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ORIGINAL ARTICLE

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Normothermic blood perfusion of isolated rabbit kidneys

III. In vitro physiology of kidneys after perfusion with Euro-Collins solution or 7.5 M cryoprotectant (VS4)

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All procedures and experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, DHHS, Publication No. (NIH) 86-23 (1985) and USDA guidelines and were approved by the Animal Care and Use Committee of the Naval Medical Research Institute

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Abstract Cryopreservation of solid organs might be possible using a mixture of cell-permeable agents, cryoprotectants (CPA), which are designed to completely preclude ice crystal formation during cooling to cryogenic temperatures. The effects of a specific prototype solution (VS4) were evaluated by normothermic blood perfusion in vitro. Rabbit kidneys were divided into three groups: untreated controls ($n=7$), Euro-Collins (EC)-perfused controls ($n=6$) and VS4 (49%, w/v) CPA-perfused kidneys ($n=7$). After a 2-h blood perfusion, five of the seven CPA-perfused kidneys developed polyuria (0.21 $ml \times min^{-1} \times g^{-1}$) relative to untreated controls (0.07 $ml \times min^{-1} \times g^{-1}$) or EC-perfused kidneys (0.06 $ml \times min^{-1} \times g^{-1}$), owing to the lower reabsorption of water (34.3%), Na^+ (34.2%) and glucose (35.6%). Furthermore, two kidneys were non-functional with virtually no urine production. Reduced tubular function was associated with reduced oxygen consumption (3.6 versus 2.3 versus 2.0 $\mu mole \times min^{-1} \times g^{-1}$ for controls, EC- and CPA-perfused kidneys, respectively) and increased weight gain (17% versus 20% versus 30%, respectively) after blood perfusion. Therefore, the current results provide insight into both the physiological effects of VS4 and the limits of reversibility of renal pathophysiological states. Our results also indicate that in vitro monitoring of oxygen consumption and weight gain of perfused organs could be used as predictors of renal function.

Keywords Organ cryopreservation · In vitro perfusion · Vitrification · Transplantation · Oxygen consumption · VS4

Abbreviations *CPA* Cryoprotectant · w/v Weight per volume · *KHL* Krebs Henseleit-like solution · *EC* Euro-Collins · *VS4* Vitrification solution containing 49% (w/v) = 7.5 M CPA – *WBC* White blood cells · *GFR* Glomerular filtration rate · qO_2 Oxygen consumption

Introduction

Long-term cryopreservation of solid organs that are intended for transplant, such as kidneys, would allow improved storage and transportability. Storage at low temperatures might be possible with the use of vitrification [9]. Vitrification solutions usually consist of a mixture of different agents designed to prevent ice crystal formation when cooling and to inhibit crystal growth during re-warming [4], while simple freezing will result in the total loss of viability. A solution containing a mixture of cryoprotective agents with a high glass

transition temperature (T_g) and relatively low toxicity, known as VS4, provided a good recovery of rabbit cortical slices at low temperature [8]. VS4 consists of the minimum concentration (49% w/v, or 7.5 M) of cryoprotectant (CPA) capable of forming a glass at 1,000 atm. Concentrations of at least 55% CPA are required to permit vitrification at 1 atm [8, 13].

Several vitrification solutions and procedures have been tested in this laboratory to achieve organ vitrification using an autologous transplant model with rabbit kidneys or rat livers [7, 11, 13, 14, 20]. Rabbit kidneys perfused with this cryoprotective solution and cooled to sub-zero temperatures could subsequently be transplanted with 100% survival at 3 weeks postoperatively[11, 12], although graft function was delayed for a few days. Renal function after VS4 perfusion was also evaluated using an ex vivo blood reperfusion model [7], which showed that 50% of the CPA-perfused kidneys yielded appreciable amounts of urine ex vivo. All kidneys experienced transient reductions in renal blood flow, and the severity of the vascular response appeared to determine the ability of the kidney to produce urine and function within the period of evaluation. However, the pathophysiologic basis of this transient hypoxia (vascular crisis) was not elucidated in either the ex vivo or the transplant model [11, 14, 15, 16].

In order to better understand these events, we turned to blood perfusion as a suitable technique to measure the function of the isolated, perfused kidney [21]. A computer-controlled apparatus has been previously developed to assess in vitro renal function under normothermic conditions using blood as a perfusate [1]. This apparatus has allowed us to monitor, quantify and record key processes such as renal blood flow, glomerular filtration and tubular reabsorption soon after reperfusion. This normothermic blood perfusion device does not perturb organ viability since control kidneys have been shown to retain normal function upon transplantation of the organ following 2-h blood perfusion [2].

The objective of this study was to evaluate the effect of VS4 on renal function in vitro in order to separate the intrinsic effects of VS4 from effects that may be dependent upon the in vivo environment [7, 11] and to establish a frame of reference for further studies intended to test interventions designed to ameliorate observed deficits. A further objective was to seek correlations between functional recovery and readily monitored end points that might be useful in the eventual prediction of post-transplant survival. Results obtained with CPA perfusion are compared to those with Euro-Collins (EC) perfusion since EC is the vehicle solution commonly used for the delivery of the CPA to the organs [11, 15].

Material and methods

Experimental design

Experiments were designed to assess in vitro function of kidneys perfused with 49% CPA vitrification solution compared to untreated controls and kidneys perfused with EC, the vehicle solution. Therefore, three groups of kidneys were examined separately: untreated controls, EC-perfused controls and CPA-perfused controls. All kidneys were excised from the rabbit and stored briefly in EC. In the first group, the untreated controls, seven kidneys were immediately tested by 2-h normothermic blood perfusion [2]. In the second group, six kidneys were perfused with EC at +3° C for 5 h, the same duration as the CPA perfusion, and then blood perfused; they served as perfused controls. In the third group, seven kidneys were perfused with gradually increasing concentrations of CPA as the temperature was gradually lowered to –3° C, and the process was then reversed, with the entire perfusion lasting for 5 h. Despite the perfusion temperature discrepancy between the EC and VS4 groups, this experimental design is most appropriate as experiments at –3° C cannot be performed without CPA and as experiments at 3° C with CPA could induce some toxicity [11]. After this procedure, the kidneys were promptly submitted to evaluation by blood perfusion.

Kidney procurement

The kidneys were removed from healthy 2.8–3.1 kg male New Zealand White (NZW) rabbits as described elsewhere [2, 10]. Briefly, the right kidney was mobilized, diuretics (mannitol: 3 ml of 20% w/v, furosemide: 10 ml of 0.5 mg/ml) were administered, and Iloprost (25 μg/kg) (Berlex, Wayne, NJ) was infused directly to the renal artery over a time course of 20 min. The use of Iloprost was based on previous findings that it increased survival rates after transplantation [11, 12]. The renal artery was cannulated. The blood was flushed immediately from the organ in situ with 100 ml of cold (4° C) EC solution at a pressure not exceeding 60 mmHg. The first 50 ml contained EC supplemented with Iloprost (1 μg/ml) and heparin (10 U/ml), and the second 50 ml was solely EC. The warm ischemia time was less than 2 min. The kidney was removed and the renal vein and ureter were cannulated. The organ was mounted on a stage, the initial weight was measured, and the kidney was stored in EC at 4° C until it was further perfused with EC or 49% CPA or directly perfused with blood.

