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# The role of preservation solution on acid–base regulation during machine perfusion of kidneys

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Abstract: To meet the current clinical organ demand, efficient preservation methods and solutions are needed to increase the number of viable kidneys for transplantation. In the present study, the influence of perfusion solution buffering strength on renal pH dynamics and regulation mechanisms during kidney *ex vivo* preservation was determined. Porcine kidneys were hypothermically machine perfused for 72 h with either Unisol™-UHK or Belzer-Machine Perfusion solution, Belzer-MP solution. Renal perfusate samples were periodically collected and biochemically analyzed. The UHK solution, a Hepes-based solution (35 mM), provided a more efficient control of renal pH that, in turn, resulted in minor changes in the perfusate pH relative to baseline, in response to tissue $CO_2$ and $HCO_3^-$ production. In the perfusate of Belzer-MP kidney group a wider range of pH values were recorded and a pronounced pH reduction was seen in response to significant rises in $pCO_2$ and $HCO_3^-$ concentrations. The Belzer-MP solution, containing phosphate (25 mM) as its main buffer, and only 10 mM Hepes, had a greater buffering requirement to attenuate larger pH changes.

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Key words: acid–base regulation – hypothermic machine perfusion – kidney preservation – pH control

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There is a significant organ supply/demand imbalance in current clinical renal transplantation (1). Improved organ preservation techniques and solutions, better renal viability assessment methods (2), and reduced organ discard rates have the potential to increase the number of kidneys for transplantation.

The success of transplantation, reflected by graft immediate function at normal physiological parameters, without external support and medical complications, depends on a wide variety of factors (3). Among those, the quality and efficiency of the hypothermic preservation technique and solution play a critical role in the transplantation outcome. Essential properties of the preservation solution include minimization of the hypothermically induced cell swelling and extracellular space edema, restriction of ionic imbalance, prevention of intracellular acidosis and injury from free radicals after reperfusion, and provision of necessary substrate for the regeneration of high energy phosphates during reperfusion (3). Currently, the University of Wisconsin (UW) solution, considered the 'gold standard' solution for static cold storage of abdominal organs (4), is used extensively in clinical and experimental settings. For hypothermic machine perfusion of kidneys the Belzer's modification of the UW solution, the Belzer-Machine Perfusion solution (Belzer-MPS) (5–10) is the primary clinical perfusate.

Hypothermic pulsatile machine perfusion of kidneys has been clinically used for over 30 yr (7, 8, 10). Machine perfusion is considered superior to static cold storage because of lower incidence of delayed graft function (7, 11–13), better preservation over periods longer than 24 h, the ability to monitor renal flow rate, pressure and internal resistance and assess kidney viability, and the possibility of providing metabolic support during perfusion (13, 14).

It is well known that acid–base regulation is an important determinant of cell survival after exposure to ischemia (15–21). Low temperatures alter pH control mechanisms and reduce tissue pH. Consequently, pH regulation during hypothermic preservation of organs is crucial for their optimal functioning. The chemical composition of a preservation solution plays a major role in controlling and maintaining tissue pH-stasis at low temperatures. The solutions used for hypothermic organ storage and perfusion are multi-component solutions, and as we have reported recently, their efficiency in preventing acidosis, although influenced by the main buffer, is the result of cumulative effects of their individual chemical components (22). The ability of cold preservation solutions to control pH and combat acidosis at low temperatures has been documented on a physico-chemical basis in the absence of tissue/organs (22). We have shown that the preservation solution buffering capacity is strongly dependent upon both the nature and concentration of the main buffer (22). Moreover, solutions containing Hepes as the main pH buffer are characterized, at low temperatures, by high buffering capacities and efficiencies, and a rate of change of pH with temperature that allows these solutions to constantly maintain pH in the physiologically important range (22).

From our study (22), Unisol$^{TM}$ intracellular type solution (UHK (23), 35 mM Hepes) emerged as the solution that, by design, has the highest physiological buffering efficiency and potential in maintaining and regulating pH within the range of $7.4 \pm 0.4$ at 5°C. To confirm UHK's buffering properties in the presence of tissue/organ, in the current study, the influence of UHK solution buffering strength on renal pH dynamics and regulation mechanisms during kidney *ex vivo* hypothermic perfusion was determined. The UHK solution is a proprietary solution (23) and is relatively new to the field of renal preservation (2). For comparison purposes and results validation, Belzer-MP solution was also employed to machine perfuse kidneys, this solution being currently clinically used for machine preservation of human kidneys for transplantation.

