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Retrograde oxygen persufflation in combination with UW solution enhances adenine nucleotide contents in ischemically damaged rat kidney during cold storage

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Abstract Retrograde oxygen persufflation (ROP) has been reported to be beneficial to kidney preservation. The purpose of this study was to investigate whether use of ROP during cold storage (CS) with Universita of Wisconsin (UW) solution could ameliorate energy metabolism and functional recovery of ischemically injured rat kidneys and, moreover, to study the particular role of adenosine (ADO) in CS with ROP. Kidneys subjected to 30 min of warm ischemia (WI) were preserved for 24 h in 4 °C UW solution with or without ROP and with or without ADO. Measurements of tissue high-energy phosphate levels showed that reduced total adenine nucleotides (TAN) after 30 min of WI further declined during the subsequent CS. In ROP kidneys, however, TAN were less reduced, suggesting that even during CS, TAN can still be regenerated in the injured kidneys when ROP is combined with UW solution. When UW did not contain ADO, regeneration of TAN by ROP was slightly less than in the case of UW with ADO. This indicates that the supply of molecular oxygen is a significant factor in TAN resynthesis during CS. There was no statistically significant difference in survival rate between the ROP and CS groups, indicating that an improved energy status is not the sole determinant of functional recovery. We conclude that the gaseous oxygen supply provided by ROP during CS in UW solution ameliorates the energy state of ischemically injured rat kidneys and that exogenous ADO from the UW solution contributes to the improvement of energy metabolism to a limited extent.

Key words Kidney, preservation, retrograde oxygen · Preservation, kidney, retrograde oxygen · Retrograde oxygen, preservation, kidney · Adenosine, kidney, preservation

Introduction

An improvement in renal viability by therapeutic interventions during hypothermic preservation is a prerequisite to the recovery of post-transplant renal function when using kidneys from non-heart-beating donors (NHBD). Kidneys from these donors are characterized by a period of warm ischemia (WI) prior to hypothermic preservation. It is known that preservation of renal viability becomes more difficult when the kidney has suffered from a previous period of WI. Moreover, the protective effect of hypothermic storage is reduced if it is preceded by WI [6].

Nowadays, simple cold storage (CS) and hypothermic machine perfusion (MP) are the two most commonly used methods for preserving kidneys. It is generally accepted that CS and MP are equally effective in 24-h hypothermic preservation of undamaged kidneys and that MP is superior to CS in renal preservation longer than 48 h [1]. With respect to preservation of the is-

chemically damaged kidney, we have shown that MP is able to negate the detrimental effects of a serious warm ischemic injury up to 30 min in dogs [2]. The disadvantage of MP is its relative complexity and high cost. Conversely, CS is easy to perform and inexpensive, but it cannot provide the organ with the substances necessary for ongoing metabolism. It fails to prevent progressive deterioration during the period of storage and is less effective after a significant period of WI [3].

Retrograde oxygen persufflation (ROP) adjunct to CS is simple and cheap, and might therefore become an alternative to machine preservation of the ischemically damaged kidney. ROP was reported as having a beneficial effect on both experimental and clinical hypothermic preservation of kidneys [5, 11, 12–15]. This method is based on supplying the kidney with gaseous oxygen via the renal vein during CS and allowing the gas to escape at the renal surface through "pin-prick" perforations.

Following cessation of the oxygen supply to the kidney due to circulatory arrest, oxidative phosphorylation in the mitochondria ceases, whereas the need for energy continues. As a consequence, the adenylate pool starts to decrease and the tissue cells lack energy for maintenance of their normal function [4, 16]. When the duration of WI is prolonged, the resynthesis of adenosine triphosphate (ATP) is further impaired. Hypothermia does not stop metabolism entirely, but it slows down reaction rates and delays cell death [1, 10]; oxygen and energy substrate are still required and consumed during hypothermic preservation. Therefore, the supply of oxygen and energy substrates is important for the maintenance of cellular energy of the cold-preserved organs. ROP during CS can provide the preserved kidney with a sufficient amount of oxygen and UW solution contains ATP precursors such as adenosine (ADO). We hypothesized that the combined use of ROP with UW solution would improve the energy state of the ischemically injured kidney during CS.