Blood collection and handling

Approximately 40 ml of blood was collected from three healthy (3.5 kg) NZW rabbits through the median ear artery using a 50 ml syringe containing 1.5 ml anticoagulant (0.75 ml of 1 mg/ml aspirin and 0.75 ml of 1,000 U/ml heparin). The blood was filtered free of white blood cells (WBC) and platelets using a R-500 Leukocyte type filter (Sepacell R-500, Fenwal, Deerfield, IL). Removal of WBC and platelets has been reported to be necessary for stable blood perfusion [22]. The hematocrit was adjusted to 30% with the addition of Krebs Henseleit-like solution (KHL). A volume of approximately 120 ml of blood perfusate was required to complete a 2-h perfusion.

Solutions

Krebs Henseleit-like solution [1] is an extracellular solution consisting of 3 mM KCl, 0.5 mM KH_2PO_4, 94 mM NaCl, 0.7 mM NaH_2PO_4, 20 mM $NaHCO_3$, 12.5 mM Na acetate, 15 mM Na lactate, 6.3 mM Na butyrate, 1 mM $MgSO_4$, 1 mM alanine, 5 mM

glycine and 6 mM glucose supplemented with 0.35 mM creatinine (pH: 7.4 and osmolality: 310 mOsm/kg).

The Euro-Collins solution contained 15 mM KCl, 42 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $NaHCO_3$ and 194 mM dextrose (pH: 7.4 and osmolality: 360 mOsm/kg).

The fixative solution, a low-osmolality Karnovsky's solution (LOK), contained 10 g/l paraformaldehyde, 25 g/l glutaraldehyde, 24 mM Na_2HPO_4 and 34 mM NaCl (pH: 7.4 and osmolality: 700 mOsm/kg).

The vitrification solution (49% CPA, VS4) consisted of 2.76 M dimethyl sulfoxide, 2.76 M formamide and 1.97 M propylene glycol. This cryoprotective solution was prepared with EC as the vehicle solution. Viability experiments performed on kidney slices leading to the development of the cryopreservation solution have indicated that EC yielded superior results as compared to UW in the presence of VS4 (unpublished data). Therefore, EC was used as the carrier solution in all subsequent experimentation. The pH was adjusted to 8.0 using HCl; the total molarity was 7.5 M, and the freezing point depression was –33° C. All chemicals were obtained from either the Sigma Chemical Company (St Louis) or Fisher Scientific (Pittsburgh).

Cryoprotectant perfusion protocol

The cryoprotectant perfusion apparatus has been previously described in detail [6]. Briefly, a computer-controlled apparatus was developed for the introduction and removal of the cryoprotectant under constant perfusion pressure. The perfusate flow and perfusate concentration were continuously monitored. The difference between arterial and venous cryoprotectant concentration (A-V) was continuously measured by a differential refractometer. The desired cryoprotectant concentration was obtained by adequate mixing of CPA and EC in gradient formers, which were controlled by solenoid valves responding to the refractometer readings. Immediately after removal of the kidney, the cannulated kidney artery was connected to the perfusion apparatus. Effluent from the venous line was allowed to drip into the chamber and was recirculated to the perfusate line, except when the perfusion protocol required it to be discarded (see [6] for a full description). After an initial 4 min perfusion equilibration of the kidney with EC at 3° C, the full addition and removal of CPA was performed stepwise (Fig. 1). CPA was gradually introduced to achieve a concentration of 4 M, and the temperature was progressively lowered to –3° C. This permitted intracellular equilibration of 4 M permeant CPA. Full permeation of the cells and their content is necessary in order for the cells to be protected at lower temperatures [9]. Then, 7.5 M CPA was perfused directly (a single step change in concentration). The intracellular equilibrium of the 7.5 M CPA was achieved within a period of 20 min by a combination of cryoprotectant uptake and water exosmosis, resulting in intracellular concentrations sufficient to assure the protection of the cell [9]. Washout of the cryoprotectant was then initiated by dropping the CPA concentration to 4.5 M in one step. Thereafter, the CPA concentration was decreased gradually, and the temperature was raised back to 4° C. EC supplemented with 250 mM mannitol was used for the washout to counteract any cell swelling. The perfusate pressure was maintained at 40 mmHg, and the temperature was continuously measured by copper constantan thermocouples on the kidney surface as well as in the venous outflow. The procedure took approximately 5 h to be completed. Data for temperature, flow rate, pressure, resistance and CPA concentration were displayed on a computer screen and stored for further analysis. This apparatus was also used to perfuse kidneys for 5 h with EC solution. In this case, the temperature was maintained at 3° to 4° C. After the 5-h perfusion, the kidney weight was measured, and the organ was placed back in EC before the normothermic blood perfusion.

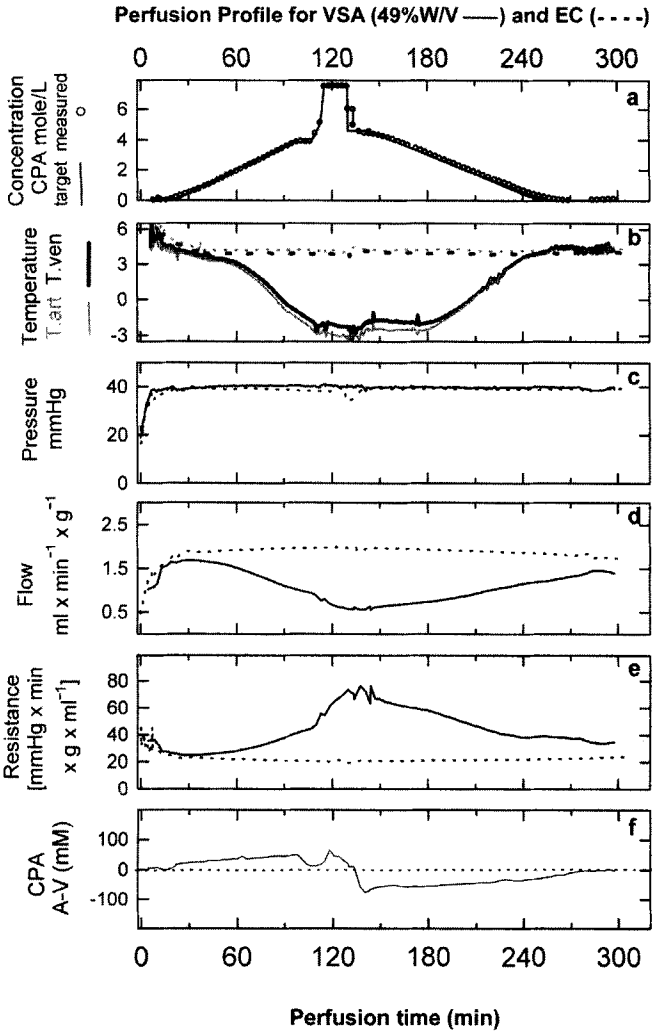

Fig. 1a–f. Perfusion profile of parameters obtained using the cryoprotectant perfusion apparatus. Both EC and 49% CPA perfusion are represented on the same graph, 49% CPA (*w/v*) (*solid lines*) and EC perfusion (*dashed lines*). **a** CPA concentration. **b** Temperature profile. **c** Perfusion pressure. **d** Perfusate flow rate. **e** Apparent vascular resistance of the kidney (pressure/RBF). **f** Arterio-venous cryoprotectant concentration difference as determined by differential refractometry (*A– V*)

