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Original Article

Prevention of cold ischaemia–reperfusion injury by an endothelin receptor antagonist in experimental renal transplantation

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Abstract

Background. Endothelin (ET) is known to play a role in the pathogenesis of warm ischaemic renal damage, however, little is known about its involvement in renal cold ischaemia. This study was designed to investigate the response of ET after kidney cold ischaemia, and to assess the potential protective effect of bosentan, a dual, non-selective ET_A/ET_B receptor antagonist, against cold ischaemia–reperfusion injury in a rat model of syngeneic renal transplantation.

Methods. Kidneys from Lewis rats were transplanted, either immediately or after 5 h of cold preservation. After 48 h, contralateral nephrectomy was performed. Rats were organized into three groups: Tr-NoISC, no cold ischaemia; Tr-ISC, 5 h cold ischaemia; and Tr-BOS, 5 h cold ischaemia plus bosentan (100 mg/kg/day, from the day before transplantation until the seventh day post-transplantation). On day 7, plasma and tissue immunoreactive ET (irET), as well as ET mRNA tissue expression, were evaluated. Renal function was measured by means of serum creatinine on days 3, 4, 5 and 7, and by creatinine clearance on day 7. Conventional histology was performed.

Results. The ischaemic group had significantly higher plasma irET levels than the non-ischaemic group and significantly lower levels than the bosentan group. Tissue irET levels and ET mRNA expression were similar in the ischaemic and bosentan groups and were higher than in the non-ischaemic group. Throughout the follow-up, serum creatinine was significantly higher in the ischaemic group than in the bosentan group. Moreover, creatinine decreased rapidly in the bosentan group after nephrectomy, whereas it continued to increase for 48 h in the ischaemic group. Kidneys from the ischaemic group showed a higher degree of tubular-cell necrosis and epithelial-cell detachment than kidneys from the bosentan group.

Conclusions. We conclude that cold ischaemia and preservation damage induces an increase in renal ET mRNA and irET expression in the reperfusion phase, contributing both to the deterioration of renal function and to tubular necrosis. Bosentan is effective in protecting kidneys from this cold ischaemia–reperfusion damage. Non-selective ET_A/ET_B receptor antagonists might be potentially useful in clinical renal transplantation.

Key words: cold ischaemia–reperfusion injury; endothelin; ET_A/ET_B receptor antagonist; rat renal transplantation

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Introduction

Ischaemic damage during kidney transplantation is responsible for a 20–30% worldwide incidence of delayed graft function and may increase the incidence of acute rejection, as well as favour chronic transplant nephropathy [1,2]. Nowadays, dialysis is the only accepted treatment for acute renal failure, although in some instances it can aggravate ischaemic renal injury, prolonging the renal impairment [3]. Thus, better therapeutic regimes for the period following the initial injury are needed.

Although the precise mechanisms of ischaemic acute renal failure have not been clarified, some chemical mediators, such as oxygen radicals [4], eicosanoids [5] and platelet activating factor [6,7] accompanied by vasculoendothelial dysfunction, have been suggested to play a role [8]. Endothelins are a family of three highly vasoactive and mitogenic 21 amino acid residue peptides (ET-1, ET-2, ET-3) with multiple biological actions in a variety of organs under physiological conditions and during disease [9,10]. ET-1 (ET), the most active peptide, is a potent renal vasoconstrictor with direct glomerular and tubular effects [10] mediated through specific cell-surface receptors (ET_A and ET_B receptors). There is increasing evidence to indicate

the role of ET in the pathogenesis of warm ischaemic renal damage. Indirect evidence includes the increased plasma [11,12] or tissue levels [13] of ET, or the increased affinity of the renal ET receptor [14], which have been observed in post-ischaemic kidney. Direct evidence has been obtained from studies using ET antibodies [12,15,16] or receptor antagonists [11,13,17,18] as protecting agents in models of renal warm ischaemia.

Some data regarding the involvement of ET in cold ischaemia in organ transplantation have been reported [10]. For example, an increased concentration of ET in the preservation solution after a long period of cold storage has been found [19]. These ET levels, as well as the levels of other vasoconstrictive substances, were higher if kidneys had been subjected to warm ischaemia prior to renal harvesting in cold solution [19]. Elevated ET plasma levels have also been observed in transplant recipients suffering from acute tubular necrosis [20]. Lastly, high ET plasma levels, as well as lipid peroxidation and protein oxidation tissue products, have been reported in an experimental renal auto- and allotransplantation model after cold preservation [21]. In this study, the addition of BQ-123, a specific ET_A receptor antagonist, normalized these changes. However, this study only analysed the acute role of ET in cold ischaemia making the results difficult to extrapolate to the clinical setting.

The present study assessed the response of ET in cold ischaemia–reperfusion damage using a rat model of renal syngeneic transplantation. The absence of alloreactivity in this model allowed us to isolate the effect of cold ischaemia from immunological factors. We also evaluated whether the administration of bosentan, a non-selective ET_A/ET_B receptor antagonist, protects the kidney from the detrimental effects caused by ET after renal transplantation.

