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Effect of IGL-1, a new preservation solution, on kidney grafts (a pre-clinical study)

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Abstract Ischemia-reperfusion injury conditions short-term and long-term graft function. The effects of the inversion of K^+ and Na^+ concentrations and substitution with polyethylene glycol for hydroxyethyl starch in University of Wisconsin (K-UW) solution were evaluated in isolated perfused rat kidneys and in autotransplanted pig kidneys. In the rat model kidneys were cold-stored for 24 h in K-UW or Na-UW or Na-PEG UW solutions (IGL-1 solution). Fractional sodium reabsorption and glomerular filtration rate was better in kidneys preserved in Na-UW and IGL-1 solution than those preserved in K-UW solution. In the pig model the left kidney was harvested and preserved in K-UW or IGL-1 solution for 24 h and then transplanted. In the autotransplanted pig model, kidneys preserved in IGL-1 solution showed a better function and a significant reduction of MHC class II expression, cellular apoptosis and interstitial fibrosis. In conclusion, kidneys preserved in IGL-1 solution tolerated ischemia/reperfusion injury better than those preserved in K-UW solution.

Keywords Ischemia-reperfusion injury · Preservation solution · Extracellular solution · Polyethylene glycol · Kidney transplantation

Introduction

Ischemia-reperfusion injury is a crucial problem in transplantation as it may induce delayed graft function. Although many grafted kidneys are lost because of immune-mediated mechanisms, there is clear evidence that non-immune factors, such as ischemia-reperfusion injury, contribute to short-term and long-term graft dysfunction [1, 2, 3].

Hypothermic storage of kidneys remains the major approach to human kidney preservation because low temperature decreases metabolism of preserved organs [4]. Although University of Wisconsin (UW) solution was introduced to decrease ischemic graft damage [5], the beneficial effects of storage in this solution do not completely prevent cellular and interstitial swelling and reperfusion-induced oedema. Our previous studies [6] demonstrated that it is possible to improve cold-preservation solutions by modifying the UW solution composition: the simple inversion of K^+ and Na^+ concentrations in UW solution improves rat liver function [7] as well as rat kidney function [8]. In addition, an improvement of all functional parameters of the liver has been demonstrated using a cold preservation solution where polyethylene glycol (PEG) was substituted for hydroxylethyl starch (HES) in the extracellular UW solution [9].

This study presents the results obtained with a new preservation solution, a Na-PEG-UW solution called IGL-1, manufactured in Lyon (France). We firstly evaluated the efficacy of IGL-1 solution in isolated perfused rat kidney, then in a model of autotransplanted pig kidneys. We have therefore compared the effects of IGL-1 solution versus UW solution on renal structure and function.

Materials and methods

Preservation solutions

The composition of the tested preservation solutions is shown in Table 1. The K-UW solution is the original Belzer liquid without dexamethasone, insulin and antibiotics and was modified in a stepwise fashion. The Na-UW solution [6] contains all the ingredients of the K-UW solution but with inverted concentrations of K^+ and Na^+. IGL-1 solution is the result of PEG-35 (0.03 mM) substitution for HES (0.25 mM, MW 200,000) in the Na-UW solution.

Isolated perfused rat kidney model

Experimental protocol

Male inbred Sprague-Dawley rats (Iffa-Credo, France) weighing 200–250 g were used as organ donors. Before the experiments they had free access to water and a standard pellet diet. All animals were handled in compliance with French regulations.

The study comprised 24 rats randomly divided between a control group without cold preservation ($n=6$) and 3 groups ($n=6$ for each group) with cold storage in K-UW solution, Na-UW solution or IGL-1 solution, respectively. The control kidneys were catheterized, isolated without interruption of blood flow and perfused at 37°C immediately after harvesting. The other kidneys from the different groups were perfused with the chilled storage solution (4°C) for 10 min at 3 ml/min and stored in the same solution at 0–4°C for 24 h.

