caused by ischemic damage still poses a clinical problem, and renewed efforts have been devoted to improving methods of preservation. The cause of post-transplant renal failure is multifactorial, which has made it difficult to pinpoint individual mechanisms involved in the preservation damage. Systematic studies focusing on biochemical and pathophysiological mechanisms are thus necessary.

In order to carry out research in the field of renal preservation, it is important to be able to measure the degree of ischemic damage. In this report, we have tried to make use of a well-known observation, namely of the hemorrhagic zone that is seen in the juxtamedullary areas in experimental models of warm ischemia. The hemorrhagic zone is the result of densely packed erythrocytes in the capillaries (see descriptions by Karlberg et al. [3] and Mason [5]). Karlberg et al. found that rat kidneys that had been subjected to 45 min of warm ischemia showed significantly increased trapping of ^{51}Cr-labelled erythrocytes in the inner stripe of the outer zone after 10 min of recirculation. This phenomenon has been further studied by Öjteg et al. [8] and Hellberg and Källskog [2] in a warm ischemia model in the rat.

The aim of this investigation was to study whether this erythrocyte trapping also occurs after

reperfusion of rat kidneys damaged by varying periods of *cold* ischemia and to investigate the possible correlation between the extent of erythrocyte trapping and cold ischemia time (CIT).

Materials and methods

The experiments were performed on male, Sprague Dawley rats, weighing 225 to 350 g (ALAB, Sollentuna, Sweden) with free access to a standard pellet diet, R3 (ALAB, Sollentuna, Sweden), and tap water. Anesthesia was induced by an intraperitoneal injection of Inactin (Byk-Gulden, Konstanz, FRG) in a dose of 120 mg/kg body weight. The rats were placed on a servo-controlled heating pad to keep their body temperature at 37.5 °C.

Kidney harvesting procedure

One hour before harvest of the kidney, the donor animal was pretreated with phenoxybenzamine i.v. in a dose of 3 mg/kg body weight. Heparin was given i.v. in a dose of 300 IU, 10 min before harvesting. The left renal vein and artery were dissected free. Ligatures were placed around the aorta proximally and distally to the renal vessels. A catheter with modified Sack's solution [10] at +4 °C was introduced into the aorta and the renal vein was cut off. The kidney was perfused with at least 6 ml of perfusion solution for 10 min at a hydrostatic pressure of 70-80 cm of water. The modified Sack's solution consisted of 1.26 g $NaHCO_3$, 2.30 g $KHCO_3$, 4.76 g KH_2PO_4, 7.41 g K_2HPO_4, 37.50 g mannitol, and ad 1000 ml sterile water. The kidneys were stored at 4 ± 1 °C in Sack's solution until transplanted.

Transplantation procedure

The recipient's left femoral artery and vein were catheterized, the artery for continuous monitoring of blood pressure and for blood sampling, the vein for continuous infusion of saline at a rate of 0.5 mg/h per 100 g body weight. The left kidney was mobilized and removed after clamping of the renal vein and artery. These vessels were then prepared using the "cuff" technique as described elsewhere [9]. In brief, short polyethylene tubes ("cuffs"), about half the length of the vessels, were placed outside each vessel. The edges of the vessels were subsequently pulled backward over the tubes and fixed with ligatures around these cuffs and vessels. The arterial cuffs were made of nylon (ID 0.75 mm, OD 0.94 mm) and the vein "cuffs" were made of polyethylene tube PP 190 (ID 1.19 mm, OD 1.70 mm). The anastomosis was performed by slipping the graft vessels over the cuffs and tying them in position. The time for anastomosis varied between 2 and 20 min with a mean of 5 min. To avoid warming during the anastomosing procedure, the graft was placed in a Lucite cup filled with crushed ice. Finally, the contralateral kidney was removed. Animals with a systemic blood pressure below 80 mm Hg during the anastomosing procedure were excluded.