Normothermic blood perfusion

The blood perfusion apparatus has been described in detail in a previous publication [1]. Briefly, the untreated kidney or previously perfused kidney was placed inside a small humidity- and temperature-controlled chamber. The kidney was flushed with 30 ml of KHL solution. It was then connected to the blood loop of the apparatus through the cannulas placed in the renal artery and renal vein. Upon activation, a computer program initiated the circulation of blood in the kidney through a roller pump (Masterflex, Cole Parmer). The pressure was elevated stepwise under computer-controlled incrementation until it reached 90 mmHg. Blood gases (measurements of oxygen, carbon dioxide, pH and temperature) were monitored in line with optical sensors (CDI 400, 3M-health care) and were continuously recorded during perfusion. The computer also monitored perfusion pressure

(measured by pressure sensor), blood flow (measured as blood pump rate) and urine production (measured by drop detector). The computer-controlled addition of KHL was introduced into the blood perfusate to compensate for the volume of urine that was produced and removed from the system. This maintained the hematocrit and plasma protein concentration at their initial values during the perfusion. Serum chemistry was performed on arterial blood samples drawn from a collection port every 15 min, and urine samples were collected over these time intervals and pooled.

Blood and urine assays

RBC counts, hematocrit (Hct) and total hemoglobin (Hb) in whole blood samples collected during perfusion were determined using an electronic cell counter (Hematology analyzer 9000, Serono Baker, Allentown, PA). Residual platelet and WBC counts were also determined. Subsequently, plasma was obtained from these samples after 10 min centrifugation at 2,500 g. Plasma and urine chemistry profiles were obtained using an Ektachem Analyzer (Johnson and Johnson).

Hemolysis determination

Plasma hemoglobin was measured by a colorimetric method using 3,3′–5,5′ tetramethylbenzidine (TMB) (Sigma kit no. 527-11); 0.05 ml of plasma was added to 1 ml of TMB. The reaction was initiated by adding hydrogen peroxide at very precise time intervals, and the samples were read exactly 10 min later at 600 nm (OD_t) on a spectrophotometer (55B, Perkin-Elmer, Norwalk, CT) with respect to a hemoglobin standard (OD_s). The free hemoglobin concentration in test samples was calculated as:

$$Hb_t = OD_t \times Hb_s/OD_s$$

Plasma hemoglobin concentration of the test sample and the standard (Hb_t and Hb_s, respectively) were determined as mg/100 ml blood. The percentage of hemolysis (Hm) was related to the total hemoglobin of the sample (THb) adjusted for the hematocrit as follows:

$$Hm(\%) = 100 \times (Hb_t \times (1 - Hct))/THb.$$

Kidney histology

Following blood perfusion, the kidneys were flushed with 50 ml cold saline, the weight was measured, and the organ was then perfused-fixed with 50 ml of LOK solution under a pressure not exceeding 40 mmHg. The kidneys were sectioned, and the pieces were embedded, sliced and stained with periodic acid Schiff (PAS) for histology examination. Histological processing of kidney sections was performed at the pathology laboratory at NMRC or American Histolabs, Inc. (Gaithersburg, MD).

Renal function indices

All clearances (Cl) were calculated by using the standard formula:

$$Cl_t(ml/min) = (U_t \times V)/Pt$$

where t refers to the substance of interest, U_t is urine concentration of substance t, P_t is the plasma concentration of t, and V is the average urine flow rate over the period of urine collection. The glomerular filtration rate (GFR) is calculated based upon the creatinine clearance.

The method to calculate other indices such as the fraction reabsorption (FR_t), the water volume reabsorption (Vr), the filtration fraction (FF), the fractional protein exclusion from the urine (Ex_{pr}) and the vascular resistance have been described previously [2].

Oxygen consumption (qO_2) was calculated as the product of the differential arterial-venous oxygen content and the renal blood flow, assuming that qO_2 was solely due to kidney metabolism [1]. Oxygen content was calculated from the hemoglobin concentration and blood saturation. The saturation (Sat) was calculated from the pO_2 measurements by the CDI sensors and adjusted to rabbit blood. Plasma dissolved oxygen was also taken into account. The qO_2 was expressed in $\mu mole \times min^{-1} \times g^{-1}$. The full equation was described in [2]. The fractional oxygen extraction (Fr_{O2}) was calculated as the ratio of the oxygen consumed (qO_2) divided by the oxygen delivered to the kidney (dO_2) and was expressed as a percent; dO_2 was calculated as the product of renal blood flow and the arterial oxygen content (CaO_2).

Statistical analysis

Blood perfusion pressure data were acquired every 45 s, and the other parameters (temperature, blood gases, urine flow and RBF) were acquired every 4 min. All data of each type were averaged within each 4 min time frame for a given experiment. This allowed standardized times frames to be averaged over different experiments for all parameters over the 2 h run (Microsoft Excel software). Chemistry data, GFR and reabsorption data were obtained from the blood samples collected every 15 min and averaged over the same time frame. Results were compared using a Student's *t*-test, one factor ANOVA or Fisher test. Linear regression was calculated by the method of the least squares. The confidence level for statistical significance was equal or greater than 95%.

Results

Perfusion profiles of EC- and CPA-perfused kidneys

The CPA-perfusion profile presented in Fig. 1 is representative for all 49% CPA perfused kidneys. While increasing the CPA concentration (Fig. 1a) and reducing the temperature to –3° C (Fig. 1b) during the first 2 h, the viscosity rises. This causes the resistance to increase (Fig. 1c) and the flow rate to decrease (Fig. 1d). The initial flow rate was 1.57 ± 0.37 $ml \times min^{-1} \times g^{-1}$. At 7.5 M CPA perfusion, the arterio-venous difference (A-V) (Fig. 1f) reached zero, indicating that osmotic equilibrium was attained in the kidney. After an initial step change and equilibration at 4.5 M CPA, CPA washout was performed linearly with time, and this was similar to ex vivo [7] or in vivo [11] procedures. The measured rate of change of CPA concentration followed the computer-programed rate very closely. During this phase the flow rate gradually increased and approximated the original value at the end of the perfusion (1.49 ± 0.43 $ml \times min^{-1} \times g^{-1}$ for the last 1/2 h, no significant difference with the initial flow).