Subjects and methods

Animals and surgical technique

Donors and recipients were male Lewis rats (250 g body weight; Charles River, Spain) maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals and Good Laboratory Practice. Transplantation was performed according to a modification of a method described elsewhere [22–24]. For kidney harvesting, anaesthesia was induced and maintained by ether inhalation. After a midline incision, the left kidney was gently exposed and a cannula introduced into the infrarenal aorta to the ostium of the renal artery. A single dose of sodium heparin (1000 UI) was administered and the kidney was immediately washed with EuroCollins solution (2 ml, 4°C, 20 ml/h flow rate). The renal artery was excised with an aortic patch, the renal vein excised proximal to its union with the cavae and the urether cut next to the bladder. The kidney was either transplanted immediately or embedded in EuroCollins solution and preserved for 5 h at 4°C.

For kidney transplantation, anaesthesia was induced and maintained by ether inhalation. After a midline incision, the left kidney was gently exposed, the urether of the recipient was cut next to the kidney pelvis and nephrectomy was performed. The donor kidney was grafted heterotopically. For this purpose, the recipient aorta and cavae were occluded with vascular clamps and the donor renal artery patch and the renal vein were anastomosed, respectively, to the receptor vessels with a 9-0 monofilament. After the vascular clamps had been removed, allowing the graft to be conveniently reperfused, the urether was anastomosed end-to-end with four individual 11-0 monofilament stitches. Finally, the laparotomy was closed with 3-0 silk suture and a single intramuscular dose of Cyprofloxacine (5 mg) was administered. Reanastomosis and total surgical time took no more than 30 and 60 min, respectively.

The animals were placed in a warm cage in a light/dark cycle chamber and allowed free access to tap water and rat chow. Forty-eight hours after transplantation, the recipient was again anaesthetized by ether inhalation and right native nephrectomy was performed through a 2-cm lumboiliac incision. The wound was closed and a single intramuscular dose of Cyprofloxacine was administered.

Follow-up and design of the study

Two days prior to surgery and on the seventh day after transplantation, rats were placed in metabolic cages for 24-h urine collection (Figure 1). Prior to surgery, and on days 3, 4, 5 and 7, a blood sample was obtained from the tail vein. Creatinine levels were quantified on plasma and urine samples, and creatinine clearance was calculated using standard formulas. On the seventh post-transplant day, the animal was anaesthetized by ether inhalation and the grafted kidney nephrectomized through a midline laparotomy. Two millilitres of blood were collected in EDTA for ET measurement. The kidney was processed for conventional histology, tissue ET studies and molecular biology.

Twenty five rats with kidney grafts were studied and divided into three groups: Tr-NoISC group, kidneys immediately transplanted without cold ischaemia ($n=7$); Tr-ISC group, 5 h of cold ischaemia before renal transplantation ($n=9$); and Tr-BOS group, 5 h of cold ischaemia before renal transplantation plus bosentan ($n=9$).

Bosentan was administered by oral gavage at a dose of 100 mg/kg/day beginning on the day before transplantation up to the seventh day after transplantation.

Plasma and tissue levels of endothelin

Blood for the quantification of plasma immunoreactive ET (irET) was collected into tubes containing 15% EDTA on

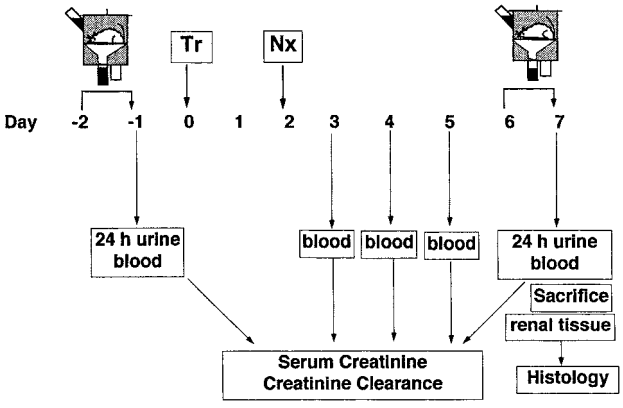

Fig. 1. Design of the study.

ice. Tubes were centrifuged at 4°C and the plasma was frozen until assayed. For quantification of renal tissue irET, 500 mg of kidney were rinsed with ice-cold phosphate buffered saline (PBS), and individually homogenized (Ultra Turrax T8, IKA Labortechnik, Staufen, Germany) in 1 mol/l acetic acid (1:3 wt : vol.) and centrifuged at 15 000 *g* for 10 min at 4°C. Supernatants were frozen at −20°C until the concentrations of irET and proteins were analysed.