^{51}Cr Labelling of erythrocytes

For labelling of erythrocytes, 10 ml of blood was withdrawn from one rat and placed into 1 ml ACD solution (Travenol Laboratories, Tretford, UK) to which 100 IU heparin was added. After centrifugation at 1500 rpm for 10 min and removal of the supernatant, 100 μCi of ^{51}Cr (sodium chromate, Amersham Int., Amersham, UK) was added. Following incubation at room temperature for 20 min, the erythrocytes were washed three times in saline and resuspended in an equal volume of saline. The labelled erythrocytes were stored in a refrigerator for a maximum of 5 days. Immediately before each experiment, the blood was washed twice to eliminate free chromium.

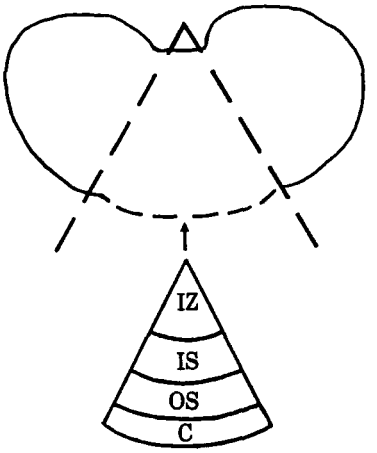

Fig. 1. Dissection of the kidney. A triangular core was obtained, which was then further microdissected into cortex (*C*), outer stripe (*OS*), inner stripe (*IS*), and inner zone (*IZ*)

Experimental protocol

Two series of experiments were performed.

Group A. In this experimental group of 44 transplantations, kidney grafts with CIT varying between 0.3 and 30.1 h were transplanted into recipient animals. Ten minutes before the vascular clamps were removed, 1.5 ml of ^{51}Cr-labelled erythrocyte suspension was given i.v. to the recipient. After 20 min of reperfusion, the graft was removed and microdissected into cortex (C), outer stripe of the outer zone (OS), inner stripe of the outer zone (IS), and inner zone (IZ) of the medulla, as described by Karlberg et al. [4] and illustrated in Fig. 1. The specimens were weighed and the radioactivity measured in a gammaspectophotometer (LKB-Wallac, Stockholm, Sweden). After the reperfusion period, two blood samples were taken, the radioactivity was measured, and the mean value of these samples was used as reference. At the same time, the hematocrit (Hct) was measured.

The amount of trapping (%T) was calculated as the percentage erythrocyte content according to the formula:

$$\%T = \frac{\text{cpm/g tissue}}{\text{cpm/g blood}} \cdot \text{Hct} \cdot 100$$

cpm = counts per minute

Background radiation was subtracted from the cpm values for each sample. The values obtained from this formula had to be corrected by subtracting the "normal" erythrocyte content, i.e., the nontrapped erythrocyte content in control kidneys. This normal content of erythrocytes in rat kidneys was determined in eight kidneys. The animals were pretreated with phenoxybenzamine as described above and were given 1.5 ml ^{51}Cr-labelled erythrocyte suspension, which was allowed to circulate for 10 min before removal of both kidneys. The erythrocyte content was calculated as above. The following values for the different regions were: cortex 1.44 ± 0.52%, outer stripe 1.40 ± 0.37%, inner stripe 2.10 ± 0.38%, inner zone 1.09 ± 0.30%, and, for the whole

kidney, 1.57 ± 0.32%. These mean values for each zone were subtracted from the corresponding values in the experimental group to adjust for the normal erythrocyte content in these kidneys.

Group B. To estimate the amount of erythrocytes that could not be washed out during the perfusion of the donor kidney, 1.5 ml ^{51}Cr-labelled erythrocyte suspension was given to five donor rats before the in situ perfusion. After the perfusion, the kidneys were microdissected as outlined above and the radioactivity in the respective specimens was measured.

Statistics

Values are given as means ± SD. Statistical analysis was made by linear regression analysis. A P value of < 0.01 is considered statistically significant.

Results

Mean arterial blood pressure during anastomosis and reperfusion was 110 (range 80–120). The hematocrit at the end of the reperfusion period varied between 0.45 and 0.50. In group B, no blood could be detected in the kidneys, indicating that the "washout" procedure was highly effective in removing residual blood from the organ.

Results from the experimental group A are displayed in Table 1 and Fig. 2. These values are the "corrected" values, i.e., the normal erythrocyte content (see Materials and methods) has been subtracted.

