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Evidence for a protective role of trimetazidine during cold ischemia: targeting inflammation and nephron mass

Jean-Pierre Faure[a], Herve Baumert[a], Zeqiu Han[b], Jean Michel Goujon[a], Frederic Favreau[a], Delphine Dutheil[a], Isabelle Petit[a], Michel Barriere[a], Claude Tallineau[a], Jean Paul Tillement[c], Michel Carretier[a], Gerard Mauco[a], Vassilios Papadopoulos[b], Thierry Hauet[a,*]

[a]*Unité de Transplantation Expérimentale, Département de Génétique Animale, Institut National de Recherche Agronomique, Domaine du Magneraud, BP 52, Surgères 17700, France*
[b]*Division of Hormone Research, Department of Cell Biology, Medical/Dental Building, Georgetown University Medical Center, 3900 Reservoir Road NW, Washington, DC 20007, USA*
[c]*Laboratoire de Pharmacologie and Centre National de la Recherche Scientifique, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, Créteil F-94010, France*

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Abstract

Ischemia–reperfusion injury (IRI) is associated with an increased risk of acute rejection, delayed graft function, or chronic graft dysfunction. Mitochondria plays a central role in this process. Using an autotransplant pig kidney model, changes in renal function and morphology were determined after different periods of cold ischemia in kidneys preserved in the University of Wisconsin solution (UW), high-Na^+ version of UW (HEH) or Celsior (CEL) a newly developed high-Na^+ solution, with or without trimetazidine (TMZ). Kidney function was better preserved in HEH after 24 hr and particularly 48- and 72-hr cold storage than in CEL and UW. TMZ improved the preservation quality when added to the different solutions tested, particularly after 48- and 72-hr cold storage. Interstitial fibrosis and tubular atrophy were reduced in HEH with TMZ. $CD4^+$ T-cell infiltration was also modulated by the preservation conditions. Peripheral-type benzodiazepine receptor (PBR) positive cells infiltration was also modulated by preservation conditions. TMZ was efficient to reduce IRI when added in the various preservation solutions. These results suggest that protection of the mitochondrial function should be a major target to limit IRI. In addition, this study outlines the role of $CD4^+$ T cells and PBR expression in inflammatory responses after IRI.

Keywords: Ischemia–reperfusion injury; Cold preservation; Trimetazidine; Inflammation; Peripheral benzodiazepine-type receptor; Renal protection

1. Introduction

Organs used for transplantation undergo varying degrees of cold ischemia and reperfusion injury after transplantation. The central focus of organ transplantation therapy has been the prevention of acute rejection. Therapies have evolved to reduce the recognition of allopeptides by helper T cells. As a result, early rates of acute rejection have lowered to below 20% and increased one-year renal allograft survival to well above 80%. Unfortunately, this

* Corresponding author. Present address: INSERM ERM 324, BP 570, Rue de La Milétrie, CHU de Poitiers, Poitiers 86000, France. Tel.: +33-5-49-44-44-44; fax: +33-5-49-44-38-34.
E-mail address: t.hauet@chu-poitiers.fr (T. Hauet).

Abbreviations: CEL, Celsior solution; CEL24 hr, 24 hr of cold ischemia in CEL; CEL48 hr, 48 hr of cold ischemia in CEL; CEL72 hr, 72 hr of cold ischemia in CEL; CELTMZ24 hr, 24 hr of cold ischemia in CEL plus TMZ; CELTMZ48 hr, 48 hr of cold ischemia in CEL plus TMZ; CELTMZ72 hr, 72 hr of cold ischemia in CEL plus TMZ; C_{Cr}, creatinine clearance; HEH, Hopital Edouard Herriot solution; HEH24 hr, 24 hr of cold ischemia in HEH; HEH48 hr, 48 hr of cold ischemia in HEH; HEH72 hr, 72 hr of cold ischemia in HEH; HEHTMZ24 hr, 24 hr of cold ischemia in HEH plus TMZ; HEHTMZ48 hr, 48 hr of cold ischemia in HEH plus TMZ; HEHTMZ72 hr, 72 hr of cold ischemia in HEH plus TMZ; IRI, ischemia–reperfusion injury; Nef, uninephrectomy; PBR, peripheral-type benzodiazepine receptor; TMZ, trimetazidine; UW, University of Wisconsin solution; UW24 hr, 24 hr of cold ischemia in UW; UW48 hr, 48 hr of cold ischemia in UW; UW72 hr, 72 hr of cold ischemia in UW; UWTMZ24 hr, 24 hr of cold ischemia in UW plus TMZ; UWTMZ48 hr, 48 hr of cold ischemia in UW plus TMZ; UWTMZ72 hr, 72 hr of cold ischemia in UW plus TMZ.

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improved early graft survival has not translated in improved long-term graft survival, as graft half-life and the effects of chronic allograft nephropathy have remained relatively constant throughout the eras of cyclosporine and monoclonal antibody therapies. One of the most critical problems in transplantation today is the relatively high incidence of delayed graft function after surgery, affecting 20–30% of kidney transplants. Delayed graft function causes reduced short and long-term renal allograft survival and influence the incidence of acute rejection [1]. Recent works, both in human renal tubular cells in culture and clinical transplant settings, indicate that cold temperature causes necrosis, while rewarming causes apoptosis [2,3]. Such apoptosis is now shown to occur through the mitochondrial pathway and involves cold-induced mitochondrial permeability transition pore opening and, consequently, mitochondrial swelling, mitochondrial membrane rupture, and cytochrome *c* translocation to the cytosol [4]. Consequently, in organ transplantation, there is increasing evidence that mitochondria play a central role in IRI because of its implication in ATP synthesis, ionic homeostasis and production of reactive oxygen species.

PBR is an 18,000 Da transmembrane protein that localizes to the outer mitochondrial membrane where it shares a close physical association with the voltage-dependent anion channel and adenine nucleotide translocator, constituents of the PTP [5]. There is growing evidence of the importance of PBR in the transport of the substrate cholesterol into mitochondria in steroidogenic and liver tissues [6,7]. Previous studies demonstrated also the role of PBR in the mitochondrial protection against oxygen radical damage and mitochondrial protein import [8–10]. More recently, PBR and its ligands were shown to be involved in inflammatory processes [11,12]. Consequently, there is reasonable rational to study PBR expression in cold ischemia–reperfusion, a situation where oxidative stress and neutrophil activation are the two keystones of IRI.

