SDC, Materials and Methods

Studies were performed in 276 male Fischer rats aged 7-8 weeks provided by Charles (River Spain, Madrid, Spain). Rat CT-1 was provided by DRO Biosystems (San Sebastian, Spain). CT-1 was dissolved in cold (4ºC) University of Wisconsin solution, at the concentrations described in the study.

Experimental design

Effect of CT-1 on renal damage after cold preservation. Preservation protocol 1.

Male Fischer rats (7-8 weeks of age) were anesthetized by intraperitoneal injection of ketamine hydrochloride (2 mg/kg, Ketolar, Pfizer) combined with diazepam (10 mg/kg, Valium, Roche) and atropine (1 mg/kg, Atropina, Braun). Isoflurane (Isoba ® vet, Schering-Plough) was used at 5% mixed with a percentage 50-50 oxygen/nitrogen. After the induction of anesthesia, the isoflurane concentration was decreased in a step-like manner to 2% and continued through a nose cone.

Once anesthetized, a standard laparotomy was performed and the retroperitoneum was dissected to expose the abdominal aorta and renal arteries to the aortic bifurcation. Renal vessels and the proximal half of the ureter were dissected and isolated by ligation of the adrenal and gonadal branches. For kidney perfusion and washing procedure, an occlusion over the left renal artery was performed followed by aortic catheterization. At the same time, the infrarenal clamp was withdrawn to start in situ renal perfusion through the aorta with 5 mL of chilled (4ºC) University of Wisconsin (UW) solution with or without 0.2 μg/mL CT-1, at a flow rate of 1 mL/min. Once perfused, the kidney was removed and stored at 4 ºC in UW solution (with or without 0.2 μg/mL CT-1).
After different time points of cold preservation (0.5, 6, 24 and 48 hours), the kidneys were harvested and a piece of tissue including cortex and medulla were trimmed down, snap frozen in liquid nitrogen and stored at −80°C (Figure 1). The remaining renal tissue was immediately used for SOA measurement. Two ml of the preservation fluid were also collected and stored at -80°C for several measurements. In a simulated group (Sham group), kidneys were perfused with 1.5-2 mL of UW solution and immediately harvested and stored as above described, without subjecting them to preservation.

**Effect of CT-1 in the perfusion fluid on kidneys previously cold-preserved for 6 hours. Preservation protocol 2.**

Kidney isolation was conducted as described above (for protocol 1). In this case, the kidneys were perfused in situ with 5 mL cold UW solution, harvested and preserved in cold UW solution for 6 hours, and then reperfused at a flow rate of 1 mL/min with 1.5-2 mL of UW solution containing 16, 32, or 64 μg/mL CT-1. Then, the kidneys were immediately returned to the preservation fluid for an additional period of 3 or 18 hours (total preservation time 9 and 24 hours, respectively). In a control group, the kidneys were perfused in situ with 5 mL cold UW solution, harvested and preserved in cold UW solution for 6 hours, and then reperfused at a flow rate of 1 mL/min with 1.5-2 mL of UW (without CT-1). In a simulated group (Sham group), the kidneys were perfused in situ with 5 mL of UW solution and immediately harvested as above described without preservation. In a positive control group (Ctrl+ group), kidneys were perfused in situ with 5 mL of UW solution containing 0.2 μg/mL CT-1,
immediately harvested and preserved in UW solution containing 0.2 μg/mL CT-1 for 9 or 24 h. After different time points of cold preservation (2 and 24 hours), the kidneys were harvested and a piece of tissue including cortex and medulla were trimmed down, snap frozen in liquid nitrogen and stored at -80ºC (Figure 1). The remaining renal tissue was immediately used for SOA measurement. Two ml of the preservation fluid were also collected and stored at -80ºC for several measurements.

*Transplantation of kidneys preserved with CT-1*

Renal transplantation was performed as previously described, both after preservation protocols 1 and 2. In brief, donor rats (male Fischer rats of 7-8 weeks of age) were anesthetized by intraperitoneal injection of ketamine hydrochloride (2 mg/kg, Ketolar, Pfizer) combined with diazepam (10 mg/kg, Valium, Roche) and atropine (1 mg/kg, Atropina, Braun). Isoflurane (Isoba ® vet, Schering-Plough) was used at 5% mixed with a percentage 50-50 oxygen/nitrogen. After the induction of anesthesia, the isoflurane concentration was decreased in a step-like manner to 2% and continued through a nose cone. The animals were placed in a thermostated surgical pad, and after laparotomy, the donor left kidney was isolated by ligating and dividing the adrenal and testicular vessels with microsutures. The aorta and inferior vena cava were mobilized at their junction, with the left renal artery and vein. The aorta was ligated below the renal vessel. An elliptical patch of bladder containing the left ureterovesical junction was excised. The graft was perfused in situ with cold UW solution (with or without CT-1), removed and preserved during 24 hours in cold UW with or without CT-1 as above described, according to either protocol 1 or protocol 2 (Figure 1). Recipient rats (from the same strain and age) were
anesthetized as above described. After laparotomy, the recipient’s right native kidney was removed immediately before transplantation. The infrarenal aorta and inferior vena cava were carefully isolated and cross-clamped. Then, the preserved kidney was positioned orthotopically into the recipient and an end-to-side anastomosis between the donor renal vein and the recipient inferior vena cava was made using continuous 10/0 suture. The arterial anastomosis between the donor aortic cuff and recipient aorta was made in the same manner as the venous anastomosis, with the exception that only 2 or 3 sutures were required for each side because the aortic diameter is smaller than the vein. After successful anastomosis, the kidney graft was perfused instantly. The grafts that did not reperfuse correctly were rejected. These grafts were not included in the study. The frequency of bad reperfusion was similar in the kidneys preserved or not with CT-1. Contralateral urinary reconstruction was then performed by a bladder-to-bladder anastomosis. Peritoneum and abdominal muscle wound was closed in a single plane with continuous 4/0 suture. Skin was closed with skin staples and the animals were temporarily maintained under a heating lamp, and then, returned to their cages for recovery.