Table 1 Composition of the three preservation solutions used in the study

Component	K-UW	Na-UW	IGL-1
HES (mM)	0.25	0.25	–
PEG-35 (mM)	–	–	0.03
Lactobionic acid (mM)	100	100	100
Raffinose (mM)	30	30	30
$MgSO_4$(mM)	5	5	5
KH_2PO_4(mM)	25	25	25
Glutathione (mM)	3	3	3
Adenosine (mM)	5	5	5
Allopurinol (mM)	1	1	1
Na^+(mM)	30	125	125
K^+(mM)	125	30	30
Osmolality (mOsm/Kg)	320	320	320
pH	7.2–7.4	7.2–7.4	7.2–7.4

Renal harvesting

Rats were anesthetized by an intraperitoneal injection of ketamine chlorhydrate (35 mg/kg). The surgical technique has been previously described [6]. Briefly, after an i.v. injection of heparin (1,000 U), 4 polyethylene catheters were inserted into (i) the ureter to collect urine, (ii) the infrarenal aorta to measure the renal perfusion pressure, (iii) the mesenteric artery facing the origin of the right renal artery to ensure kidney perfusion and finally (iv) the suprarenal vena cava to collect the renal venous effluent. The right kidney was then excised, trimmed of adhering tissue and completely isolated.

Organ perfusion and renal function study

All kidneys were perfused at 37°C via the mesenteric artery in a closed and controlled pressure circuit for 75 min. The perfusion liquid consisted of a cell culture medium (William's medium E, BioWhitaker) with a Krebs-Henseleit-like electrolyte composition [7], 5% albumin (bovine serum albumin fraction V, Sigma) as oncotic supply, and polyfructusan (Inutest, Laevosan, Linz, Austria) to measure glomerular filtration rate. The solution was perfused in a thermostatically controlled closed circuit at a constant rate providing a perfusion pressure of 90–100 mmHg. Renal perfusion pressure and the perfusion flow were continuously monitored. The medium was continuously gassed with a 95% O_2-5% CO_2 mixture.

After a 30 min equilibration period, urine and perfusate samples were collected for 3 consecutive 15 min periods from the control and cold-preserved kidneys. At each period, polyfructosan clearance (GFR), fractional sodium reabsorption (FRNa) and renal pressure were measured.

Autotransplanted pig kidney model

Experimental protocol

Experiments were performed using white pigs (mean weight 25 kg), housed according to the national guidelines at least 7 days before the experimental procedure. The study included 16 pigs randomly divided into 3 groups: group 1 included 6 animals transplanted with kidneys flushed and stored with K-UW solution, group 2 included 6 animals transplanted with kidneys flushed

and stored with IGL-1 solution, group 3 included 4 pigs that underwent sham surgery and were considered controls.

The removed kidneys were immediately flushed either with 250 ml of cold K-UW solution or 250 ml of cold IGL-1 solution and stored in the same solution for 24 h before transplantation.

In all animals renal function was investigated by serum creatinine and urea levels before the transplantation and daily until sacrificed. Creatinine clearance and tubular sodium reabsorption (FRNa) were also assessed. Measurements were performed using urine samples collected by bladder catheter over 30 min. The clearance of creatinine was calculated from the respective urine and plasma concentration ratios. FRNa was used to assess the tubular function. All the measurements were performed on day 7 in groups 1 and 2 as well as in control group.

Biopsies from transplanted kidneys were performed before and after reperfusion and on day 7. In addition, a biopsy was performed in the controlateral kidney before nephrectomy. In control animals the biopsies were performed only on day 7.

Surgical procedure

All pigs were pre-medicated with tiletamine (10 mg/kg), zolazepam (7.5 mg/kg) and atropine sulfate (10 μg/kg) before being anesthetized with a mixture of halothan and oxygen. A catheter was inserted in an ear vein. All surgical procedures were performed under sterile conditions. The left kidney was harvested by lombotomy. The removed kidneys of animals from groups 1 and 2 were flushed with the randomized cold preservation solution and stored with the same solution at 4°C for 24 h then the controlateral kidney was removed via a midline incision preserving the right renal artery and an ortothopic transplantation was performed. The left renal artery and vein were respectively anastomosed with a termino-terminal suture to the right renal artery and with a termino-lateral suture to the inferior vena cava. All the anastomoses were performed using 7.0 propylene sutures. Warm ischemia time was standardized at 60 min in all animals.