In previous studies, using current preservation methods (Euro-Collins: EC, UW), we have demonstrated the role of TMZ in reducing cold ischemia injury from kidneys preserved for 48 hr [13–16]. TMZ is an anti-ischemic agent which exerts cytoprotection by shifting metabolism from fatty acid to glucose oxidation, which highlights the mitochondrial impact of this drug [17]. We have also demonstrated the role of TMZ in CD4-positive cells infiltration after cold ischemia and reperfusion. Recently, several studies have demonstrated the TMZ contributions in renal protection after hindlimb ischemia–reperfusion and glycerol-induced acute renal failure [18,19]. TMZ was also demonstrated to positively influence tissue regeneration after partial hepatectomy under hepatic blood inflow occlusion [20].

UW continues to be considered the gold standard by which new cold-storage preservation solutions are evaluated. However, clinical and experimental data demonstrate that this solution is not completely successful in preventing cold ischemic graft injury. New concepts are emerging about preservation solutions. Recent solutions using high Na^+ and low K^+ ratio were successfully assessed in multi-organ preservation. CEL was recently developed as a multi-organ preservation solution [21–23]. The high Na^+–low K^+ version of UW was also evaluated in different preservation conditions [24–27]. This study was designed to identify the individual contributions of different times of cold ischemia and the impact of preservation solutions with or without TMZ. In addition, we tested the hypothesis that PBR expression in inflammation areas could be modulated by preservation conditions.

2. Materials and methods

2.1. Surgical procedures and experimental design

We used an established autotransplanted pig kidney model of cold ischemia and reperfusion injury [5,16]. Briefly, following nephrectomy, kidneys were immediately cold-flushed and preserved at 4° for 24, 48 or 72 hr, after which the organs were autotransplanted. Ureteneocystostomy and contralateral nephrectomy were performed. All surgical procedures were performed aseptically. The preservation solutions were the UW, the low-K^+ version of UW (HEH) and a recently low-K^+ developed CEL solutions. The animals were divided into 20 groups: control and uninephrectomized (Nef) age-matched group ($N = 6$, respectively), group UW24 hr (UW, 24-hr preservation, $N = 8$), UW48 hr (UW, 48-hr preservation, $N = 8$), and group UW72 hr (UW, 72-hr preservation, $N = 8$), group HEH24 hr (HEH, 24-hr preservation, $N = 8$), group HEH48 hr (HEH, 48-hr preservation, $N = 8$), group HEH72 hr (HEH, 72-hr preservation, $N = 8$), EC24 hr (CEL, 24-hr preservation, $N = 8$), CEL 48 hr (CEL, 48-hr preservation, $N = 8$) and group CEL 72 hr (CEL, 72-hr preservation, $N = 8$). TMZ (10^{-6} M/L) was added to UW, HEH and CEL solutions and kidneys were preserved for 24, 48 and 72-hr ($N = 8$). This study was performed in accordance with the Guidelines of the French Agricultural Office and the legislation governing animal studies.

2.2. Renal function

Pigs were placed in metabolic cages for 24 hr to allow urine and blood samples collections. Endogenous C_{Cr} (mL/min), and urine proteins excretion were measured before kidney preservation and on postoperative days 1, 3, 5, 7 and 14 (D1–D14) and 4–12 weeks after autotransplantation (W4–W16). Blood and urine parameters were measured with an automatic analyser (Hitachi). Twenty-four-hour protein excretion was measured after precipitation by using a colorimetric reaction with pyrogallol (Laboratoire Biorea).

2.3. Immunohistochemical studies

Indirect immunocytochemistry was also performed using the mouse anti-porcine CD4 (MCA1749; Serotec Product Data Sheet), and the mouse anti-porcine MC1218 macrophage/monocyte and neutrophils markers (Serotec Product Data Sheet) for 30 min at room temperature. In all cases, the sections were rinsed in PBS and incubated with biotylated antispecies (Dako Ltd.) for 20 min (1:100) at room temperature. As controls, omitting the primary antibodies, indirect immunofluorescence was performed. Phosphatase alkaline activity was revealed using freshly prepared Fas red substrate solution (Sigma) in Tris-buffered saline (TBS). Sections were counterstained in hematoxylin and mounted in Aquamount (Gurr). Immunolocalization of PBR was determined as previously described using an affinity purified anti-PBR peptide antiserum raised against an amino acids sequence (amino-acids 9–27, VGLTLVPSLGGFMGAYFVR) conserved across species. Paraffin embedded sections were incubated with rabbit anti-PBR (1:400, dilution with 10% FBS-PBS) for 1 hr at room temperature. After rinsing the sections in PBS, horseradish peroxidase conjugated goat anti-rabbit IgG (Transduction Laboratory), diluted 1:500. PBR staining was determined 30 min to 1 hr after reperfusion, at day 7 and 2 weeks after reperfusion. All sections were examined under blind conditions and photographed. The number of PBR, CD4 and MC1218-labeled cells per surface area ($10^4\ \mu m^{-2}$) was counted on five to ten different tissue sections in each of the experimental conditions. The Degree of MHC class II (mouse anti-porcine MHC class II, MCA1335, Serotec Products Data Sheet) and VCAM-1 (anti-human vascular cell adhesion molecule-1) staining was also semiquantitatively determined.

2.4. Histological study

Kidney fragments obtained by ultrasonography-guided biopsy performed at various times after surgery were processed for light microscopy. Biopsy samples from the deep cortex-outer medulla region of the kidney were fixed in Dubosq-Brazil and 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin and stained with hematoxylin and eosin, periodic acid-Schiff. Tubular atrophy was graded as follows: 0, no abnormality; 1, discrete lesions (<10% of kidneys samples); 2, mild lesions (<10–25% of kidney samples); 3, lesions affecting 50–75% of kidney samples; 4, lesions affecting more than 75% of kidney samples. Tissue sections (5 μm) were also labeled with Picro sirius, known to stain collagens I and III deposited within the interstitium and recommended for the diagnosis of renal injury. The degree of interstitial fibrosis stained with Picro Sirius was determined by an imaging technique and the percentage of Picro Sirius stained surface was measured on five different tissue sections viewed at 100× magnification for each experimental condition, and expressed as a percentage of the total surface area examined.