By comparison, the EC-perfusion profile (Fig. 1) shows steady resistance and flow because of the absence of CPA in the perfusate and the constant perfusion temperature in this group. Perfusate flow was constant starting initially at 1.91 ± 0.46 $ml \times min^{-1} \times g^{-1}$ and reaching 1.79 ± 0.41 $ml \times min^{-1} \times g^{-1}$ during the last hour (no significant difference). Overall, the flow rate averaged

1.83 ± 0.43 ml×min^{-1} giving a resistance of the kidneys of 22.6 ± 5.3 mmHg×min×g×ml^{-1}, comparable to what has been described previously [7]. The arterial and the venous temperatures were maintained at $4.0 \pm 0.1°$ C and $4.3 \pm 0.3°$ C, respectively, during the perfusion. The perfusion pressure was well regulated at 40 mmHg for both EC- and CPA-perfused kidneys (Fig. 1c).

Renal function

Renal functional indices are presented in Table 1 and Table 2 and in Fig. 2, Fig. 3 and Fig. 4. The temperature and perfusion pressure were well controlled close to 36° C and 90 mmHg, respectively, in all groups. The hematocrit was successfully maintained at 30% during blood perfusion. The hemolysis level of the blood perfusate of EC- or CPA-perfused kidneys at the end of the blood perfusion was low and comparable to that of the controls (Table 1).

Even though the perfusion conditions were maintained with excellent reproducibility, the CPA-perfused kidneys fell into two distinct categories: a functional subgroup ($n = 5$) and a non-functional subgroup ($n = 2$) (Fig. 3, Fig. 4, Fig. 5). The non-functional kidneys produced virtually no urine for the first 40 min and very little thereafter. Consequently, their other renal parameters neared zero, which justifies regarding these kidneys as a separate group.

Following treatment but prior to blood perfusion, the weight gains seen in the EC- and CPA-perfused groups were within the limits typically seen in our laboratory, with the exception of the non-functional kidneys perfused with 49% CPA (Table 1, $P < 0.005$). The weight gain in

Table 1. Initial in vitro parameters and weight gain of blood-perfused kidneys. Parameters reflecting initial or final status of the kidneys undergoing 2 h blood perfusion in the three groups of kidneys studied: *untreated control*, *EC-perfused* and *CPA-perfused* (mean and standard deviation). Weight increase is relative to the initial weight measured after organ procurement. Student's *t*-test comparison between EC and 49% CPA-perfused kidneys after asanguineous perfusion and after blood perfusion

Parameters	Untreated control ($n = 7$)	EC-perfused ($n = 6$)	CPA-perfused ($n = 7$)	
Initial hematocrit (%)	29.2 ± 3.1	31.3 ± 3.3	29.8 ± 2.3	
Average hematocrit (%)	30.6 ± 2.3	32.6 ± 1.4	32.6 ± 2.5	
Perfusion pressure (mmHg)	89.1 ± 3.9	87.6 ± 3.7	86.3 ± 6.8	
Arterial perfusion temperature (average) (°C)	36.6 ± 1.2	35.8 ± 0.3	36.4 ± 0.7	
Venous perfusion temperature (average) (°C)	36.2 ± 0.9	35.1 ± 0.6	35.6 ± 0.9	
Final blood hemolysis (%)	0.34 ± 0.14	0.28 ± 0.09	0.33 ± 0.09	
Initial weight (g)	12.4 ± 1.9	12.3 ± 1.8	12.5 ± 1.5	
Kidney weight increase (%)			Functional ($n = 5$)	Non-functional ($n = 2$)
After EC- or CPA-perfusion	–	12.9 ± 7.3	19.6 ± 10.2	26.9 ± 8.7*
After blood perfusion	17.4 ± 10.9	19.6 ± 10.8	29.8 ± 8.7*	48.6 ± 2.0*

*$P < 0.05$, **$P < 0.001$

Table 2. In vitro parameters and function of blood-perfused kidneys. Functional parameters illustrating the renal function after 5 h perfusion with either EC or 49% CPA and tested with a 2 h blood perfusion (mean and standard deviation). Data were averaged after 15 min from the start of the blood perfusion or 40 min from the start for the CPA-perfused kidneys

Parameters	Units	Control group $n = 7$	EC-perfused $n = 6$	49% CPA-perfused	
				Functional $n = 5$	Non-functional $n = 2$
RBF	ml/min/g	2.91 ± 0.57	$4.07 \pm 0.75^{\#}$	4.65 ± 1.64(*)	4.32 ± 2.7^{S}
Urine flow	ml/min/g	0.068 ± 0.05	$0.056 \pm 0.022^{\#}$	0.208 ± 0.08(*)	0.008 ± 0.007^{S}
GFR	ml/min/g	0.30 ± 0.06	$0.15 \pm 0.06^{\#}$	0.30 ± 0.13(*)	0.007 ± 0.007^{S}
FF	%	12.82 ± 3.22	$5.80 \pm 1.71^{\#}$	10.9 ± 2.9(*)	0.16 ± 0.12^{S}
Vr	%	74.7 ± 3.8	$60.9 \pm 3.5^{\#}$	34.3 ± 7.7(*)	4.0 ± 6.0^{S}
FR[Na]	%, [µg/min/g]	78.1 ± 2.8, $[30.8 \pm 6.1]$	$64.1 \pm 3.7^{\#}$, $[13.3 \pm 5.0]$	34.2 ± 4.1(*), $[14.4 \pm 6.7]$	6.4 ± 6.0^{S}
FR[Glu]	%, [µmole/min/g]	90.6 ± 1.4, $[557 \pm 138]$	$81.8 \pm 7.2^{\#}$, $[294 \pm 123]$	35.6 ± 8.2(*), $[181 \pm 103]$	0
EX[protein]	%	99.8 ± 0.1	98.2 ± 0.7	90.9 ± 7.9(*)	44.7 ± 24.7^{S}
QO2	µmol/min/g	3.58 ± 1.77	2.3 ± 0.8	2.04 ± 0.5(*)	1.51^{S}

Anova comparison (* between 49% CPA-perfused and EC-perfused kidneys ($P < 0.01$), # between EC and untreated controls ($P < 0.05$), S between non-functional and functional CPA-perfused kidneys) ($P < 0.01$)