The removed kidneys invariably showed a darker zone in the juxtamedullary areas. Trapping of erythrocytes in the renal vasculature is illustrated in a scanning electron micrograph (Fig. 3).

For the cortex (Fig. 2A), no significant correlation between CIT and the amount of erythrocyte trapping (%T) could be found. From Fig. 2B–D, describing the results from the medullary areas and whole kidney, two separate observations can be made: (1) for CIT > 15 h, there is no correlation between CIT and %T, and (2) for CIT < 15 h, all areas show a significant ($P < 0.01$) correlation between CIT and %T. The best correlation was obtained for the IS ($r = 0.97$, $P < 0.001$), followed by the whole kidney ($r = 0.82$, $P < 0.01$).

Table 1. Values for trapping in the different zones in the kidney for each individual rat (expressed as percent as described in the methods section). C, Cortex; OS, OZ, outer stripe of the outer zone; IS, OZ, inner stripe of the outer zone; IZ, inner zone; Tot, total trapping for the whole kidney

CIT (h)	T (%)				
	C	OS, OZ	IS, OZ	IZ	Tot
0.3	0.0	0.0	0.3	0.0	0.0
0.4	0.0	0.0	0.6	0.0	0.0
0.5	0.0	0.5	1.5	0.0	0.0
1.1	0.0	0.5	0.7	0.0	0.2
1.2	0.2	2.6	4.6	1.0	0.3
1.6	0.6	3.1	4.0	1.1	0.6
2.5	1.8	2.4	5.5	1.3	4.3
3.5	0.4	2.4	5.4	2.0	1.2
4.5	0.0	1.3	6.6	1.1	1.7
6.0	1.0	3.6	8.0	2.6	1.7
7.4	0.0	3.4	7.6	2.1	1.7
7.6	1.4	8.5	9.5	6.4	5.8
8.0	3.6	6.2	13.5	5.0	6.6
8.2	0.9	6.4	11.1	3.3	3.6
9.2	4.2	8.4	18.5	8.6	8.3
9.6	0.1	5.3	13.5	5.9	2.6
9.8	0.0	7.5	19.4	8.2	4.6
10.0	1.4	9.6	15.2	4.0	4.3
10.5	3.4	8.8	16.4	5.7	7.7
11.6	0.9	6.7	18.1	6.5	4.3
12.0	0.0	0.0	21.1	23.7	10.4
12.6	2.3	12.6	21.9	10.6	6.5
14.5	6.0	13.1	24.3	8.9	9.2
14.8	1.8	6.4	18.3	8.7	6.1
18.8	4.8	10.6	14.8	5.0	6.9
18.8	2.4	6.1	12.9	6.2	5.6
19.0	4.9	6.6	7.4	3.7	2.2
19.5	0.0	0.0	5.7	0.0	1.6
20.0	0.0	3.9	17.7	13.3	3.5
22.2	1.8	6.3	13.9	8.1	5.2
23.0	3.1	7.2	13.3	5.6	5.8
23.2	1.1	9.9	30.3	10.4	6.9
23.5	2.8	6.4	18.8	8.9	7.5
23.6	2.7	8.2	14.3	7.1	5.5
24.0	19.0	28.7	58.6	10.1	17.4
24.0	1.8	3.4	7.9	0.0	3.6
24.8	2.2	5.0	26.9	5.4	1.0
29.2	7.0	8.5	14.7	7.0	8.3
30.0	0.0	1.7	2.5	0.0	3.6
30.0	2.6	10.1	24.3	10.4	7.8
30.0	3.2	7.5	20.1	8.0	8.4
30.0	6.9	11.6	21.5	8.8	9.6
30.1	4.2	9.8	24.3	8.4	9.7
30.1	6.2	12.6	23.6	7.9	10.1

Discussion

The pathophysiological significance of erythrocyte trapping and medullary congestion after warm ischemia has been thoroughly reviewed by Mason [6]. It seems conceivable that the resulting obstruction of the peritubular capillaries might contribute to and aggravate the medullary ischemia found after recirculation of ischemically injured kidneys [4]. This concept is further supported by the finding that renal function improved considerably when the medullary congestion was reduced by a lowering of the hematocrit [2], or pretreatment with allopurinol [8] or superoxide dismutase [1].

