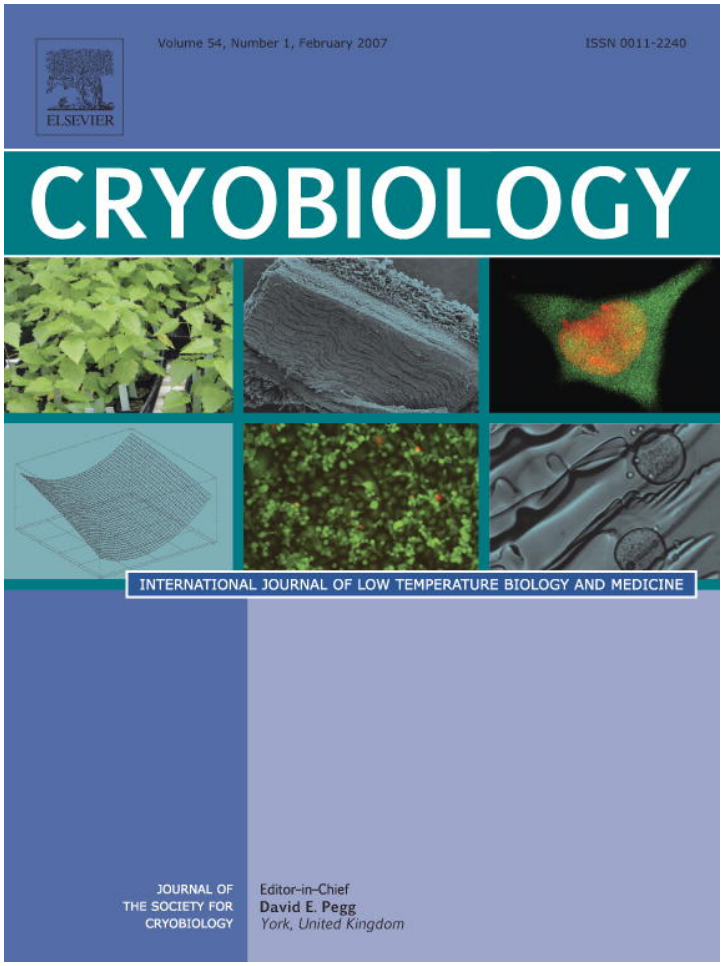

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Brief communication

Modulating biochemical perturbations during 72-hour machine perfusion of kidneys: Role of preservation solution ☆

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Abstract

This study documents renal biochemistry during hypothermic machine perfusion of kidneys. It is intended to demonstrate that a comprehensive evaluation of organ viability during *ex-vivo* preservation is needed to increase the number of organs available for transplantation and to reduce the current renal discard rate. Porcine kidneys were hypothermically machine perfused for 72 h with either Unisol™-UHK or Belzer-Machine Perfusion Solution, (Belzer-MPS). Renal perfusate samples were periodically collected and biochemically analyzed. Significant differences were measured in the renal metabolic activity between the two experimental groups while similar values for traditional parameters such as renal flow rate and vascular resistance values were recorded. The effluent of UHK perfused kidneys showed strong metabolites and NH_4^+ dynamics ($P < 0.05$ vs. baseline), while the Belzer-MPS kidneys metabolic activity led to little or no change of the effluent biochemistry relative to baseline.

Keywords: Kidney preservation; Hypothermic machine perfusion; Renal biochemistry

In recent years, the demand for transplantable, good quality human organs has grown dramatically. Nearly 70% of the current transplant waiting list patients are in need of a kidney [1]. Efficient *in-vitro* machine preservation coupled with high quality hypothermic preservation solutions and inclusive organ viability assessment can considerably increase the number of kidneys available for transplantation. Machine perfusion improves immediate post-transplant graft function [4,9,12,16], provides better survival rates and enhanced preservation of microcirculatory integrity [5,6]. Hypothermic machine perfusion allows for both extended storage and transport times and the possibility of kidney performance assessment and/or metabolic support during perfusion [7,21]. Presently, during *ex-vivo* machine perfusion, renal flow rate and vascular resistance are the only accepted indicators of kidney viability [13,15,20]. A small number of biochemical parameters and ischemic injury markers have been quantified in the renal effluent [20,11,19], however they are not commonly employed and their role in predicting kidney *in-vivo* function is still controversial. Thus, the need for a complete and diagnostically valuable evaluation of kidney function *ex-vivo* still remains and is feasible as this pilot study illustrates.

