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Ischemia-Reperfusion Injury in the Transplanted Kidney Based on Purine Metabolism Markers and Activity of the Antioxidant System

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1. Introduction

The pathophysiology of ischemia and reperfusion stress linked with the generation of reactive oxygen species (ROS), as well as the activation of antioxidant defense mechanisms are an integral part of non-immune factors implicated in the early and delayed graft function (DGF). Oxygen free radicals are central mediators of cellular injury that occurs upon postischaemic reperfusion. Studies on the mechanisms of reperfusion injury in the cold-preserved kidney transplant model have suggested an important role for free radicals generated at reperfusion from oxygen by activated xanthine oxidase. Generation of ROS is the main mechanism inducing ischemic/reperfusion damage of the organ. Oxygen burst is a trigger for complex biochemical reactions leading to generation of oxygenated lipids and changes in microcirculation with recruitment of neutrophils to the graft. Recently, radical generation has been measured in postischaemic tissues using electron paramagnetic resonance spectroscopy. Electron paramagnetic resonance techniques have demonstrated that presence of oxygen burst after postischaemic reperfusion of the graft (Hirayama et al.,2004). Moreover, it has been shown that free radical generation is correlated with the activity of the anti-oxidative system. Many markers have been researched to prove the presence of ROS in the transplanted tissue including malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). They are involved in protection against free radicals. Elucidation of inter-relations between these factors is important for our understanding of the phenomena and for implementation of perioperative procedures aimed at prolonging graft survival. Organ preservation seeks to ensure the functional viability of transplanted organs. Preservation during ischemia includes steps against acidosis, steps to maintain cell volume and for optimal utilization of anaerobic energy reserves. Previous studies have demonstrated that apart from ischemic damage, additional tissue injury evolves as a result of reperfusion and reoxygenation (Tilney et al., 2001). Energy-dependent processes occurring during cold ischemia of the graft require adequate levels of ATP and other high-energy compounds generated in the majority during oxidative catabolism of various substrates. Because oxygen is required for such reactions, the ability to maintain adequate levels of high-energy phosphates would appear to depend on oxygen delivery to graft cells (Chien et al., 2001a). Oxygen delivery during reperfusion is

insufficient to maintain an aerobic environment in graft tissue. It is plausible that such tissue hypoxia may be associated with alterations in concentrations of high-energy phosphates and their metabolites (Grinyo et al., 2001). Various molecular mechanisms of ischemic changes in the graft tissue have been demonstrated. The graft's adenine nucleotide metabolism is directly linked to high-energy phosphate turnover and graft functioning. Some studies have shown that prolonged graft ischemia is accompanied by nucleotide degradation, but clinical data concerning nucleotide pool changes in renal and peripheral veins is lacking (Harris et al., 1996; Rabb et al., 1997). ATP is catabolized to adenosine and then to hypoxanthine. Nucleosides and oxypurines are released to plasma, but part of them are transported to erythrocytes where they are metabolized to other nucleosides and nucleotides. Ischemia-reperfusion injury is a not only a major cause of acute renal damage but also affects its clinical expression, mentioned earlier: delayed graft function. DGF has a significant impact on short- and long-term graft survival (Chien et al., 2001b). DGF is defined as a requirement for dialysis in the first week post-transplantation and includes a spectrum of clinical manifestations, ranging from borderline function to a complete absence of graft function. According to recent studies (Azevedo et al., 2005; Carter et al., 2005; Woodle et al., 2005), the rate of DGF varies between 20% and 50% in patients receiving a first cadaver graft. Consequently, estimation of the ischemic damage is important and it is essential to define reliable markers in order to properly assess renal ischemic damage and predict DGF. Determination of such parameters should improve the early follow-up of renal transplantation and the management of patients who receive nephrotoxic immunosuppressive compounds such as cyclosporine, which may enhance acute tubular injury and reduce the long-term prognosis of the graft functions. The pathophysiology of acute ischemia and the pathophysiology of reperfusion of the kidney graft require further studies and more focus on clinical parameters.

2. Objectives of ischemia-reperfusion injury analysis in the transplanted kidney

The impairment of organ function derived from ischemia–reperfusion injury is still an important problem in solid organ transplantation. Cell alterations induced by ischemia prime the tissue for the subsequent damage that occurs during the reperfusion phase. Ischemia–reperfusion injury affects early graft function and influences the development of chronic graft dysfunction.

Therefore the objective of the study was to determine:

- The concentration profile of oxypurines and purine nucleosides in the renal vein of the kidney graft in humans during reperfusion as a marker of the energy status of kidney tissue and as a prognostic factor for graft function;
- The activity of superoxide dismutase, catalase, and glutathione peroxidase in erythrocytes during reperfusion of the kidney graft in humans and their effect on graft function;
- The relationship between purine concentrations in blood and the activity of anti-oxidative enzymes in erythrocytes during reperfusion of the renal graft in humans;
- The effect of trimetazidine on the concentration of purine nucleotides as markers of ischemia in rat kidney with ischemia-reperfusion injury.

2.1 Oxypurine and purine nucleoside concentrations in renal vein of allograft as potential markers of energy status of renal tissue

Purine nucleosides and oxypurines are products of adenine nucleotides degradation. Reperfusion and reoxygenation are accompanied by production of reactive oxygen species and free radicals, which lead to damage of graft tissue. The aim of our study was to measure concentrations of adenine nucleotides and their metabolites in renal allograft vein as well as in recipient's peripheral veins during the reperfusion period and to evaluate their usefulness as markers of tissue metabolism in kidney allografts.

The study included 20 deceased donor kidney transplant recipients (11 males, 9 females, mean age 52 ± 8 years, cold ischemia time 19.9 ± 8.8 h, warm ischemia time 19.3 ± 5.7 min). The harvested grafts were perfused with EuroCollins preservation solution. All patients received standard immunosuppressive protocol with triple drug therapy including cyclosporine A, azathioprine, and steroids. The first blood sample (UV0) was taken from the recipient's ulnar vein before anastomosing of the kidney allograft vessels with recipient's iliac vessels. Next, samples were taken from the graft's renal vein (RV1) and recipient's ulnar (UV1) vein at 5 min of graft reperfusion. Five ml of blood was aliquoted into heparin-containing tubes kept on ice. Reperfusion of the transplanted kidney was followed precisely with ThermaCAM SC500 thermovision camera (FLIR Systems), which detects infrared radiation and records digital images presenting surface temperature distribution of the object. We assumed that the process of total reperfusion was completed when thermal scans showed the homogeneous distribution of graft's temperature. Samples RV1 and UV1 were taken from plastic microcatheters inserted into the renal allograft and ulnar veins after total tissue reperfusion when the temperature of the graft reached 35.0ºC. Two mL of blood was mixed with the same volume of 1.3 mol/L HClO4 and stored at -80ºC until assayed. The remaining blood was centrifuged within 15 min from collection at 4ºC for 10 min. Three hundred mL of plasma was supplemented with 300 mL of 1.3 mol/L perchloric acid and vortexed. Subsequently, plasma and blood samples were centrifuged at 20,000 x g and 4ºC for 5 min. Four hundred mL of the acid supernatant was removed and neutralized with 130-160 mL of 1 mol/L potassium phosphate to pH 5-7. Centrifugation was repeated and the supernatant was withdrawn for chromatography. High-performance liquid chromatography (HPLC) was done to measure blood and plasma concentrations of adenosine triphosphate (ATP), adenosine monophosphate (AMP), guanosine (Guo), inosine (Ino), hypoxanthine (Hyp), xanthine (Xan), uric acid (UA), and uridine (Urd). Analyses were performed with Hewlett-Packard 1050 series chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Rheodyne 7125 manual injection valve with 20 uL loop, UV-VIS detector, and series 1100 thermostatted column compartment. Separations were achieved on Hypersil BDS 125 x 3 mm, 3-mm particle size column (Agilent Technologies). Modifications were introduced into the original method (Smolenski et al., 1990). The mobile phase flowed at a rate of 0.5 mL/min and column temperature was 20.5ºC. Buffer composition remained unchanged (A: 150 mmol/L, phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 5.05 min, 100% at 5.35 min, 100% at 7.00 min, 0% at 7.10 min. Samples of 100 mL were injected every 12 min into the injection valve loop. Absorbance was read at 254 nm. Concentrations were

expressed as mmol/liter of blood or plasma. Adenylate energy charge (AEC) was calculated using the following formula: AEC = (ATP + ADP/2)/(ATP + ADP + AMP).