Endothelin was measured by a radioimmunoassay [25] (Nichols Institute Diagnostics B.V., Wijchen, The Netherlands) after ET extraction on Sep-Pak C18 cartridges (Waters Associates, Milfort, MA, USA). Briefly, plasma samples (1 ml) were acidified with 4% acetic acid (4.5 ml) and applied to cartridges pre-activated with methanol, distilled water and 4% acetic acid. Cartridges were then washed with distilled water and 25% ethanol and irET was eluted twice with 1 ml of 4% acetic acid in 86% ethanol. The eluted ET was then concentrated to dryness (Speed Vac Concentrator, Savant Instruments Inc, Farmingdale NY, USA) and reconstituted for radioimmunoassay. The recovery rate for the extraction procedure was 85%, as determined by the addition of labelled ET-1 (3500 c.p.m.) to plasma. Cross-reactivity of the antiserum for ET-1, ET-2, ET-3 and big ET was 100%, 52%, 96% and 7%, respectively. Intra-assay and interassay coefficients of variation were 6.9% and 12.1%, respectively. Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as standard.

Tissue ET mRNA expression

In order to analyse the ET-1 mRNA expression in renal tissue, a semiquantitative multiplex reverse transcription polymerase chain reaction (RT–PCR) protocol was performed using *β*-actin as a housekeeping gene. Total kidney RNA was isolated with TriPure™ Isolation Reagent (Boehringer Mannheim, Germany), according to the Chomczynski and Sacchi method [26]. The final RNA pellet was resuspended in diethylpyrocarbonate-treated water and quantified by spectrophotometry at 260 nm. All samples had a 260/280 OD ratio > 1.8.

For random-primed first-strand cDNA synthesis, a sample of 1–2 μg total RNA was incubated with 10 U of DNase I RNase-free (Boehringer Mannheim) and 20 U Recombinant RNasin Ribonuclease Inhibitor (Promega Corp, Madison, WI, USA) in a total volume of 10.5 ml at 37°C for 30 min. The reaction was stopped by heating at 95°C for 15 min. Fifty nanograms of random hexamers were then added and the mixture was kept for 10 min at 65°C and put on ice immediately thereafter. Two hundred units of SuperScript™ reverse transcriptase (GIBCO BRL, Eggenstein, Germany) were then added and RT was carried out according to standard manufacturer's specifications for 50 min at 42°C in a final volume of 20 μl. The RT reaction was stopped by heating at 95°C for 5 min. Negative controls without RT were included for all individual samples.

For PCR analysis, non-looping, non-overlapping deoxyoligonucleotide primers from separate exons were obtained for each analysed gene. Rat *β*-actin primer pair was purchased from Clontech (Palo Alto, CA, USA). A 740-bp fragment was amplified using a 5′-forward primer 5′-TTGTAACCAACTGGGACGATATGG-3′; and a 3′-reverse primer 5′-GATCTTGATCTTCATGGGTGCTAGG-3′. Endothelin-1 primer pairs were 5′-forward 5′-CTAGGTCTAAGCGATCCTTG-3′ and the 3′-reverse 5′-TTCTGGTCTCTGTAGAGTTC-3′, which amplify a 318-bp fragment as reported previously [27].

One microlitre of the RT product was amplified in a final volume of 50 μl containing 1.25 U of BIOTaq DNA polymerase (Progenetic, Vista, CA, USA), 67 mM Tris–HCl buffer (pH 8.8), 16 mM $(NH_4)_2$ SO_4, 0.1% Tween-20, 2.2 mM $MgCl_2$, 200 μM of dNTP and 0.125 μM of *β*-actin oligonucleotide primer, as well as 0.5 μM of ET-1 primer pairs in the same tube to minimize variability between PCR reactions. All PCR reactions were performed at the same time in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc). After analysing the number of PCR cycles at the linear phase of PCR amplification and following an initial denaturation step at 94°C for 5 min, PCR was conducted for 40 cycles at 94, 60 and 70°C for 45 s, 45 s and 2 min, respectively as denaturation, annealing and extension steps. A final extension at 72°C was performed for 7 min. Lastly, the PCR products were analysed semiquantitatively. Briefly, after amplification, 10 μl of each PCR reaction mixture were electrophoresed through a 1% agarose gel in 1 × TBE buffer containing 0.1 μg/ml of ethidium bromide. The gel was photographed with Polaroid Type 665 positive/negative film (Polaroid Corp, Cambridge, MA, USA) under ultraviolet light exposure. The bands on the negative film were scanned by laser densitometry (Epson GT-8500). The signal intensity for ET-1 was counted and expressed as a ratio to *β*-actin.

Histological studies

For conventional histology, kidneys were fixed in formaldehyde embedded in paraffin and stained with both haematoxylin–eosin and Schiff periodic acid. Light-microscopy was reviewed by a pathologist blinded to the treatment groups. The sections were examined for tubular necrosis, tubular dilation, intratubular cell detachment, interstitial oedema and interstitial cellular infiltrate. Abnormalities were graded using a semiquantitative scale; 0 being no abnormality, and 1+, 2+ and 3+ representing mild, moderate and severe abnormalities, respectively.

Statistical analysis

Statistical analysis between groups was performed by using one-way analysis of variance and subsequent Scheffe's test. When needed, the Kruskall Wallis test was used. All *P* values were two-tailed, and a *P* value < 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

Plasma and tissue endothelin

Tissue and plasma irET concentrations are shown in Figure 2 (A and B). The tissue content of irET was similar between the Tr-ISC and Tr-BOS groups, and was significantly higher than in the Tr-NoISC group (Tr-ISC, 6.4 ± 1.3; Tr-BOS, 5.9 ± 1.1; and Tr-NoISC, 2.5 ± 0.3 pg/mg protein). In the Tr-NoISC group, the tissue irET concentration was similar in the transplanted and non-transplanted kidneys (control: 2.9 ± 0.4 pg/mg protein).