Progression-free survival probabilities were evaluated using the Kaplan–Meier method, from the first day after transplant until death or euthanasia (30 days). In the latter case, the survival time was censored. All groups were compared and statistical differences were confirmed by the log-rank test.

At the end of the experimental time, after laparotomy, a sample of blood was obtained from the abdominal aorta, immediately centrifuged and plasma stored at -80°C. The transplanted kidney was removed, washed with cold saline solution, and divided in 2 halves. One half was immediately stored at -80°C for
further analysis, and the other half was used immediately for SOA determination. A specific group of animals was devoted to assess survival rate and renal function.

For renal function studies, once fully recovered from surgery, rats were placed in individual metabolic cages to collect urine free of food and faeces. Blood and urine samples were obtained at days 3, 7 and 14 after renal transplantation.

**Analysis**

Plasma and urinary creatinine concentrations were assessed using an automated method (Reflotron Plus®; Roche Diagnostics, Barcelona, Spain) with commercial diagnostic kits (Roche Farma, Spain). Creatinine clearance (CrCl), an indirect marker of glomerular filtration rate (GFR) was calculated with the formulae: 

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\text{CrCl} = \frac{\text{UF} \times \text{UCr}}{\text{PCr}}
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where UF is the 24 hour urine output (mL/min), UCr is the urinary concentration of creatinine (mg/dL), and PCr is the plasma concentration of creatinine (mg/dL).²

Levels of SOA in renal tissues were measured in the soluble fraction of renal tissue extracts as previously described.³ SOA production was expressed in nmol/mg protein/minute. Protein content was determined spectrophotometrically using the Lowry’s method.

Tumor Necrosis Factor-alpha (TNF-α) and IL-6 levels were quantified using a specific enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions and guidelines (rat cytokines, R&D Systems, Minneapolis, MN), and expressed as pg/mL.
Extracts from kidney tissue were prepared and analyzed by Western blot according to standard protocols. Briefly, extract samples that contained 150-200 μg proteins were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membranes. After being blocked with 5% BSA, the membranes were incubated at 4ºC overnight with antibodies (dilution 1:500). Then, membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG for 1 hour at RT (dilution 1:10 000; Bio-Rad Laboratories). After washing, the immune complexes were detected with chemiluminiscent (ECL) HRP substrate using an image reader (ImageQuant RT ECL, GE Healthcare, Spain), and the intensity of the bands were measured. Membranes were also reproved with mouse monoclonal anti-β-actin antibody (1:20 000; Sigma-Aldrich) to verify equal loading of protein in each lane. Levels of soluble ICAM-1 and VCAM-1 in plasma were also measured by western blot. Antibodies used for this technique were: anti-VCAM-1, anti-phosphoSTAT-3, anti-STAT-3, antinuclear factor κB (NFκ-B), antiphospho NFκ-B, anti-iNOS/NOS-2, anti-NFκB inhibitor (IκB) and anti-gp130, from Cell Signaling, CA, USA; anti-CT-1 from R&D Systems, CA, USA; and anti-LIFR from Santa Cruz Biotech, CA, USA.

**Data management and statistical analysis**

Statistical analysis was performed using the NCSS 2007 Statistical System Data (Dr. Jerry L. Hintze, Kaysville, Utah, USA). Values from data with a normal distribution were expressed as mean ± standard deviation (SD). Scheffe’s correction test was used for multiple comparisons. For data not conforming to a normal distribution the Kruskal-Wallis test followed by Dunn's test was used for multiple comparisons. In general, statistical significance was considered when
p<0.05 or Z >1.96, for normal and non-normal data, respectively. Progression-free survival probabilities were evaluated using the Kaplan–Meier method, from the first day after transplantation until death or euthanasia (30 days). In the latter case, the survival time was censored. All groups were compared and statistical differences were confirmed by the log-rank, Mantel–Cox test.

REFERENCES