The present study is a part of an ongoing program that investigates kidney preservation technologies with the objectives of reducing the current renal discard rate and improving the use of marginal donor kidneys. We have designed a new solution, Unisol™-UHK [27], with higher buffering capacity than the commonly used preservation solutions [2] that provides superior protection of the vascular endothelium [28], and has proved optimal for acid–base regulation during prolonged hypothermic machine perfusion of kidneys [3].

The purpose of this study was to investigate the role of the perfusate in modulating kidney biochemical perturba-

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tions during lengthy machine perfusion, and to emphasize that a comprehensive assessment of kidneys *ex-vivo* performance is needed for a reliable screening of viable organs for transplantation. A more complete assessment of viability has to consider renal biochemistry in addition to the traditional biophysical parameters of flow rate and vascular resistance. For this pilot study the new Unisol™-UHK solution, an intracellular base solution [27,2,26] designed for applications at profound hypothermic temperatures (<15 °C), and Belzer's-MPS were compared. The latter is the current clinical standard for machine perfusion of kidneys [20,23].

Porcine kidneys were harvested from heart-beating donors (25–32 kg adult male farm pigs, Hambone Farms, SC) and hypothermically machine perfused. Fourteen kidneys were recovered, cold flushed at low pressure for 30 min with the perfusion solution, then immersed in the flushing solution, placed on ice, and transported to the perfusion lab. Pre-perfusion cold ischemic time (CIT) was limited to 1 h and warm ischemia was less than 3 min. Two experimental groups were considered: (i) UHK group, $n = 7$, included kidneys flushed and perfused with the Unisol™-UHK solution (Organ Recovery Systems (ORS), Des Plaines, IL), CIT = 55.57 ± 4.2 min, and (ii) Belzer-MPS group, $n = 7$, included kidneys flushed and perfused with the Belzer-MP solution (manufactured and commercialized by ORS), CIT = 60.71 ± 2.57 min. The kidneys were hypothermically perfused for 72 h at 5–8 °C and 30–50 mmHg arterial pressure using the continuous pulsatile perfusion prototype of LifePort™ Kidney Preservation System (ORS, Des Plaines, IL). The UHK solution (Table 1), prior to its use, was supplemented with 3 mM of reduced form glutathione (Sigma–Aldrich), in accordance with its chemical formulation [27,26]. The perfusate was renewed every 24 h without interrupting organ perfusion. Kidney weight was documented before and after perfusion. Renal arterial pressure, flow rate, internal vascular resistance and kidney bath temperature were constantly monitored and recorded. Renal perfusate samples were periodically collected and biochemically analyzed (BioProfile200, Nova Biomedical, Waltham MA). A reference baseline sample was collected from the organ bath prior to placing the kidney into the organ cassette. Histopathological analysis (HistoPathology Associates, Mechanicsville, VA) was performed on wedge tissue biopsies using light microscopy.

At low temperature the rate of biophysical processes [25] and chemical reactions is significantly reduced. However, the effect of cooling on renal metabolism is complex and it is more than a simple slowing-down of biochemical reactions. Preservation temperature strongly influences the nature of the preferred substrate for energy metabolism [10]. During deep hypothermic kidney preservation glycolysis is the principal source of energy, the balance between glycolysis and complete oxidation of fatty acids being controlled by oxygen tension and temperature [18]. During hypothermic preservation the high-energy reserves must be protected and the metabolism needs to be continued, however at reduced levels. The latter relies on the preservation oxygen tension, temperature and perfusion solution chemistry.