Hyp concentrations in whole blood were significantly increased in renal allograft vein after reperfusion as compared with peripheral (ulnar) vein before (1.82 times higher) as well as after reperfusion (1.61 times higher). Moreover, there was no statistically significant difference in Hyp peripheral vein concentrations before and after reperfusion. Similar differences were observed for Hyp concentrations in plasma. Plasma Hyp concentrations in renal vein after reperfusion were 155% and 162% higher than in peripheral vein before and after reperfusion, respectively. Xan concentrations in whole blood and plasma were significantly higher in renal vein in comparison with peripheral vein. Xan concentrations in plasma and whole blood from the renal vein were 155% and 163% higher before reperfusion and 162% and 129% after reperfusion, respectively. Ino concentrations in plasma and whole blood were significantly higher in renal allograft vein compared with peripheral vein in the post-reperfusion period. Moreover, peripheral vein Ino concentrations were significantly decreased after reperfusion. Guo concentrations in plasma and whole blood were higher in peripheral vein than in renal allograft vein after reperfusion. There was no significant difference in Guo peripheral vein concentrations before and after reperfusion. There was no significant difference between concentrations of uric acid and uridine in renal and peripheral veins before and after reperfusion. There was no significant difference in whole blood concentrations of ATP in renal and peripheral veins before and after reperfusion. AMP concentration in peripheral vein was decreased by 24% after reperfusion, which was associated with a slight increase in AEC. There was found many strong positive correlations between concentrations of purines in plasma and whole blood. Positive correlations between concentrations of most purines in plasma from the renal allograft vein and cold ischemia time, as well as warm ischemia time were showed. Correlations were most significant for Xan (with cold and warm ischemia time) and Ino and Guo (warm ischemia time only).

Long ischemia time can alter electron transport in the respiratory chain. All complexes involved in the process show a reduction in their activity associated with structural damage. ATP hydrolysis during ischemia causes a rise in free inorganic phosphate, which increases membrane permeability. ATP hydrolysis and anaerobic glycolysis decrease the intracellular pH which is a factor that may protect cells from death during ischemia (Smoleński et al., 1989). Extended ischemia causes progressive reduction of the iron-sulfur proteins associated with NADH dehydrogenase. During renal ischemia, ATP is degraded via ADP, AMP, adenosine and inosine to hypoxanthine, which is next oxidized to xanthine and uric acid. Catabolism of adenine nucleotides results in accumulation of hypoxanthine in ischemic cells. Ischemia also promotes the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (Arduini et al., 1988). Xanthine acts as a substrate for xanthine oxidase and enhances superoxide generation (Li et al., 1995). When xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen, superoxide radicals are released. Reactive oxygen species generated during ischemia-reperfusion play a major role in microvascular dysfunction and exert direct tissue damage, leading to lipid peroxidation, denaturation of proteins, and oxidation of DNA (Dowell et al., 1993). During ischemia, elevated concentrations of inosine, hypoxanthine and xanthine may occur due to the breakdown of ATP (Kurokawa et al., 1996). Administration of cyclosporine, which inhibits

adenosine uptake by erythrocytes but not adenosine catabolism to inosine mediated by adenosine deaminase, is an additional factor for increasing concentrations of these nucleotides in recipient's plasma (Guieu et al., 1998). However, we were not able to verify this hypothesis because all our patients were treated with cyclosporine. Previous studies on the effect of xanthine or xanthine oxidase as inducers of renal injury have produced inconsistent results. It was found that infusion of xanthine into isolated perfused kidneys increases the generation of oxygen free radicals and impairs renal function (Galat et al., 1989). Another study showed that xanthine oxidase depletion improved renal function after reperfusion (Linas et al., 1990). However, some authors have revealed a lack of effect of inhibitors of xanthine oxidase or of xanthine itself (Zager & Gmur, 1989). Purine metabolism during kidney transplantation in humans were assessed (Vigues et al., 1993). ATP levels in renal bioptates were decreased, whereas degradation products (IMP, inosine, adenosine, hypoxanthine) increased during cold storage. Moreover, adenylate energy charge was reduced by half. Kidneys with subsequent acute tubular necrosis (ATN) had significantly lower levels of the total pool of adenine nucleotides at reperfusion, but there was no correlation between incidence of ATN and concentrations of ATP and other metabolites in the kidneys before and during cold preservation. Lipid peroxidation during ischemia-reperfusion of rat kidney (Akcetin et al., 1999, 2000) was observed with decreased ATP levels and consecutive accumulation of hypoxanthine at the end of the ischemic period as well as a subsequent decline of hypoxanthine during reperfusion. In our study we measured levels of hypoxanthine, xanthine, inosine, guanosine, uric acid, uridine, ATP, and AMP in renal allograft and ulnar veins during reperfusion of kidney allografts. All these purines are present physiologically in the blood (Chouker et al., 2005). After reperfusion, Hyp and Xan concentrations in whole blood and plasma increased to the greatest extent. The catabolism of adenine nucleotides leads to generation of Hyp, which is metabolized to Xan. Xanthine is metabolized to uric acid, the final product of purine degradation in humans (Colpaert & Lefebvre, 2000). In our study we did not observe differences in the concentrations of uric acid between peripheral and renal veins. This finding can be explained by high basal concentrations of uric acid in blood and relatively low xanthine oxidoreductase activity in renal grafts (Sun et al., 2004). The relatively higher Hyp concentrations (which is a substrate for xanthine oxidoreductase) in renal vein than Xan concentrations (product of xanthine oxidoreductase) seem to confirm this hypothesis. Under conditions of low xanthine oxidoreductase activity, hypoxanthine is phosphoribosylated by hypoxanthine-guanine phosphoribosyltransferase to IMP (salvage pathway). We have shown that guanosine and uridine are not synthesized during ischemia and reperfusion and therefore are not useful for monitoring of kidney graft metabolism during reperfusion. A relatively stable erythrocyte adenine nucleotide concentration in the pre- and post-reperfusion period suggests that the process does not lead to disturbances of purine metabolism in erythrocytes. We found increased concentrations of oxypurines and nucleosides in plasma as well as in whole blood. Therefore, plasma or whole blood may be used to determine the release of these metabolites by the graft. However, when quick centrifugation of blood after collection is not possible, the only way to measure purine content is precipitation of whole blood with perchloric acid, which can be done immediately at the collection site. In the present study we also examined differences in concentrations of purine metabolites in renal allograft and peripheral veins. Concentrations of Hyp and Xan were significantly higher in the renal vein compared with peripheral vein. Higher Hyp and Xan concentrations in the renal vein were associated with

increased ischemic duration and can represent accelerated high-energy product breakdown. Therefore, we suggest that the differences in Hyp and Xan concentrations between renal and peripheral veins reflect changes and damage to renal tissue during reperfusion and might be useful for monitoring graft function during reperfusion.

