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Karri Norio
Mårten Wikström
Kaija Salmela
Lauri Kyllönen
Leena Lindgren

Ascorbic acid against reperfusion injury in human renal transplantation

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K. Norio (✉) · L. Lindgren
Department of Anaesthesia,
Fourth Department of Surgery,
Helsinki University Central Hospital,
P.O. Box 263, 00029 HUS
Helsinki, Finland
E-mail: karri.norio@hus.fi
Tel.: +358-9-47162362

M. Wikström
Helsinki Bioenergetics Group,
Programme for Structural Biology
and Biophysics, Institute of Biotechnology,
University of Helsinki, Helsinki, Finland

K. Salmela · L. Kyllönen
Division of Transplantation,
Fourth Department of Surgery,
Helsinki University Central Hospital,
Helsinki, Finland

Abstract The cadaveric renal graft is exposed to ischaemic injury during preservation and to oxidative damage during reperfusion. Both these mechanisms are known to cause cell damage, which may impair graft function. Reperfusion injury (RPI) is mediated by reactive oxygen species (ROS). Ascorbic acid (AA) is a potent physiological extracellular scavenger of ROS. We perfused 31 renal grafts immediately before implantation with a solution of Euro-Collins containing 0.5 mg/ml of AA to diminish RPI. From every donor, the contralateral kidney served as a control. The control grafts were perfused with the same perfusion as those of the AA group, only without the AA substitution. We assessed the effect of AA by recording serum creatinine, creatinine clearance, initial graft function and early rejections. The incidence of delayed graft function (DGF) was 32% in the AA group, and 29% in the control group. Other parameters were also similar in both groups, except for the length of DGF, which showed a trend towards a shorter duration in the AA group. The pre-operative systemic AA concentration was significantly ($P=0.01$) lower in the haemodialysis patients than in those on peritoneal dialysis. In conclusion, this clinical study could not demonstrate significant benefits of AA in renal transplantation.

Keywords Kidney transplantation
Ascorbic acid · Delayed graft function

Introduction

Delayed graft function (DGF) after renal transplantation is still a significant clinical problem [1] and every attempt should be made to minimise its frequency and severity. DGF predisposes to acute postoperative pulmonary oedema. Prolonged dependence on dialysis treatment after transplantation is costly [28], and the situation is confusing for the patient. The diagnosing of acute rejection, urinary leak and graft thrombosis becomes difficult, and infections are frequent [24]. Patients with DGF more often have acute rejections, and these two conditions together exert an additive adverse effect on allograft survival [10, 23]. In some studies, DGF per se is not thought to reduce the long-term outcome of cadaveric allografts [18, 31]; however, opposing evidence exists [7, 13, 23].

The incidence of DGF is increased by a prolonged cold ischaemia time of the graft [23] and by high donor age [25]. Also, insufficient peri-operative fluid management of the recipient [4] and HLA mismatching [23] have been associated with DGF. The main cause of DGF is thought to be renal injury caused by renal ischaemia during preservation and by reperfusion injury (RPI) after reperfusion. At reperfusion, the re-introduction of oxygen into tissues may result in oxidative

tissue damage due to a high concentration of reactive oxygen species (ROS), namely the hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and the superoxide anion (O_2^-). An association has been reported between a high concentration of a marker (malondialdehyde) that is released during free-radical damage, and clinically compromised early renal graft function [3]. On the other hand, cyclosporine (CyA) is known to inhibit the opening of the mitochondrial permeability transition pore, which has a major pathogenetic role in ROS-induced cell injury [2, 20]. The clinical importance of reperfusion injury in human renal transplantation still needs to be clarified.

The minimisation of cold ischaemia time would undoubtedly be rewarding [12], but this approach is limited, for practical reasons. In an experimental setting, the restricting of the delivery of oxygen at reperfusion has induced some favourable effects [19]. The use of extra antioxidants at the site of ROS-assault has been considered to be a potential option [9, 14]. The local demand of antioxidants may be significantly increased at reperfusion and might exceed the capacity of natural circulating antioxidants. This may apply especially to renal transplant recipients, in whom the concentration of antioxidants in the blood may be low after dialysis treatment before surgery [26, 32].