Table 1
Solutions chemical composition

Chemical components (mM)	Belzer-MPS	UHK
Ionic		
Na^+	100.0	62.5
K^+	25.0	70.0
Ca^{2+}	0.5	0.05
Mg^{2+}	5.0	15.0
Cl^-	1.0	30.1
pH buffers		
$H_2PO_4^-$	25.0	2.5
HCO_3^-	—	5.0
Hepes	10.0	35.0
Impermeants		
Lactobionate$^-$	—	30.0
Sucrose	—	15.0
Mannitol	30.0	25.0
Glucose	10.0	5.0
Gluconate	85.0	70.0
Ribose	0.5	—
Colloids		
HES	5%	—
Dextran 40	—	6%
Pharmacologics		
Adenosine	—	2.0
Adenine	5.0	—
Glutathione	3.0	3.0
Relevant baseline values[a]		
$K^+/Na^+(-)$	0.240 ± 0.005[#]	1.390 ± 0.042
NH_4^+ (mM)	1.067 ± 0.080[#]	0.324 ± 0.056
Glucose (g/L)	1.728 ± 0.025[#]	0.940 ± 0.004
Glutamine (mM)	0.075 ± 0.048[#]	1.241 ± 0.081
Glutamate (mM)	0.447 ± 0.034	0.445 ± 0.097

Complete solution formulations were used throughout the study.
[a] Baseline values, of perfusate samples collected from the organ bath, were measured (after glutathione addition to UHK) prior to kidneys attachment to the pump. Data were averaged over seven kidneys and presented as mean ± SEM.
[#] $P < 0.001$ Belzer-MPS vs. UHK .

Both UHK and Belzer-MP solutions were supplemented with 3 mM of reduced form glutathione (Table 1) either prior to solution use or at the time of solution manufacture, respectively. Glutathione, a powerful antioxidant and an important component of the preservation solutions, is a tripeptide formed of glutamic acid, glycine and cysteine [22,30]. Belzer-MP solution is Belzer's modification of University of Wisconsin (UW) solution for machine perfusion [2]. In the UW solution and therefore Belzer-MPS, glutathione, added at the time of solution manufacturing, is spontaneously converted to an oxidized form, yet it still remains effective in protecting the mitochondria and preserving the integrity of plasma membrane of cold stored renal tubules cells [22]. In the absence of glutathione in the UW solution cold stored kidneys have a slow return to normal physiological function and have high levels of serum creatinine post

transplantation [22]. Moreover, the endogenous renal cortex glutathione content drops at a rate of 20% per day [30]. Although kidney cells appear to be permeable to glutathione, it has not been established with certainty that glutathione can enter the cells during cold storage, thus it either stays in the extracellular space, or it is hydrolyzed to its amino acids [22,30]. Also, it has not been confirmed if oxidized glutathione or the amino acid components of glutathione provide organ protection during hypothermia [22]. During hypothermic preservation, in the absence of oxygen, the regeneration of glutathione from glutamine is limited by reduced cellular ATP levels however, the catabolism of glutathione can still take place [30].

In our study baseline glutamine was detected (Table 1) only in the UHK group, presumably due to freshly added glutathione. In the Belzer-MP solution the glutathione was added at the time of solution manufacture and it was already oxidized (partially or totally) at the time of solution use. After 4 h of perfusion glutamine concentration was considerably reduced in the effluent of UHK perfused kidneys relative to baseline ($P < 0.01$). At six hours glutamine content decreased to 0.14 ± 0.07 mM ($P < 0.01$ vs. baseline), and for the rest of perfusion it oscillated slightly around this value.

