ABSTRACT

Delayed graft function remains an important complication after renal transplantation. In this study, we investigated the influence of trimetazidine (TMZ), a cytoprotective agent, on renal medullary damage after prolonged preservation and autotransplantation. Pig kidneys were cold-flushed and preserved (48 h at 4°C) with two standard renal preservation solutions Euro-Collins and University of Wisconsin supplemented or not with TMZ (10⁻⁶ M). Analysis of plasma and urine from 48-h-cold-stored and autotransplanted kidneys was performed with biochemical methods and proton NMR spectroscopy. Histological study by light and electron microscopy was performed after reperfusion (30–40 min) and on day 14. The results showed that the preservation in either Euro-Collins or University of Wisconsin solution containing TMZ improved significantly glomerular filtration rate compared with kidneys preserved without TMZ. TMZ significantly reduced renal medullary damage, evidenced by decreased excretion of trimethylamine-N-oxide, dimethylamine, dimethylglycine, and acetate in urine. Proximal tubular injury in TMZ-free groups was assessed by significantly greater Na⁺ excretion, amino aciduria, and lactic aciduria than in TMZ-supplemented groups. Urinary concentrating ability was significantly improved in TMZ-treated groups compared with TMZ-free groups. In TMZ-supplemented groups, there was also a greater excretion of citrate, which is a citric acid cycle metabolite. An extensive reduction in apical brush border of tubular cells, notably those of the proximal tubules, was noted in TMZ-free groups. This study clearly shows that TMZ has a beneficial action on in vivo renal preservation and its major impact is the vulnerable renal medulla.
During reperfusion, the capillaries become plugged, which results in a state of marginal oxygenation. This enhances the vulnerability of the medullary thick ascending limb and the S3 segment of the proximal tubule. Consequently, pharmacological interventions that maintain endothelial cell integrity, or prevent cell swelling may efficiently reduce ischemia-reperfusion injury.

Trimetazadine (TMZ) has been described as a cellular anti-ischemic agent (for review, see Harpey et al., 1989). The effects of ischemia-reperfusion injury are well correlated with alterations of mitochondrial function, namely, a decrease of ATP synthesis, NADPH levels, and mitochondrial membrane potential (Mac Dougall, 1988). Significant opening of the mitochondrial permeability transition pore (MTP) might occur during posts ischemic reperfusion after mitochondrial calcium overload (Leduq et al., 1998). MTP opening also is potentiated by free radicals. Reactive oxygen species occurring during the ischemia-reperfusion process seem to be a major determinant of tissue injury (Rao et al., 1983). Recent experimental data demonstrated that TMZ prevented the ischemia-reperfusion deleterious effects at both the cellular and mitochondrial levels (Elimadi et al., 1998). Recently, two families of \[^{3}H\]TMZ-binding sites, located both on the outer and the inner mitochondrial membranes, have been evidenced (Morin et al., 1998). These sites are distinct from all the other mitochondrial sites described to date and may be involved in the closure of MTP. This set of data indicates that mitochondria could be the main target of TMZ. Other recent study demonstrated that the pH partition profile of TMZ may explain its effect to export protons to extracellular space and to reduce the deleterious intracellular acidity (Reymond et al., 1999). We have recently demonstrated that TMZ is efficient against cold ischemia and reperfusion injury in an isolated perfused pig kidney model (Hauet et al., 1997b). This drug is efficient against lipid peroxidation and reduces intracellular acidity during cold storage (CS) and reperfusion of isolated perfused rat kidneys (Hauet et al., 1998). Because this agent efficiently improves renal function in ex vivo models, this study was undertaken to assess whether TMZ added to two standard renal preservation solutions, Euro-Collins (EC) and University of Wisconsin (UW) also reduced in vivo ischemia-reperfusion injury.