2.2 Oxypurine and nucleoside concentrations in renal veins during reperfusion as predictors of early graft function

Perfusion is a process which creates the possibility of graft injury. A high perfusion pressure or an improper fluid composition may cause diffuse endothelial damage, creation of thrombi in small vessels and neutrophil infiltration (Gulec et al., 2006). Hypothermia downregulates the metabolism and prevents protease-dependent cell degranulation. Negative consequences of hypothermia and ischemia include cessation of ATP synthesis and Na-K-ATP synthase inhibition (Giligan et al., 2004). ATP is catabolized intracellularly to adenosine and then to hypoxanthine (Princemail et al., 1993; Hower et al., 1996). Both products migrate from the cell by means of a sophisticated membrane transport system. Under such conditions, xanthine oxidoreductase oxidizes hypoxanthine to xanthine and xanthine to uric acid. Hypoxanthine, xanthine and uric acid have only oxo substituents in their purine rings and are therefore called oxypurines. Uric acid is the final product of purine catabolism in humans. Superoxide radical is another product of reactions catalysed by xanthine oxidoreductase. It is toxic to the cell membrane, may cause structural damage to proteins and enzymes and finally may activate the arachidonic acid cascade, leading to production of inflammatory mediators (Grinyo et al., 2001; Skrzycki & Czeczot, 2004). The aim of this study was to examine whether purine and pyrimidine nucleoside concentrations as well as oxypurine concentrations in renal allograft and peripheral veins correlate with graft function.

The study population comprised 25 recipients of cadaver kidney transplant. A first blood sample was taken from the recipient's peripheral vein before anastomosing the kidney allograft vessels with the recipient's iliac vessels. Subsequent samples were taken from the allograft renal vein and the recipient's peripheral vein 5 min after beginning reperfusion. High-performance liquid chromatography was done to measure plasma concentrations of the oxypurines: hypoxanthine, xanthine and uric acid and the nucleosides: guanosine, inosine and uridine. Concentrations of Hyp, Xan and Ino were significantly higher in the renal than the peripheral vein. The differences between the Xan, Hyp, Ino and Urd plasma concentrations in the renal and peripheral veins before and 5 min after reperfusion correlated positively and significantly with serum creatinine concentrations 24 and 72 h after graft transplantation. Moreover, the concentrations of Hyp were significantly increased in renal transplant recipients with delayed graft function.

Preservation of the harvested organ constitutes a prerequisite for organ transplantation. For kidney preservation, hypothermic storage remains the commonest technique in use. However, hypothermic organ preservation is associated with oxygen deprivation, which inevitably leads to some degree of ischemia-reperfusion injury upon transplantation (Smolenski et al., 1989). During renal storage before transplantation, hypothermic swelling of the medullary thick ascending tubules results in mechanical constriction of the peritubular capillaries and vasa recta (Corner et al., 2003). During reperfusion, a large amount of reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen

peroxides) is produced by the re-entry of oxygenated blood into the ischemic tissue. A long period of ischemia can alter the electron transport complexes. All of the complexes show a reduction in their activity associated with structural damage. ATP hydrolysis during ischemia causes a rise in free inorganic phosphate, which increases membrane permeability. ATP hydrolysis and anaerobic glycolysis decrease the intracellular pH, a factor that may protect cells from death during ischemia (Arduini et al., 1988). During renal ischemia ATP, as mentioned earlier, is degraded via adenosine diphosphate, adenosine monophosphate, adenosine and Ino to Hyp, which is oxidized to Xan and UA. The catabolism of adenine nucleotides results in an accumulation of Hyp in ischemic cells. Also, ischemia is associated with the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (Dowell et al., 1993). When xanthine oxidase converts Hyp to Xan in the presence of molecular oxygen, superoxide radical is generated. Reperfusion and reactive oxygen species which are generated during ischemia play a major role in microvascular dysfunction and cause direct tissue damage, leading to lipid peroxidation, denaturation of protein and oxidation of DNA (Chouker et al., 2005). During ischemia, elevated concentrations of Ino, Hyp and Xan may occur due to the breakdown of ATP (Colpaert & Lefebvre, 2000). Xan acts as a substrate for xanthine oxidase and enhances superoxide generation (Li et al., 1995). These two events may influence renal injury, but previous studies on the effect of Xan or xanthine oxidase as inducers of renal injury are inconsistent. It was shown in some studies (Galat et al., 1989) that infusion of Xan into isolated perfused kidneys increases the generation of oxygen free radicals and impairs renal function. It was found that xanthine oxidase depletion improved renal function after reperfusion (Linas et al., 1990), while other authors (Zager et al., 1989) revealed a lack of effect of inhibitors of xanthine oxidase. Assessement of purine metabolism during kidney transplantation in humans showed (Vigues et al., 1993), that ATP levels in renal bioptates were decreased whereas levels of the degradation products (inosine monophosphate, Ino, adenosine, Hyp) increased during cold storage. Moreover, the adenylate energy charge was reduced by half. Kidneys with acute tubular necrosis (ATN) had significantly lower levels of the total pool of adenine nucleotides at reperfusion, but there was no correlation between the incidence of ATN and concentrations of ATP and other metabolites in the kidneys before and during cold preservation. In another study (Lopez-Marti et al., 2003), tissue levels of adenosine in rats decreased significantly 30 min following ischemia, whereas Xan/Hyp levels increased concomitantly with renal dysfunction and histological damage. In our study, which to our knowledge is the first to evaluate the purine and pyrimidine nucleoside as well as oxypurine concentrations in renal and peripheral veins during reperfusion of kidney allografts in humans, we examined plasma levels of Hyp, Xan, Ino, Guo, UA and Urd. After reperfusion we observed increased concentrations of all metabolites, with the greatest increase occurring in the concentrations of Hyp and Xan in renal allograft veins. This finding is in concordance with those of previous studies carried out in animal models and may result from disturbances in purine metabolism during kidney allograft reperfusion. The catabolism of adenine nucleotides leads to the generation of Hyp, which is metabolized to Xan. Xan is metabolized to UA, the final product of purine degradation in humans (Sun et al., 2004). A lack of significant differences in UA concentrations between the studied blood samples may have been due to high basal plasma concentrations of UA in the systemic circulation. Hyp and Xan are the factors that may be modified depending on the duration and severity of ischemia. The renal content of Hyp and Xan changes after renal ischemia as a consequence of ATP breakdown (Li et al., 1995).

Increased Hyp and Xan concentrations in the renal compared with the peripheral vein are manifestations of ATP breakdown in renal tissue, which occurs during reperfusion. Therefore, the differences between metabolite concentrations in the renal and peripheral veins may most precisely reflect the degree of metabolic changes in renal tissues during reperfusion. Moreover, we correlated the differences between metabolite concentrations in renal and peripheral veins with graft function and creatinine concentrations at follow-up. The Hyp concentrations correlated with creatinine concentrations during the first 3 days after transplantation, whereas there was no correlation with long-term graft function. The differences in Guo concentration correlated negatively with the recipients' serum creatinine concentration 6 and 12 months after transplantation. Guo is metabolized to Xan by the kidney. Therefore, decreased metabolism of Guo to Xan may correlate with improved late graft function. Moreover, Hyp concentrations correlated with DGF in graft recipients. These results suggest that Hyp may be a useful predictor of early graft function. Another aim of the study was to determine whether disturbances of purine metabolism in the graft are associated with graft function. At transplantation it is not known when the graft will be fully functional, but a high concentration of Hyp in the graft's renal vein may indicate an increased risk of DGF. We conclude that disturbances in purine metabolism may be involved in the pathomechanisms of DGF.