The present study was designed to answer the following questions: Are ATP and/or other adenine nucleotides in the ischemically injured rat kidney consumed during CS with UW solution? Does ROP in combination with UW solution improve ATP resynthesis and enhance the total adenine nucleotide (TAN) content in these kidneys during CS? If so, is the exogenous ADO present in UW solution involved in ATP regeneration and recovery of the adenine nucleotide content? Finally, does ROP, when added to CS, provide the rat kidney, which has been subjected to 30 min of WI, with an immediate life-sustaining post-transplant function?

Materials and methods

Adult male inbred Lewis rats weighing 250–300 g were used. The animals were housed in a room at a constant temperature (22 °C) with free access to tap water and food. The rats were anesthetized with ether and placed on a heating device to maintain body temperature at 37 °C. Both kidneys were exposed through a midline abdominal incision and dissected free. WI of both kidneys was induced by crossclamping the aorta above the renal arteries for 30 min. A cannula was inserted into the aorta below the renal arteries. At the end of the warm ischemic period, the kidneys were flushed with 4 °C UW solution (Dupont Pharma, USA) through the aortic cannula while the outlet was secured by opening the inferior vena cava (IVC) at the diaphragm level. The technique of harvesting both kidneys was performed as described previously [20]. In brief, the right kidney was procured with an upper segment of the aorta and IVC. The left renal vein was severed at its junction with the IVC, and the left kidney was harvested with a lower segment of the aorta and its long renal vein. The ureters were cut as close to the bladder as possible. The procured kidneys were stored in 4 °C UW solution for 24 h.

Technique of the ROP

ROP was performed as described earlier [12, 14]. In short, the graft's IVC or renal vein was cannulated, the anterior and posterior renal surfaces were perforated at evenly distributed sites to a depth of 1 mm with a 33-gauge needle, and filtered gaseous oxygen with a flow rate of 20 ml/min was administered through the IVC or renal vein into the kidney under a pressure of 15–25 mmHg. It was found that after the first few minutes the oxygen perfusion pressure remained constant. The oxygen supply by ROP was continued during the 24 h of UW preservation.

Tissue adenine nucleotides

Fifty kidneys were divided equally into five groups (Table 1). Group 1 included the kidneys removed before induction of ischemia and frozen in liquid nitrogen (nonischemic kidneys). In group 2 (WI group), the kidney was subjected to 30 min of WI by crossclamping the aorta and immediately frozen in liquid nitrogen at the end of WI. In group 3 (CS group), the kidneys were procured after the 30 min of WI, subsequently cold-flushed with UW solution, and preserved for 24 h in UW solution. In group 4 (ROP group), kidneys were treated in the same way as in group 3, but ROP was added during 24 h of CS. In group 5, kidneys were treated like those in group 4, but the UW solution used did not contain adenosine (ADO); this was the non-ADO group. At the end of 24 h of hypothermic storage, the kidneys were frozen in liquid nitrogen and stored at –80 °C for biochemical assessments of tissue adenine nucleotides and related compounds.

The concentration of energy metabolites in the renal tissue was determined by high-performance liquid chromatography (HPLC) [19]. Before analysis, the tissue samples were lyophilized at –30 °C. Adherent blood was subsequently removed. The samples were weighed and extracted in 25–50 µl/mg tissue $HCLO_4$ (2 mol/l). The tissue $HCLO_4$ mixture was centrifuged at 1200 *g* for 5 min at 4 °C and the supernatant was frozen in liquid nitrogen. Following the addition of 40–80 µl/mg tissue $KHCO_3$ (2 mol/l) to the supernatant, this mixture was allowed to thaw during centrifugation at 1200 *g* at 4 °C for 1 h. A Varian Vista 5000 (Varian, Wallnut Creek, Calif., USA) equipped with a narrow bore, stainless steel column filled with spherical liChrosorb RP-18 particles of

Table 1 Treatment used in each of the experimental groups

Group	Treatment
1	No ischemia (normal kidneys)
2	30 min of warm ischemia (WI9
3	30 min of WI, 24 h of cold storage (CS) in UW solution
4	30 min of WI, 24 h of CS in UW solution with retrograde oxygen persufflation (ROP)
5	30 min of WI, 24 h of CS in UW solution not containing adenosine (ADO) with ROP