A pyelo-ureteral anastomosis between the left transplanted kidney and the right native ureter was performed and a double J stent was placed during the procedure.

After 7 days all animals were sacrificed and the transplanted kidney removed for histological studies.

Histology and immunohistochemistry

Histology was performed on paraffin-embedded sections from day 7 after autotransplantation specimens, 4 μ sections were prepared and stained with hematoxylin eosin. Several sections of each kidney were examined and graded according to independent criteria of injury such as cytoplasmic vacuolization of tubular epithelial cells, epithelial necrosis, tubular dilatation, glomerular ischemia and inflammatory cellular infiltration. Scores from 0 (normal kidney) to 4 (maximal involvement) were used for all criteria.

Immunohistochemistry was performed on biopsies obtained at day 7 after autotransplantation and were processed using two monoclonal antibodies, anti-porcine major histocompatibility complex (MHC) class II antibody on frozen sections (MCA 1335, Serotec, Oxford, UK) and anti-α smooth muscle actin (α SMA) (clone1A4, Sigma, Saint Louis, MO) on paraffin sections to evaluate early fibrosis. Negative controls were performed with monoclonal antibodies of the same isotype. Sections (3 μ) were firstly incubated with the monoclonal antibody (dilution 1:20 and 1:400, respectively) for 60 min, then consecutively with the biotinylated secondary antibody for 30 min and streptavidin biotinylated peroxidase complex (streptABC/HRP, Dako) for 30 min. Peroxidase activity was visualized using a 3,3' diaminobenzidine tetrahydrochloride substrate solution in phosphate-buffered saline containing H_2O_2 for 5–7 min.

The terminal dUTP-transferase-mediated nick end labeling (TUNEL) method was used to identified apoptotic cells [10]. Briefly, deparaffined sections (5 μ) were rehydrated in an ethanol series. After a microwave oven heating pre-treatment, sections were rinsed and a non-specific blocking was performed in PBS containing 3% BSA. Then, the slides were incubated with biotinylated dUTP and TdT enzyme for 1 h at 37°C in a humid atmosphere as suggested by the manufacturer (In situ cell death detection kit, POD ref 1684817, Roche Diagnostics, Meylan, France). Converter-POD was added 30 min at 37°C and the reaction was visualized using diaminobenzidine tetrahydrochloride (DAB+, Dako ref K3468). No counterstaining was performed in order to facilitate image analysis.

For quantification of immunostaining the percentage of immuno-labelled surface area was evaluated by image analysis using Quantimed 600 (Leica, Cambridge, UK) as previously described [11]. Briefly, the percentage of stained area was calculated as the ratio of suitable binary thresholded image to the total field area. The mean staining value, for each kidney, was obtained by analysis of at least 10 different fields (×400).

Statistical analysis

Results were expressed as mean ± SE, except for the results of image analysis, which were expressed as median numbers. Renal function parameters (GFR and FRNa)

were expressed as the mean of the 3 consecutive 15 -min collection periods. Data between groups were compared using the ANOVA test, followed by the Fisher's protected least-significant difference test (PLSD). Statistical significance was defined as $p < 0.05$.

Results

Isolated perfused rat kidney model

As shown in Fig. 1, there was a significant decrease in FRNa values in kidneys preserved in K-UW solution compared to control kidneys and those preserved in Na-UW solution; there was also a significant decrease in GFR values (Fig. 2) between kidneys preserved in K-UW solution and control kidneys but not with those preserved in Na-UW solution. When PEG 35 was substituted for HES, there was a significant increase in FRNa and GFR values between kidneys preserved in IGL-1 and those preserved in K-UW solution.