2.5. Statistical analysis

Mean values were calculated for each group (mean ± SEM) and compared for statistical significance by ANOVA for repeated measures, using the Instat (v.2.04) software package from GraphPad. The unpaired *t*-test was used for cellular infiltration and the Mann–Whitney *U*-test was used for histological data analyses and immunohistochemical data. Differences at a P value of less than 0.05 were considered to be significant.

3. Results

3.1. Effect of cold ischemia time and TMZ on renal function, survival and on renal medulla injury

Total body weights and kidneys weight were not significantly different between control (49.2 ± 2.5 kg), Nef (50.4 ± 2.4 kg) and experimental groups (UW24 hr: 49.1 ± 3.4 kg and 134 ± 7 g; HEH24 hr: 51.2 ± 3.1 kg and 135 ± 3 g; CEL24 hr: 48.5 ± 3.3 kg and 131 ± 2.2 g; UWTMZ24 hr: 51.3 ± 3 kg and 130 ± 3 g; HEHTMZ24 hr: 49.3 ± 3.3 kg and 129 ± 2.5 g; CELTMZ24 hr: 49.0 ± 2.8 kg and 130 ± 3.8 g; UW48 hr: 50.5 ± 4.1 kg and 129 ± 3 g; HEH48 hr: 50.4 ± 2.6 kg and 131 ± 3 g; CEL48 hr: 51 ± 2.9 kg and 132 ± 2.5 g; UWTMZ48 hr: 50.5 ± 4.1 kg and 129 ± 3 g; HEHTMZ48 hr: 50.4± 2.6 kg and 131 ± 3 g; CELTMZ48 hr: 51 ± 2.9 kg and 132 ± 2.5 g; UW72 hr: 45.7 ± 4.2 kg and 135 ± 9.7 g; HEH72 hr: 50.1 ± 3.4 kg and 132 ± 3.6 g and CEL72 hr: 50.1 ± 3.4 kg and 132 ± 3.6 g; UWTMZ72 hr: 49.8 ± 2.6 kg and 131 ± 2.6 g; HEHTMZ72 hr: 50.5 ± 3.2 kg and 127 ± 3 g and CELTMZ72 hr: 49.4 ± 3.8 kg and 132 ± 3 g). Three pigs died on postoperative days 6 and 10 in group UW48 hr and CEL48 hr and 6 pigs died on postoperative days 5 and 8 in UW72 hr group and 4 pigs died on postoperative days 5 and 9 in group HEH72 hr. Three pigs and two pigs in UWTMZ72 hr and CELTMZ72 hr, respectively, died between days 7 and 14. All animals from group CEL72 hr died on postoperative days 4 and 7. All these animals developed primary non-function. Survival was 100% in the control group, 100% in the Nef group, UW24 hr, HEH24 hr, CEL24 hr, UWTMZ24 hr, HEHTMZ48 hr, CELTMZ48 hr and HEHTMZ72 hr groups. Functional data were not determined in groups UW48 hr, EC48 hr and HEH72 hr, UW72 hr (<100 mL/24 hr) related to a prolonged anuria before D3, D5 and D7, respectively. As shown in Fig. 1, cold ischemia and reperfusion affect the renal functions after autotransplantation and correlated with time preservation. The highest C_{Cr} occurred in experimental groups HEH and TMZ improved renal function in all preserved

groups particularly when combined with HEH (Fig. 1A). There was a transient proteinuria in all experimental groups, which decreased progressively between D1 and W4 (Fig. 1B). Progressive proteinuria developed again after week 4 following surgery in urine from Nef group and kidneys preserved and transplanted particularly in groups UW48 hr and CEL48 hr and UW72 hr. Proteinuria was significantly lower in HEH groups than those cold flushed and preserved for 24, 48 and 72 hr in UW and CEL solution (Fig. 1B).

3.2. Effect of cold ischemia and TMZ on histopathological long-term consequences

While vascular lesions were barely appreciable, tubular atrophy and interstitial fibrosis were observed particularly in preserved groups for 72 hr and without TMZ. HEH was the more efficient preservation solution in reducing fibrosis and tubular atrophy particularly after 48 hr of preservation (Fig. 2). TMZ was efficient to reduce chronic injury particularly in combination with CEL and HEH (Fig. 2).