5 μm (Merck, Darmstadt, Germany) was used for a gradient HPLC analysis. The injection volume of standard or sample extract was 10 μl. After each single run, which took 30 min, the column was re-equilibrated for 15 min. Peaks were quantitated at 254 nm with a Varian 604 data system using a conversion factor of peak area per concentration of a known external standard. Standards were of the highest purity available. Adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inosine monophosphate (IMP) were purchased from Boehringer (Mannheim, Germany), hypoxanthine (HX) and ADO from Merck (Darmstadt, Germany), and ATP, inosine (INO), and xanthine (XAN) from Sigma (St. Louis, Mo., USA). Total adenine nucleotide (TAN) levels were calculated as the sum of the ATP, ADP, and AMP. The total degradation product (TDP) levels were determined by summing IMP, ADO, INO, HX, and XAN. The total pool of purine compounds was considered as the sum of TAN and TDP. Data are presented as mean ± SEM. The Mann-Whitney U-test was used to test for significant differences between the groups. Differences were regarded to be statistically significant when P was below 0.05.

Functional recovery of kidneys after syngeneic transplantation

Ten rats were used as donors. Anesthesia and renal harvesting were performed as described above. Both kidneys were harvested after the induction of WI for 30 min and flushed with cold UW solution. Ten kidneys were preserved with CS in UW solution at 4 °C (CS group); ROP was used during the preservation of the other ten kidneys (ROP group). After 24 h, the preserved kidneys were transplanted in the heterotopic position on the right side, followed by bilateral native nephrectomy of the recipient rats. During reperfusion with blood, the ROP kidneys lost some drops of blood from the surface puncture holes, which closed in 1–2 min. The ureter was implanted directly into the bladder, and thereafter the kidney was anchored to the posterior abdominal wall. After transplantation, the animals were observed for a period of 2 weeks to assess recovery of renal function. Blood samples were taken for determination of serum creatinine levels on days 2, 4, 7, and 14 postoperatively. The rats were sacrificed under ether anesthesia if they had a concentration of serum creatinine higher than 900 μmol/l or showed very severe clinical deterioration. The rats that survived for 14 postoperative days were considered survivors. Fischer's exact test was used to calculate the differences in survival rate between the groups. Histological studies under the light microscope were performed in the nonsurvivors to detect any vascular thrombosis.

Results

Tissue adenine nucleotides and degradation products

In control, nonischemic kidneys (group 1), the TAN content amounted to 16.1 μmol · g^{-1} dry weight (Fig. 1), whereas ATP and ADP together contributed to 95 % of TAN (Fig. 2). The level of TDP was very low in the nonischemic kidneys (0.35 μmol · g^{-1} dry weight), with HX, XAN, INO, and ADO below the level of detection (Fig. 3). The total pool of purine compounds in the nonischemic kidneys was 16.5 ± 1.1 μmol · g^{-1} dry weight (Fig. 1).

Compared with that in the nonischemic kidneys, TAN content in group 2 (30 min of WI) decreased to 4.3 μmol · g^{-1} dry weight ($P < 0.001$; Fig. 1), and ATP and ADP levels significantly declined, contributing to only 40 % of TAN (Fig. 2), whereas AMP levels were found to be elevated ($P < 0.05$). All of the degradation products tested in this group significantly increased and, as a result, TDP levels dramatically increased to 8.2 μmol · g^{-1} dry weight ($P < 0.001$; Fig. 3). Although the decrease in TAN levels was accompanied by an increase in TDP levels, the total pool of purine compounds (12.5 ± 0.5 μmol · g^{-1} dry weight) was significantly decreased ($P < 0.05$; Fig. 1).

Compared to that in group 2, the TAN content of kidneys in the CS group (group 3) was further reduced to 2.6 μmol · g^{-1} dry weight ($P < 0.05$) by the end of 24 h of CS. This was mainly caused by a further decline in ATP and AMP ($P < 0.05$; Fig. 2). The tissue TDP contents increased to 12.2 μmol · g dry weight ($P < 0.001$; Fig. 1). ADO, INO, and HX contents showed a significant increase, IMP did not change, and XAN decreased ($P < 0.05$; Fig. 3). Because of the slight decrease in TAN content and the substantial increase in TDP level ($P < 0.05$), the sum of purine compounds (14.8 ± 1.9 μmol · g^{-1} dry weight) in the CS group was significantly higher than that after the 30 min of WI (group 2; $P < 0.05$) and eventually reached the nonischemic levels.