Under physiological conditions, glucose is an important cell nutrient, energy source and metabolite, both *in-vivo*

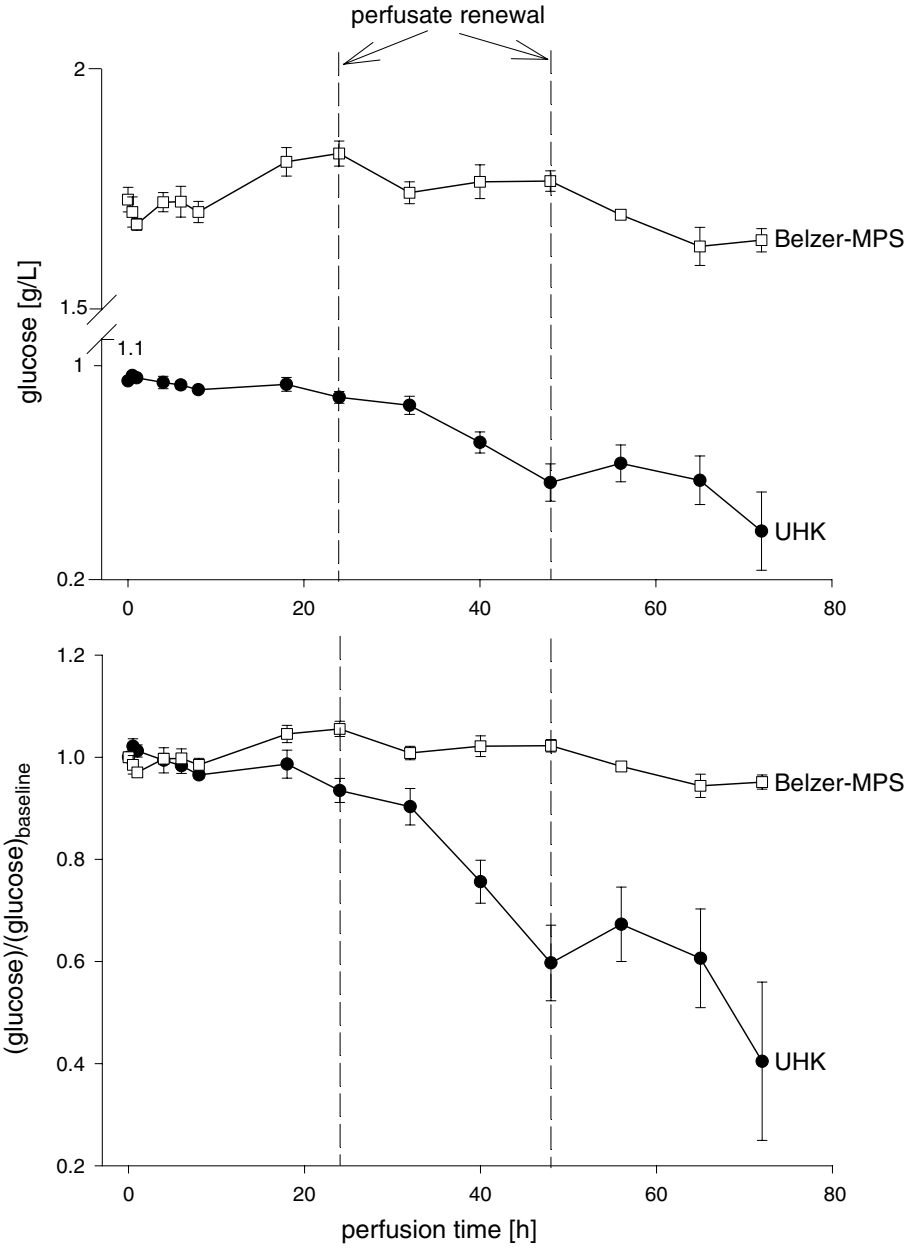

Fig. 1. Variation of glucose concentration with perfusion time in the renal perfusate. Continuous uptake of glucose was recorded throughout perfusion only in the UHK group kidneys. Perfusate renewal was performed after 24 and 48 h of perfusion, as indicated by the two corresponding dashed lines. In each group data are averaged for 7 kidneys and are presented as mean ± SEM.