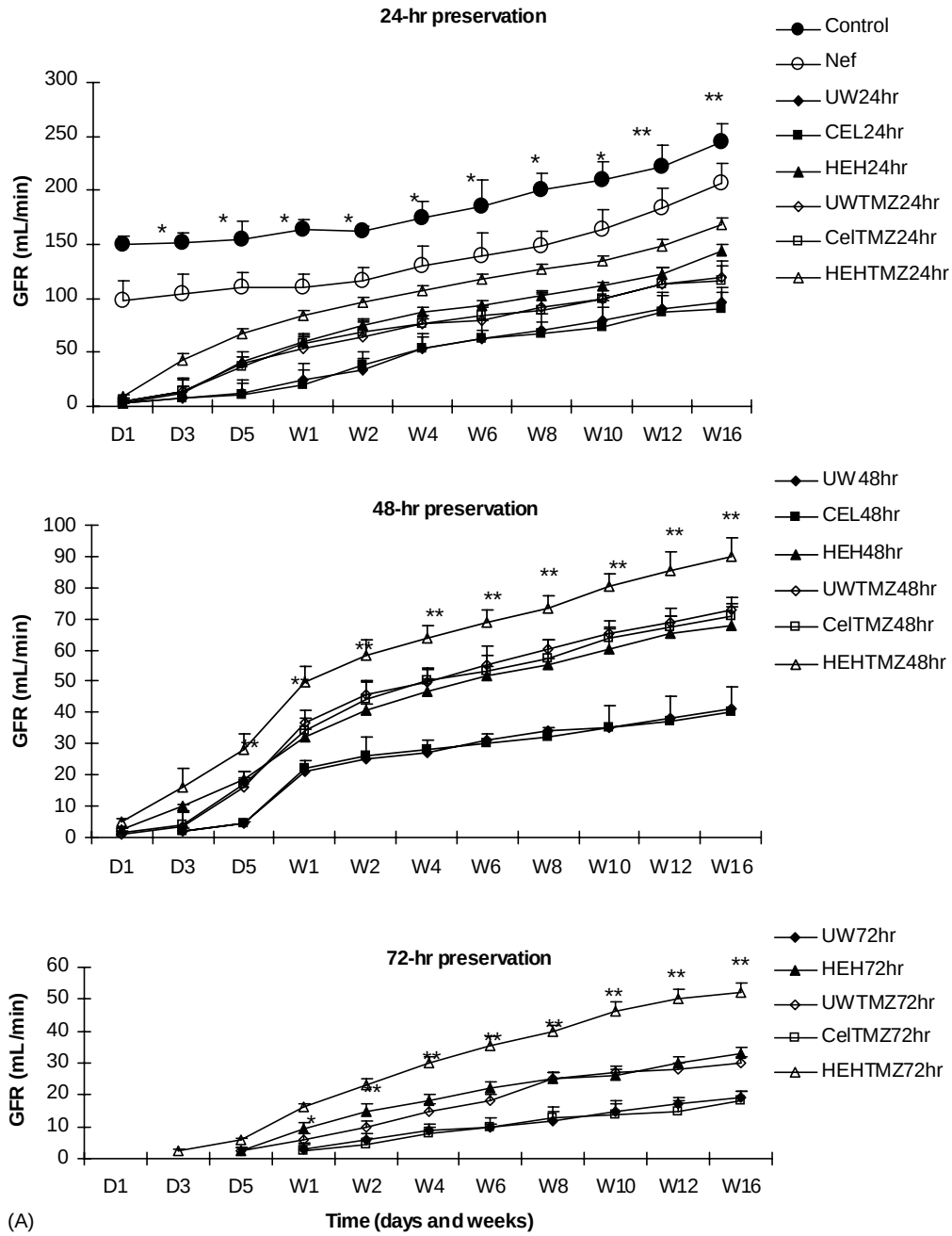

Fig. 1. Effect of cold ischemia and TMZ on the glomerular filtration rate (A) and proteinuria (B). Renal function was also determined in control and uninephrectomized animals (control, black circle and Nef, open circle). Autotransplanted kidneys were cold-flushed and preserved with University of Wisconsin (UW) solution for 24 hr (UW24 hr), 48 hr (UW48 hr), and 72 hr (UW72 hr) and HEH for 24 hr (HEH24 hr), 48 hr (HEH48 hr) and 72 hr (HEH72 hr) and CEL for 24 hr (CEL24 hr), 48 hr (CEL48 hr), and 72 hr (EC72 hr) or with the same preservation solution plus TMZ ($^{*}P < 0.05$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ, $^{**}P < 0.01$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ).

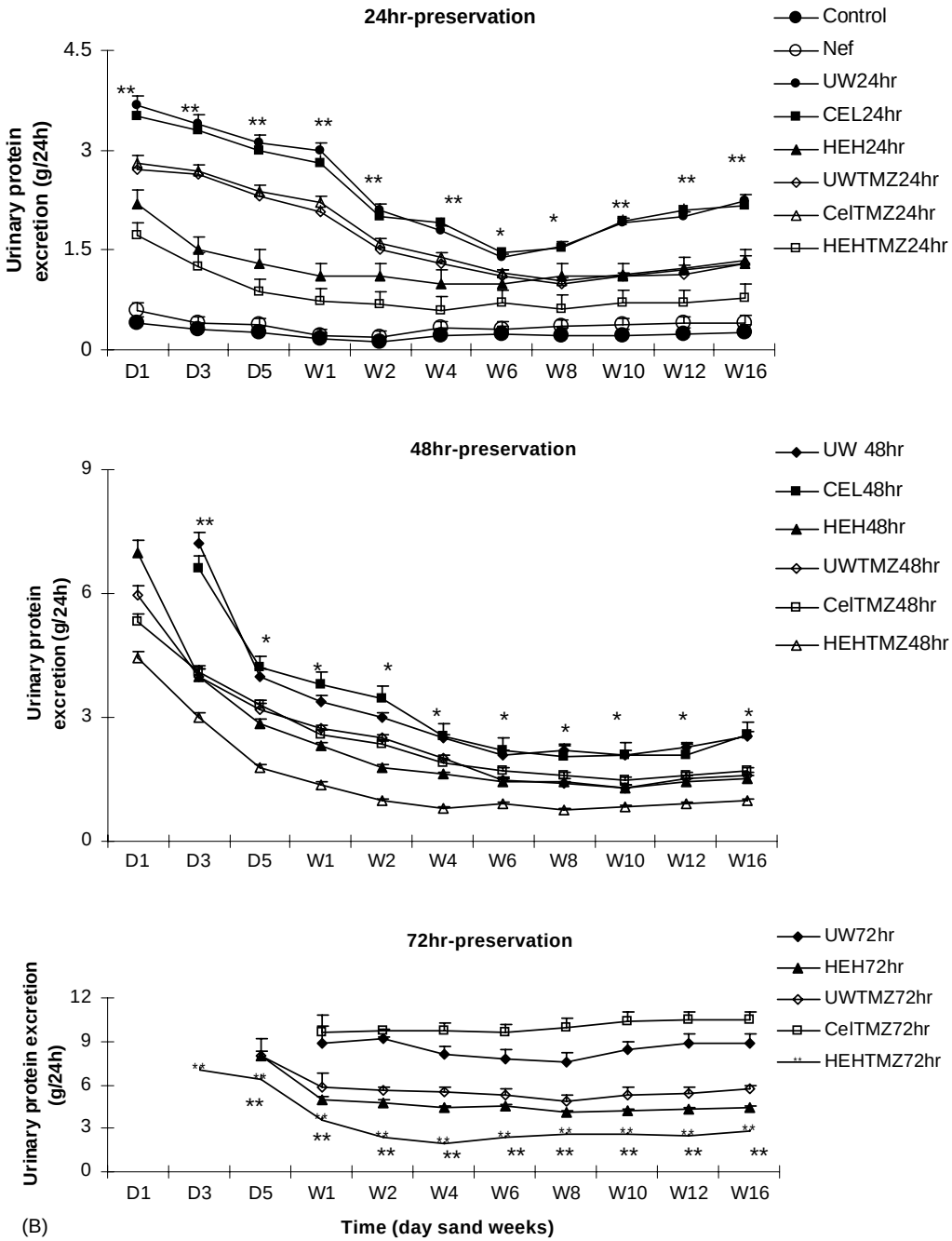

Fig. 1. (*Continued*).