#### Materials and methods

An experimental heart-beating porcine kidney donor model was employed. 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). Fourteen kidneys obtained from adult male pigs (25–30 kg, Hambone Farms, SC, USA) were used. Following aseptic excision, renal artery was cannulated and the organs were cold flushed at low pressure, for 30 min, with the perfusion solution, until the venous effluent was clear and transported on ice, in the flushing solution to the perfusion site. Cold ischemic time (CIT) was limited to 1 h while warm ischemia was kept below 3 min. Subsequently, kidneys were hypothermically perfused through the renal artery at 5–8°C and 30–50 mmHg arterial pressure for 72 h using the continuous pulsatile perfusion prototype of the LifePort$^{TM}$ kidney preservation system [Organ Recovery Systems (ORS), Des Plaines, IL, USA]. The preservation system allowed for a closed loop perfusion. The perfusate entered the kidney through the renal artery, and emerged through the renal vein into the organ bath where the kidney was immersed in the perfusion solution. Fresh perfusate was replaced every 24 h without interrupting the perfusion. Medical grade 100% $O_2$ was passed through an oxygenator directly into the organ cassette, to regulate the oxygen partial pressure of renal perfusate between 200 and 300 mmHg (at 37°C). Sterile, disposable filters were used (0.2 μm, Fisher Scientific, Norcross, GA, USA) for the first 6 h of perfusion to remove the remnant blood cells from the flow system.

Two preservation solutions were employed for the flushing and perfusion of kidneys, Unisol$^{TM}$-UHK (23) (ORS) and Belzer-MPS (manufactured and commercialized by ORS as KPS-1, the Kidney Perfusion solution-1). The UHK, a Unisol$^{TM}$-intracellular base solution (21, 23–26), is a hypertonic solution with high concentration of potassium and reduced sodium content (Table 1), for application at profound hypothermic temperatures (<15°C).

Immediately prior to use the UHK solution was supplemented with 3 mM of reduced form glutathione (Sigma-Aldrich, St. Louis, MO, USA), as required by its chemical formulation (23). Also, 62.5 mg/L Primaxin I.V. (Merck & Co., Inc., West Point, PA, USA) was added to both preservation solutions to minimize the risk of contamination. No other pharmacological agents (i.e. vasodilator, muscle relaxant and osmotic diuretic) were used during organ perfusion.

The kidneys were divided into two experimental groups, based on the preservation solution employed: (i) UHK group (n = 7) included kidneys flushed and perfused with the UHK solution (CIT = $55.57 \pm 4.2$ min) and (ii) KPS-1 group (n = 7) included kidneys flushed and perfused with Belzer MPS (CIT = $60.71 \pm 2.57$ min). Kidney weight was documented before and after perfusion. Renal arterial pressure, flow rate, internal vascular

Table 1. Solutions chemical composition, buffering capacity and efficiency

| Chemical components (mM) | Belzer-MPS (KPS-1) | UHK |
|---|---|---|
| Ionic | | |
| $Na^+$ | 100.0 | 62.5 |
| $K^+$ | 25.0 | 70.0 |
| $Ca^{++}$ | 0.5 | 0.05 |
| $Mg^{++}$ | 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% |
| Physico-chemical properties (5°C) [22] | | |
| Dissociation constant, p*Ka′* | 6.99 ± 0.01 | 7.66 ± 0.01 |
| Temperature coefficient, $\Delta$p*Ka′* per°C [pH units/°C] | –0.0046 | –0.0126 |
| Buffer capacity, $\beta_{conv}$ (Slykes) | 12.7 | 14.0 |
| Physiological buffer capacity, $\beta_{phys}$ (Slykes) | 13.4 | 15.5 |
| Maximum buffer capacity, $\beta_{max}$ (Slykes) | 14.9 | 16.5 |
| Buffering efficiency, $\eta_{conv} = \beta_{conv}/\beta_{max}$ | 0.853 | 0.846 |
| Physiological buffering efficiency, $\eta_{phys} = \beta_{phys}/\beta_{max}$ | 0.897 | 0.937 |