ROP treatment during CS (group 4) resulted in increased TAN (7.2 μmol · g^{-1} dry weight) as compared to that in groups 2 and 3 ($P < 0.001$; Fig. 1). This increased TAN content was associated with higher tissue levels of ATP and ADP (Fig. 2) which, together, contributed to 90 % of TAN. The AMP content in the ROP group was reduced ($P < 0.05$). The TAN level was, however, still lower than that of the nonischemic kidneys ($P < 0.001$). With respect to the degradation products (Fig. 3), ROP treatment significantly increased the level of IMP, whereas the content of HX and XAN was found to be reduced ($P < 0.05$) compared to group 3. No significant difference was found in the levels of TDP ($P < 0.05$), ADO, INO, or HX. The total pool of purine compounds in the ROP-treated kidneys amounted to 20.4 ± 0.7 μmol · g^{-1} dry weight, which was signifi-

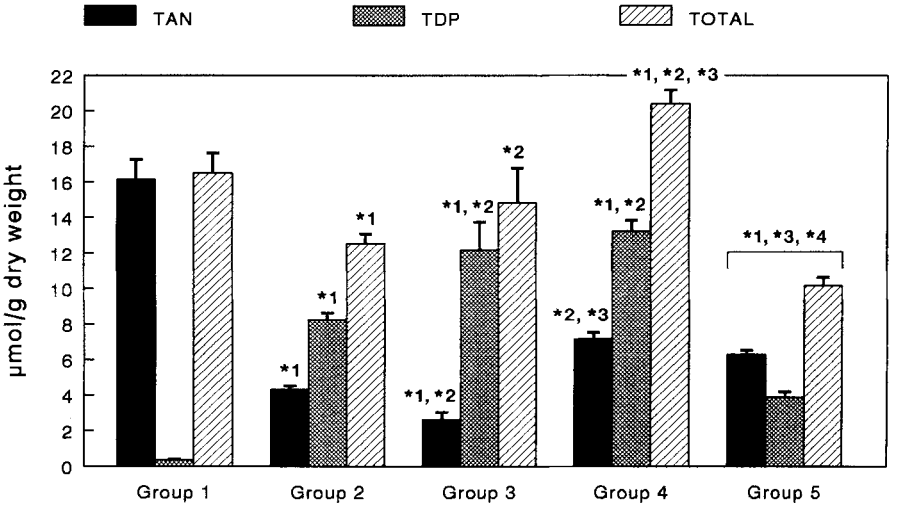

Fig. 1 The levels of total adenine nucleotides (*TAN*), total degradation products (*TDP*) and total pool of purine metabolites (*TOTAL*) in kidney tissue of groups 1–5. Data are presented as means ± SEM. Group 1, nonischemic kidneys; group 2, 30 min of WI; group 3, 30 min of WI followed by 24 h of CS in UW solution; group 4, 30 min of WI followed by 24 h of CS in UW solution with ROP treatment; group 5, 30 min of WI followed by 24 h of CS in UW solution in absence of ADO with ROP treatment. ^{*1} $P < 0.05$ compared to group 1; ^{*2} $P < 0.05$ compared to group 2; ^{*3} $P < 0.05$ compared to group 3; ^{*4} $P < 0.05$ compared to group 4

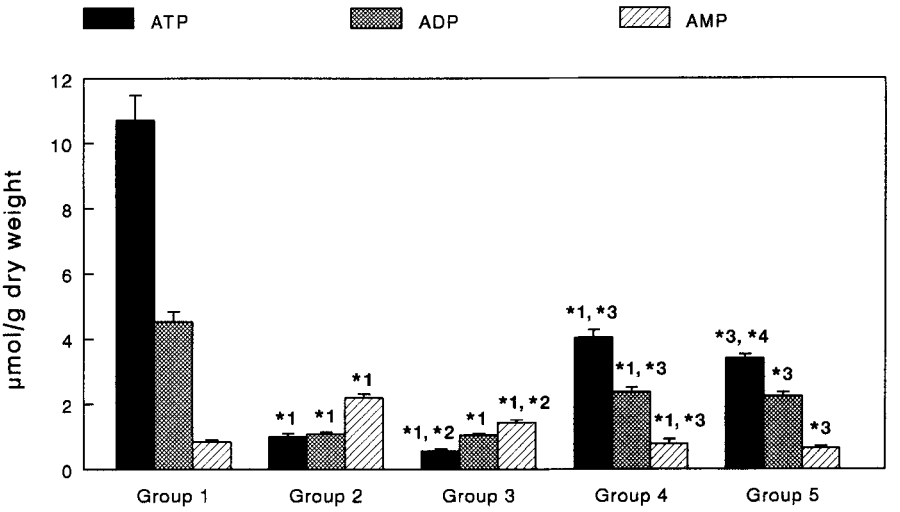

Fig. 2 Levels of ATP, ADP, and AMP in kidney tissue of groups 1–5. Data are presented as means ± SEM. Symbols as in Fig. 1