Pig autotransplanted kidney model

After 24 h of cold ischemia, plasma creatinine and urea increased in animals with the autografted kidney preserved in K-UW solution as well as in IGL-1 solution with a peak at 72 h after the reperfusion. These values decreased only in pigs from group 2 and they were close to those of the control group on day 7 (Figs. 3 and 4). Moreover, on day 6 and 7 after transplantation, creatinine and urea values were statistically lower in group 2 ($p < 0.05$) compared to those of group 1. These data included only 5 pigs from group 1 because 1 animal died for acute renal failure on post-operative day 2. Creatinine clearance was higher, but not statistically significant, in animals from group 2 compared to those

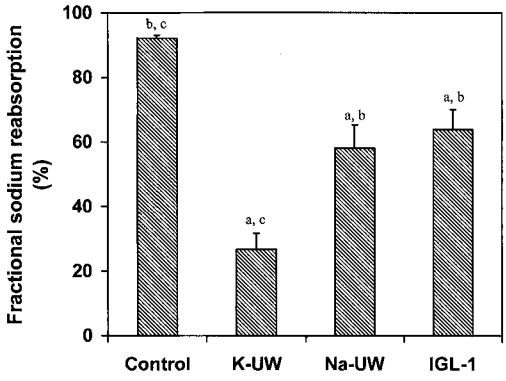

Fig. 1 Renal function parameters in isolated perfused rat kidney: tubular sodium reabsorption. All values are mean ± SEM; $p < 0.05$: *a* vs controls, *b* vs K-UW solution, *c* vs Na-UW solution

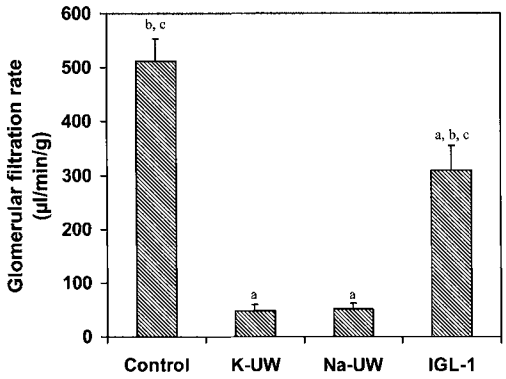

Fig. 2 Renal function parameters in isolated perfused rat kidney: glomerular filtration rate (GFR). All values are mean ± SEM; $p < 0.05$: *a* vs controls, *b* vs K-UW solution, *c* vs Na-UW solution

from group 1 while there was a significant difference between creatinine clearance of animals from group 1 and those from the control group (Fig. 5). FRNa values were not significantly different between animals from the different groups.

By light microscopy of hematoxylin and eosin-stained sections, no alterations were shown in right kidneys. In the grafted kidneys glomerular damage was absent while cytoplasmic vacuolization, dilatation of tubular lumens and tubular necrosis were found after reperfusion in both groups without statistical differences between kidneys preserved in IGL-1 and K-UW solution while a slight inflammatory infiltration was shown only in K-UW preserved kidneys. Kidneys preserved in IGL-1

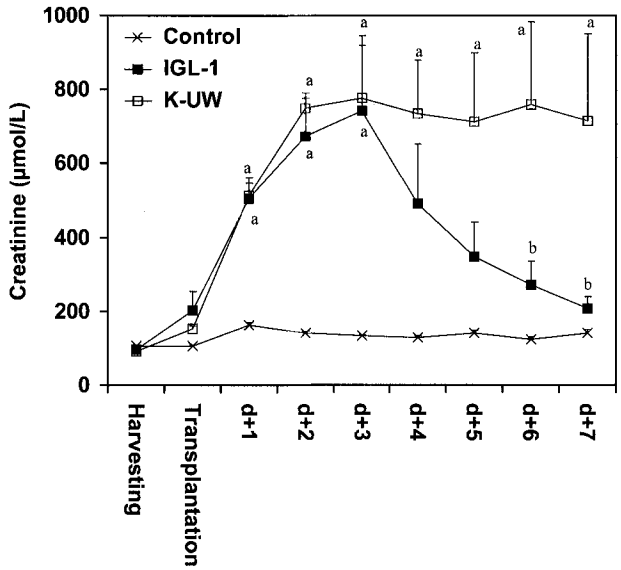

Fig. 3 Renal functional parameters in autotransplanted pigs: serum urea concentration, before and after transplantation, in group 1, group 2 and control group. All values are mean ± SEM; $p < 0.05$: *a* vs control, *b* vs K-UW solution