KPS-1, the Kidney Perfusion solution-1; UHK, Unisol$^{TM}$-intracellular base solution.

resistance, and kidney bath temperature were constantly monitored and recorded by the LifePort$^{TM}$ perfusion machine data acquisition system. Renal perfusate samples were periodically collected and biochemically analyzed (BioProfile200; Nova Biomedical, Waltham, MA, USA). A reference baseline sample was collected from each organ bath prior to placing the kidney into the organ cassette.

### Results and data analysis

The current study aimed to validate UHK solution *in vitro* buffering properties (22) while used for kidneys *ex vivo* hypothermic machine perfusion. Thus, the study was focused on renal pH dynamics and control, and the time variation of biochemical parameters directly related to pH regulation are reported.

Renal venous effluent/perfusate pH, $pCO_2$, $pO_2$ and $HCO_3^-$ concentration were periodically measured and recorded as temperature corrected values (at 37°C). Data normalization was applied as appropriate. The coefficients of variation (CV) of pH, $pCO_2$ and $HCO_3^-$ were calculated using the following relationships:

$$CV_{pH} = \frac{(pH_{base} - pH)}{pH_{base}} (\%),$$

$$CV_{pCO_2} = \frac{abs(pCO_{2base} - pCO_2)}{pCO_{2base}} (\%) \text{ and}$$

$$CV_{HCO_3} = \frac{abs(HCO^-_{3base} - HCO^-_3)}{HCO^-_{3base}} (\%),$$

respectively (subscript 'base' refers to baseline). The coefficient of variation represents the percentage change of a chemical concentration in the renal perfusate relative to its baseline value. The absolute value (abs) was considered for $CV_{pCO_2}$ and $CV_{HCO_3}$ for a better illustration of their variation with perfusion time, since during perfusion the perfusate $pCO_2$ and $HCO_3^-$ content increased above baseline value (Fig. 1).

After 8 h of perfusion kidneys perfused with UHK experienced a significant ($p < 0.05$) reduction in the perfusate pH relative to baseline value (Fig. 1). At 24 h, just before the first perfusate renewal, the pH value relative to baseline was $7.27 \pm 0.02$ vs. $7.34 \pm 0.01$ ($p < 0.01$). Following perfusate replacement, at and after 32 h, the pH of the renal venous effluent increased to a value comparable with baseline ($p > 0.05$) and continued to fluctuate around that value for the rest of perfusion, without being significantly affected by the second perfusate renewal at 48 h of perfusion. After 4 h of perfusion the pH of the KPS-1 group perfusate dropped considerably ($p < 0.01$) relative to baseline (Fig. 1). In this group the increase in acidity continued up to 24 h when perfusate pH reached a minimum. Perfusate renewal at 24 and 48 h resulted in a temporary yet substantial increase in the effluent pH (i.e. from $7.22 \pm 0.01$ at 24 h to $7.30 \pm 0.01$ at 32 h, $p < 0.001$) but did not correct the pH, the value remaining markedly below baseline ($p < 0.0001$). After 72 h of perfusion the pH of the KPS-1 group perfusate was significantly lower when compared with baseline ($7.25 \pm 0.02$ vs. $7.40 \pm 0.01$, $p < 0.0001$) and significantly lower than the corresponding mean pH value of UHK perfused kidneys.

In the UHK group, increases in perfusate content of $pCO_2$ and $HCO_3^-$ relative to baseline were recorded near, yet just prior to the perfusate renewal time point, i.e. at 18, 24, 40 and 48 h for $pCO_2$ ($p < 0.05$ vs. baseline) and at 40, 48 and 56 h for $HCO_3^-$ ($p < 0.05$ vs. baseline), as shown in Fig. 1. For the