2.3 Early phase of reperfusion of human kidney allograft and the erythrocyte anti-oxidative system

Although the problem of anti-oxidant defence is often brought up in the literature, authors are not precise in describing the activity of the enzymes during the first minutes after reperfusion. Previous studies on the animal model have shown increased free radicals generation during the first minutes of kidney allograft reperfusion with simultaneous activation of protecting mechanisms (Singh et al., 1993; Davies et al., 1995). The erythrocyte anti-oxidative system plays an important role in the protection against free radicals generated during the first minutes of reperfusion. The majority of reports regarding the erythrocyte anti-oxidative system derive from animal studies. Studies in humans are inconsistent. Therefore, the aim of our study was to examine the activity of the erythrocyte anti-oxidative system: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) during reperfusion of the transplanted kidney.

The study included 40 renal transplant recipients (22 men, 18 women, mean age 51 ± 7 years, cold ischaemia time 25 ± 5 h). The grafts were perfused with EuroCollins preservation solutions. All patients received standard immunosuppressive protocol with triple drug therapy including cyclosporine A, azathioprine and steroids. The '0' blood sample was taken from the iliac vein before anastomosing of the kidney vessels with recipient's iliac vessels. Then, the renal vein of the graft was cannulated and blood samples I, II and III were taken. The reperfusion of the transplanted kidney was measured precisely with ThermaCAM SC500 (AGEMA, Infrared System AB, Danderyd, Sweden) thermovision camera, which detects infrared radiation and records digital images presenting surface temperature distribution of tested objects. The process of total reperfusion was completed when the scans from thermovision camera showed the whole organ filled with recipient's blood. Sample I was taken from the inserted catheter after total tissue reperfusion had been demonstrated on the scan monitor and after the temperature of the graft had reached 34 °C.

Blood samples II and III were taken 2 and 4 min after blood sample I. The erythrocytes were separated by centrifugation (300 *g*, 10 min), washed three times with buffered 0.9% NaCl solution (PBS: 0.01 mol phosphate buffer; 0.14 mol NaCl, pH 7.4) chilled to 4 °C and finally frozen at −70 °C. Before the analysis, erythrocytes were thawed and the haemolysate of washed red blood cells was diluted with distilled water and chilled to 4 °C. SOD, CAT, GPx activity and GSH concentrations were measured with Bioxytech (Oxis Research, Portland, OR, USA) kit using UV/ VIS Lambda 40 (Perkin Elmer, Wellesley, MA, USA) spectrophotometer. Friedmann ANOVA was used to assess statistical significance of changes of studied parameters. Spearman's rank correlation coefficient was used to measure correlations between measured parameters. There were no statistically significant differences in the activity of superoxide dismutase, catalase, glutathione peroxidase as well as glutathione concentrations during the first 4 min after total graft reperfusion and after having reached the temperature of 34.0 °C by the graft. Nevertheless, there was a positive correlation between the activity of superoxide dismutase and glutathione peroxidase ($P < 0.005$). The activity of glutathione peroxidase correlated positively with the concentration of glutathione in the fourth minute after total reperfusion ($P < 0.005$). Moreover, there was negative correlation between superoxide dismutase activity and glutathione concentration. This correlation was statistically significant before and 2 min after reperfusion ($P < 0.05$).

Oxidative reperfusion injury is thought to be a central mechanism of cellular damage affecting all organs and tissues after ischaemia. The mechanisms of this damage, however, are still not fully understood. Ischaemia results in the impairment of mitochondrial anti-oxidant defences and thereby renders cells more susceptible to oxidative stress (Arduini et al., 1988). The activity of anti-oxidative enzymes seems to be relatively stable during ischaemia (Kurokawa et al., 1996). However, glutathione peroxidase levels may decrease during both warm ischaemia and cold storage (Jassem et al., 1996). It has been demonstrated in isolated perfused kidney that oxygen free radicals are generated on postischaemic reperfusion (Paller et al., 1984). Several sources may be responsible for oxygen free radical production during ischaemia-reperfusion. These include alterations in mitochondrial electron transport, arachidonic acid metabolism, activation of xanthine oxidase, catecholamines or haemoglobin oxidation, as well as massive release of iron (Jassem et al., 2002). The results from previous studies indicate that leucocytes play a key role in ischaemia-reperfusion injury (Harris et al., 1996). The phenomenon also involves components of a typical inflammatory reaction. Oxygen free radicals can directly trigger the activation of leucocytes and adhesion molecules. Leucocyte activation is significantly enhanced within minutes after the onset of reperfusion and remains elevated for hours (Rabb et al., 1997). The measurement of the activity of enzymes involved in the anti-oxidative system is considered to be one of the reliable and sensitive assays of ischaemia-reperfusion period. Nevertheless, only a few studies have been conducted in humans to investigate the activity of the anti-oxidative system in erythrocytes during ischaemia and reperfusion. In the present study changes in activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione concentrations in erythrocytes during the first 4 min after total kidney graft reperfusion were examined. The authors did not find statistically significant changes in the activities of enzymes involved in the anti-oxidative system. However, there was significant positive correlation between the activities of superoxide dismutase and glutathione peroxidase. This correlation may indicate the presence of mechanisms coordinating the activity of these enzymes. This mechanism might be of clinical

importance increasing the effectiveness of superoxide radical inactivation, because H2O2, which is the product of superoxide dismutase, is at the same time the substrate for glutathione peroxidase. Moreover, there was no significant association between the erythrocyte anti-oxidative system and transplant outcome such as delayed graft function and acute rejection. In the present we analysed the enzymatic anti-oxidative system in the renal vein only and have not detected any changes in their activity in first 4 min of graft reperfusion. We have not analysed the enzymatic and non-enzymatic anti-oxidative systems in serum, as well as markers of biological membranes oxidative damage such as lipid peroxidation products. The lack of unfavourable changes during the early period of reperfusion such as decreased glutathione concentration, decreased glutathione peroxidase or SOD activity may indicate the very high capacity of anti-oxidative systems. It is possible that besides the erythrocyte anti-oxidative system other anti-oxidative systems such as plasmatic (Muzakova et al., 2000; Pechan et al., 2003;) or tissue antioxidative system (Dobashi et al., 2000) are involved in the defence against free radicals during allograft reperfusion. Nevertheless, a better understanding of changes in the anti-oxidative systems protecting against free radicals generation during allograft reperfusion requires further investigations.

2.4 Activity of CuZn-superoxide dismutase, catalase and glutathione peroxidase in erythrocytes in kidney allografts during reperfusion and delayed graft function

Many markers have been researched to prove the presence of ROS in the transplanted tissue. Some of them, like superoxide dismutase, catalase, and glutathione peroxidase are considered to play a major role in graft protection against oxygen stress during reperfusion. The SODs appear to be the most important enzymes involved in the defense system against ROS, particularly against superoxide anion radicals. Three distinct isoforms of SOD have been identified in mammals and their genomic structure, c-DNA, and protein structure have been described (Skrzycki & Czeczot, 2004). Two isoforms of SOD have Cu and Zn in their catalytic center and are localized in either intracellular cytoplasmatic compartments (CuZn-SOD or SOD1) or in extracellular elements (EC-SOD or SOD3). SOD1 is a homodimer with a molecular mass of about 32 kDa (Fattman et al., 2003; Zelko et al., 2002). It has been found in the cytoplasm, nuclear compartments and lysosomes of mammalian cells. SOD3 is a homotetramer of 135 kDa with a high affinity to heparin (Lookene et al, 2000; Oury et al., 1996). Manganese (Mn) is the cofactor of the third SOD isoform (Mn-SOD or SOD2) which is localized in aerobic cells' mitochondria (Petersen et al., 2003; Tibell et al., 1997). Early reperfusion is definitely associated with upregulating of SODs activity, but is also a factor that may lead to their rapid depletion (Davies et al., 1995; Singh et al., 1993) as huge amounts of ROS are released during that process. The aim of the study was to examine the activity of erythrocyte antioxidative system (SOD1, GPx, and CAT activity as well as GSH concentration) among patients with or without DGF.