cantly higher than that in the CS group (group 2; $P < 0.005$) and even higher than that of nonischemic kidneys (group 1; $P < 0.05$). This was caused by the higher TAN level in the ROP-treated kidneys than in the CS group (group 3) and by the higher TDP level in the ROP-treated kidneys than in the nonischemic kidneys (group 1).

When the UW solution without ADO was used during the ROP-treated CS (group 5), the TAN level amounted to 6.3 µmol · g^{-1} dry weight (Fig. 1), which was lower than that of the ROP group (group 4; $P < 0.05$) but much higher than that of the CS group (group 2; $P < 0.001$). ATP and ADP together contributed to 90 % of the TAN content (Fig. 2). The TDP level was 3.9 µmol · g dry weight (Fig. 3), which was much lower than those of groups 2, 3, and 4 ($P < 0.001$). The level of IMP, ADO, INO, and HX in this non-ADO group was dramatically lower than ($P < 0.001$), and XAN the same as in the ROP group (Fig. 3). All of the degradation products but IMP were lower than those in groups 2 and 3. The total pool of purine compounds in group 5 was 10.2 ± 0.5 µmol · g^{-1} dry weight, which was lower than that in all of the other groups (Fig. 1). This is mainly attributed to the lower concentration of TDP in group 5 than in the CS and ROP groups (groups 3 and 4, respectively).

Functional study

None of the ten kidneys from the CS group sustained life (survival rate 0/10), while 3 out of 10 rats survived in the ROP group. This difference was not significant according to Fischer's exact test. The deaths of the rats

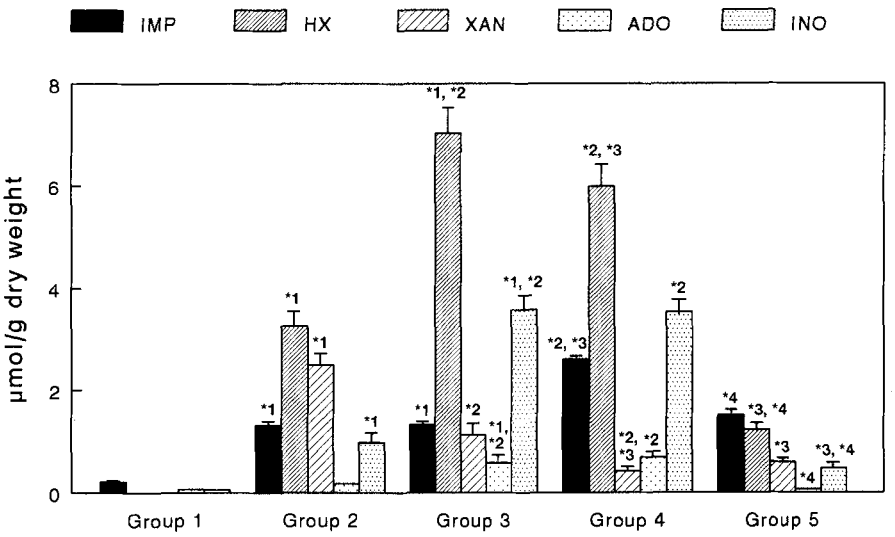

Fig. 3 Levels of different degradation products in kidney tissue of groups 1–5. Data are presented as means ± SEM. *IMP* inosine monophosphate, *HX* hypoxanthine, *XAN* xanthine, *ADO* adenosine, *INO* inosine. Symbols as in Fig. 1