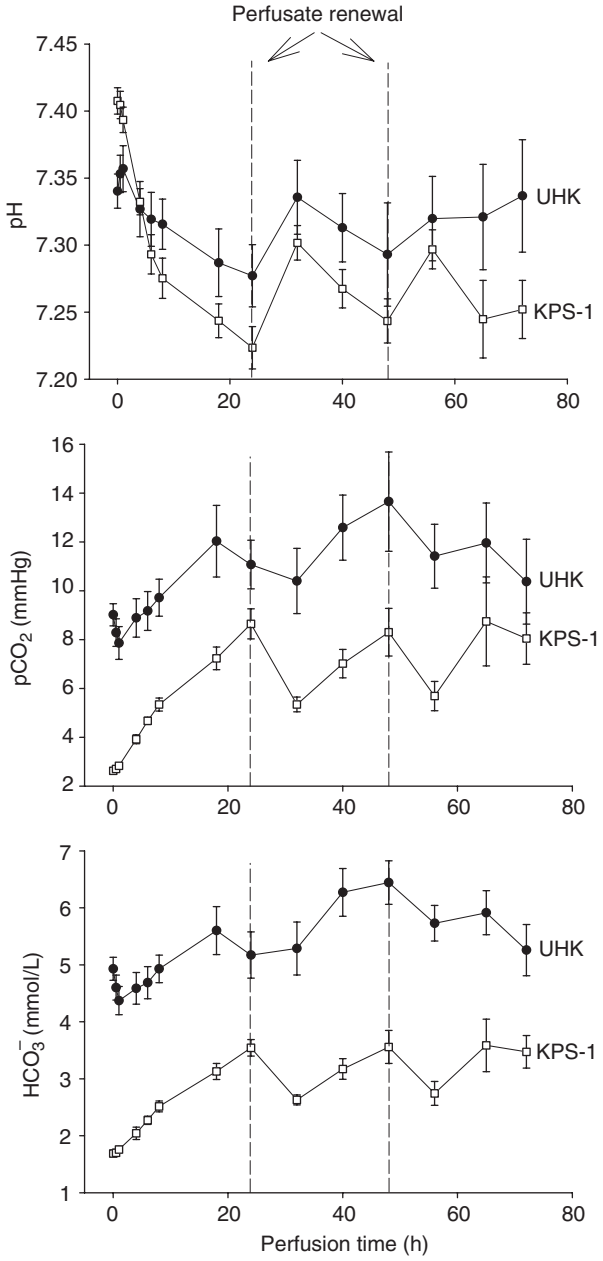


*Fig. 1.* Variation of renal perfusate pH, $pCO_2$ (at 37°C) and $HCO_3^-$ concentration with perfusion time. Perfusate renewal was performed twice during 72-h machine perfusion, after 24 and 48 h, as indicated by the two corresponding dashed lines. In each group, data was averaged for seven kidneys and is presented as mean ± standard error of the mean (SEM).

rest of perfusion time these two parameters were maintained in the vicinity of their baseline value in the UHK buffer solution. The $pCO_2$ and $HCO_3^-$ dynamics in the venous effluent of the KPS-1 group was influenced by and correlated well with perfusate renewal (Fig. 1). After 4 h of perfusion $pCO_2$ and $HCO_3^-$ content in the renal perfusate was significantly higher relative to baseline ($p<0.01$), and continued to increase until it reached a maximum value at 24 h. Rates of hourly increase of 9.52% (0.250 mmHg/h) and 4.58% (0.077 mM/h) were recorded for $pCO_2$ and $HCO_3^-$, respectively, in the KPS-1 perfusate. The perfusate renewal (at 24 and 48 h) triggered an immediate, yet temporary, reduction (at 32 and 56 h, respectively) in the $pCO_2$ and $HCO_3^-$ concentration of renal effluent. However, the value remained significantly higher than baseline ($p<0.01$). Following this brief decline, renal effluent $pCO_2$ and $HCO_3^-$ resumed a continuous increase up to the next perfusate renewal.