Plasma irET was significantly higher in the Tr-

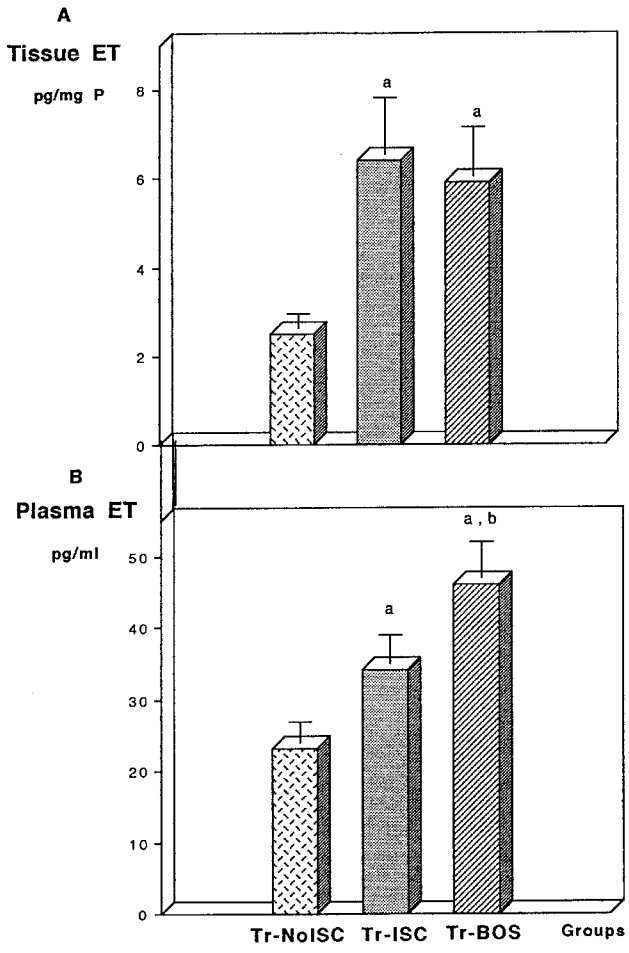

Fig. 2. Tissue irET (**A**) was similar between the Tr-ISC and Tr-BOS groups and was significantly higher than in the Tr-NoISC group (*a*: $P<0.05$). Plasma irET (**B**) was significantly lower in the Tr-NoISC group than in the other two groups (*a*: $P<0.05$). The value in the Tr-BOS group was significantly higher than in the Tr-ISC group (*b*: $P<0.05$).

NoISC group compared with plasma from normal rats (Tr-NoISC, 23 ± 3; control, 10 ± 3 pg/ml). This difference can be explained by the model itself and that irET may come from extra-renal sources. Rats exposed to 5 h of kidney cold ischaemia had higher plasma irET concentrations than kidney-transplanted rats without cold ischaemia (Tr-ISC: 34 ± 4 pg/ml). Rats exposed to 5 h of kidney cold ischaemia and treated with bosentan showed significantly higher plasma irET than rats not treated with bosentan (Tr-BOS: 46 ± 5 pg/ml).

Tissue ET mRNA expression

To test whether the expression of tissue ET production is paralleled by changes in ET mRNA level, multiplex semiquantitative RT–PCR analysis of ET transcription was performed. Representative RT–PCR amplification products for both ET and β-actin for the different experimental groups are shown in Figure 3. Endothelin and β-actin were co-amplified and the densitometric analysis of bands revealed that the Tr-ISC and Tr-BOS kidneys had similar expression of ET relative to β-actin, and both were significantly higher than Tr-NoISC kidneys (0.49 ± 0.07 in Tr-ISC group, 0.55 ± 0.07 in Tr-BOS group and 0.15 ± 0.06 in Tr-NoISC group; Figure 4).

Functional studies

Functional studies are shown in Figures 5 and 6. The Tr-ISC group had significantly higher creatinine levels than rats from the Tr-NoISC group throughout the whole study. In the Tr-NoISC group, the creatinine level on day 3 was transiently slightly higher than the basal value. This informs us that the transplantation technique was correctly performed and that our model, in the absence of cold ischaemia, only induces mild renal failure. Throughout the whole study, Tr-BOS animals had significantly lower creatinine levels than Tr-ISC animals. Moreover, creatinine levels in the Tr-ISC group increased further after contralateral nephrectomy until they peaked on day 4. Afterwards, the creatinine level began to ameliorate, but these animals still showed moderate–severe renal failure on day 7 with significantly higher creatinine levels than both the Tr-BOS and Tr-NoISC groups. On the contrary, Tr-BOS animals had rapid amelioration of renal failure after nephrectomy and a higher creatinine level with respect to Tr-NoISC only on days 4 and 5. Serum creatinine level on day 7 was similar in the Tr-BOS and Tr-NoISC groups.