Fibrosis was dramatically reduced by TMZ in all experimental groups and correlated with an improved preservation of nephronic mass even after 72-hr cold storage (Figs. 3 and 4).

3.3. Effect of cold ischemia and TMZ on CD4 and PBR-positive cells and monocytes and macrophage infiltration and the expression of MHC class II and VCAM-1

We have previously demonstrated that T-lymphocyte infiltration was prominent during the early phase following autotransplantation [13,14]. This study demonstrated that the preservation influences the cellular infiltration in autotransplanted pig kidneys (Fig. 5). As previous described, the number of CD4-positive cells gradually increased from D5 to D14, decreased from weeks 2 to 5 and gradually increased from weeks 4 to 5 to weeks 10 to 12 in the groups CEL24 hr, UW24 hr, CEL48 hr and UW48 hr. HEH-preserved kidneys exhibited a significantly reduction of inflammatory cell inflammation. After 72-hr cold storage, the number of $CD4^+$ cells increased from D5 to weeks 4 to 5 and from weeks 4 to 12 following surgery (Fig. 5). HEH remained also efficient in reducing $CD4^+$-cells infiltration. Kidneys preserved in UW and CEL exhibited a reduction

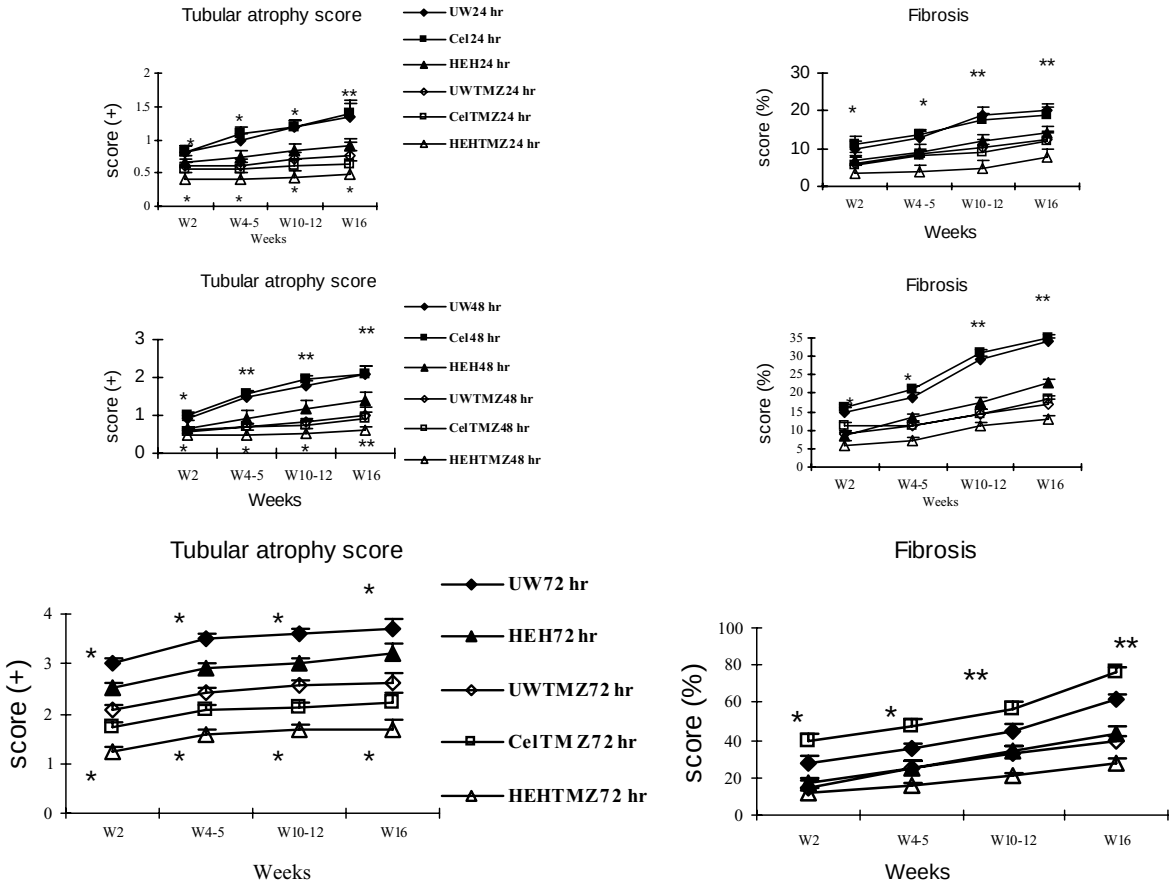

Fig. 2. Effect of cold ischemia and TMZ on tubular atrophy and interstitial fibrosis at weeks 2, 4–5, 10–12 and 16. HEH combined to TMZ is particularly efficient in reducing tubular atrophy and interstitial fibrosis. Autotransplanted kidneys were cold-flushed and preserved with University of Wisconsin (UW) solution for 24 hr (UW24 hr), 48 hr (UW48 hr), and 72 hr (UW72 hr) and HEH for 24 hr (HEH24 hr), 48 hr (HEH48 hr) and 72 hr (HEH72 hr) and CEL for 24 hr (CEL24 hr), 48 hr (CEL48 hr), and 72 hr (EC72 hr) or with the same preservation solution plus TMZ ($^{*}P < 0.05$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ, $^{**}P < 0.01$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ).