and *in-vitro*. Besides glucose, glutamine and glutamate, 5-carbon amino acids, are important for cell function proliferation, regulation of renal acid–base balance and gluconeogenesis [14,17,24]. Glutamine, an important ammonia donor in the kidney, is the prevailing amino acid of the extracellular space (0.7 mM vs. 20 μM glutamate), while glutamate, the most abundant intracellular amino acid (2–20 mM), is an immediate product of glutamine metabolism [17,24]. Glutamate is produced from glutamine by glutaminase while ammonia is released. More ammonia is released during gluconeogenesis when glutamate is partially oxidized to α-ketoglutarate, a Krebs cycle intermediate [17,24,8]. Ammonia diffuses rapidly and easily across the tubule cell membrane into the tubule lumen, combines with free protons from carbonic acid dissociation and forms ammonium ions (excreted in urine) [17,8]. Glutamine catabolism generates two molecules of ammonium and two molecules of bicarbonate per one molecule of glutamine [17,24,8].

Throughout machine perfusion glucose concentration of the effluent of kidneys perfused with Belzer-MPS remained unmodified relative to baseline (Fig. 1). In contrast, in the UHK group after 24 h of perfusion glucose uptake was significantly increased relative to baseline ($P < 0.05$). Glucose consumption continued to increase with perfusion time, after 72 h of perfusion perfusate glucose was reduced to 0.38 ± 0.14 g/L ($P < 0.05$ vs. baseline). The rate of glucose uptake (on a linear basis) for the last 48 h of perfusion was 10.41 mg/L/h. After 32 h of perfusion, the UHK group kidneys had a significantly lower ($P < 0.05$) normalized glucose relative to the Belzer-MPS group kidneys (Fig. 1).

As a general trend, glutamate production increased with time during kidney perfusion (Fig. 2). In the UHK group the perfusate glutamate content reached a value significantly higher than baseline ($P < 0.01$) after 4 h of perfusion. Eight hours into perfusion, the Belzer-MPS perfused kidneys showed a markedly increased production of glutamate relative to baseline ($P < 0.05$). A maximum glutamate concentration was reached after 24 h of perfusion in the perfusate of UHK group ($P < 0.001$ vs. Belzer-MPS, 3.19 ± 0.33 mM vs. 1.08 ± 0.05 mM, respectively). Glutamate production rates (on a linear basis) of 0.114 and 0.026 mM/hour were recorded in the UHK and Belzer-MPS group, respectively, for the first 24 h of perfusion. After four hours of perfusion glutamate concentration was significantly higher in the effluent of UHK perfused kidneys relative to Belzer-MPS group ($P < 0.01$) and remained higher throughout perfusion.

After 4 h of perfusion, in the UHK group, the amount of ammonium released in the perfusate was significantly higher than baseline ($P < 0.001$), and reached a value of 2.58 ± 0.14 mM after 24 h (Fig. 3, upper plot). As observed with glutamate, NH_4^+ dynamics paralleled fluid renewal in the UHK group. The 48 and 72 h ammonium concentration was not statistically different from the one at 24 h, yet immediately following perfusate renewal (i.e., at 32 and 56 h), a significant reduction in NH_4^+ concentration was registered ($P < 0.05$ vs. 24 h). The NH_4^+ production