Forty patients undergoing kidney transplantation at our center were assigned to two groups: with or without delayed graft function. Before anastomosing kidney vessels with recipient's iliac vessels, the '0' blood sample was taken from the iliac vein. Next blood samples I, II and III were taken from the graft's renal vein. The reperfusion of the transplanted kidney was evaluated precisely with the thermovision camera. Erythrocyte SOD1, CAT and GPx activity was measured with a spectrophotometric method. We did not

observe statistically significant changes in SOD1, CAT and GPx activity in erythrocytes during the early phase of reperfusion in patients with and without DGF.

In humans, only a few studies have been conducted to investigate the activity of antioxidative system in erythrocytes during ischemia reperfusion. The excess of ROS, particularly of superoxide anion radicals is the main factor, which activates SODs. These enzymes are essential for cell protection against oxygen toxicity. As a result of the reaction catalyzed by SODs, hydrogen superoxide is generated and becomes a substrate for further reactions involving GPx and CAT. In our study the activity of erythrocyte antioxidative system, after total graft reperfusion in patients with and without DGF was assessed. SOD, CAT and GPx are the most important enzymes involved in antioxidative system during reperfusion. The SOD1 gene has been mapped to chromosome 21 (region 21q22) in humans (Levanon et al., 1985). Despite the fact that SOD1 is considered to be constitutively expressed, transcriptional regulation of SOD1 is highly controlled depending on extra- and intracellular conditions (Crapo et al., 1992). SOD1 mRNA level elevates in response to a wide array of mechanical, chemical and biological messengers such as heat shock, shear stress or hydrogen peroxide (Dimmerel et al., 1999; Hass & Massaro, 1988; Inoue et al., 1996; Yoo et al., 1988). SOD1 was fund to have a widespread distribution in a variety of tissues (Crapo et al., 1992). Early reperfusion is definitely associated with upregulating of SODs activity, but is also a factor that may lead to their rapid depletion (Davies et al., 1995; Singh et al., 1993) as huge amounts of ROS are released during that process. It was shown that MDA concentration as a marker of lipid peroxidation in the renal vein after 2 min of reperfusion increased by 30% compared with the systemic baseline value (Hower et al., 1996). In rats, the endogenous scavenger SOD, especially the cytoplasm copper-zinc (CuZn) form, is rapidly depleted during ischemia and reperfusion (Davies et al., 1995; Singh et al., 1993). Numerous studies have assessed the potential benefits of exogenous SOD administration in preventing reperfusion injury, but conclusions are ambiguous. In one study (Land et al., 1994) intraoperative human recombinant CuZn-SOD administration led to a significant reduction of early and late immunological complications. The effect, according to the authors, could be related to the enzyme's antioxidant action on ischemia-reperfusion injury of the renal allograft, which is a potential factor that reduces the immunogenicity of the graft. In another experimental study protective effect of exogenous Cu/Zn SOD proved to be minimal, probably because of very short half-period of SOD counted in minutes (Bayati et al., 1988; Johnson & Weinberg, 1993). In those studies, however, the authors did not specify which form of Cu/ Zn SOD was administered. An experimental study observed (Yin et al., 2001), that increased levels of intracellular SOD in kidney induced by its transfection with an adenoviral vector minimized ischemia-reperfusion induced tubular injury and improved post-ischemic renal function. Results of the studies suggest that some changes in SOD activity occur in graft's vein during the first few minutes after reperfusion. In our experiment no significant changes in graft's vein SOD1 activity were observed either in the group of patients with DGF or in the group without DGF, as the enzyme activity in sample III was comparable with its activity in sample 0. Early period of reperfusion from clamp removal till the whole graft is filled with recipient's blood and reaches the temperature of 36.6^{o}C is essential for further graft function. Therefore appropriate assessment of the process is very important. The use of thermovision camera

was the important point of our research. In most cases the assessment of graft reperfusion is performed by the surgeon without any objective method during the operation. However, proper evaluation of the process seems to be crucial in everyday practice as well as in experimental studies. Thermovision camera helped us to determine the moment when the whole graft was perfused with recipient's blood (Gnaiger et al., 1999; Jassem et al., 2002; Ostrowski et al., 2004). We suggest that this method is an important step to objective assessment of early reperfusion. In our study the erythrocyte antioxidative system remained stable after total reperfusion. The activity of SOD, CAT, GPx as well as GSH concentrations did not change statistically significantly during the reperfusion. Taking into consideration the results of our and previous studies which proved the beneficial effect of Cu/Zn SOD on the graft in the early stages after transplantation, we suggest that extracellular antioxidative system proves to be essential for protection against ROS during early stages after reperfusion. Extracellular matrix and the cell surface is the main EC SOD (SOD3) localization (Marklund, 1984, 1994) and structural differences between SOD1 and SOD3 seem to be the main factor that influences their activity and the place of action. In experiments with fluorescently labeled SOD (Emerit et al., 2002) clearly, it was showed that the enzyme binds to cellular membranes and the intensity of binding varies according to cell type. The differences observed for binding were concomitant with decreased inhibition of stimulated superoxide production. Taking into account the results of present study we suggest that the erythrocyte antioxidative system during the reperfusion is stable, and the main protective role against free radicals plays the extracellular antioxidative system, nevertheless this hypothesis requires further investigations.

2.5 Hypoxanthine as a graft ischemia marker and catalase activity during reperfusion

It has been proposed that xanthine oxidase may be a central mechanism of postischemic free radical generation in a variety of cells. Superoxide dismutase, catalase, glutathione and glutathione peroxidase are involved in protection against free radicals (Colpaert &Lefebrve, 2000; Komada et al., 1999; Radi et al., 1997). The aim of the study was to examine the correlation between the concentrations of ischemia markers: hypoxanthine or inosine and the activity of erythrocyte SOD, CAT as well as GPx.

The study included 40 renal transplant recipients. Before anastomosis of the kidney vessels with the recipient's iliac vessels, a "0" blood sample was taken from the iliac vein. Then, after anastomosis, the renal vein of the graft was cannulated and blood samples I, II, and III were obtained. The reperfusion of the transplanted kidney was measured with a thermovision camera ThermaCAM SC500. The study included 40 renal transplant recipients (21 men, 19 women), of mean age 52 ± 8 years with mean cold ischemia time of 26 ± 3 hours. The grafts were perfused with EuroCollins preservation solution. All patients received cyclosporine, azathioprine, and steroids. The "0" blood sample was taken from the iliac vein before anastomosing the kidney vessels with recipient iliac vessels. Then, the graft renal vein was cannulated to obtain blood samples I, II, and III. The reperfusion of the transplanted kidney was measured with thermovision camera (ThermaCAM SC500), which detects infrared radiation, recording digital image presenting the surface temperature distribution of the organ. The process of total reperfusion was completed when the scans from thermovision camera showed the whole organ filled with recipient blood. The sample I

was taken from the inserted catheter after total tissue reperfusion had been shown by the scan monitor and after the temperature of the graft had reached 34°C. Blood samples II and III were taken 2 and 4 minutes after blood sample I, respectively. Blood was aliquoted into heparin containing tubes kept in ice. One tube was centrifuged within 15 minutes from collection. Plasma (300 μL) was supplemented with 300 μL of 1.3 mol/L perchloric acid, vortexed, and centrifuged (5 min/20000 *g*/4°C). The acid supernatant (400 μL) was removed and neutralized with 130 to 160 μL of 1 mol/L potassium phosphate to pH 5 to 7.7 Centrifugation was repeated and the supernatant withdrawn for high-performance liquid chromatography (HPLC) to measure plasma concentrations of Hyp and Ino. Analyses were performed with a Hewlett-Packard (now Agilent) Series 1050 chromatography System. SOD, CAT, and GPx activities were measured with spectrophotometric methods using an UV/VIS Lambda 40 (Perkin Elmer) spectrophotometer. We used Bioxytech (Oxis Research, USA) kits. The results are presented per gram of hemoglobin, measured using Drabkin's method. Friedmann ANOVA was used to assess the significance of changes in the studied parameters. Spearman's rank correlation coefficient was used to measure correlations between analysed parameters.

