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Interstitial fluid analysis for assessment of organ function

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Abstract: Evaluation methods are required for non-heart-beating donor (NHBD) kidneys to ensure the success of transplantation. In this study, the microdialysis technique was employed for the *ex-vivo* assessment of hypothermically preserved NHBD kidney function. Microdialysis probes were placed in the renal cortex of 2 h warm ischaemic porcine kidneys to monitor interstitial pyruvate dynamics during hypothermic machine perfusion with perfusate containing 29.4 mM fructose-1,6-diphosphate (FDP). The presence of exogenous FDP in the perfusate induced no changes in the renal flow rate and vascular resistance, renal artery effluent biochemistry, or pyruvate concentration relative to untreated control kidneys. Significant increases in pyruvate production ($P < 0.05$), however, were observed after 12 h of perfusion in the interstitial fluid of FDP-treated kidneys relative to control kidneys. After 24 h of perfusion, interstitial fluid concentrations of pyruvate were 149.1 ± 58.4 vs. 55.6 ± 17.9 μM ($P < 0.05$) in the FDP and control group, respectively. The microdialysis probe collected the interstitial fluid directly from the cellular sites of metabolic and synthetic activity, where perfusate dilution was minimal. Consequently, the biochemical changes induced by the organ metabolic activity were detected only at the interstitial level, in the microdialysates. Interstitial fluid pyruvate may be a good indicator of kidney function. The addition of FDP to the perfusion solution during ischaemic kidney preservation resulted in enhanced pyruvate production in the extracellular space, indirectly reflecting an increase in anaerobic ATP production. The pyruvate will be transformed during organ reperfusion into acetyl Co-A enzyme allowing an immediate start of aerobic metabolism. This in turn can increase the amount of ATP available to the cells and may help prevent reperfusion injury upon transplantation.

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In the year 2001, over 81 500 patients in need of an organ transplant were registered on the waiting list, and 51 000 of them required a kidney (1). The patient waiting list gets longer every year, outpacing organ availability. The use of non-heart-beating donor (NHBD) kidneys has been adopted as a partial solution to the severe clinical organ shortage, in spite of relatively high rates of delayed onset of post-reperfusion graft function. Machine perfusion however, has been proved advantageous for the preservation of ischaemically damaged kidneys. Relative to static storage, it results in improved immediate post-transplant graft function, better survival rates and superior preservation of microcirculatory integrity (2–8).

Reliable kidney preservation and quality control methods are needed to provide successful organ transplantation and minimization of organ discard rate. Currently, besides flow rate and vascular resistance during hypothermic machine perfusion, there are no other entirely accepted indicators of kidney viability (9–11). Attempts have been made to determine the kidney biochemical profile through the analysis of renal artery effluent

samples during organ perfusion (12). In this study, we propose the use of a new method to assess kidney metabolic activity, based upon microdialysis. The microdialysis method has been successfully employed in an extensive array of applications, from small to large animals and human, from brain and myocardium to liver, subcutaneous tissue, eye, and blood, from pharmacokinetic to metabolic studies (13–23). A very small, blood-capillary-analogous dialysis probe is implanted into the tissue and isotonic fluid is forced through the probe inlet port into the probe membrane. A low, constant flow rate is needed to allow isotonic fluid equilibration with the interstitial fluid and collection of the dialysate at the outlet probe port. Microdialysis offers the advantage of directly collecting the interstitial fluid from the extracellular space, next to the cellular sites of metabolic and synthetic activity.

Under ischaemic (hypoxic) conditions, cells revert to the anaerobic glycolytic pathway in an attempt to satisfy their energy requirements. During glycolysis fructose-1,6-diphosphate (FDP) is generated from glucose and is further transformed into pyruvate while energy is being produced (24). In the absence of oxygen, pyruvate is transformed into lactate rather than acetyl CoA, or is accumulated in the cells. The build up of pyruvic acid causes a decrease in the pH, which inhibits glucose conversion to FDP. The build up of lactate inhibits glycolysis through the inhibition of phosphofructokinase (PFK), a key enzyme in FDP synthesis. If exogenous FDP is made available to the cells, the PFK blockade can be bypassed and the glycolytic pathway can be maintained.