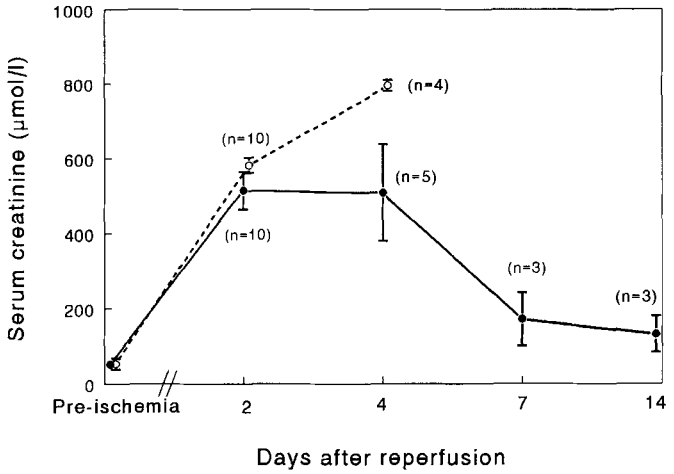

Fig. 4 Levels of serum creatinine in rats with kidneys preserved by cold storage (–○–) or retrograde oxygen persufflation (–●–) in UW solution. Data are presented as means ± SEM. There were no significant differences in the values of the serum creatinine between the two groups at day 2 and 4 ($P = 0.69$ and $P = 0.22$, respectively)

mostly occurred 2–4 days after transplantation. In all cases they were due to renal failure. There was no vascular thrombosis found in either group. Microscopy of the kidneys from the surviving rats showed residual degenerative changes and compensatory hypertrophy of the nephrons. Necrosis affecting entire proximal tubules was found in all kidneys of rats that were sacrificed as well as of rats that died spontaneously. Serum creatinine values are shown in Fig. 4. Two days after renal transplantation, the mean serum creatinine was 582 µmol/l in the CS group and 515 µmol/l in the ROP-treated rats. On the 4th postoperative day, the mean creatinine of the two groups was 795 µmol/l and 509 µmol/l, respectively. Because of the large number of animals that were died by day 4, no reliable statistical analysis with paired data could be made.

Discussion

The cellular high-energy phosphate store of the kidney is substantially reduced after 30 min of normothermic ischemia. The decline in ATP content is most likely due to both decreased mitochondrial oxidative phosphorylation and continued consumption. Our study shows that 30 min of WI depletes TAN contents to 25 % of the levels in nonischemic kidneys, increases TDP levels by 27 times that of nonischemic kidneys, and decreases the total pool of purine compounds. In line with many previous studies [3, 9, 18], this finding demonstrates that during normothermic ischemia ATP is rapidly degraded to ADP and AMP, and subsequently to IMP, ADO, INO, HX, and XAN. The increase in TDP was found to be less than the decline in TAN. At present, no satisfactory explanation can be offered for this discrepancy. Probably some end products are formed that are not detectable by our HPLC analysis.

When 30 min of WI was followed by 24 h of hypothermic storage (group 3), the injured rat kidneys lost still more ATP and TAN (up to 18 % of the nonischemic kidneys) and gained degradation products, which indicates that catabolism in the ischemically damaged kidney continues during hypothermic preservation. The decrease in TAN levels is in accordance with findings from our previous work in dogs showing that CS is unable to maintain TAN levels [8]. It should be emphasized that the quantity of degradation products accumulated in the cold-stored kidneys exceeded the amount of TAN

lost. As a consequence, the total pool of purine compounds was normalized during the CS as compared to the levels after 30 min of normothermic ischemia (group 2). This increase can most likely be explained by the fact that the ADO that is present in the UW solution is taken up by the cells and is mostly degraded into HX and INO, since the increase in TDP in this group was mainly due to the increase in these two products rather than in ADO itself.

It is generally agreed that rat kidneys can tolerate 30 min of WI with a 100 % animal survival rate [3, 7], suggesting that the drop in the ATP and TAN levels does not prevent restoration of renal function after reperfusion and indicating that the renal damage caused by 30 min of WI is reversible. However, the study of survival rate demonstrated that the cold-stored kidneys (CS group) could not sustain life after transplantation. Obviously, the renal injury induced by 30 min of WI deteriorated further and became irreversible due to the 24 h of CS. This finding indicates that although UW solution can provide ADO during CS, the ability of the kidneys to resynthesize ATP from this precursor might be limited because of the cold temperature, lack of oxygen, and previous warm ischemic insult. It is known that a proper amount of ATP is needed to maintain cellular viability and that cellular energy is required for resynthesis of ATP from purine degradation products (including ADO). Under this simple CS condition, the cells obviously lack energy for the restoration of the TAN pool.