All of these experiments were performed with kidneys that had been subjected to warm ischemia. The question may therefore be raised as to whether

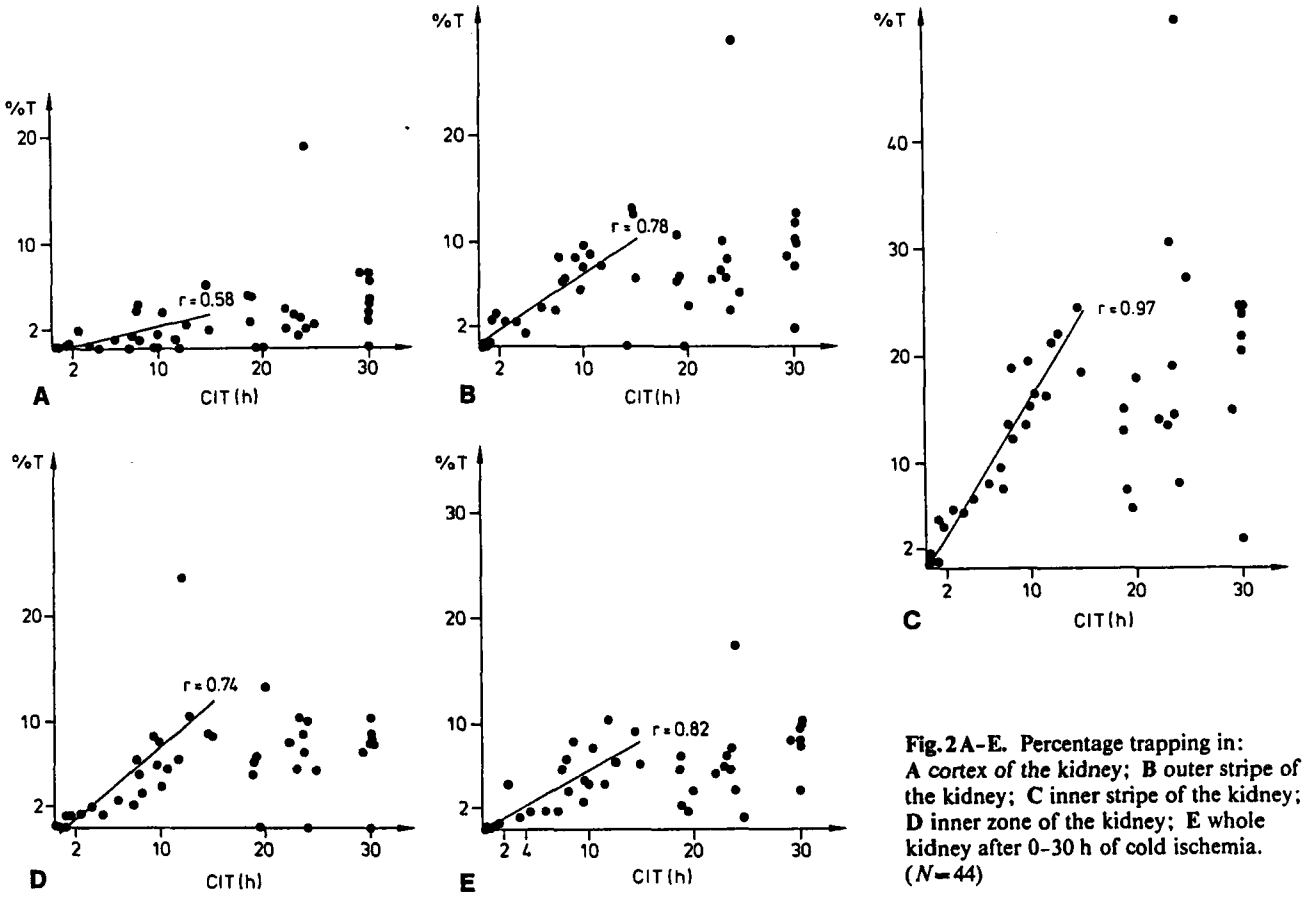

Fig. 2 A–E. Percentage trapping in: **A** cortex of the kidney; **B** outer stripe of the kidney; **C** inner stripe of the kidney; **D** inner zone of the kidney; **E** whole kidney after 0–30 h of cold ischemia. ($N = 44$)