The addition of FDP to organ preservation solution has been shown to be beneficial for the function of some organs. Hearts preserved in solutions containing FDP have better function, regain their beat faster and have lower coronary vascular resistance after normothermic reperfusion when compared to untreated control hearts (25–28). Twenty-four hour cold ischaemic livers preserved with FDP-containing solution have similar functional and histological parameters as livers preserved with plain University of Wisconsin solution (29). The addition of 20 mM FDP to UW solution results in good hypothermic preservation of rat Kuppfer cells for 12 and 24 h (30). After 18 h of cold ischaemia, kidneys flushed with a solution containing 25 mM FDP have significantly reduced histological damage, less tubular necrosis and tubular dilatation than kidneys flushed with plain EuroCollins or UW solution (31).

In this study porcine kidneys harvested from NHB donors were hypothermically machine perfused with FDP-containing perfusate. The effect of FDP on kidney metabolic performance was assessed using the microdialysis technique. The kidneys were instrumented with microdialysis probes and sampling of the interstitial fluid was periodically performed during 24 h continuous machine perfusion. We hypothesized that the presence of FDP in the kidney perfusate would result in the continuation of glycolysis, improved renal function, and that interstitial fluid biochemistry profiles would be superior to those obtained from renal artery effluent.

Materials and methods

An experimental non-heart-beating donor animal model was employed to study the influence of exogenous FDP on kidney function during *ex-vivo* hypothermic preservation. Animal care and handling complied with the 'Principles of Laboratory Animal Care' as formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' published by the National Research Council (National Academy Press, 1996). Twelve NHBD kidneys were obtained from adult male pigs (25–30 kg, Hambone Farms, SC) after sustaining 2 h of *in-situ* warm ischaemia. Following the aseptic explantation, kidneys were cold flushed for 30 min with Unisol^{TM} (UHK, Organ Recovery Systems (ORS), Chicago IL) at low pressure until the effluent was clear and residual blood was eliminated, and transported on ice, in the flushing solution to the perfusion site. The cold ischaemic time (CIT) was kept near 1h. Then, the kidneys were hypothermically perfused at 5–7°C and 30–60 mmHg arterial pressure for 24 h using the continuous pulsatile perfusion prototype of the LifePort^{TM} kidney preservation system (ORS, Chicago, IL). The control kidneys were perfused with KPS-1 solution (ORS, Chicago, IL) ($n = 4$, CIT = 53.2 ± 8.9 min). The FDP-treated kidneys were perfused with KPS-1 solution supplemented with 10 g/L (29.4 mM) FDP sodium salt (Sigma, St. Louis, MO) ($n = 8$, CIT = 70.8 ± 13.5 min).

Porcine renal interstitial fluid was sampled using flexible peripheral tissue-type calibrated microdialysis probes of 20 kDa molecular cut-off and 10 mm membrane length (CMA/20, CMA Microdialysis, Sweden). A single microdialysis probe was inserted into the renal cortex (pole) of each kidney (Fig. 1) and interstitial fluid samples were collected hourly following an initial 30 min period of local fluid equilibration. The probes were perfused with isotonic fluid at a flow rate of 2.5 μL/min and the dialysates were collected in vials placed in a 4°C