The pH coefficient of variation, $CV_{pH}$ (%), is presented as a function of perfusion time in Fig. 2. Statistically, after 6 h of perfusion the pH deviation from its baseline value was significantly higher in the KPS-1 group when compared with UHK group ($p<0.001$), an increase in the $CV_{pH}$ denoting a reduction in pH. Kidneys perfused with UHK were characterized by fairly small pH coefficients of variation. The change of pH relative to baseline covered a narrower range, 0.36 ± 0.13–1.24 ± 0.26%, when compared with the KPS-1 group kidneys, 0.33 ± 0.02–2.10 ± 0.29%. After 4 h of perfusion the coefficients of variation of $pCO_2$ and $HCO_3^-$ (Fig. 2) for the KPS-1 perfused kidneys were considerably higher ($p<0.001$) when compared with the UHK group. The change in $pCO_2$ content, relative to baseline, resided in the range of 4.73 ± 1.73–232.60 ± 69.37% for KPS-1 group and 12.06 ± 5.12–57.09 ± 16.47% for UHK perfused kidneys. The $CV_{HCO_3}$ fluctuated between 8.07 ± 4.01–30.72 ± 7.74% and 6.90 ± 2.36–112.71 ± 27.32% in the UHK and KPS-1 group, respectively.

As shown in Fig. 3, the changes in renal perfusate $pCO_2$ and $HCO_3^-$ correlated well with pH dynamics, a linear proportionality between $HCO_3^-/pCO_2$ ratio and pH being maintained throughout perfusion for both UHK and KPS-1 kidney groups,

$$pH = 6.94 + 0.74^{*}HCO_3^-/pCO_2 \ (R = 0.93) \text{ and}$$
$$pH = 6.92 + 0.77^{*}HCO_3^-/pCO_2 \ (R = 0.99),$$

respectively. Applying the Henderson–Hasselbach equation (27, 28),

$$pH = pKa\prime + \log\left(\frac{HCO_3^-}{CO_2}\right),$$

with $pKa'$ measured at 5°C (Table 1), and using the two empirically derived aforementioned relationships, $CO_2$ concentration in the perfusate was calculated as a function of perfusion time (Fig. 4).

To validate the study from the point of view of kidney *in vitro* performance, it has to be mentioned