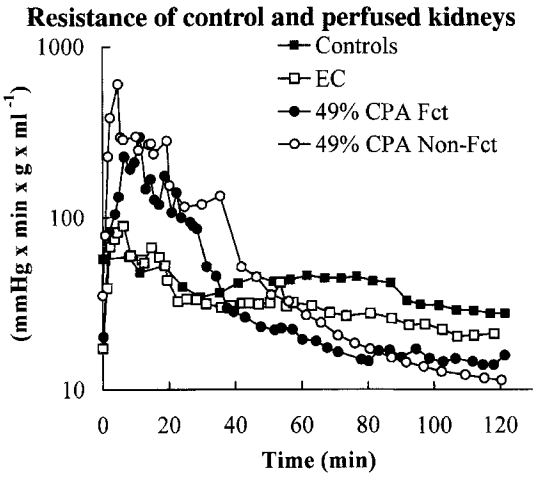

Fig. 2. Vascular resistance (mmHg×min×g×ml^{-1}) of the kidneys during blood perfusion, Untreated controls (*filled squares*), EC perfused controls (*empty squares*), 49% CPA perfused functional (*fct*) (*filled circles*) and 49% CPA perfused non-functional (*non-fct*) (*empty circles*). SEMS ranged from 8.9 min×mmHg×g×ml^{-1} (controls) to 89 min×mmHg×g×ml^{-1} (49% CPA) during the highest resistance phase, and from 3.1 min×mmHg×g×ml^{-1} to 4.4 min×mmHg×g×ml^{-1} at the end of the perfusion

the EC-perfused group after blood reperfusion (20%) was not significantly different from that in the untreated control group (17%), suggesting that EC perfusion per se was not damaging to the microcirculation.

The edema observed in the non-functional subgroup was reflected in a dramatic initial increase in vascular resistance (vascular crisis). These kidneys experienced a greater peak resistance (reaching higher than 400 mmHg×min×g×ml^{-1}) as compared to the functional subgroup kidneys (200 mmHg×min×g×ml^{-1}) (Fig. 2). This elevated resistance fell after 40 min, whereas resistance fell after 30 min for the functional CPA group. The final resistance reached 15.3 ± 1.4 and 15.7 ± 10.5 mmH×min×g×ml^{-1} for non-functional and functional kidneys, respectively, during the last hour of blood perfusion. At the time of vascular crisis, renal blood flow was initially twofold lower in the CPA-perfused group than in the EC-perfused or untreated control groups (Fig. 3a). After 40 min RBF increased to higher than the control values, and final RBF in the non-functional subgroup exceeded that of the functional subgroup. Also consistent with the pattern of weight gain, CPA-perfused kidneys lost considerable protein to the urine (Fig. 3b), a problem that was reversed after 60 min blood perfusion in the functional group. However, this proteinuria remained severe to the end of perfusion in the non-functional subgroup where protein exclusion from urine was less than 65%. In contrast, EC-perfused kidneys showed proteinuria only at the onset of blood reflow and less than 2% proteins was found in urine at the end of perfusion.

The filtration fraction of the functional CPA-perfused kidneys (Fig. 3c) and untreated controls were identical in contrast to that of the EC-perfused kidneys, which was reduced. The filtration fraction of non-functional kidneys remained at or near zero during the entire observation period. The GFR of EC-perfused kidneys was lower than untreated controls (Fig. 3d) and seemed to be governed by the filtration fraction. The GFR of functional CPA-perfused kidneys was initially lower than the untreated controls but increased above normal during the last hour of blood perfusion, following in this case the pattern of RBF. Although the RBF of EC-perfused kidneys was about 1.5 times higher than the controls, GFR was reduced, indicating a filtration defect.

The CPA-perfused kidneys filtered a load similar to the controls but reabsorbed less water volume (Fig. 3f), sodium (Fig. 3g) and glucose (Fig. 3h), indicating a generally reduced tubular reabsorptive capacity, reflected in a disproportionate urine flow (Fig. 3e). Loss of function was severe in the non-functional kidneys as filtration and reabsorption capabilities were almost nonexistent during the 2-h blood perfusion.

Deficiencies of tubular reabsorption were related to oxygen consumption in the different groups. The oxygen consumption of the EC- and CPA-functional perfused kidneys was similar until about 60 min, after which the oxygen uptake of EC-perfused kidneys plateaued whereas that of CPA-perfused kidneys deteriorated (Figure 4a). There was an initial delay of 20 min before any visible indication of oxygen consumption for non-functional kidneys, and thereafter, the pattern followed that of the functional subgroup. Figure 4b presents oxygen consumption as a percentage of oxygen delivered to the kidneys (FrO_2) to account for the variation of RBF between the groups. The fractional oxygen extraction ratio for untreated controls was steady after the first 10 min and fell within the 10% to 15% range known for kidneys [24]. EC-perfused kidneys showed a lower level of extraction than controls. The fractional oxygen utilization was identical for EC, functioning CPA and non-functioning CPA kidneys for the first 20 min of perfusion, showing that differences between oxygen consumption in these groups initially resulted entirely from differences in renal blood flow. Thereafter, oxygen uptake by the non-functioning kidneys lagged despite a remarkable extraction fraction from the blood available, reflecting the prolonged "vascular crisis" in this group alone. By 60 min, oxygen uptake and extraction stabilized in the EC-perfused group at values lower than those observed for non-perfused controls and stabilized at still lower and similar values for both CPA groups.

A trend between oxygen consumption and weight gain could be detected (data not shown). Interestingly, there was a significant correlation between the weight gain after blood perfusion and the ratio of the percent sodium reabsorption to oxygen consumption ($r^2 = 0.68$)