Ascorbic acid (AA) is a water-soluble, physiological, extracellular antioxidant that acts as a rapid primary defence against aqueous radicals in the blood [22, 30]. It has been used in human cardiopulmonary bypass [5] and in renal transplantation models in animals [6, 15] with promising results. Studies on AA in human renal transplantations are scarce [9]. We designed a prospective randomised pilot trial to study the effect of AA against RPI. We tested the hypothesis that an administration of AA via a re-flush immediately before implantation might reduce the damage to the renal cells, which could clinically be manifested as better initial graft function.

Patients and methods

Donor and recipient management

Sixty-two adult renal transplantation recipients were enrolled in the study. The grafts were harvested from 31 heart-beating cadavers that donated paired grafts. During the study period, six pairs of grafts could not be implemented in the study because of a temporary lack of study personnel. Seven grafts were excluded from the study because the contralateral kidney was needed for transplantation in another centre. Donor and recipient characteristics are shown in Table 1. Care of the donors was managed according to our national recommendations. The kidneys were perfused either in situ or on the back-table immediately after they had been removed, depending on the surgical team. Our kidney allocation policy required the sharing of at least two antigens in the HLA–AB and one in the HLA–DR loci, a negative T-cell crossmatch test against donor spleen cells, and the avoidance of repeated HLA class I mismatches. All recipients underwent a blood transfusion before they were accepted on the waiting list, unless they were pregnant or had undergone blood transfusion before. The donor and recipient characteristics were similar in both groups (Table 1).The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital. Individual informed consent was obtained from every patient.

The study group

Only paired cadaveric renal grafts were accepted. From every pair, one kidney was allocated to the ascorbate group (AA group) in a random, double-blind manner, while the contralateral kidney served as control. Randomisation was performed before surgery by means of a coded card in a sealed envelope, which was sent to the operating theatre, where the perfusion solution was prepared accordingly.

Anaesthesia

One hour before anaesthesia was induced, the recipients underwent pre-medication with oral diazepam 0.15 mg kg^{-1} and cyclosporine 5 mg kg^{-1}. Pre-anaesthetic intravascular volume loading was started with potassium-free Ringer's acetate and 4% human albumin solution (1:1) until a central venous pressure (CVP) of 5 mmHg was reached. Thereafter, standardised anaesthesia was induced. After induction of anaesthesia, standard immunosuppressive medication, azathioprine 50 mg i.v. and methyl prednisolone 40 mg i.v., was given. During the operation we maintained the systolic blood pressure at above 110 mmHg by keeping CVP >6 mmHg with administration of the Ringer/4% albumin combination described above. Infusion of dopamine was started, if necessary, to maintain the target blood pressure.

Perfusion protocol of AA

Before implantation, all the grafts were re-flushed through the renal artery with ice-cold (0–4 °C) Euro-Collins solution (EC). In the study group, 500 mg of AA (Orion Pharma, Finland) was added to the EC to produce an AA concentration of 0.5 mg/ml. The infusion was continued until 300 ml of fluid was obtained from the graft vein, to ensure complete perfusion. The flushed grafts were then implanted via routine surgical procedures. Blood samples were drawn from the central venous catheter for measurement of systemic concentration of AA according to Table 3. Renal blood samples were drawn from the graft vein with a 24 G needle immediately after reperfusion and 10 min thereafter. The study protocol is shown in Table 2. The method of determination of serum AA has been described earlier [17].

Clinical endpoints

The primary endpoint was the occurrence of DGF. We applied the criteria described by Halloran et al. [8] to define DGF: serum creatinine concentration higher than 500 μmol/l throughout the first post-transplant week, the need for more than one dialysis session in the first week, or oliguria of less than 1 l/24 h for more than 2 days. The day of first spontaneous decrease of serum creatinine concentration was defined as the day of onset of graft function.

We assessed the patients' serum creatinine concentration preoperatively and once a day postoperatively for 21 days and at postoperative day (POD) 100, and creatinine clearance at PODs 7 and 14. To compare the initial graft function between the groups

Table 1 Donor and recipient demographics. Data are presented asmean ± SD or number. *SAH* subarachnoid haemorrhage, *ICH* intracerebral haemorrhage, *UW* University of Wisconsin solution