On day 7, creatinine clearance in the Tr-BOS group was clearly higher than in the Tr-ISC group and significantly lower than in Tr-NoISC animals. On day 7, creatinine clearance in the Tr-NoISC group was not different to basal values.

Histological studies

Contralateral non-transplanted kidneys were used to evaluate normal tubulointerstitial parameters. The light-microscopy results are shown in Table 1. Tr-NoISC kidneys had greater tubular dilation than contralateral kidneys, but the other histological parameters were not different.

Kidneys from the Tr-BOS and Tr-ISC groups had significantly more tubular cell necrosis and intra-tubular cell detachment than kidneys from the Tr-NoISC group. However, it is noteworthy that kidneys from bosentan-treated animals (Figure 7A) displayed significantly less tubular cell necrosis and intra-tubular cell detachment than kidneys from ischaemic non-treated animals (Figure 7B). No differences in tubular dilatation were observed among the Tr-NoISC, Tr-ISC or Tr-BOS groups. Oedema and interstitial-cell infiltrate were similarly scarce in all the study groups.

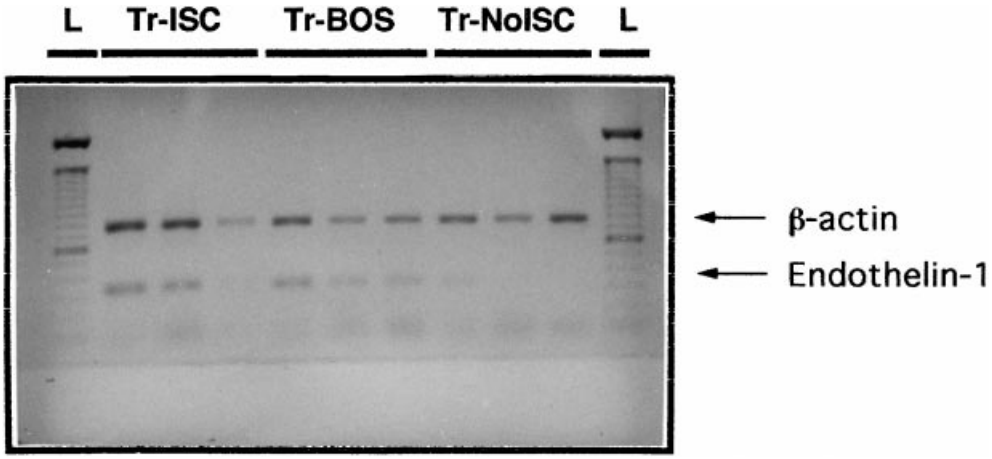

Fig. 3. Endothelin-1 mRNA expression by multiplex RT–PCR in three representatives rats (one lane for rat) for each group. L, ladder 100 bp (GIBCO BRL, Eggenstein, Germany, no. catalogous 15628–019). The upper band (740 bp) reflex β-actin expression, and the lower band (318 bp) represents ET-1 expression.

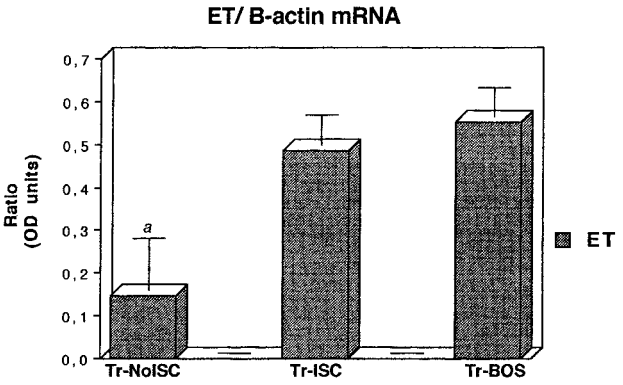

Fig. 4. Endothelin expression measured in OD units and normalized by the OD units of β-actine mRNA. ET mRNA levels in the Tr-NoISC group was significantly lower than in the Tr-ISC and Tr-BOS groups (*a*: $P=0.01$). ET mRNA levels were not statistically different between the Tr-ISC and Tr-BOS groups.

Discussion

We clearly show that endothelin contributes to renal cold ischaemia–reperfusion injury in an experimental rat model of renal transplantation, and that treatment with the non-selective ET receptor antagonist, bosentan, attenuates this injury. The observations supporting these findings are: (i) animals with ischaemic kidneys had significantly higher plasma irET and tissue ET mRNA than non-ischaemic animals 7 days after transplantation; and (ii) the administration of bosentan during the whole transplant period resulted in an amelioration of the functional and morphological pathologies induced by preservation cold ischaemia injury.