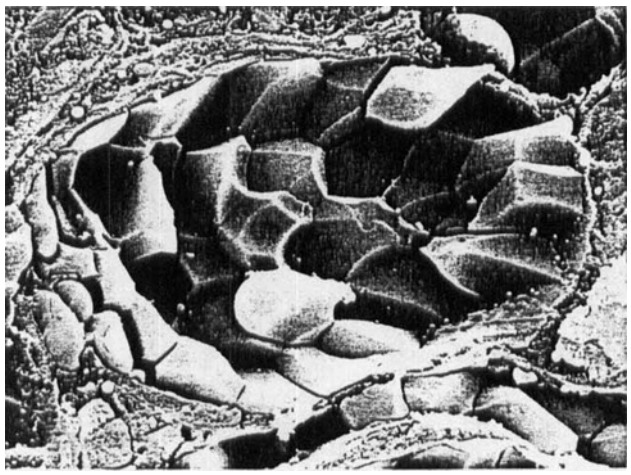

Fig. 3. Scanning electron micrograph showing extensive trapping of erythrocytes in a capillary of the inner stripe of a kidney after 20 min reperfusion following 18 h of cold storage ($\times 3.005 \cdot 10^3$)

a similar mechanism of erythrocyte trapping may be responsible for injuries observed after reperfusion of kidneys preserved by cold storage. The present study shows that a substantial trapping occurs in this situation. The results indicate that the trapping occurs predominantly in the medullary areas. Furthermore, for kidneys stored for less than 15 h, there is a good correlation between CIT and the extent of trapping and, therefore, presumably also between the degree of ischemic injury and the extent of trapping. This was especially evident where trapping was most extensive, i.e., in the inner stripe. It is difficult to evaluate functional parameters, for example, glomerular filtration rate in the immediate post-transplant (revascularization) period. Results from this study, however, show that the degree of trapping is a reproducible parameter, strongly correlated to CIT. It should, therefore, be evaluated as a possible, and easily determined, indicator of damage caused by cold storage and reperfusion.

For kidneys stored cold for more than about 15 h, the pattern in terms of medullary trapping of erythrocytes is more complex. As can be seen in Fig. 2, there tends to be a lower degree of trapping for some kidneys stored for 15–30 h. One reason for this could be an inhomogeneity of reperfusion of these kidneys, something which was already evident upon macroscopic examination. This concept is supported by the findings of Norlén et al. [7] showing nephron heterogeneity in rat kidneys stored cold for 16 h.

Some kidneys stored cold for 15-30 h tended to give especially high values for erythrocyte trapping. A possible explanation for this is that vascular disruptions with extravasation of blood occurred in addition to trapping in these severely damaged kidneys. This phenomenon has been observed under both light and scanning electron microscopy (unpublished observations). Thus, these observed values are not a reflection of "true" trapping.

Thus, it is obvious that quantification of medullary erythrocyte trapping can be used as an indicator for ischemic injury caused by cold storage of rat kidneys for less than 15 h, but that it is not suitable for CIT beyond this time.

Because of similarities between these findings and those obtained from models of warm ischemia, it is conceivable that the obstruction of medullary vessels - primarily peritubular capillaries - by trapped erythrocytes is also of pathophysiological importance for post-transplant renal function. Thus, future research efforts should be directed towards reducing the amount of trapping in the reperfusion phase of preserved kidneys. Judging from the experiences obtained in warm ischemia models, lowering the hematocrit of the recipient [2] or treatment with allopurinol [8] or superoxide dismutase [1] could improve renal function by reducing the trapping.

In summary, we have found that the trapping of erythrocytes, previously demonstrated after warm ischemic damage, also occurs after cold ischemia and that there is a strong correlation between the duration of cold ischemia and the amount of trapping, especially in the inner stripe of the outer zone.

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