Materials and Methods

**Surgical Procedures.** This study was approved by our review committee. The animals were taken care of in accordance with university and national guidelines. Large white male pigs weighing 45 to 52 kg (Institut National Reseurches Agronomique, Le Magnacud, Surgères, France) were used. All surgical procedures were performed aseptically. This study was carried out as a prospective randomized trial with strict exclusion criteria, where animals that died from causes other than renal failure during the 2-week follow-up period were excluded, and any animal that developed renal artery, vein, or ureteric problems also was excluded. Rapid preoperative tranquilization was provided by a quick and atraumatic nasal administration of 0.2 mg/kg midazolam (Laboratoire Roche, Neuilly-sur-Seine, France). Afterward, the animals were anesthetized with halothane (Laboratoire Belamont, Paris, France) and 100% oxygen. A 20 gauge plastic catheter (Becton Dickinson Vascular Access Inc., Sandy, UT) was inserted in an ear vein. Atropine sulfate 10 µg/kg was given i.v. to reduce pharyngeal and tracheal secretions and to prevent postintubation bradycardia. The left renal vascular pedicle and ureter were atraumatically isolated and 100 U/kg sodium heparin was adminis-
lesions affecting 25 to 50% of kidney samples; 3, lesions affecting 50 to 75% of kidney samples; 4, lesions affecting >75% of kidney samples. The brush border lining the apical membrane of tubular cells was evaluated and classified as follows: 0, normal; 1, focal reduction; 2, moderate reduction; 3, extensive reduction; or 4, entirely absent.

**Statistical Analysis.** Mean values from TMZ-supplemented groups were calculated for each group (means ± S.E.) and compared with non-TMZ-treated groups for statistical significance by the unpaired t-test or ANOVA followed by the Student-Newman-Keuls test for multiple comparison. Differences at a P value <.05 were considered to be significant. When a nonparametric test was needed, the Kruskall-Wallis analysis was used. Control group values are shown in figures and tables as reference values but they were not used for statistical analysis.

**Results**

**Effect of Trimetazidine on Functional Results and Survival.** The outcomes following 48-h CS, autotransplantation, and contralateral nephrectomy differed markedly (Figs. 1 and 2). Pigs died on postoperative day 3 and 5 in group A and on postoperative day 6 and 11 in group C. All these animals developed acute renal failure, confirmed by histological analysis. As shown in Fig. 1A, Ct clearance was identical in all experimental groups before surgery. The levels of Ct clearance from the uninephrectomized animals (control group) was lower than in intact animals but remained stable over the 2 weeks following surgery. Cold ischemia and reperfusion or nephrectomy affected Ct clearance after autotransplantation in experimental groups. However, the Ct clearance for the animals autotransplanted with kidneys preserved with the TMZ-supplemented solutions were significantly higher than those for the pigs autotransplanted with kidneys preserved with TMZ-free solutions. The highest Ct clearance occurred in experimental group D after autotransplantation. As shown in Fig. 1B, these results were associated with prolonged oligoanuria in nontreated EC and UW groups (<100 ml/24 h from day 1 to day 4 in group A and from day 1 to day 2 in group C). Because of this prolonged oligoanuria, the assessment of renal tubular function was not strictly conceivable before day 3 in groups B and C and day 5 in group A. The recovery of renal tubular function was more rapid in the TMZ-supplemented groups as determined by Na+ excretion and amino aciduria. Fractional excretion of sodium (FE\textsubscript{Na}) significantly increased in the TMZ-free groups after cold preservation and autotransplantation, consistent with increased reabsorption of Na+ from damaged tubules compared with TMZ-supplemented groups (Fig. 2A).

The excretion was also significantly higher in group UW compared with EC + TMZ-preserved kidneys. Under normal conditions, alanine and valine excretion are almost completely reassorbed in the proximal tubule. Their excretion declined significantly in TMZ-supplemented groups compared with TMZ-free groups and were related to severe impairment of proximal tubule function (Fig. 2, B and C). Proximal tubular injury also is associated with lactate excretion, which is an acid normally reassorbed in the proximal tubule. Lactate excretion also declined significantly in TMZ-supplemented groups (Fig. 2E). The ability to concentrate urine also was impaired in TMZ-free groups and correlated with the severe polyuria particularly in group A after day 5 (Figs. 1B and 2D). This severe diuresis can lead to severe volume depletion and is related to the suppression of the medullary osmotic gradient. These findings indicated that the addition of TMZ to the preservation solution ameliorates the renal function after cold-ischemia and reperfusion.