The plasma concentrations of Hyp and Ino increased in statistically significant fashion immediately after total tissue reperfusion ($P < .0001$) then gradually decreasing. There were no statistically significant differences in the activities of CAT, SOD, and GPx. The activity of catalase at 4 minutes after total tissue reperfusion (sample III) correlated positively with hypoxanthine concentrations immediately after total tissue reperfusion (sample I; Rs = +0.49), 2 minutes after total tissue reperfusion (sample II; Rs = +0.47), and 4 minutes after total tissue reperfusion (sample III; Rs = +0.46). There were no statistically significant correlations between CAT activity and Hyp concentrations in the iliac vein before transplantation. Similar correlations were observed between CAT activity and Ino concentrations; those values, however, did not reach statistical significance ($P < .07$). Moreover, there were no statistically significant correlations between Hyp and Ino concentrations and SOD and GPx activities.

For kidney preservation, hypothermic storage remains the most common technique. However, hypothermic organ preservation is associated with oxygen deprivation, which inevitably leads to some degree of ischemia-reperfusion injury upon transplantation (Hower et al., 1996). During reperfusion, a large amount of reactive oxygen species (superoxide anions, hydroxyl radicals, and hydrogen peroxides) are produced by the reentry of oxygenated blood into the ischemic tissue (Grinyo, 2001). The catabolism of adenine nucleotides results in an accumulation of Hyp in ischemic cells. Ischemia is also associated with the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (Sermet et al. 2000). When xanthine oxidase converts Hyp to xanthine in the presence of molecular oxygen, superoxide radical is generated. SOD, CAT, GSH, and GPx are involved in defense against these processes (Dowell et al., 1993). In the present study we evaluated the correlation between the concentration of ischemia markers (Hyp or Ino) and the activity of erythrocyte SOD, CAT, as well as GPx. Immediately after total reperfusion we observed a statistically significant increase in Hyp and Ino plasma concentrations, changes which retreated after reperfusion. Elevated concentrations of Hyp and Ino during ischemia may occur due to ATP breakdown. The elevated concentrations of Hyp and Ino correlated with increased activity of catalase in erythrocytes. We did not, however, observe a correlation

with SOD activity. Characterization of the human SOD gene has revealed several potential transcriptional regulatory sites, none of which have been demonstrated to be active in the human SOD gene (Foltz & Carpo, 1994). The presence of those possible regulatory sequences suggests that SOD expression may be regulated by multiple stimuli. Recently SOD expression has been shown to be regulated by several cytokines. Interferon-γ results in an increased expression of SOD, whereas tumor necrosis factor-α and transforming growth factor- β decrease enzyme expression (Marklund 1992). Various forms of direct oxidative stress do not, however, seem to affect SOD expression (Stralin & Marklund, 1994). The results of the present study suggested that CAT activity may correlate with the concentration of Hyp and other mediators of oxidative stress in the graft renal vein. We hypothesize that catalase activity is regulated by factors that protect against a burst of free radicals upon postischemic reperfusion. The hypothesis requires further investigation.

2.6 Effect of trimetazidine on the nucleotide profile in rat kidney with ischemia–reperfusion injury

Total adenine nucleotide concentration (TAN) is a measure of pool of all (high- and low-energy) nucleotides containing adenine moiety (ATP + ADP + AMP). The balance between these three nucleotides is maintained by the action of adenylate kinase which catalyzes the reversible transphosphorylation of two molecules of ADP to one ATP and one AMP (Saiki et al., 1997). Adenylate energy charge is a measure of the balance between adenine nucleotides. Its value (Atkinson & Walton, 1967) is between 0 (when only low-energy AMP is present in the cell) and 1 (when only high-energy ATP is present). Dephosphorylation of AMP to adenosine catalyzed by 5′-nucleotidases is responsible for loss of AMP and decrease in TAN concentration (Welsh & Lindinger, 1997). Preservation of TAN pool is crucial to maintenance of normal purine metabolism in the cell since the replenishment of the lost adenine nucleotide molecules (via synthesis "de novo" or salvage reactions) would require additional energy, which is lacking during ischemia. Therefore considerable loss of adenine nucleotides during ischemia results in vicious circle leading to irreversible deficit of energy which cannot be recovered during reperfusion (Hiraoka et al.,1993). The catabolism of adenine nucleotides results in an accumulation of hypoxanthine in ischemic cells. Ischemia is also associated with proteolytic conversion of xanthine/hypoxanthine dehydrogenase to xanthine/hypoxanthine oxidase (Colpaert & Lefebvre, 2000; Komada et al., 1999; Sun et al., 2004). With the supply of molecular oxygen upon reperfusion of ischemic tissues, xanthine oxidase metabolizes xanthine and hypoxanthine to uric acid and free radicals are generated. The generation of oxygen free radicals during reperfusion may overcome the capacity of physiologic scavengers. The excess of these highly reactive species results in cytotoxicity and induces peroxidation of the lipid cell membrane (Akcetin et al., 2000; Pincemail et al., 1993). It has been shown that the free radicals generation correlates with the activity of antioxidative system (Singh et al., 1993). The excess of ROS, particularly of superoxide anion radicals is the main factor which activates superoxide dismutases. These enzymes are essential for cell protection against oxygen toxicity. As a result of the reaction catalyzed by SODs, hydrogen superoxide is generated and becomes a substrate for further reactions involving glutathione peroxidase and catalase (Gulati et al., 1993; Yoo et al., 1999). In experimental systems of ischemia–reperfusion injury, it has been shown that mitochondria may play a key role in oxidative injury (Vlessis & Mela-Riker, 1989). Mitochondria are the powerhouse of the cell and provide ATP through the oxidative phosphorylation process.

Moreover, mitochondria were identified as an important source of hydrogen peroxide (Jassem et al., 2002). Ischemia causes also extensive cytoskeletal and mitochondrial damage and uncoupling of oxidative phosphorylation (Akcetin et al., 1999; Caraceni et al., 2005). The effect of trimetazidine on the mitochondrial function of ischemic Wistar rat was studied (Monteiro et al., 2004). In this model, trimetazidine had a preferential action on the oxidative system, increasing its enzyme activity and decreasing O2 consumption after phosphorylation; this could decrease oxygen free radical production and increase mitochondrial integrity, thus allowing the maintenance of the electrical potential. Trimetazidine (TMZ) is an anti-ischemic agent. Its anti-ischemic effects have been experimentally assessed in various models including cell cultures, isolated and perfused organs, and also in vivo (Baumert et al., 1999; Hauet et al., 2000). TMZ mostly acts on mitochondria by restoring ATP synthesis, which was blocked by the Ca overload, by releasing Ca accumulation in the matrix, and by restoring the mitochondrial membrane impermeability and its affinity for protons. Moreover, TMZ inhibits the excessive release of oxygen free radicals, increases glucose metabolism, limits intracellular acidosis, protects ATP stores, reduces membrane lipid peroxydation and inhibits neutrophils infiltration after ischemia and reperfusion (Catroux et al., 1990; Hauet et al., 1998a). The aim of the study was to examine the effect of TMZ on nucleotide profile in rat kidney with ischemia–reperfusion injury.