Functional, biochemical and molecular tissue results from our study with cold ischaemia are similar to the results of the majority of studies analysing warm ischaemic damage [11–18,28]. In addition, our histological studies demonstrated that the tubular necrosis in ischaemic kidneys treated with bosentan was less severe compared with ischaemic non-treated kidneys 7 days after transplantation. Most studies with ET antagonists in kidney warm ischaemic injury only evaluate functional parameters and there are few studies evaluating both functional and histological parameters [12,29,30]. In one of these studies [12], the morphological changes in the early reperfusion period (120 min) after renal warm ischaemia were evaluated in rabbits. No tubular damage was seen, but the authors reported significant medullary erythrocyte congestion and polymorphonuclear accumulation which were ameliorated by ET antibody infusion. It has been reported that ischaemia not only damages parenchymatous cells but also has a prolonged affect on the function and reactivity of the vasculature of the kidney [31]. ET and nitric oxide have been shown to mediate these sustained vasomotor effects on kidneys. Likewise, constriction of afferent arterioles has been shown to be an important mechanism of post-ischaemic acute renal failure in renal transplantation [32]. Thus, bosentan, both by blocking the functional effects exerted by ET, i.e. the constrictor effect on renal arterioles, and reducing the early polymorphonuclear recruitment, leads to morphological protection in these treated animals as our results indicate.

There are a couple of reports suggesting that ET contributes to the development of post-ischaemic acute renal failure in clinical transplantation [20,33]. There are no clinical studies evaluating the effect of an endothelin-receptor antagonist on cold ischaemia–reperfusion injury, and there is only one study in experimental rat-kidney transplantation [21]. In this work the ET_A selective receptor antagonist, BQ-123, was highly effective in both reversing the increased ET-1, ET-2 plasma levels and abolishing the tissue damage induced by oxygen free radicals after cold ischaemic injury. This study is highly interesting in terms of evaluating the pathophysiological mechanisms of cold ischaemia–reperfusion-induced damage, and the role that ET plays in it, but it does not support

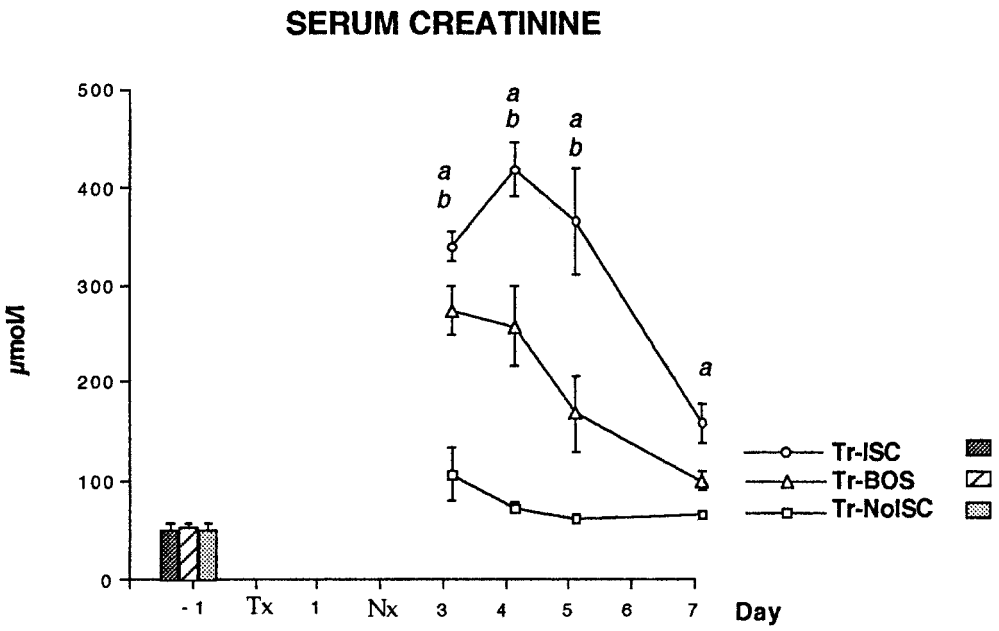

Fig. 5. Serum creatinine in the Tr-BOS group was significantly lower than in the Tr-ISC group (*a*: $P<0.05$) for the whole follow-up. Serum creatinine in the Tr-NoISC group was significantly lower than both in the Tr-ISC and the Tr-BOS groups (*b*: $P<0.05$). On day 7, serum creatinine was similar between the Tr-NoISC and the Tr-BOS groups.

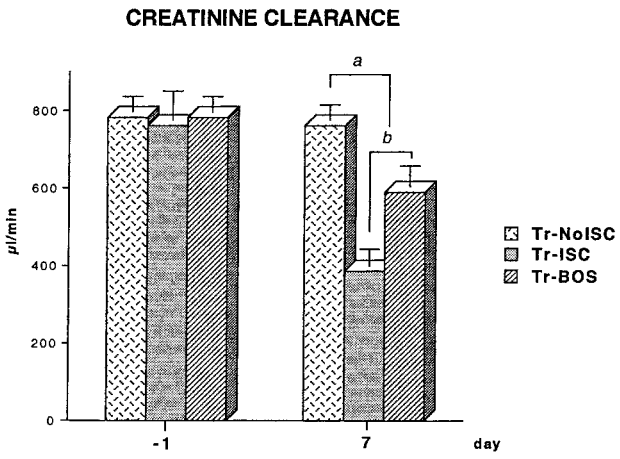

Fig. 6. Basal creatinine clearance was similar among all groups. On the seventh day, the Tr-BOS group had higher creatinine clearance (*b*: $P<0.05$) than the Tr-ISC group. Neither the Tr-ISC nor the Tr-BOS group returned to basal values as did the Tr-NoISC rats (*a*: $P<0.05$).

the use of ET antagonists in clinical transplantation. Cold ischaemia time in that study was very short (45 min). The follow-up was only 120 min and no information regarding urine production or the renal function was provided. We used a longer cold ischaemia time (5 h) that allowed us to obtain a degree of renal dysfunction which resembles that observed in the clinical setting. The follow-up period of 1 week was sufficient to evaluate the effect of a protecting agent in post-transplant renal failure.