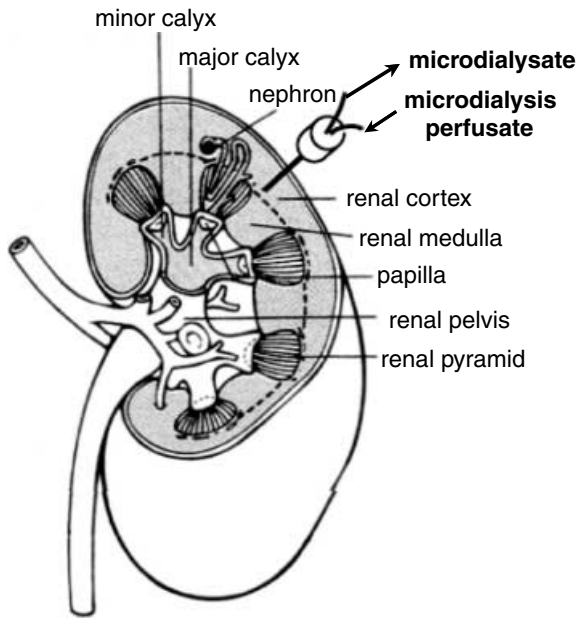

Fig. 1. Graphical illustration of the microdialysis probe (not at scale) placement into the renal cortex for the sampling of interstitial fluid. Kidney schematic adapted from Guyton and Hall (24).

refrigerated fraction collector. Renal artery effluent samples were also collected hourly, and pH, gases, ions, metabolites and osmolality were measured using a biochemical analyser (BioProfile200, Nova Biomedical, Waltham, MA). An enzyme-linked immunosorbent assay, ELISA (Sigma, St. Louis, MO) was employed to measure pyruvate concentrations in both renal artery and microdialysis samples. Kidney bath temperatures, arterial pressures and flow rates were constantly monitored and recorded. Biochemistry baseline values were recorded prior to placing the kidneys onto the perfusion system.

Results

Throughout the entire study, similar flow rate and internal vascular resistance values (the ratio of pressure to flow rate) were recorded in both control and FDP treated kidneys. In each group, the flow rate and internal vascular resistance remained unchanged relative to the 30 min perfusion value. Average (over 24 h) flow rates and vascular resistances of 35.31 ± 0.53 mL/min and 1.033 ± 0.016 mmHg min/mL, and 36.09 ± 0.97 mL/min and 1.038 ± 0.026 mmHg min/mL, were obtained in the control and FDP-treated kidneys, respectively. During perfusion all kidneys had similar renal artery effluent biochemical profiles (in terms of pH, pCO_2, pO_2, Ca^{++}, Na^{+}, K^{+}, NH_4^{++}, glucose, glutamine, glutamate, and osmolality). With the exception of glutamate, all the other biochemical concentrations did not statistically depart from baseline during kidney perfusion. Eight and 12 h into perfusion, considerably higher concentrations of glutamate were detected in the renal effluent of FDP-treated and control kidneys, respectively, relative to baseline ($P < 0.05$, Fig. 2).

Regardless of kidney group, the pyruvate concentration of renal artery effluent kept a constant low value throughout perfusion. It fluctuated within a range of 5–27 μM, with average values of 8.95 ± 1.1 μM and 9.02 ± 0.56 μM, in the control and FDP groups, respectively. By marked contrast, interstitial fluid pyruvate production increased with time, significantly higher concentrations being detected in the extracellular space than in the renal effluent ($P < 0.05$) for all perfused kidneys (Fig. 3). Statistically significant increases in pyruvate production, relative to baseline, were detected after 14 and 9 h of perfusion in the interstitial fluid of control and FDP-treated kidneys, respectively ($P < 0.05$). After 24 h of perfusion, the extracellular space pyruvate concentration measured in the microdialysate was 149.12 ± 58.4 μM in the FDP group and 55.69 ± 17.9 μM in the control group. For the last 12 h of perfusion, noticeably higher concentrations of pyruvate were measured in the interstitium of FDP-treated kidney relative to control kidneys.