of $CD4^{+}$-cells infiltration when TMZ was added to preservation solution (Fig. 5). The more beneficial effect of TMZ was observed when TMZ was added to HEH particularly after 48 and 72 hr of preservation. Positive staining with the MC1218 macrophage/monocyte was detected in all kidney biopsies taken 5 days after transplantation (Fig. 5). However, there were more MC1218-positive cells in posttransplanted kidneys from groups CEL and UW after 24 and 48 hr of preservation and after 72 hr in UW group. This macrophage/monocyte infiltration was reduced in biopsy samples performed 2 weeks after transplantation from 48 and 72-hr cold-stored kidneys. In contrast, MC1218-positive cells were detected on biopsy samples performed 12 weeks following transplantation. The number of MCA1218-positive cells was much lower in cold flushed kidneys and preserved for 24 and 48 hr in UW and CEL solutions with TMZ than those preserved with UW and CEL without TMZ. TMZ was also efficient to improve CEL preservation efficiency after 72 hr of preservation when compared to CEL alone. PBR immunoreactivity was detected in few infiltrating mononuclear cells and their number paralleled with the level of injury and time of preservation. PBR staining was reduced in TMZ preserved groups when compared to standard solutions. The more efficient combination was HEH and TMZ particularly after 72 hr of preservation in reducing $CD4^{+}$, monocytes–macrophages and PBR-positive cells. MHC class II expression was reduced in HEH preserved groups particularly when combined with TMZ (Table 1). VCAM-1 expression was also reduced in HEH and HEH plus TMZ preserved groups (Table 2).

4. Discussion

The mechanism underlying the detrimental effect of cold ischemia and thermal injury on graft survival remains unclear and the factors that trigger and control the repair process are poorly understood. However, it is becoming clear that mitochondria play a critical role through their central role in cellular bioenergetics by the production of reactive oxygen species and the control of cell death. In addition, mitochondrial complexes have different susceptibilities to cold ischemic and reperfusion damage and cold storage and rewarming are involved in the mitochondrial pathway of apoptosis [28,29].

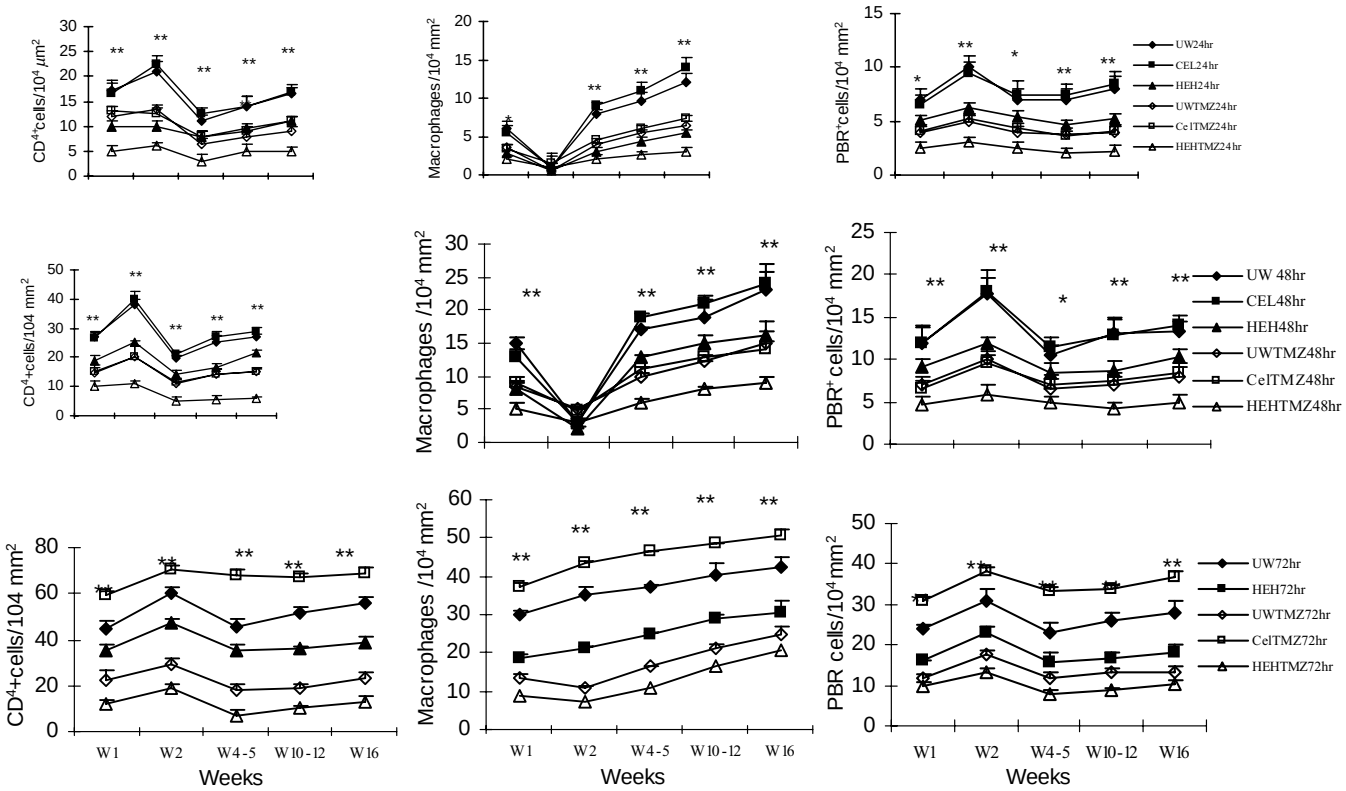

Fig. 3. Interstitial fibrosis in long-term post transplanted kidneys. Biopsy samples from week-10 posttransplanted kidneys initially cold-flushed with UW, HEH or CEL solutions with or without TMZ were stained with Picro sirius. Important interstitial fibrosis was detected in UW and CEL groups when compared to HEH. The effect of cold ischemia and TMZ interstitial fibrosis was also measured. HEH combined to TMZ is particularly efficient in reducing interstitial fibrosis or HEH vs. HEHTMZ, $^{**}P < 0.01$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ.