Most of the studies on renal warm ischaemia have shown a role for ET in the first 24 h after ischaemia, reporting increases in renal ET [34], plasma ET [33] and ET receptor affinity [14]. Emerging evidence indicates that ET may be an important negative factor during the maintenance phase of acute renal failure, beyond 24 h. First, infusion of an ET antibody in the renal artery 48 h after ischaemia attenuated the characteristic post-ischaemic vasoconstriction [16]. Secondly, an increase in ET mRNA after unilateral ischaemia, which peaked within 24–48 h and was still maintained

Table 1. Histological parameters

Groups	Tubular dilation	Cell detachment	Necrosis	Oedema	Infiltrate
Tr-NoISC	1.14 ± 0.14	0.00 ± 0.00	0.29 ± 0.29	0.14 ± 0.14	0.29 ± 0.29
Tr-ISC	1.44 ± 0.18	$1.78\pm0.15^{a,b}$	$2.00\pm0.24^{a,b}$	0.22 ± 0.15	0.33 ± 0.17
Tr-BOS	1.55 ± 0.24	0.89 ± 0.35^{a}	1.22 ± 0.22^{a}	0.33 ± 0.17	0.22 ± 0.15
P	n.s.	0.0002	0.0004	n.s.	n.s.

The light-microscopy study showed significantly greater intra-tubular cell detachment and tubular cell necrosis in the Tr-ISC and the Tr-BOS groups compared with the Tr-NoISC group (*a*: $P<0.05$). These parameters were also statistically higher in the Tr-ISC group than in the Tr-BOS group (*b*: $P<0.05$).