Discussion

It has been documented that kidney supply for transplantation can potentially be increased by a factor of 2–4.5 (32) by the use of viable NHBD kidneys. Once blood circulation stops, these

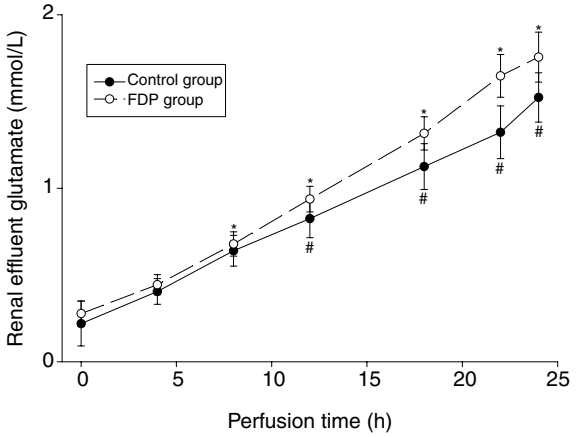

Fig. 2. Variation of renal artery glutamate concentration with perfusion time. The data are presented as mean ± SEM. *,#$P < 0.05$ vs. baseline value.

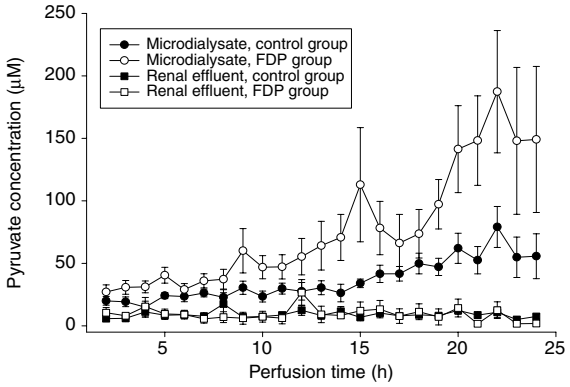

Fig. 3. Renal pyruvate concentration during machine perfusion, as a function of time. Statistically significant higher pyruvate concentrations ($P < 0.05$) were detected in the interstitial fluid relative to renal effluent. The data are presented as mean ± SEM.

kidneys sustain a period of warm ischaemia (33,34). The warm ischaemic period leads to rapid loss of high-energy metabolites, altered calcium metabolism, vascular ischaemia, and ultimately to graft failure in the first month after transplantation (35–37). From the moment of cardiac arrest, the organs start to deteriorate. Although this decay cannot be stopped completely, the rate of deterioration can be decreased by cooling. The cold ischaemic time (hypothermic preservation) alters membrane viscosity and permeability, cellular metabolism (depletion of energy-rich phosphates) and free-radical regulation. Free radicals are responsible for reperfusion injury, which is commonly observed in warm ischaemic kidneys, when basic cell functions are distorted and reactive oxygen species are generated post-reperfusion (38).

Cellular damage due to warm and cold ischaemia is triggered mainly by the imbalance between energy production and consumption. With reduced oxygen, the anaerobic glycolytic pathway becomes the cells' primary source of adenosine triphosphate (ATP). In the glycolysis phosphorylation stage FDP is generated from glucose through the use of two molecules of ATP per molecule of glucose (24). Following this, the FDP is transformed into pyruvate and four molecules of ATP per molecule of FDP are produced. However, the accumulation of lactate (from anaerobic conversion of pyruvate) inhibits PFK and implicitly the dephosphorylation energy generating phase of glycolysis.

In the present study, 2 h warm ischaemic porcine kidneys were hypothermically machine perfused for 24 h. FDP was included in the renal perfusate in an attempt to maintain the glycolytic pathway (by avoiding the PFK blockade) and help improve kidney function during preservation. The ability of sugar diphosphates, particularly FDP to penetrate the cells membrane and the mechanism by which it enters within the cytoplasm is still under debate. However, there have been studies to prove that FDP can permeate the membrane and serve as a substrate for glycolysis (25,39–41). In the present study interstitial fluid pyruvate measured in the microdialysates had significantly higher concentrations in the FDP treated kidneys relative to control kidneys. This suggests that the exogenous FDP made available to the cells was used during glycolysis and the process of pyruvate synthesis. This validates the study hypothesis that during hypothermic organ perfusion glycolysis can be maintained by the presence of exogenous FDP in the perfusate. The lactate concentration in the interstitium was not quantified. In addition to pyruvate concentration, the lactate/pyruvate ratio would be a better indicator of the extracellular space pyruvate dynamics. It would also help quantify the pyruvate left in the tissue to be used by the cells at the onset of reperfusion, for an immediate start of aerobic metabolism (Krebs cycle).