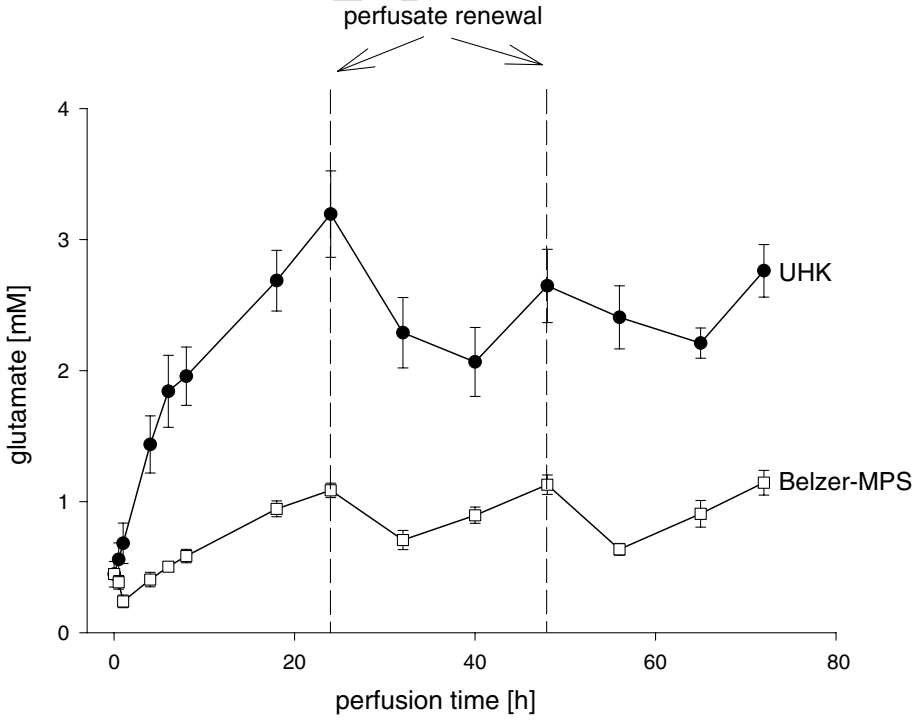

Fig. 2. Glutamate concentration dynamics during kidneys machine perfusion. The changes of glutamate concentration measured in the renal effluent correlated with perfusate renewal. Perfusate renewal was performed after 24 and 48 h of perfusion, as indicated by the two corresponding dashed lines. In each group data are averaged for 7 kidneys and are presented as mean ± SEM.