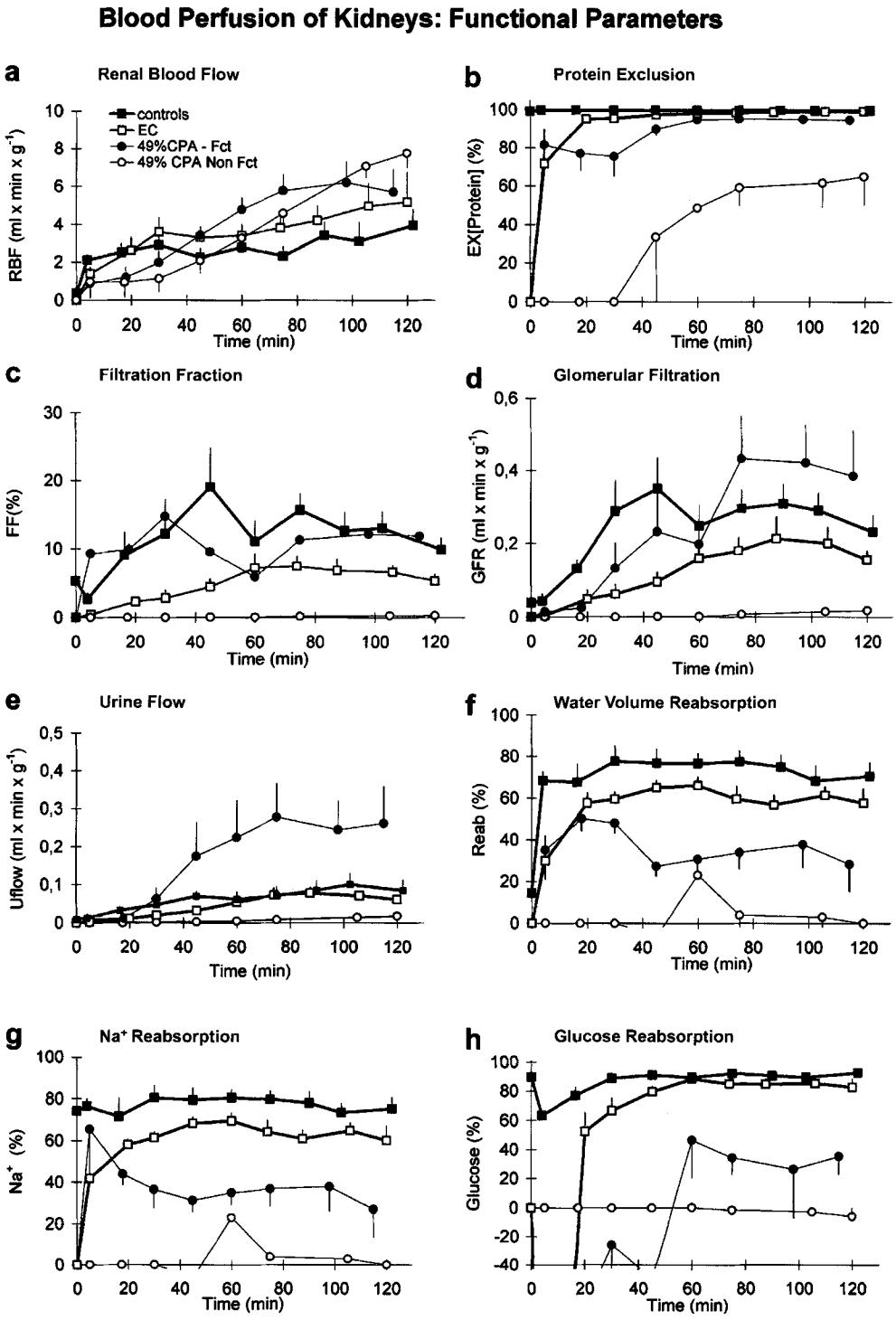

Fig. 3a–h. Functional parameters obtained during 2-h blood perfusion for control and treated kidneys. Each panel represents a parameter as indicated. Untreated controls (*filled squares*), EC perfused controls (*empty squares*), 49% CPA perfused functional (*fct*) (*filled circles*) and 49% CPA perfused non-functional (*non-fct*) (*empty circles*). (Mean and standard error of the mean)

[2] (Fig. 5) and, to a lesser degree, with the ratio Glu/qO_2 ($r^2 = 0.41$; data not shown).

Urine osmolalities averaged 295 ± 25 mOsm, 312 ± 8 mOsm and 305 ± 9 mOsm/kg for untreated, EC- and CPA-perfused kidneys, respectively. Over the course of the blood perfusion urine remained close to isotonicity showing no signs of concentration. This perhaps reflects the water diuresis (isotonic urine) caused by the absence of vasopressin supplementation in our standard baseline blood perfusion system [2, 5].

The histology of the kidneys after 2-h blood perfusion is presented in Fig. 6. The control kidneys ap-

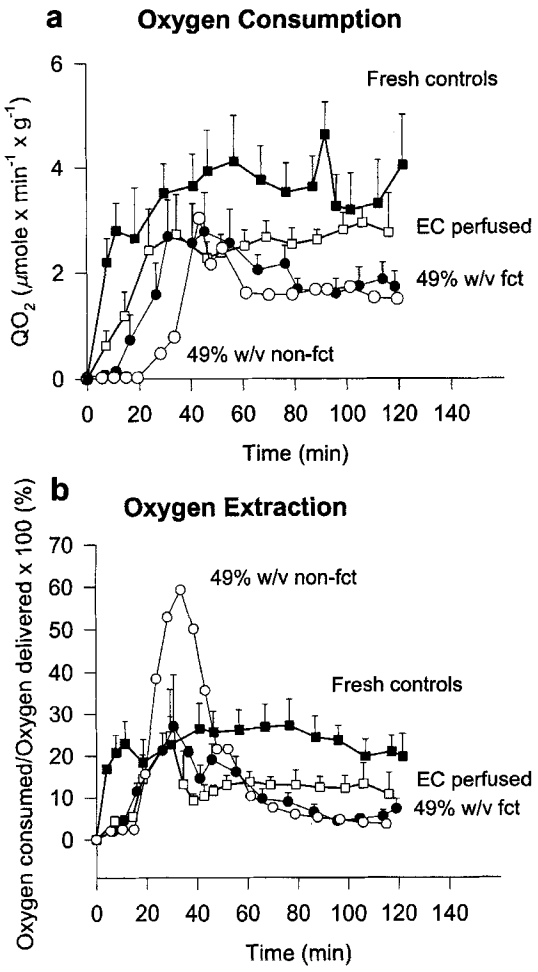

Fig. 4. **a** Oxygen consumption, qO_2 ($\mu mole \times min^{-1} \times g^{-1}$) obtained during blood perfusion for kidneys in groups 1, 2 and 3. **b** Oxygen extraction, FrO_2 (%). Untreated controls (*filled squares*), EC perfused controls (*empty squares*), 49% CPA perfused functional kidneys (*fct*) (*filled circles*) and 49% CPA perfused non-functional kidneys (*non-fct*) (*empty circles*). (Mean and standard error of the mean)

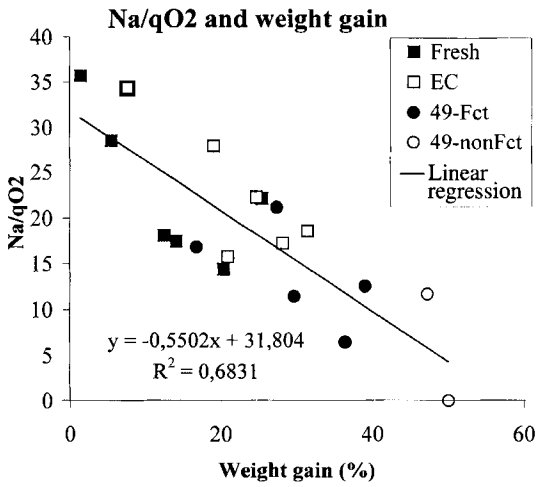

Fig. 5. The global relationship between the ratio of sodium reabsorbed to oxygen consumed (Na/qO_2) and kidney weight gain across all groups. Na/qO_2 is calculated as the percent of sodium reabsorption, and qO_2 is in $\mu mole \times min^{-1} \times g^{-1}$. Values taken at the end of the blood perfusion for individual experiments. Untreated controls (*filled squares*), EC perfused controls (*empty squares*), 49% CPA perfused functional (*fct*) (*filled circles*) and 49% CPA perfused non-functional (*non-fct*) (*empty circles*). The *trendline* represents the linear regression calculated by the method of the least squares