Characteristic	AA group ($n=31$)	Control group ($n=31$)
Donor		
Age (years)	39 ± 16	39 ± 16
Cause of death		
SAH or ICH	17	17
Traumatic cerebral injury	13	13
Other	1	1
Perfusate at harvesting		
UW/EC	25/6	25/6
Perfusing technique		
In situ/backbench	27/4	27/4
Recipient		
Age (years)	48 ± 14	51 ± 12
Mode of dialysis		
HD/CAPD	19/12	17/4
Duration of dialysis therapy (months)	16 ± 16	13 ± 8
Primary/re-transplantation	27/4	27/4
Recipient disease leading to transplantation		
Primary renal disease	25	19
Systemic disease	6	12
Other	0	0
Highest multi-specific panel reactive antibodies		
< 20%	20	24
20–80%	10	6
> 80%	1	1
HLA-AB mismatch		
0	1	0
1	12	9
2	18	20
3	0	2
HLA-DR mismatch		
0	5	10
1	23	19
2	3	2
Cold ischemia time (h)	24.6 ± 5.6	25.0 ± 5.5
Regular AA-medication	87% (27/31)	77% (24/31)

Table 2 The study protocol during the pre-operative and intra-operativeperiod

Time	Event
−1 h	Cyclosporine, 5 mg/kg p.o.
0 h	Enter the operating theatre
+0–30 min	Volume loading ad CVP = 5 mm Hg
At CVP = 5 mmHg	1. Systemic blood sample (S1)
Induction of anaesthesia	
After approximately 1 h of surgery	Perfusion of the graft with EC ± AA
Before declamping of graft circulation	2. Systemic blood sample (S2)
Immediately after declamping	1. Renal vein blood sample (R1)
10 min after R1	2. Renal vein blood sample (R2)

quantitatively, we assessed the spontaneous decrease of serum creatinine in patients with early graft function (EGF). Acute rejection episodes were recorded for the first 100 days. One-year graft and patient survival was registered.

Statistical analysis

Fisher's exact test and Student's *t*-test were used for comparisons between groups. All calculations were performed with StatView SE software (StatView, Brain Power, Calabasas, Calif., USA). A probability of $P < 0.05$ was considered significant. The data are given as mean ± SD or range.

Results

The pre-operative mean systemic AA concentration of the recipients was 54 ± 43 μmol/l (range 10–239 μmol/l), both groups combined, and the mean concentration was similar in the AA and the control group (Table 3). The pre-operative AA concentration was significantly lower in the haemodialysis (HD) patients than in the patients on peritoneal dialysis (continuous ambulatory peritoneal dialysis, CAPD) (Table 4). In the AA group, the

Table 3 Concentration of serum ascorbic acid (μmol/l) in the AA and control groups, measured from the systemic vein samples before induction of anaesthesia (*S1*), before reperfusion (*S2*), and from graft renal vein 30 s (*R1*) and 10 min (*R2*) after reperfusion. Data are presented as mean ± SD

Group	S1	S2	R1	R2
AA	61 ± 37	56 ± 33	402 ± 289***	79 ± 37**
Control	57 ± 55	51 ± 52	57 ± 44	48 ± 48

***$P < 0.001$, **$P < 0.01$ compared to S2

Table 4 Concentration of AA measured from the systemic vein samples before induction of anaesthesia (*S1*) and before reperfusion (*S2*) in all patients, who were divided into two groups depending on the mode of dialysis. Data are presented as mean ± SD

Group	S1	S2
HD patients	41 ± 35	42 ± 34
CAPD patients	77 ± 55**	70 ± 49*

**$P < 0.01$, *$P < 0.05$ between the groups

renal venous AA concentration was high immediately after reperfusion, but it returned close to systemic levels in 10 min (Table 3).

The incidence of DGF was 32% in the AA group and 29% in the control group (NS). The overall incidence of DGF was 31%. The mean duration of DGF was 9.7 days (range 5–22) in the AA group, and 22 days (4–70) in the control group ($P = 0.096$). All grafts functioned. Serum creatinine clearance (patients with DGF censored) at PODs 7 and 14 was 0.67 ± 0.39 and 0.75 ± 0.41 ml/s per 1.73 m^2 in the AA group and 0.62 ± 0.40 and 0.72 ± 0.39 ml/s per 1.73 m^2 in the control group (NS). In the patients with EGF, the daily pattern of spontaneous decrease of serum creatinine concentration (ad POD 21) was similar in both groups. At POD 100 all grafts were functioning, and the mean serum creatinine level was similar in both groups. The incidence of acute (< 100 days) rejection was 22% (7/31) in both groups (Table 5).