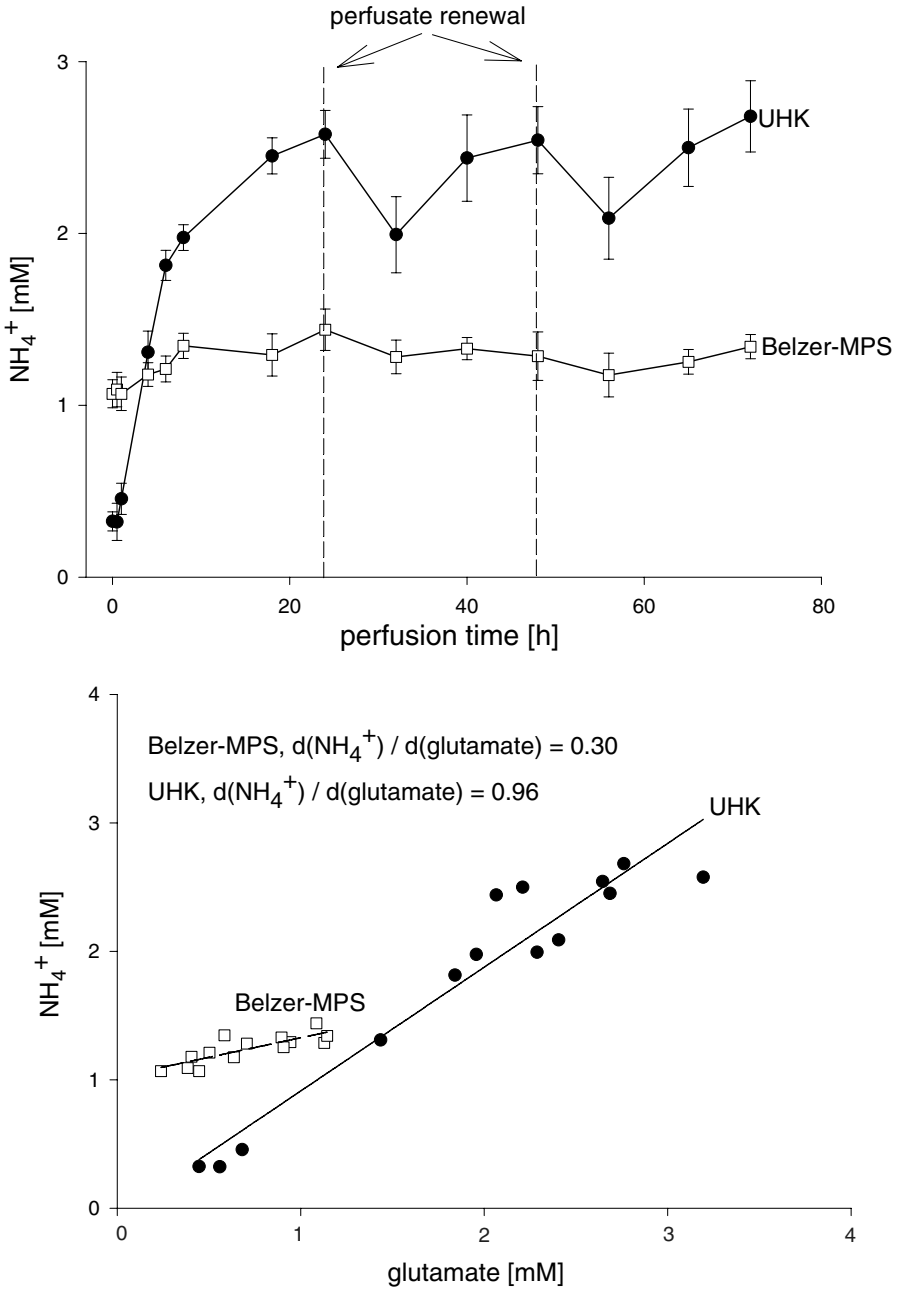

Fig. 3. Variation of ammonium content with perfusion time and the correlation of glutamate and ammonium concentrations in the renal effluent. Increased amount of NH_4^+ was generated by the UHK perfused kidneys relative to the perfusate baseline and in comparison with the Belzer-MPS group kidneys (upper plot, in each group data were averaged for 7 kidneys and are presented as mean ± SEM). During perfusion, higher concentrations of glutamate were recorded in the UHK perfused kidneys group, relative to Belzer-MPS group (lower plot), suggesting that some, if not all, of available glutamine was catabolised. As a result, in the UHK group ammonium concentration was substantially elevated in the renal effluent, in correlation with glutamate production.

in the Belzer-MPS group became significantly higher ($P < 0.05$) than baseline 6 h into perfusion. After 4 h of perfusion, there was rise in ammonium production relative to baseline in the perfusate of the UHK group kidneys when compared to the Belzer-MPS group ($P < 0.001$).

Early studies have emphasized the importance of documenting renal metabolism under ischemic/hypothermic conditions, as a functional indicator of kidneys *ex-vivo* overall performance [14,29]. Kidney metabolism, although considerably restrained at low temperature, is strongly dependent on the solution chemistry, levels of oxygen tension, temperature and time length of perfusion. It has been shown that pulsatile hypothermic perfusion of kidneys with a human albumin based solution reduces glucose and raises ammonia and glutamate content of renal effluent at a rate of 1.71 μM/h and 0.67 μM/day, respectively [14,29]. If glucogenic amino acids are added to the perfusion solution (i.e., glutamine 800 μM), renal glucose uptake rate is

decreased due to a higher rate of gluconeogenesis, while glutamine is consumed (42.29 μM/h) [14]. Moreover, glutamine consumption correlates with ammoniagenesis, while high production rates are established for glutamate and ammonia, 4.13 and 9 μM/h, respectively [14].