Male Wistar rats weighing 300–350 g were used in this experiment. They were allowed to acclimatize for a minimum of 10 days prior to the study. The rats were housed in the room maintained at 21 ± 1 °C with 12 h light–dark cycle with the light cycle beginning at 6:00 a.m. All animals were fed standard rat chow and water ad libitum. Food was withheld overnight before surgery. Animals were divided into two groups: animals treated with TMZ (n = 14) and control group receiving placebo (n = 13). The aqueous solution of trimetazidine 10 mg/kg/day was administrated by gavages vehicle twice a day at 8.00 a.m. and 18.00 p.m. for 30 days. In this study, the effect of trimetazidine on adenine nucleotide profile was examined after ischemia-reperfusion as well as by normal renal blood flow. Therefore the left kidney artery was clamped to induce the ischemia-reperfusion injury, whereas the blood flow in right kidney artery remained unchanged. The rats were anesthetized with ketamine (Ketolar). The abdominal cavity was opened via middle incision. The aorta, vena cava inferior, and finally left and right renal vessels were atraumatically isolated. An atraumatic microvascular clamp was placed on the left renal artery for 15 min to induce the ischemia of renal tissue. The catheter (Becton Dickinson Vascular Access Inc., Sandy, Utah, USA) was inserted in aorta. After clamp removal from the left renal artery, the aorta and vena cava superior were clamped over the right and left renal artery, and then vena cava inferior was catheterized to enable the outflow of perfusion solution. The EuroCollins solution was perfused continuously at 100 ml/h during 12 min (temperature of the solution, +4 ◦C; volume of injected solution, 20 ml). Finally, bilateral nephrectomy was performed. After obtaining tissue samples from both kidneys for histological analysis the right and left kidneys were immediately frozen in liquid nitrogen within a few seconds from collection and transferred to 1.5 mL airtight tubes immersed in liquid nitrogen. Subsequently, they were minced with a metal homogenizer pre-cooled in liquid nitrogen, transferred to capped 1.5 mL tubes containing 500 µL of 0.4 mol/L cold perchloric acid, and homogenized for 15 s with a knife microhomogenizer (Dispergierstation T8.10, IKA). Centrifugation followed at 4 ◦C (16,000 × g, 5 min), 400 µL of the supernatant was transferred to a test tube and

neutralized with 115 μL of 1 mol/L potassium phosphate. Centrifugation was repeated and the supernatant was taken for high performance liquid chromatography. HPLC was done to measure tissue and whole blood concentrations of the following 17 nucleotides, nucleosides, and oxypurines: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (Ado), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (Guo), inosine monophosphate (IMP), inosine (Ino), hypoxanthine (Hyp), xanthine (Xan), uric acid (UA), uridine (Urd), nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Analyses were performed with Hewlett–Packard Series 1050 chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Rheodyne 7125 manual injection valve with 20 μL loop, UV–vis detector, and series 1100 thermostatted column compartment. Separations were achieved on Hypersil BDS 125 mm × 3 mm, 3 μm particle size column (Agilent). The mobile phase flowed at a rate of 0.5 mL/min and column temperature was 20.5 ^{o}C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 5.05 min, 100% at 5.35 min, 100% at 7.00 min, 0% at 7.10 min. Samples of 100 μL were injected every 12 min into the injection valve loop. Absorbance was read at 254 nm. Concentrations were expressed as median in nmol/mg protein. For histology, kidneys were fixed in buffered formalin and 2 μm paraffin-embedded sections were stained using hematoxylin–eosin and periodic acid-Schiff (PAS). Twenty randomly selected areas in a renal cortex were examined under light microscopy at 400×. Severity of acute tubular damage was semi-quantitatively scored by estimating the percentage of tubules that showed epithelial necrosis or had necrotic debris as follows: 0, none; 1+ (mild), <10%; 2+ (moderate), 10–50%; 3+ (severe), >50%. Mann–Whitney test was performed to compare each parameter between two groups. The significance of the differences between the parameters in the left (clamped, with ischemia) and the right (non-clamped, without ischemia) kidney was assessed using Wilcoxon test. Severity of acute tubular damage was compared between groups with x2-test.

Tissue concentrations of ATP, ADP, AMP, TAN and AEC were significantly increased in kidneys from rats treated with TMZ in comparison with rats receiving placebo. Concentrations of products of nucleotide degradation: inosine, guanosine and uridine, as well as oxypurines: Hyp and Xan, were significantly decreased in rats treated with trimetazidine. Moreover, significantly less pronounced acute tubular necrosis was observed in kidneys of rats treated with TMZ. Tissue concentrations of ATP, ADP, AMP, TAN and AEC values were significantly increased in kidneys with or without ischemia in rats treated with trimetazidine in comparison with rats receiving placebo. Moreover, the concentrations of GTP and GDP were increased, although the concentrations of GMP were decreased in kidney from rats treated with trimetazidine. The concentrations of products of nucleotide degradation: Ino, Guo and Urd, as well as oxypurines: Hyp and Xan, were significantly decreased in rats treated with trimetazidine. These results suggest the protecting activity of trimetazidine against the dephosphorylation of nucleotides. Nevertheless, the concentration of Ado was significantly increased in rats treated with trimetazidine. The increased Ado concentrations may be the result of increased adenine nucleotide synthesis or inhibition of adenosine to inosine degradation. The increased tissue adenosine concentrations may be the

important kidney preconditioning factor improving the tissue metabolism during ischemia. Concentrations of NAD were significantly increased in kidneys from rats treated with trimetazidine. It might be associated with enhanced ATP availability, essential for NAD synthesis. NADP concentrations were decreased in rats treated with trimetazidine. Decreased NADP concentrations may suggest the increase in NADPH/NADP ratio. NADPH is involved in the antioxidative system; therefore the increased NADPH concentration may have beneficial effects in ischemic tissues. Analyzing tissue concentrations of ATP, ADP, AMP, GDP, GMP and TAN between kidneys with and without ischemia we observed the decrease of these nucleotide concentrations associated with ischemia in rats treated with TMZ, although the concentrations in rats receiving TMZ remained significantly higher as compared to values in rats from control group. There were no statistically significant differences in NAD concentration between kidney with and without ischemia, the NADP concentrations were significantly decreased in kidney with ischemia in rats treated with trimetazidine as well as placebo. Tissue concentrations of Guo were significantly decreased in kidneys after ischemia in rats treated with trimetazidine. It may be the result of decreased guanylate degradation in kidneys treated with trimetazidine during ischemia. Hyp concentrations were significantly decreased in kidneys after ischemia in rats treated with trimetazidine, but in rats receiving placebo these values were increased. Decreased tissue concentrations of Hyp may be associated with diminished degradation of adenine nucleotides during ischemia in rats treated with trimetazidine. The histological changes of ischemic acute tubular necrosis depends on the intensity of ischemic trigger and varies with the evolution of the lesion in relationship to the onset of initial injury. Among many histologic features of ATN individual, cell necrosis with denudation of the basement membrane, shedding of epithelial cells and necrotic debris into the lumen are most characteristic whereas glomerular morphology typically remains unchanged. The commonest finding in kidneys from control group was moderate ATN followed by complete necrosis of epithelial tubular cells and denudation of basement membranes seen in many tubules. Mild ATN characterized by the presence of fragments of cells within the tubular lamina of single tubules was significantly more frequent in kidneys from rats treated with trimetazidine than in control groups ($p < 0.001$ and <0.05, respectively). Severe ATN in kidneys from the trimetazidine treated rats was not detected. The intensity of ATN did not differ significantly between kidneys with and without ischemia. The significantly less pronounced ATN observed in kidneys of rats treated with TMZ suggests that TMZ protects the tubules from ischemic damage.