Discussion

In this study we aimed to prevent reperfusion injury in human renal transplantation by increasing the amount of the antioxidant ascorbic acid at the site of reperfusion, by performing a re-flush with AA immediately before implantation. It is reasonable to assume that in reperfusion, the local consumption of antioxidants may be increased beyond the capacity of natural circulating antioxidants and thus, a local supplement of AA could be beneficial. RPI is associated with the activation of the inflammatory cascade in the kidney [11, 21]. Extracellular ROS are generated due to intravascular adhesion and infiltration of leukocytes and platelets. Ascorbic acid radicals have been detected in the hepatic venous blood in rats immediately (5 min) after reperfusion, suggesting that AA scavenges oxygen radicals during reperfusion [30]. This supports the use of AA, which acts mostly in the extracellular space. Within the lipid compartment, AA cannot scavenge lipophilic radicals by itself, but it acts as a synergist with tocopherol for the reduction of lipid peroxyl radicals [22]. Furthermore, we assumed that the antioxidant system of dialysed uraemic patients might be compromised, since, e.g. AA, is removed in dialysis [26, 32] before surgery. The technique of a re-flush (without AA) has been used earlier, achieving a reduction in postoperative dialysis requirement [16]. However, an increased incidence of rejection and some controversial histological changes have also been described [27].

The initial systemic concentration of AA in our patients was within normal limits on average (normal limits 45–57 μmol/l) [29]. This was most likely due to adequate regular substitution therapy. In HD patients,

Table 5 Results of transplantations. Data are presented as mean ± SD or number. *ARR* acute steroid-reversible rejection, *SRR* steroid resistant rejection, *AVR* acute vascular rejection

Parameter	AA group ($n = 31$)	Control group ($n = 31$)
Graft function		
EGF/DGF	21/10	22/9
Day of onset of graft function in patients with DGF; $P = 0.096$	10 ± 6	22 ± 12
Early rejections (< 100 days)		
ARR/AVR	6/1	5/2
Patient survival (1 year)	29 (94%)	27 (87%)
Graft survival (1 year). Deaths with a functioning graft censored	29 (100%)	26 (96%)
Serum creatinine at POD 7 in patients with a functioning graft (μmol/l)	169 ± 80	167 ± 95
Serum creatinine at POD 14 in patients with a functioning graft (μmol/l)	122 ± 47	135 ± 65
Serum creatinine at POD 100 in patients with a functioning graft (μmol/l)	126 ± 33	126 ± 32
Serum creatinine clearance at POD 7 in patients with a functioning graft (ml/s per 1.73 m^2)	0.76 ± 0.36	0.70 ± 0.35
Serum creatinine-clearance at POD 14 in patients with a functioning graft (ml/s per 1.73 m^2)	0.74 ± 0.36	0.73 ± 0.40

the AA concentration was significantly lower than in patients on CAPD, showing that HD removes AA more effectively than CAPD. With our scheme, we were unable to decrease the incidence of DGF. Also, the decrease of creatinine in the patients with EGF was similar in both study groups. In the patients with DGF, AA induced a trend toward a shorter duration of DGF, indicating possibly a smaller injury in these grafts. This is consistent with our presumptions—one might expect the benefits of this intervention to be expressed more in the compromised grafts with "more to be done".

It is possible that the AA concentration in the AA group was still too low to meet the raised requirements of antioxidants at the site of reperfusion. Nevertheless, we assume that the levels of AA in all patients may have been adequate. This suggestion is supported by the fact that in the control group, the renal vein concentration of AA after reperfusion was not lower than the systemic concentration. This can be considered as a sign of minimal consumption of AA at reperfusion (Table 3). This observation suggests that there was no massive production of ROS in these conditions, at least not in the extracellular space. Furthermore, even patients with the lowest systemic AA concentrations managed as well as other patients. Therefore, it appears that *extracellular* ROS may not play a substantial role in human renal transplantation. It is even possible that the major cause of DGF is ischaemia per se during kidney preservation, while the role of RPI may be very limited in kidney transplantation. This suggestion is consistent with the fact that CyA is a powerful blocker of mitochondrial permeability transition, a key mechanism of cell damage in RPI [2, 20]. Thus, the use of CyA may subdue the manifestation of RPI in renal transplantation.

In summary, we found that with our study protocol with rather demanding clinical endpoints, we could not show major benefits of AA in renal transplantation. It might have a slight beneficial effect on renal graft function, especially in grafts with compromised function, but to affirm this theory one would need an excessive number of patients.

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