Against this background, the UHK and Belzer-MP solution baselines had similar glutamate concentration, while glutamine was detected only in the baseline sample of UHK solution. The latter is considered to be the result of glutathione addition immediately prior to UHK solution use. For Belzer-MP solution, by the time of its use, it is assumed that glutathione, added at the time of manufacture, was partially or totally oxidized (hydrolyzed to its amino acids, glutamine being one of the three constituents). During perfusion, higher concentrations of glutamate were recorded in the UHK perfused kidneys group, relative to Belzer-MPS group, which suggests that some, if not all, of available glutamine was catabolised. Thus, even at the low temperature of 5–8 °C, glutamine, endogenous and/or from glutathione, was reduced, yet at a different rate than at physiologic conditions [14,17,24,8]. As a result, in the UHK group, ammonium and glutamate concentrations were substantially elevated in the renal effluent, at correlated rates and levels (Fig. 3, lower plot). The ammonium was the result of either glutamine deamination and reduction to glutamate or the deamination of glutamate to α-ketoglutarate [17,24,8] contributed also to the increased levels of ammonium content in the perfusate. In the UHK group the glucose uptake was considerably enhanced after 24 h of perfusion, and glucose consumption continued until perfusion completion, while in the Belzer-MPS group glucose was just slightly reduced relative to baseline. For a better understanding of glucose concentration dynamics, more experiments are required to document the perfusate pyruvate/lactate ratio as indicator of the glycolysis process rate [8].

A pulsatile pressure driven flow was applied to continuously perfuse the organs. The two experimental group kidneys had similar arterial flow rate and internal vascular resistance, the latter being somehow lower in the UHK perfused kidneys. During machine perfusion renal flow rate increased for the first 4–6 h of perfusion and started to slightly decrease after 36 h. However, these variations were not significant relative to the 30 min baseline value. Accordingly, internal vascular resistance showed reduction in the first 4–6 h and an increase toward the end of perfusion, yet the values were not markedly changed relative to the 30 min value. The flow rate acceptance limit for clinical transplantation of pumped kidneys [13,15,20,23] was met by all perfused kidneys. Moreover, in the UHK group, after 4 h of perfusion, the perfusate K^+/Na^+ ratio decreased markedly to 1.30 ± 0.04 ($P < 0.05$), and remained at this value until perfusion completion.

In the present study, the hypothermia/perfusion-induced morphological damage to renal tissue was minimal to none. Arterial and arteriolar narrowing were not seen, two biopsies showed one or two small blood vessels with minimal vasculitis, without intimal thickening. Tubular atrophy (less than 20%) was more pronounced in the UHK group relative to Belzer-MPS group. Tubular necrosis was minimal (<5%) in most of the kidneys, and was predominantly seen in the medullary region. In spite of the relatively good pathological results, renal hypothermia/perfusion-induced edema could not be avoided, after 72 h of perfusion the weight gain was markedly more pronounced ($P < 0.01$) in the UHK group relative to Belzer-MPS perfused kidneys ($92.3 \pm 5.6\%$ vs. $55.4 \pm 4.4\%$, respectively). Dextran (40 kDa molecular weight) and HES were the two colloids used with the current study perfusion solutions (Table 1) to prevent cellular water uptake and reduce osmotic cell swelling. The role of these two colloids on renal edema needs further investigation.

Kidney perfusion flow parameters, with similar values between the two experimental groups, satisfied the published acceptance criteria for transplantation [13,15,20,23]. Thus, from a renal flow point of view, the two employed perfusates, UHK and Belzer-MPS were equivalent. However, marked differences were monitored between the two experimental groups in terms of kidney biochemical and metabolic activity. These data serve to emphasize that a comprehensive assessment of kidney *ex-vivo* function during the preservation interval would facilitate a greater degree of discrimination and allow for an accurate reliable selection of viable kidneys for transplantation. These experiments are reported as a prelude to further studies designed to evaluate transplantation outcome. The validation of the aforementioned *ex-vivo* renal preservation findings by subsequent kidney transplantation and *in-vivo* viability assessment is necessary and accepted as the next step in these studies. Moreover, the influence of warm ischemia (NHBD kidneys) on kidneys *in-vitro* biochemistry needs to be investigated and correlated with renal biophysical parameters.

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