peared normal, indicating no adverse effect of the 2-h blood perfusion (Fig. 6a). The EC-perfused kidneys exhibited mild tubular swelling with no other obvious lesions. Brush borders were comparable to the controls (Fig. 6b). Functional CPA-perfused kidneys after blood perfusion showed tubular cell thinning and enlarged tubular lumens (Fig. 6c). Hyaline deposits and some debris were present in a small number of tubules. The non-functional CPA-perfused kidneys showed marked cortical alterations and in some areas cells were enucleated. Bleeding into several tubules was apparent, and cellular debris were present in the lumens, suggesting disintegration of the glomerular basement membrane. Adjacent to the damaged tubules, some tubules could be seen that had the appearance of those of the functional CPA-perfused kidneys (Fig. 6d). No signs of bacterial contamination in the blood or kidneys were detected.

Discussion

Normothermic blood perfusion is a useful tool to document renal function after various treatments and offers greater opportunities of interaction with the system than transplantation [1, 21]. This method was used here to assess the function of kidneys after perfusion with cryoprotective solutions. The ex vivo model, which was used by Fahy et al. [7] to study events occurring at blood reflow, has provided more quantitative information than the transplant model (referred to as the in vivo model in the following text). The present study confirms and complements these results, and the effects of 49% w/v CPA on the isolated rabbit kidney have now been evaluated using in vitro, in vivo [11] and ex vivo [7] models.

As blood perfusion starts, there is a mild increase in vascular resistance in EC-perfused kidneys, which is more pronounced with 49% CPA-perfused kidneys and becomes extremely marked in case of non-functioning kidneys. This leads ultimately to reactive hyperemia (RHE) where, depending on the degree of the trauma, RBF tries to compensate for the oxygen debt [3]. EC-perfused kidneys were able to compensate rapidly for temporary occlusion, whereas CPA-perfused kidneys showed an obvious delay. Our results show that CPA-

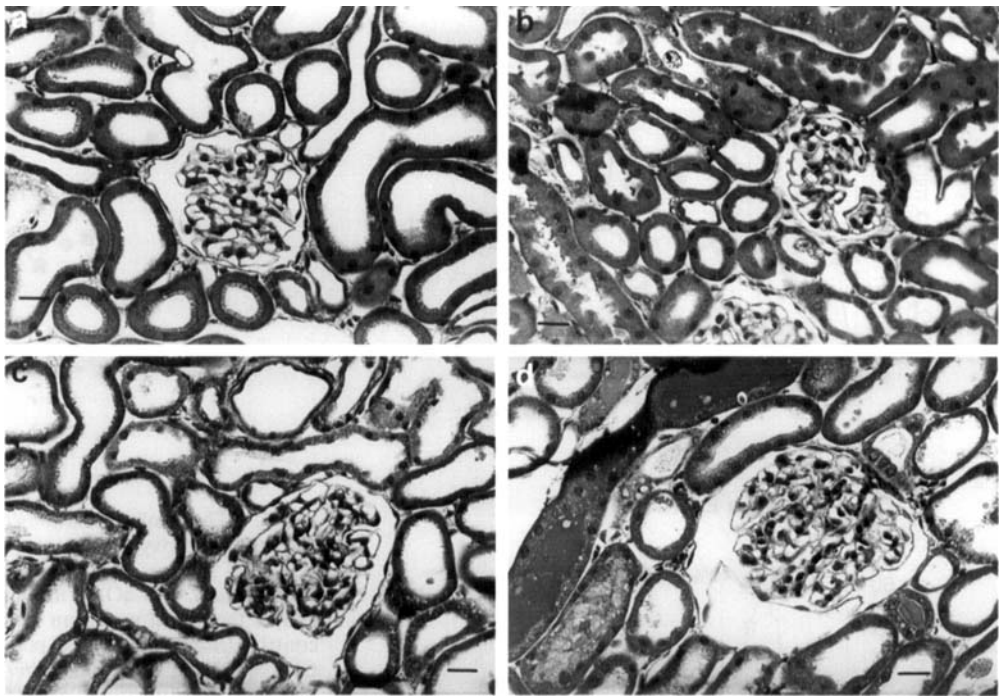

Fig. 6a–d. Histology of cortical tissue after blood perfusion. **a** Untreated control kidneys, **b** EC-perfused kidneys, **c** 49% CPA-perfused functional kidneys, **d** 49% CPA-perfused non-functional kidneys. Scale bar: 20 μm

perfused kidneys consume less oxygen at all times than kidneys not perfused with CPA.

In vitro versus ex vivo and in vivo models

Table 3 outlines similarities and dissimilarities between the present results and ex vivo results that have been previously reported [7]. As in the present experiments, following 49% CPA in ex vivo perfusion, kidneys fell into two distinct categories: kidneys that could function adequately and kidneys that could not. In the ex vivo study, the failure rate was 7 of 13, whereas in the present study, the failure rate was only 2 of 7 ($P < 0.003$). The reason for the different failure rates is not clear. The difference may reside in the fact that Iloprost was administered prior to the kidney procurement for in vitro perfusion, whereas Iloprost was not given in the ex vivo experiments. Perfusion pressure was similar in vitro and ex vivo, but in the functional group blood flow was three times higher in vitro than ex vivo. This may reflect protection from Iloprost in the present experiments. When Iloprost was not administered in the in vivo model, 5 of 13 kidneys tolerated 49% CPA-perfusion, which is comparable to what is found ex vivo. Those numbers contrast with the 100% survival obtained in vivo [11] when Iloprost was used. This confirms the beneficial effect of Iloprost on kidney function whether

Table 3. Parameters pertinent to the comparison of the present in vitro to previous ex vivo [7] Model for the last 60 min perfusion. In vitro: present experiments using the blood perfusion apparatus to test renal function. Ex vivo: rabbit model developed in [7]. RPS-2: vehicle solution used to perfuse kidney instead of EC

Parameters	Units	EC-perfused	RPS-2-perfused	49%-perfused functional		49%-perfused non-functional	
		In vitro	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo
Renal blood flow	$ml \times min^{-1} \times g^{-1}$	4.1	1.9	4.7	1.5	4.3	2.3 and increasing
Peak resistance	mmHg×min ×g×ml^{-1}	60	35	200	450	>300	800
FR[Glu]	%	82	95	36	75	0	0
FR[Na]	%	64	80	34	60	6	20
Water volume reabsorption	%	61	88	34	61	4	0
Urine flow	$ml \times min^{-1} \times g^{-1}$	0.06	0.075	0.21	0.09	0.008	0.05
GFR	$ml \times min^{-1} \times g^{-1}$	0.15	0.37	0.3	0.23	0.007	0.02
Filtration fraction	%	5.8	~30	11	~22.5	0.16	~6
Protein exclusion	%	98	99	91	95	45	22

or not this is related to anti-thrombic effects, as Iloprost inhibits the function of platelets, which were present in vivo. The effect of blood cells such as platelets and leukocytes on renal function at the time of reflow could have dramatic effects in reperfusion injury [18]. A second and perhaps equally important difference is that during asanguineous perfusion, arterial temperature was maintained between 0° C and 1° C in the ex vivo series, in contrast to the subzero perfusion temperatures employed in vivo and presently in vitro with Iloprost. Collectively, the current results support the value of the combination of Iloprost pretreatment and subzero perfusion as previously concluded [11].