The first major result of the present study was that cold ischemia, particularly long 48–72 hr, induced further functional deterioration compared to 24 hr of preservation. Renal functions were dramatically impaired after cold preservation particularly after 72 hr of cold ischemia. These functional results were related to tissue damage, which were more important in UW and CEL groups. HEH preservation groups exhibited more efficient renal functions. TMZ improved renal function after cold preservation, particularly in HEH groups. TMZ improved CEL

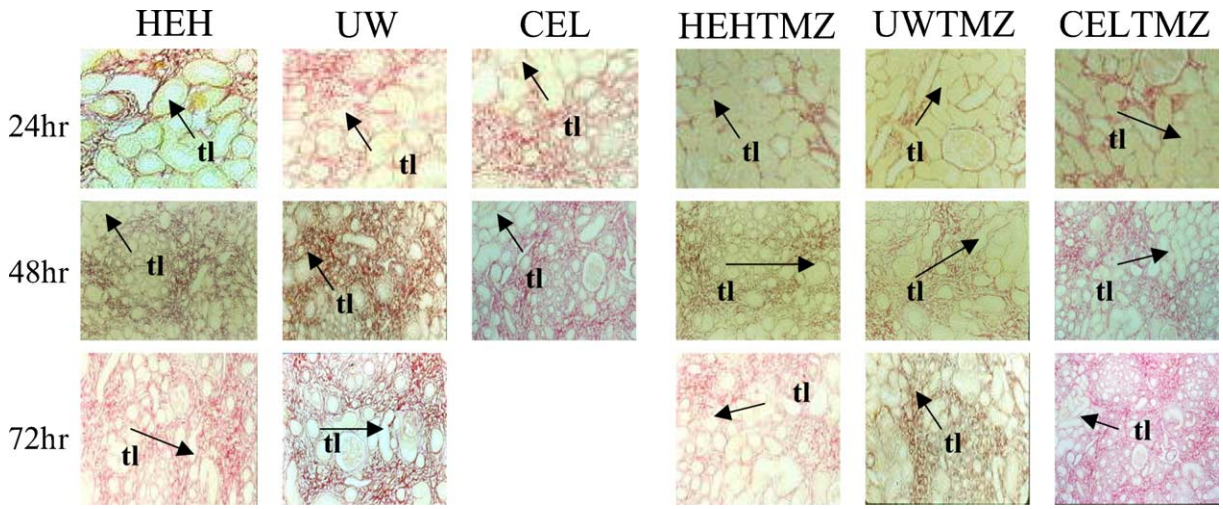

Fig. 4. Interstitial fibrosis in long-term post transplanted kidneys by Trichrome Masson. Biopsy samples from week-10 posttransplanted kidneys initially cold-flushed with UW, HEH or CEL solutions with or without TMZ were stained with Trichrome Masson. Important interstitial fibrosis was detected in UW and CEL groups and interstitial fibrosis decreased in kidneys from HEH. TMZ was also efficient to limit interstitial fibrosis particularly when combined to HEH.

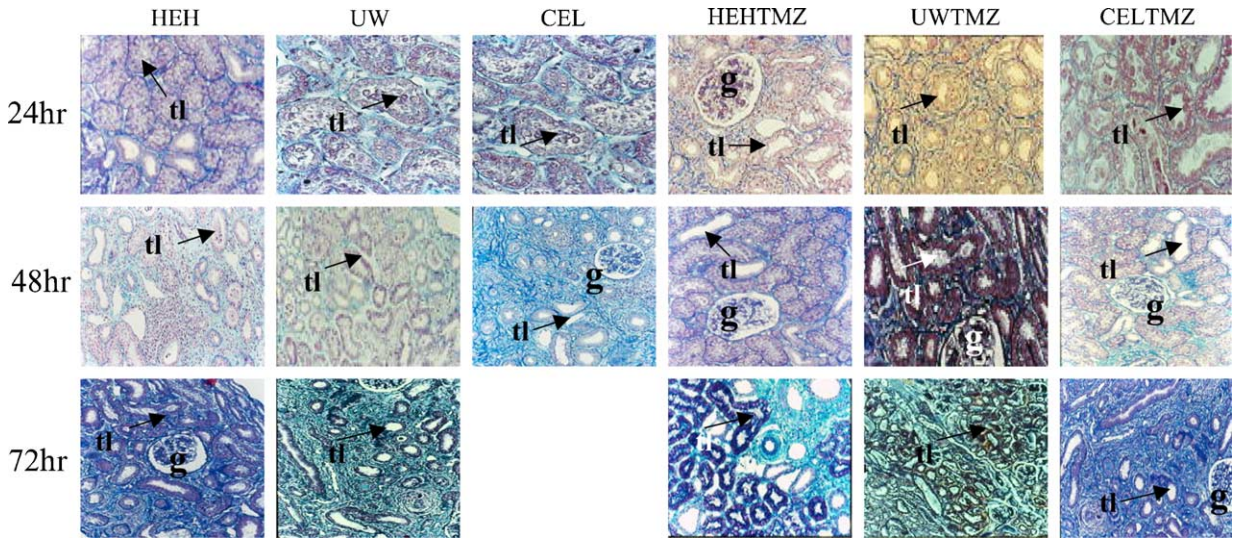

Fig. 5. Identification of $CD4^+$, macrophages and PBR-positive cells in post transplanted pig kidneys. Autotransplanted kidneys were cold-flushed and preserved with University of Wisconsin (UW) solution for 24 hr (UW24 hr), 48 hr (UW48 hr), and 72 hr (UW72 hr) and HEH for 24 hr (HEH24 hr), 48 hr (HEH48 hr) and 72 hr (HEH72 hr) and CEL for 24 hr (CEL24 hr), 48 hr (CEL48 hr), and 72 hr (EC72 hr) or with the same preservation solution plus TMZ ($^*P < 0.05$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ, $^{**}P < 0.01$ CEL and UW vs. HEH, CEL vs. CELTMZ, UW vs. UWTMZ or HEH vs. HEHTMZ).

efficiency after 72 hr cold storage. The development of significant and progressive proteinuria was also related to renal damage and reduction of nephron mass. As expected, the present study shows that cold ischemia produced tubulointerstitial damage and tubular atrophy and support previous data [15,17,30–32]. Our data support that cold ischemia is mainly responsible for tubulointerstial damage and correlated with the time duration of cold ischemia and preservation conditions.