After 24 h of *ex-vivo* hypothermic perfusion all kidneys were characterized by low flow rate and high vascular resistance. The transplantation acceptance limit values of 40 mL/min flow rate and 0.8 mmHg min/mL vascular resistance (9–11) were not met, exogenous FDP did not improve these two parameters and did not reduce the effects of prolonged warm ischaemia. The low renal flow rate and high vascular resistance of ischaemically damaged kidneys are, in common clinical practice, addressed through the use of muscle relaxants and vasodilators. These were avoided in the present study to evaluate solely the effect of FDP. It has been proven, however, that FDP has rheologic and antioxidant effects in addition to its ability to promote glycolysis. Several studies have shown that FDP improves red blood cell deformability, consequently decreasing blood viscosity (42–44). FDP also has the ability to restrain the oxygen-radical related reperfusion injury, due to induced high ATP levels that in turn result in the inhibition of NADPH-oxidase, and to limit ischaemia induced conversion of xanthine dehydrogenase to xanthine oxidase (26).

A validation of the present study results using a transplantation–reperfusion model is needed. The FDP remaining in the tissue is expected to help reduce vascular resistance and increase renal flow post-reperfusion. It is anticipated that the amount of pyruvate accumulated in the extracellular space will be transformed during reperfusion into acetyl Co-A enzyme that will facilitate an immediate start

of the aerobic metabolism. This in turn will increase the amount of ATP available to the cells and minimize normothermic reperfusion injury.

The novelty of the present study consists in the use of microdialysis for kidney function evaluation. To our knowledge, no research studies of kidney preservation have employed the microdialysis technique to monitor renal extracellular space electrolytes or metabolites during either *ex-vivo* organ perfusion or static cold storage. For the last 12 years (45), however, the microdialysis method has been a useful technique in monitoring endogenous and exogenous compounds of the extracellular space, a practical method for both free moving and anaesthetized animal research, and clinical investigations. There is ample justification, therefore, to adopt this minimally invasive technique for the evaluation of kidneys for transplantation. Implementation of the method allowed accuracy in the measurement of kidney interstitial fluid components. The microdialysis probe was placed in intimate contact with the tissue, and therefore interstitial fluid pyruvate was collected from the immediate vicinity of its site of production, while its degradation prior to collection was minimized. As a result, the changes in pyruvate concentration induced by the FDP were recorded only in the microdialysates. The fall in pyruvate concentration due to transport across the extracellular space into the blood vessels, and dilution in the perfusion solution (constant volume of renal perfusate circulated in a closed loop) resulted in almost no detectable pyruvate in the renal artery effluent.

In the current study, FDP served as a substrate for anaerobic glycolysis, it was metabolized resulting in an increase in extracellular space pyruvate concentration that was detected by microdialysis. In contrast to microdialysis, biochemical analyses of the renal artery effluent were not able to detect changes in kidney metabolism. To fully evaluate the influence of exogenous FDP on the function of hypothermic kidneys *ex vivo*, a more comprehensive evaluation of renal metabolic activity needs to be performed by microdialysis, and the *in vitro* results need to be validated by an *in vivo* transplantation–reperfusion model. The interstitium biochemical profiles might be a better indicator of kidney viability, enabling more effective, valid decisions to be made on suitability of NHB donor-derived kidneys for transplantation.

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