The fractional reabsorption of sodium, glucose and water ex vivo was higher than what was observed in vitro, and this seems unlikely to result from the greater filtered load in vitro, since GFR was similar in vitro and ex vivo for 49% CPA-perfused kidneys (Table 3). Consistent with this observation, polyuria developed sooner and was more extreme in the functioning CPA group in vitro than ex vivo. In both the ex vivo and the current series, the extend of injury was related to the severity of the vascular crisis. It may therefore be that the greater proportion of functioning kidneys in vitro is related to improved blood reflow, but the greater fractional reabsorption in the functioning kidneys ex vivo is related to humoral factors not present in vitro.

The pattern of protein exclusion over time in vitro and ex vivo was nearly identical in all groups of kidneys. On the other hand, the GFR of functional 49% CPA-perfused kidneys exceeded that of control perfused kidneys in vitro but not ex vivo. This reflects in part an apparent detrimental effect of EC perfusion on GFR (Fig. 3d), which is apparently overcome by cryoprotectant perfusion. EC may not be the ideal carrier solution, as high sodium solutions have been shown to be equally effective in preserving stored organs [23]. UW-gluconate solution is currently preferred over EC for many, although not for all, clinical applications [19]. However, what is appropriate in the absence of cryoprotectants is often not appropriate in the presence of cryoprotectants. Prior to the initiation of the present experiments, it was determined that UW is approximately equivalent or inferior to EC for use as a carrier solution for introducing and removing cryoprotectant ([16], unpublished results). Therefore, EC was used in the present experiments.

An increase in CPA concentration would improve the conditions of long-term storage. However, the introduction of 55% CPA concentration, for instance, produces a detrimental effect, as observed in transplantation, and poses challenges [14, 15, 16]. The renal failure rate after transplantation has been approximately 58% (32 of 55) of kidneys perfused with 55% CPA (unpublished results), in contrast to 100% survival with 49% CPA [11]. This dose-dependent effect was also observed in vitro in the case of one kidney perfused with 55% CPA (unpublished data). Elution of proteins was observed during the cryoprotectant perfusion, and this was particularly exacerbated as CPA concentration increased (the amount of eluted protein was 7.7-fold higher during the washout than during the CPA introduction phase with 55% CPA; unpublished data).

Mechanisms of injury

The linear correlation found between weight gain and the ratio Na/qO_2 (Fig. 5), and, to a lesser degree, with the ratio Glu/qO_2 suggests a link between weight gain and active fluid regulation to the overall renal injury. Weight gain during perfusion is generally the result of fluid accumulation in the interstitial space. Increased interstitial pressure has the effect of compressing the vascular compartment and reducing RBF. This may lead to the vascular crisis that is characteristic of the present model, which in turn reduces oxygen delivery to the tissue and thereby impairs the active transport required for both renal function and cell volume regulation. In agreement with this interpretation, weight gain was directly related to the severity of the injury sustained in the current study, where the final weight gain after blood perfusion was 17% for controls, 20% for EC-perfused controls, 30% for 49% CPA-perfused functioning kidneys and nearly 50% for 49% CPA-perfused non-functioning kidneys. Oncotic pressure exerted by plasma proteins contained in intact capillaries inhibits edema, as seen in control kidneys, but CPA perfusion could increase capillary permeability to plasma proteins. This would support the marked proteinuria observed in CPA-perfused kidneys in this study and ex vivo, which implies a lesion at the glomerular level, such as a loosening of the glomerular basement membrane or of its attachments to associated cells [17]. Loss of tubular cells, as illustrated in Fig. 6, further suggests weakened cell-cell junctions and the disruption of cell-basement membrane adhesion, as previously observed by electron microscopy [8]. At the end of the blood perfusion, the tubular cells from CPA-perfused kidneys appear shrunken and the tubule lumens enlarged, implying that weight gain is associated not with cellular swelling but with the accumulation of extracellular fluid.

In vitro prediction of in vivo life support function

These mechanistic observations strengthen the general possibility that the in vitro approach might be useful for predicting the ability of kidneys to survive after transplantation. The fact that oxygen consumption and extraction remained depressed to the end of the 2-h evaluation period in kidneys previously perfused with

cryoprotectant, despite an above-normal oxygen supply by hyperemia, implies serious mitochondrial impairment that might form another basis for discriminating between viable and non-viable kidneys. Evidently, non-functioning organs produce very little urine and sustain weight gains in excess of 40%, and both of these end points could readily be observed prior to transplantation. At present, we have no direct evidence that the non-functional kidneys in this study would have failed to support life if they had been transplanted. However, the similar failure rate of kidneys perfused with 49% CPA and then tested either ex vivo or in vivo supports a link between acute and chronic renal failure after perfusion with vitrifiable media. Further, the fact that the failure rate in vitro was mildly higher than the failure rate in vivo is not completely unexpected, given the inability of in vitro perfusion to supply the homeostatic support and mechanisms for healing that are provided in vivo. In vitro testing, as a quality control measure prior to or in lieu of transplantation, may be conservative, but it shows more accurate evaluation of experimental damage because it is unaffected by some of the repair functions occurring in vivo. Organs that pass in vitro testing may do so with a margin of safety since even organs that fail the test in vitro may be viable in some cases.

Conclusion

Kidneys perfused with a 49% CPA model vitrification solution, VS4, sustain injury in the form of elevated weight, transiently reduced blood flow, proteinuria, impaired oxygen consumption, impaired tubular reabsorption and tubular cell detachment from the peritubular basement membrane. Much of this injury may be related to a loosening and weakening of extracellular protein structure and adhesion. Decreased energetic efficiency of tubular transport was linearly related to weight gain, in support of this view. Even perfusion with the vehicle solution alone (EuroCollins solution) led to a reduced filtration capacity. It is likely that improvement of both the vehicle and the vitrification solution is necessary before kidney function can be fully preserved for an indefinite time. The study of isolated kidneys by normothermic blood perfusion can produce information in real time and has potential utility for the further elucidation and treatment of cryoprotectant perfusion injury. Impaired oxygen consumption, excessive weight gain and virtual anuria were identified as potentially simple in vitro tests for failed viability prior to transplantation.

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