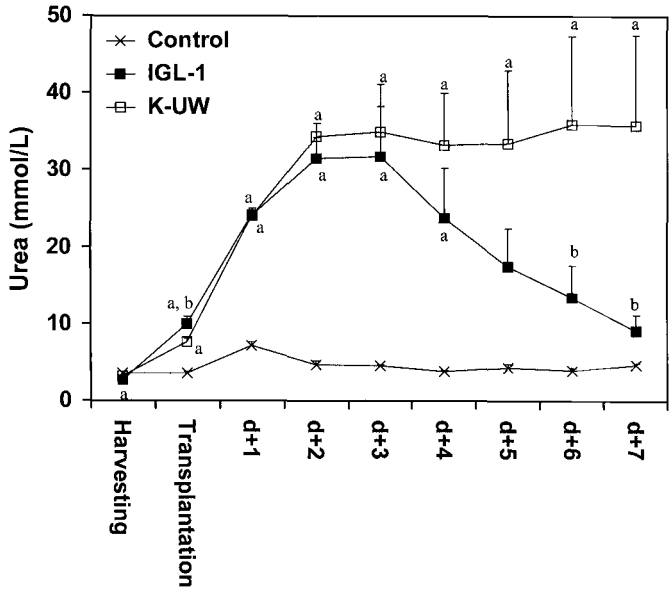

Fig. 4 Renal functional parameters in autotransplanted pigs: serum creatinine concentration before and after transplantation in group 1, group 2 and control group. All values are mean ± SEM; $p < 0.05$: *a* vs control, *b* vs K-UW solution

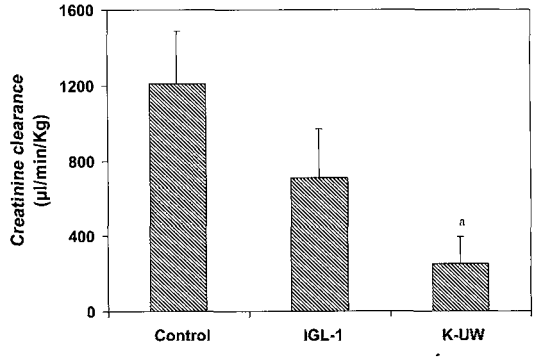

Fig. 5 Renal functional parameters in autotransplanted pigs: creatinine clearance values 7 days after transplantation in group 1, group 2 and control group. All values are mean ± SEM; $p < 0.05$: *a* vs control

solution presented a significant decrease in the expression of MHC class II molecules and in the number of apoptotic cells compared to kidney preserved in K-UW solution ($p < 0.01$ and $p < 0.006$, respectively). The percentage of surface area labelled by α-SMA antibody 7 days after transplantation was significantly higher in kidneys preserved in K-UW solution than in IGL-1 solution ($p < 0.03$).

Discussion

Experimental and clinical studies indicate that ischemia/reperfusion injury has cardinal implication in the pathogenesis of rejection playing an important role in graft function and survival. [1, 2, 3]. Cold ischemia and reperfusion induce injury of transplanted kidneys characterized by cell death (apoptosis as well as necrosis), infiltration of mononuclear cells, activation of monocyte/macrophage pathways, up-regulation of major histocompatibility complex class II antigens and overexpression of T-cell associated cytokines. [12, 13]. Renal tubular apoptosis is an established attribute of ischemia/reperfusion injury in kidneys [14]. The intracellular biochemical cascade of apoptosis induces activation of caspases, which determine a cellular disassembly as final result with release of intracellular proteolytic enzymes and other cytosolic material. The caspase activation cascade seems to contribute to inflammatory events, which are characterized by neutrophil recruitment and release of proinflammatory mediators. Moreover, there is a late inflammatory response characterized by enhanced expression of MHC class I and II antigens. Indeed, apoptosis and necrosis as well as acute and delayed inflammation are interdependent aspects of the ischemia/reperfusion injury.