Furthermore, the data presented herein indicate that the cellular infiltration strongly correlates with the intensity of renal dysfunction and damage which was significantly reduced after implantation of kidneys preserved for 24 hr when compared to 48 and 72 hr preservation. Our data also suggest that T cells play a major role in the development of renal IRI mediated probably by adhesion of infiltrating T cells to renal tubular cells. Recently, using pig kidney autotransplant model, we have demonstrated the role of 48-hr cold storage and the implication of T cells as pathogenic factors in ischemic injury [33]. However, a role for lymphocytes in this autotransplanted pig kidney model is not immediately intuitive based on classic immunologic paradigms. Classically, T-cell activation has been thought to require foreign antigen bound to a self-major histocompatibility complex molecule together with costimulatory signals by antigen-presenting cell. The absence of foreign antigens in this autotransplant model suggests that alloantigen-independent T-cell activation may be involved in

Table 1
Semi-quantitative analysis of MHC class II expression

Week	Groups		
	UW/UWTMZ	CEL/CELTMZ	HEH/HEHTMZ
24-hr cold storage			
2	++/+	++/+	+/0 to +
4–6	++/0 to +	++/0 to +	+/0 to +
10–12	+/0	+/0	0 to +/0
48-hr cold storage			
2	+++/++	+++/++	++/+
4–6	++/+	++/+	++/0 to +
10–12	+/0	+/0	0/0
72-hr cold storage			
2	++++/+++	ND/++++	+++/++
4–6	++++/+++	ND/++++	+++/++
10–12	+++/++	ND/++	++/+

The degree of MHC class II staining was determined semiquantitatively, using a scale from 0 to 4+. MHC class II was not detected in control and Nef.

Table 2
Semi-quantitative analysis of VCAM-1 expression

Week	Groups		
	UW/UWTMZ	CEL/CELTMZ	HEH/HEHTMZ
24-hr cold storage			
2	+/0 to +	+/0 to +	0 to +/0
4–6	++/+	++/+	0 to +/0
10–12	++/+	++/+	0/0
48-hr cold storage			
2	+/0	+/0	0/0
4–6	++/+	++/+	+/0
10–12	+++/++	+++/++	++/+
72-hr cold storage			
2	++/+	ND/++	++/+
4–6	+++/++	ND/+++	++/+
10–12	++++/+++	ND/++++	+++/++

The degree of VCAM-1 staining was determined semiquantitatively, using a scale from 0 to 4+. VCAM-1 was not detected in control and Nef.

renal IRI. An antigen-independent mechanism of T-cell activation has been described that involves chemokines ([34], for a review, see [35]). Recent evidence suggests that T cells also can be activated by antigen independent pathways. Oxygen-free radicals, RANTES and cytokines have all been implicated in directly causing T-cell activation. Under ischemic conditions, there is a depletion of glutathione and superoxide dismutase and insufficient levels of glutathione and superoxide dismutase can produce toxic species. Oxygen-free radicals are one of the most widely recognized products of tissue injury and they are able to up-regulate pro-inflammatory genes [35]. Our data are strongly supported by recent reports, which demonstrated that the T cells and more particularly $CD4^+$ T cells is an important mediator of ischemic injury. In addition, we demonstrate also the protective effect of TMZ is directly related to mitochondrial protection.

We demonstrate the modulation of VCAM-1 expression by preservation conditions and TMZ. VCAM-1 regulates leukocyte migration from the blood into tissues. Recent review focused on the important role of the endothelial cell in VCAM-1-dependent lymphocyte migration and [36]. VCAM-1 stimulates endothelial cell NADPH oxidase activity that is required for the lymphocyte migration. As a consequence, the endothelial cells retract, opening an ''endothelial cell gate'' and allowing lymphocytes to migrate from the blood and into tissues. It is suggested that reactive oxygen species from the VCAM-1 signaling pathway activate endothelial cells matrix metalloproteases that can degrade extracellular matrix and membrane proteins. However, the function of the cytoplasmic domain of VCAM-1 need to be delineated. Up-regulation of MHC-class II was increased by cold ischemia and related to an increased in the development of chronic renal injury determined by fibrosis and tubular atrophy. These data outline the role of allo-independent factors such as cold ischemia. TMZ is also efficient to modulate renal damage and is an important adjunct in the prevention of renal IRI.

The second major finding is the relationship between inflammation and PBR expression. PBR was shown to be expressed in monocytes and in natural killer cells, B and T lymphocytes [37]. Previous reports have documented the PBR involvement in the regulation of inflammatory processes, apoptosis and cutaneous photoinduced mechanisms [11,12,38]. In addition,, the effects of PBR ligands in inflammatory processes modulation suggest that this receptor is involved in this process [11,12,37,38]. In this study, PBR-positive cells detected within damaged kidneys must be involved in inflammatory process and/or in renal injury/repair processes as previously suggested [39]. Further studies are necessary to clarify this point. We also demonstrated the role of TMZ in inflammatory process of IRI. Kidneys from TMZ-preserved groups exhibited reduced cells infiltration. HEH and TMZ were very efficient to reduce one of the major negative effects of IRI that can influence the outcome of the grafts. Moreover, PBR was not detected in interstitial fibrosis and necrotic tissue, supporting the fact that PBR expression in tissue after IRI must be related to functional integrity of mitochondria and tissue.

Consequently, the function of PBR is not restricted to the steroidogenic tissues. Because mitochondria are involved in IRI, and above all, in the processes of cell damage and death, PBR could be a target of interest in IRI and mitochondria protection. Moreover, the results point to the potential role of TMZ in attenuating IRI and its potential role to modulate PBR expression. This study demonstrates also the important role for $CD4^+$ T cells in renal IRI and its possible modulation by preservation conditions. $CD4^+$ T-cells infiltration was followed by fibrosis via an up-regulation of fibrogenic growth factors associated with interstitial fibrosis and organ dysfunction. In addition, we demonstrate also the potential role of extracellular solutions. Clinically, the intracellular-type solutions are the most widely used solutions to preserve organs, but extracellular solutions could be also useful to improve organs preservation.

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