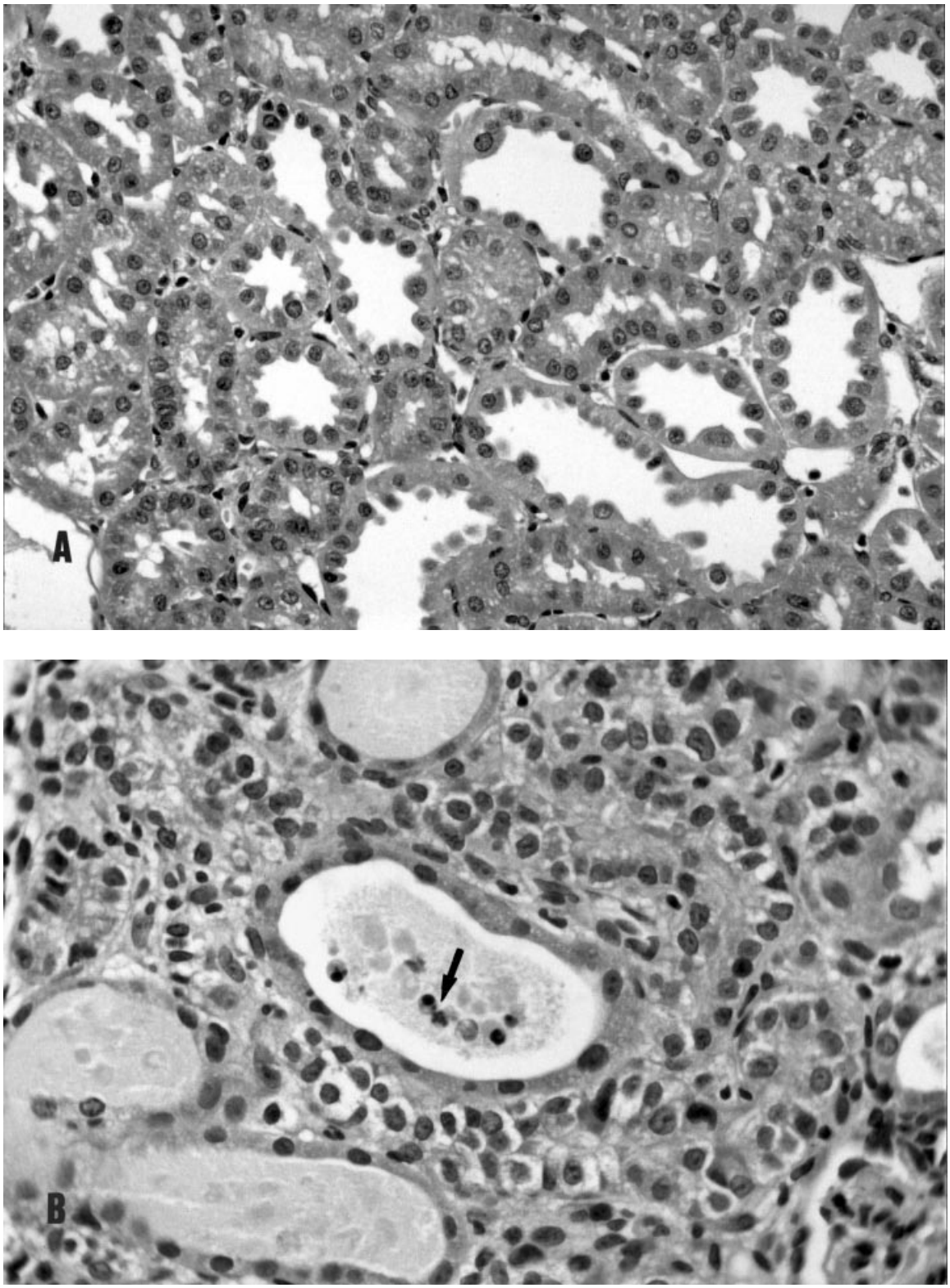

Fig. 7. Haematoxylin and eosin stained 4-μm sections of grafted kidneys from Tr-BOS (A ×400) and Tr-ISC (B ×630) groups. Animals receiving the daily dose of bosentan (**A**) had only mild tubular dilation, whereas ischaemic non-treated rats (**B**) had noticeable tubular dilation, flattened tubular epithelium and intra-tubular apoptotic bodies (arrow).

at 7 days [28]. Results from our study concerning the tissue expression of ET mRNA contribute to this evidence. A higher expression of ET mRNA in cold ischaemic kidneys compared with non-ischaemic ones was observed, and rats treated with bosentan showed similar expression of ET mRNA. This suggests that cold ischaemia induces ET mRNA expression which is prolonged 7 days after initial damage. If we assume that rat ET mRNA is as labile as human, then the increased levels of mRNA observed in our study are due to continuing induction of gene expression which is in agreement with Firth and Ratcliffe [28].

Endothelin-receptor antagonists have shown to be effective in both preventing and reversing warm-ischaemia-induced acute renal failure. In the prevention of acute renal failure, ET receptor antagonist caused a moderate, dose-dependent normalization of renal blood flow and sodium reabsorption [11], but only a partial improvement in glomerular filtration rate was observed [11,12]. Conversely, Gellai *et al.* [17,18] have shown that administration of an ET receptor antagonist 24 h after ischaemic damage was highly effective in reversing acute renal failure. In our study, bosentan was given for a broad period acting both in the prevention of and in reversing acute renal failure, thus leading to a significant protection of kidney function and morphology. Further studies are needed to design an optimal strategy to treat cold ischaemic injury in the clinical setting. We stress that prolonged administration of the drug must be carried out, beginning before transplantation and lasting for 7 days or more.

Although promising results have been reported for both selective and non-selective ET receptor antagonists in ischaemic acute renal failure [12,17,18], it may be beneficial to block both types of receptors to obtain the greatest protection of renal function. In the kidney, each receptor subtype has a different role and it differs depending on the animal species. In the rat, the ET_A receptor seems to mediate tubular urinary excretion of sodium [17,18,35], while ET_B mediates renal vasoconstriction and the control of GFR [35]. In contrast, in the dog [36,37] and in humans [38], ET-mediated renal vasoconstriction is probably primarily dependent on activation of the ET_A receptor. Therefore, results from studies using non-selective ET antagonists in rat models, as we have done, may be extrapolated to the clinical setting.

Recently, ET receptor antagonists have been demonstrated to increase the circulating levels of ET. Infusion of dual ET_A/ET_B receptor antagonists into experimental animals [34,39] or humans [40] increased plasma ET levels, possibly by displacing ET bound to tissue ET_B receptors. It has been shown that besides its haemodynamic effects, ET_B receptors possess a local clearance mechanism of ET [41,42]. In our study we found similar tissue ET and ET mRNA levels in both the ischaemic and the bosentan group. On the contrary, the plasma ET levels were much higher in the bosentan group than in the ischaemic group. This raises the possibility that ET has been displaced by bosentan from local renal sites, as has been suggested for other ET antagonists in other peripheral tissues [39,41,42]. Despite the higher plasma levels of ET in the bosentan group, we have obtained renal protection since we interfered with the action of endogenous ET in the kidney by blocking both ET_A/ET_B receptors.

We conclude that renal cold ischaemia and preservation damage induces an increase in ET mRNA and irET in the reperfusion phase, contributing to persistent deterioration of renal function and tubular necrosis. Bosentan, a non-selective dual ET_A/ET_B receptor antagonist, has been shown to be effective in protecting kidneys from this cold ischaemia–reperfusion damage. Thus, bosentan as well as other non-selective ET_A/ET_B receptor antagonists appears to have substantial therapeutic potential in kidney preservation. Since bosentan has been recently introduced to the clinical setting [43] further studies must be carried out to establish its use in the routine practice of clinical renal transplantation.

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