**Effect of TMZ on Renal Medullary Cell Injury and Citrate Excretion.** Because the urine output was very low before day 3 in groups B and C and before day 5 in group A, the assessment of renal medullary cell injury was not very efficient (Fig. 3). For urine 1H NMR spectra, TMAO, DMA, dimethylglycine (DMG), and the acetate-to-Ct ratio were significantly higher in urine from kidneys preserved in EC and UW solutions than in that from kidneys preserved with TMZ (Fig. 3, A–D). Citrate was not detected during the first postoperative week in urine from kidneys preserved in EC. Its excretion was detected significantly earlier in urine from the TMZ-supplemented groups than in TMZ-free groups. These findings demonstrated that TMZ reduced renal medullary damage and consequently the release of TMAO, DMA, and DMG into the urine from renal medullary cells. Renal medullary damage was associated with impairment of Na+ reabsorption, urinary-concentrating ability, and glomerular filtration. In addition, the early detection of citrate can enable to determine which kidneys show a favorable evolution.

**Pathologic Assessment of Postischemic Renal Tissue.** After a 40-min reperfusion, kidneys flushed and pre-
served with EC and UW solutions showed a significantly higher score than kidneys flushed and preserved with EC and UW solutions containing TMZ (Table 1). Structural injury was most prevalent in the proximal tubules. Under electron microscopy, proximal tubular cells exhibited a marked reduction or complete absence of the apical brush border. On day 14, tubular alterations were more pronounced in TMZ-free groups particularly in group A.

**Discussion**

Ischemia-reperfusion injury associated with kidney retrieval, storage, and transplantation is a major issue in kidney transplantation because it has become more and more evident that its clinical expression, DGF, has a significant impact on graft survival (Cecka et al., 1992; Ojo et al., 1997). Because the prevention of reperfusion injury is limited in organ preservation, various attempts have been made to decrease ischemia-reperfusion injury. We have previously demonstrated that TMZ efficiently protected the kidney from cold ischemia-reperfusion injury in ex vivo models (Hauet et al., 1997b, 1998).

The first major result of the present study was that TMZ added to UW and EC solutions limited DGF and improved functional outcome. This was characterized by the improvement of $\text{Na}^+$ reabsorption and glomerular filtration, and by a decrease in amino aciduria and lactic aciduria. $\text{Na}^+$ reabsorption across the apical membranes of renal epithelial cells is mediated primarily by four distinct transporters (Gamba et al., 1994; Rossier et al., 1994). Most of the $\text{Na}^+$ reabsorption in the proximal tubule and ascending limb of Henle occurs via the $3\text{Na}^+/	ext{H}^+$ Exchanger (NHE) and apical $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransporter. In the distal convoluted tubule and collecting duct, $\text{Na}^+$ reabsorption is mediated predominantly via the $\text{Na}^+/-\text{Cl}^-$ cotransporter and the $\text{Na}^+$ channel. Transport across the basolateral membrane is mediated primarily by $\text{Na}^-\text{K}^+\text{-ATPase}$. A major advancement in our understanding of $\text{Na}^+$ handling in acute renal failure resulted from the demonstration that ischemic tubular damage led to a loss of $\text{Na}^-\text{K}^+\text{-ATPase}$ pump distribution from normal basal to