Ischemia–reperfusion injury affects early graft function and influences the development of chronic graft dysfunction. Ischemia favors the depletion of cellular adenosine nucleotides, alterations in membrane ATP-dependent ionic transporters, and the intracellular accumulation of Ca, Na and water. The great swelling of endothelial and tubular epithelial cells due to ischemia not only increases the acidosis caused by anaerobic metabolism, but also alters cell permeability and favors the obstruction of capillary flow (Facundo et al., 2005). The reperfusion of ischemic tissues also increases the release of intracellular enzymes. In anaerobic metabolism the energy production for essential processes is greatly reduced. Failure of ion pumping with rapid loss of electrochemical gradients results in translocation of ions. The production of oxygen-derived free radicals is considerably increased during tissue ischemia caused by dissociation of oxidative phosphorylation, which results in univalent reduction of oxygen, catabolism of ATP into hypoxanthine and uric acid (Elsner et

al., 1998). In particular, the reduced form of NADH, the essential coenzyme for all the oxidative enzymatic reactions, accumulates and is only utilized in reactions such as lactate formation from pyruvate, which under ischemia cannot be used in Krebs cycle. A general assumption is that NADP – the cofactor of anabolic enzymatic reactions – accumulates in the reduced form (NADPH), following the same pattern of the NADH/NAD redox couple. Under conditions of impaired NADPH production, the possible utilization of cofactor can remain unbalanced. There is clear evidence that a highly-reduced state of NADP and of glutathione redox couples exerts a major protective function (Hai et al., 2005). In particular glutathione is involved in the protection against free radical induced damage through glutathione peroxidase. Interruption of blood supply and lack of oxygen during ischemia lead to anaerobic metabolism that entails a depletion of ATP (Cruthirds et al., 2005). In this study we examined the effect of TMZ on nucleotide concentrations in renal tissue after ischemia–reperfusion. The animals received TMZ 4 weeks before the experiment. Tissue concentrations of the majority of nucleotides involved in energetic processes in cells were significantly increased in renal tissues from animals receiving TMZ as compared to rats receiving placebo. Although the concentrations of purine nucleotides decreased during ischemia in rats treated with TMZ, they remained still significantly increased as compared to control group. Our results suggest that TMZ increases the synthesis of purine and pyridine nucleotides leading to improvement of energy status of cells. Moreover in histological pictures the severe tubular necrosis in kidneys from animals treated with TMZ was not detected. In kidneys from animals receiving TMZ the prevalence of mild ATN was observed. Various experimental studies have shown that TMZ has preserved the intracellular concentration of ATP and inhibited the extracellular leakage of K+ during cellular ischemia (Hauet et al., 1998b). Additionally it prevents excessive release of free radicals, which are particularly toxic to phospholipid membranes and are responsible for both the fall in intracellular ATP concentration and the extracellular leakage of K+ (Catroux et al., 1990; Maupoil et al., 1990). The protective effect of TMZ on the decreased levels of NADPH caused by ischemia-reperfusion suggests that this agent has some beneficial effects against the activation of NADPH-dependent oxidases. They represent the most significant O2 source in tissues. TMZ is also efficient in preventing inflammatory cell infiltration (Baumert et al., 2004). Current immunosuppressive protocols use treatments that might worsen nonimmunological mechanisms such as ischemia, hypertension and direct drug nephrotoxicity. TMZ has been shown to restore ATP synthesis from cyclosporine-treated isolated mitochondria suspensions (Simon et al., 1997). Moreover, it has been shown that TMZ significantly reduced lipid peroxidation after 60 min warm renal ischemia followed by 15 min reperfusion (Hauet et al., 1998c). TMZ decreased also the concentrations of malondialdehyde and increased the activity of glutathione peroxidase in rat's kidney after warm ischemia (Ozden et al., 1998). Recipient treatment with trimetazidine improved graft function and protected energy status after lung transplantation (Inci et al., 2001). The effects of trimetazidine in a rat model of renal ischemia–reperfusion injury were investigated (Kaur et al., 2003). The ischemic kidneys of rats showed severe hyaline casts, epithelial swelling, proteinaceous debris, tubular necrosis, medullary congestion and hemorrhage. Trimetazidine markedly reduced elevated levels of tissue lipid peroxidation and significantly attenuated renal dysfunction and morphological changes in rats subjected to renal ischemia–reperfusion. Singh et al. studied the effect of trimetazidine on ischemia–reperfusion induced renal failure in rats as well as the protective effect of trimetazidine

against the damage inflicted by reactive oxygen species. Pretreatment of animals with trimetazidine markedly attenuated renal dysfunction, morphological alterations and restored the depleted renal antioxidant enzymes (Singh & Chopra, 2004). The results of present study have shown that trimetazidine reduced the dephosphorylation of nucleotides caused by renal ischemia-reperfusion and this correlated with a reduction of histological evidence of renal injury. Because the rejection is initiated by the response to injury sustained during the transplant process and because TMZ may diminish the reperfusion injury, it seems that this drug could be useful in limiting the complications after cadaver organ transplantation and preventing the initiation of rejection.

3. Conclusion

1. The profile of oxypurines and purine nucleosides in the renal vein of the reperfused graft in humans appears to be a reliable marker of the energy status of kidney tissue and a prognostic factor for early graft function.
2. Concentrations of hypoxanthine and xanthine in the renal and peripheral vein are useful for monitoring the energy status of the graft during reperfusion.
3. The early phase of reperfusion in humans is without significant effect on the activity of superoxide dismutase, catalase, or glutathione peroxidase in erythrocytes from the graft vein. The activity of anti-oxidative enzymes in erythrocytes from the graft vein does not correlate with early graft function and as such is without value as a prognostic factor for graft function.
4. Hypoxanthine appears to stimulate catalase activity which is an important element of the antioxidant system.
5. Trimetazidine inhibits dephosphorylation of purine nucleotides and histological changes in tubules of the ischemic rat kidney, thereby limiting the extent of ischemia-reperfusion injury.

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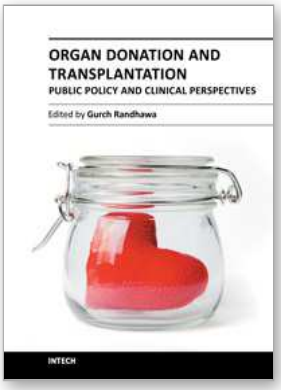

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Transplantation has succeeded in prolonging the lives of those fortunate enough to have received the gift of a body organ. Alongside this life-saving development, there lies another sadder side to the story - there are not enough organs to meet the ever increasing demand. This not only places an increasing emotional and physical burden among the waiting patients and families but heaps a great financial burden upon health services. This book provides an analysis and overview of public policy developments and clinical developments that will hopefully ensure an increased availability of organs and greater graft survival. Medical, policy, and academic experts from around the world have contributed chapters to the book.

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