The purpose of the present study was to evaluate whether IGL-1 solution was able to protect kidney grafts against cold ischemia storage and reperfusion. This solution, characterized by a high Na^+ concentration and PEG 35 substitution for HES, was tested in a rat isolated perfused kidney model and in a pig autotransplanted kidney model. As a first step in ex vivo studies, the inversion of K^+ and Na^+ concentrations determined an improvement of FRNa as tubular cells were less damaged. Several studies [7] have already demonstrated that liver preservation in high Na^+ concentration (high-Na) UW solution reduces damage to sinusoidal endothelial and hepatocellular cells as well as kidney preservation in high-Na UW solution is less deleterious to initial renal function [8, 15]. This result allows an improvement of functional outcome that is not negligible in clinical transplantation. In addition, in a pig autotransplanted kidney model a decrease in cellular infiltration and interstitial fibrosis and an improvement of GFR has been shown when kidneys were preserved in high-Na UW solution [15]. Indeed, several studies reported that extracellular solutions might determine less inhibition of Na-K ATPase, as well as preserve tubular function and decrease vasoconstriction which is due to the limitation of Ca entry through the Na-Ca exchange [16]. Thus, the prevention of vasoconstriction favors the distribution of the preservation solution within the organ.

PEG was then substituted for hydroxylethyl starch (HES) in the Na-UW solution because it has been reported that its beneficial action is enhanced when it is added to an extracellular solution [17]. In addition, HES is not [18, 19] considered essential for kidney and liver cold preservation and it has been reported to induce

erythrocyte aggregation [20]. Even if the PEG mechanism is not completely elucidated, it has been shown to have oncotic properties, and to bind to phospholipids accumulating in cell membranes [21]. Recent studies [17, 21, 22] have shown that PEG improves endothelial cell preservation and viability constituting a barrier that restricts the passage of ions during ischemia and hypothermia. Moreover, it reduces the influx of inflammatory cells and MHC class II expression. Our previous experiments (data not shown) have demonstrated the superiority of high molecular weight PEG in liver preservation showing a more effective capacity to preserve cellular function and to decrease vascular resistance.

In ex vivo experiments kidneys preserved in IGL-1 showed an improvement in FRNa and GFR values and in in vivo experiments IGL-1 solution was shown to be able to improve pig renal function after ischemia-reperfusion injury. Indeed, 6–7 days after transplantation urea and creatinine values were significantly higher in animals from group 1 (K-UW) than in control animals while the values of animals from group 2 (IGL-1) were close to those of controls. Moreover, at day 7 after transplantation there was a significant difference in creatinine clearance between animals from group 1 (K-UW) and controls while there was no significant difference between animals from group 2 (IGL-1) and controls. The beneficial effect of IGL-1 was also demonstrated by a better preservation of cellular integrity, less inflammatory response and interstitial fibrosis. An important reduction of cellular apoptosis in the kidneys preserved in IGL-1 solution was demonstrated. It is possible that PEG, which reduces lipid peroxidation [23], impairs hydrogen peroxide-induced apoptosis in epithelial cells protecting kidneys against cold ischemia. Another finding of the beneficial effect of IGL-1 solution is the significant reduction of the expression of MHC class II cells, which plays an important role in ischemia-reperfusion injury, enhancing graft immunogenicity. It has been shown by other authors [3, 13, 21] that there is a clear correlation between the degree of over-expression of MHC class II and CD4+ infiltrating cells, the peritubular expression of VCAM-1 and the development of interstitial fibrosis. Indeed, kidneys preserved in IGL-1 solution presented a degree of fibrosis similar to that of control kidneys while kidneys preserved in K-UW solution showed early appearance of fibrosis, which is specific for pig models [16]. Interstitial expression of alpha-SMA is considered an early marker of chronic renal allograft dysfunction and its evaluation in time-zero biopsies can predict this event [24]. IGL-1 solution was found to be able to significantly limit the fibrosis, which is considered to be the lesion leading to chronic renal injury and progressive loss of renal function [25].

In conclusion, IGL-1 solution was found to present at the same time the advantages of Na-UW solution and those of a solution containing high molecular weight PEG. This original solution was found to be able to improve kidney preservation and function. Whether IGL-1 will be beneficial in human kidney transplantation to decrease ischemic-reperfusion injury and improve graft function and survival will be determined in a clinical trial, which has just been started.

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