**Fig. 2.** Changes in tubular function after CS and autotransplantation or uninephrectomy. Variations in $\text{Na}^+$ reabsorption (A) and alanine and valine excretion (B and C, respectively), and changes in urine/plasma osmolality (D) and lactate excretion (E) were measured in unilaterally nephrectomized pigs. For A–E, control (●); EC (○); EC + TMZ (□); UW (■), and UW + TMZ (▲). Results from the control group (●) are presented as a reference but were not used in the statistical analysis. *$P < .05$, **$P < .01$, ***$P < .001$ EC versus EC + TMZ and °$P < .05$, °°$P < .01$, °°°$P < .001$ UW versus UW + TMZ.
inappropriate apical localization (Spiegel et al., 1989). Recent studies have contributed to our understanding of the molecular basis of natriuresis. The major proximal Na\(^{+}\) exchanger, NHE, is transcriptionally down-regulated during ischemia-reperfusion injury, as are Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\), Na\(^{+}\)-Cl\(^{-}\) cotransporter, and Na\(^{+}\)-K\(^{+}\)-ATPase (Wang et al., 1998). In contrast, the Na\(^{+}\) channel remains relatively unchanged. We demonstrated that the tubular solute transport was dramatically impaired after CS preservation particularly in EC solution after 48-h cold ischemia. Alanine and valine were well detected by \(^1\)H NMR spectroscopy. These amino acids are neutral and are reabsorbed in the first quarter of the proximal tubule via a Na\(^{+}\)-dependent cotransport systems. The activity of these electrogenic transporters is energized by the electrochemical gradient generated by basolateral Na\(^{+}\)-K\(^{+}\)-ATPase. Lactate also is normally reabsorbed in the proximal tubule via a Na\(^{+}\)-coupled transport process. Consequently, the functional results observed in this study were directly related to tubulopathy of the preserved kidneys and down-regulation of these Na\(^{+}\) transporters. Persistent down-regulation of these Na\(^{+}\)-transporters can explain why natriuresis, amino aciduria, and lactate aciduria occurred when GFR improved during the second postoperative week. Natriuresis also is associated with severe diuresis. The reduction of reabsorption of Na\(^{+}\) results in suppression of the medullary osmotic gradient, which results in the urinary concentrating defect. These functional results were related to histological morphometry that revealed considerable tubular damage in nontreated groups compared with TMZ-treated groups. These findings suggest that the mitochondrial protective ef-
effect of TMZ improves renal function after prolonged cold ischemia and reperfusion. However, further studies are necessary to examine the role of TMZ in the development of organ dysfunction during prolonged follow-up.

In recent years, 1H NMR spectroscopy has been used to detect and quantify low molecular-weight metabolites, which are present in biological fluids as a result of the cellular biochemical pathways and their dynamic modulation. This method has led to the discovery of several novel markers of nephron damage. Renal medullar damage leads to the early appearance of acetate, TMAO, DMG, and DMA in urine under different experimental and clinical conditions (for review, see Neild et al., 1997). The abnormal excretion of TMAO, DMG, and DMA determined by 1H NMR spectroscopy was noted particularly in EC groups after 48-h cold ischemia. These molecules are closely related to or, are derived from, renal medullary osmolytes (Balaban and Burg, 1987). Previous studies, combining NMR spectroscopy with other analytical techniques, have identified these osmolyte molecules in the renal inner medulla of the rat and rabbit (Balaban and Knepper, 1983). We have previously detected TMAO in urine after combining cold ischemia and normothermic reperfusion, with an isolated perfused pig kidney model (Hauet et al., 1997a). In this previous study, TMAO excretion in urine was related to ischemia-reperfusion damage in the isolated perfused pig kidney model. From the present investigation, it appears that TMAO, DMA, and DMG were either not detected in urine, or only at a very low level in control kidneys and 2 days before surgery in experimental groups. This implies that their excretion is related to ischemia-reperfusion injury. More precisely, their excretion was related to more intense renal medullary damage that led to the early appearance of TMAO, DMG, and DMA in urine followed by an increased excretion of acetate. Regarding citrate, this organic acid is normally found in mammalian urine. This acid is freely filtered into the glomerulus and then reabsorbed in the proximal tubule. Once reabsorbed, citrate is metabolized either in mitochondria through the tricarboxylic acid cycle (Simpson, 1967) or in the cytosol through ATP citrate lyase (Melnick et al., 1996). This demonstrates that citrate is a significant substrate for renal metabolism. Renal metabolism and clearance of citrate are known to be influenced by changes in the acid-base balance. Metabolic acidosis causes cytoplasmic pH and bicarbonate to decrease, thus bringing about an increase in the mitochondrial pH gradient (Simpson, 1983; Brennan et al., 1988). The present study demonstrates that citrate excretion was strongly reduced in nontreated groups (particularly group EC). A possible mechanism for this reduced excretion is an impairment of the citric acid cycle. The first step of this cycle is represented by the condensation of oxaloacetate and acetate from acetyl-CoA. The early detection of citrate in urine from TMZ-supplemented groups may be related to an efficient functional recovery of the citric acid cycle compared with standard solutions. Consequently, the reduced excretion of citrate observed in the nontreated groups is related to the impairment of oxidative metabolism after autotransplantation and delayed functional recovery of acid-base control in the proximal tubule. However, chronic metabolic acidosis causes hypocitraturia, which is associated to an adaptive increase in renal ATP citrate lyase activity (Melnick et al., 1996). Consequently, hypocitraturia in TMZ-free groups was probably related to metabolic acidosis correlated with severe renal failure and a decrease in renal proximal tubular cell pH.