We observed that during hypothermic UW preservation with a gaseous oxygen supply by ROP, ATP in the stored kidney (group 4) was resynthesized, since the ATP contents were significantly higher than those of kidneys stored in UW solution only. As a consequence, the TAN contents were enhanced (45 % of the nonischemic kidneys) and the total pool of purine compounds was increased without enhancement of TDP (as compared to group 3). We also found that IMP was increased and HX decreased. These findings suggest that gaseous oxygen in the cold-stored renal tissue promotes the conversion of HX into IMP, most likely via the salvage pathway, and that kidney cells extract more exogenous ADO from the surrounding CS medium when molecular oxygen is continuously supplied. Therefore, as far as adenine nucleotide regeneration is concerned, ROP with UW solution in fact provided better preservation than CS with UW solution only.

When ADO was taken out of the UW solution (group 5), ROP treatment still enhanced ATP regeneration (40 % of the nonischemic kidneys) as compared to the CS group. The improved ATP levels, however, were only 15 % less than those in the ROP group (group 4, UW with ADO). This indicates that oxygen is a more important factor for preservation of ATP and TAN during CS than exogenous ADO. Moreover, TDP levels in this non-ADO group became much lower than those in the ROP group with ADO, suggesting that the bulk of ADO extracted from UW solution is degraded to HX and XAN, etc.

Ischemia and reperfusion-induced renal injury is most likely caused by a multifactorial process. Oxygen free radicals, generated immediately after blood reperfusion through the ischemic kidney, are among the factors that might contribute to the damage inflicted upon renal cells [17] and, consequently, that might compromise the functional outcome of the reimplanted organs. In addition to mitochondria, the conversion of HX to XAN and uric acid is believed to be an important source of oxygen free radicals. The latter process is catalyzed by xanthine oxidase. It is worth noting that in this study, the presence of ADO in the UW solution substantially increased levels of HX at the end of 24 h of CS, irrespective of additional ROP treatment. Further studies are required to elucidate whether exogenous ADO, via high tissue HX levels, exerts adverse effects on ischemically injured kidneys stored under hypothermic conditions.

Our functional study shows that ROP, even in combination with UW solution, was still insufficient to significantly improve the immediate life-sustaining function of ischemically injured renal grafts in rats. It is known that ischemia and hypothermic preservation-induced damage to the kidney is multifactorial. The importance of ATP levels for the preserved organ still remains unclear. Our experimental results demonstrate that the levels of ATP and TAN at the end of CS are not directly related to the functional recovery of ischemically injured kidneys. Of course, we still do not know whether, during CS with ROP, the biosynthetic machinery for resynthesizing ATP is capable of meeting the energy demands of the cell. The mechanism of renal damage caused by a period of WI is still unclear. It has been suggested that damage inflicted upon the kidney during the preceding period of WI increases the rate of decline of kidney function during the cold preservation [6], which might consequently influence the use of oxygen for ATP synthesis in the damaged mitochondria. It remains to be established what additional means have to be taken to improve functional recovery of ischemically injured kidneys during ROP-treated CS.

A similar experiment performed in a dog model (also with 30 min of WI preceding 24 h of CS) by Rolles and his coworkers demonstrated a higher survival rate in the ROP-treated group [12]. The difference in results can be explained by the fact that the animal model we used in this study was relatively more critical than theirs. It is known that the rat kidney is more sensitive to ischemic insult than the dog kidney [17].

Although the use of ROP with UW solution in this study was insufficient to ameliorate the functional recovery of the damaged rat kidneys in terms of survival

rate, on the basis of this and other studies [5, 11, 12–15], we feel that ROP can and should be used in the preservation of kidneys for transplantation. We recomment it especially when simplicity and energy improvement are required during CS of ischemically injured kidneys, as with the preservation of NHBD kidneys.

We conclude that ATP in ischemically damaged kidneys is consumed during CS, and that high-energy phosphates can be generated in these kidneys when ROP is combined with UW solution. Although exogenous ADO (normally present in UW solution) enhances the resynthesis of ATP and TAN during CS with ROP, molecular oxygen has proved to be a more important factor in the maintenance of cellular ATP levels in preserved, injured kidneys than the presence of ADO.

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