Our data suggests that in the autotransplant model, TMZ efficiently protects the preserved kidneys and in particular renal medullary cells against ischemia-reperfusion injury. This is directly correlated with the protective effect of TMZ on mitochondria during ischemia-reperfusion. One of the most stimulating findings was evidence that the improvement of functional recovery is associated with the limitation of morphological damage. Loss of the proximal brush border and necrosis of tubular cells, notably those of the proximal tubule, were considerably reduced in TMZ-treated groups.

In conclusion, TMZ may be an useful alternative approach for limiting ischemia-reperfusion injury. This cytoprotective agent can be included in the future development of strategies to prevent injury in cadaver organ transplantation. It also could probably efficiently reduce delayed graft function in recipients of organs from nonheart-beating donors.

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References


**Table 1**

Quantification of morphological data from 48-h-cold-stored and normothermic perfused (perfusion) and day 14 autotransplanted kidneys (sacrifice)

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>EC + TMZ</th>
<th>UW</th>
<th>UW + TMZ</th>
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<tr>
<td><strong>Perfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Microvilli disintegration</td>
<td>4.0 ± 0.1</td>
<td>3.0 ± 0.3a</td>
<td>3.3 ± 0.2</td>
<td>2.2 ± 0.2b</td>
</tr>
<tr>
<td>Tubular necrosis</td>
<td>4.0 ± 0.1</td>
<td>3.0 ± 0.1a</td>
<td>2.8 ± 0.2</td>
<td>2.2 ± 0.2b</td>
</tr>
<tr>
<td>Cell detachment</td>
<td>4.0 ± 0.1</td>
<td>3.0 ± 0.1a</td>
<td>3.8 ± 0.2</td>
<td>3.0 ± 0.1b</td>
</tr>
<tr>
<td>Brush border reduction</td>
<td>3.8 ± 0.2</td>
<td>2.7 ± 0.1a</td>
<td>3.2 ± 0.1</td>
<td>2.3 ± 0.2b</td>
</tr>
<tr>
<td><strong>Sacrifice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvilli disintegration</td>
<td>3.2 ± 0.1</td>
<td>2.2 ± 0.4a</td>
<td>2.7 ± 0.4</td>
<td>1.6 ± 0.4b</td>
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<tr>
<td>Tubular necrosis</td>
<td>3.3 ± 0.2</td>
<td>2.7 ± 0.2a</td>
<td>3.3 ± 0.2</td>
<td>1.7 ± 0.4b</td>
</tr>
<tr>
<td>Cell detachment</td>
<td>3.6 ± 0.4</td>
<td>2.6 ± 0.2a</td>
<td>3.3 ± 0.2</td>
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<td>Brush border reduction</td>
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<td>2.1 ± 0.2a</td>
<td>2.5 ± 0.2</td>
<td>1.4 ± 0.4b</td>
</tr>
</tbody>
</table>

a P < .05 versus TMZ-free EC group. 
b P < .05 versus TMZ-free UW.


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