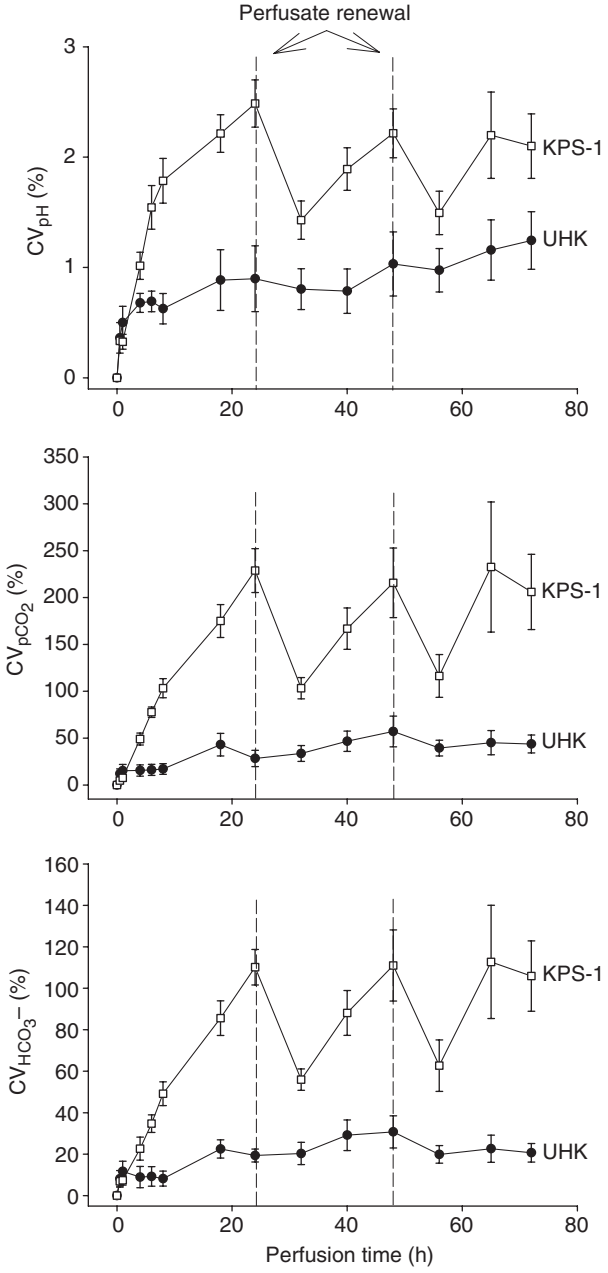


*Fig. 2.* Time dependency of the coefficients of variation for pH, $pCO_2$ and $HCO_3^-$. The percentage change of a chemical element concentration in the renal perfusate relative to its baseline value was expressed as coefficient of variation (CV): $CV_{pH} = (pH_{base-pH})/pHbase$ (%), $CV_{pCO_2} = abs(pCO_{2base} - pCO_2)/pCO_{2base}$ (%), $CV_{HCO\_3} = abs(HCO_{3base}^- - HCO_3^-)/HCO_{3base}^-$ (%) (subscript 'base' refers to baseline). In each group data is averaged for seven kidneys and is presented as mean ± standard error of the mean (SEM).

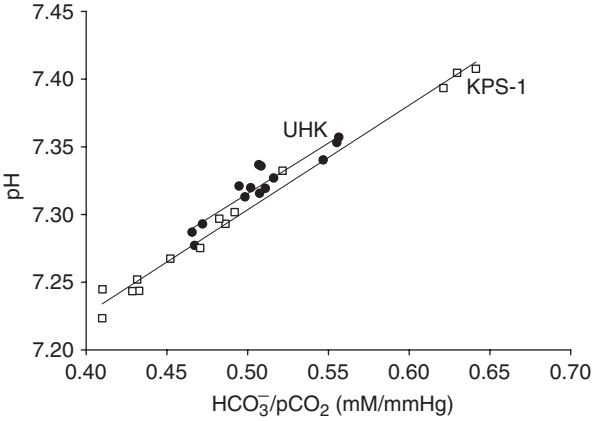


*Fig. 3.* Correlation of $HCO_3^-/pCO_2$ ratio and pH during hypothermic preservation. For the duration of kidney machine perfusion the changes in perfusate $HCO_3^-/pCO_2$ ratio were linearly related to the perfusion solution pH variation. Two correlation relationships were empirically derived, for the UHK and KPS-1 perfused kidneys: $pH = 6.94 + 0.75^* HCO_3^-/pCO_2$ ($R = 0.93$) and $pH = 6.92 + 0.77^* HCO_3^-/pCO_2$ ($R = 0.99$), respectively.

that the renal artery flow rate acceptance limit for clinical transplantation of pumped kidneys [0.45 mL/min/g (7, 29, 30)] was met by all perfused kidneys (data not shown). The average (over 72 h) internal vascular resistance had relatively high values, of 0.64 ± 0.02 and 0.73 ± 0.01 for the UHK and KPS-1 perfused kidneys, respectively. The average arterial pressure (over 72 h) measured in the UHK and KPS-1 group was 38.31 ± 0.25 and 36.13 ± 0.12 mmHg, respectively. After 72 h of perfusion, renal edema was statistically significantly ($p < 0.01$) elevated in the UHK group when compared with KPS-1 perfused kidneys. The histopathological analysis (in compliance with the International Banff Classification of Transplant Pathology (31, 32) revealed similar, yet minor, morphological changes in all perfused kidneys (not shown). Less than 5% tubular necrosis, <20% tubular atrophy, no visible glomerular sclerosis, interstitial fibrosis and arterial and arteriolar narrowing were observed after 72 h of perfusion.

## Discussion

In the current study, heart-beating donor kidneys were hypothermicaly machine-perfused for 72 h. Two solutions, Unisol$^{TM}$-UHK and Belzer-MPS (KPS-1 group) were employed to determine the influence of perfusion solution buffer composition on renal pH regulating mechanisms. The UHK solution, based on a previous experimental study (22), emerged as the solution with the highest potential in controlling and maintaining pH in the physiological range at low temperatures. Belzer-MP is the solution currently employed by the most perfusion centers for machine preservation of human kidneys for transplantation.

Systemic acid–base homeostasis is maintained either chemically, by the intracellular and extracellular buffers, or metabolically, through physiological processes [i.e. $CO_2$ excretion by the lungs and fixed

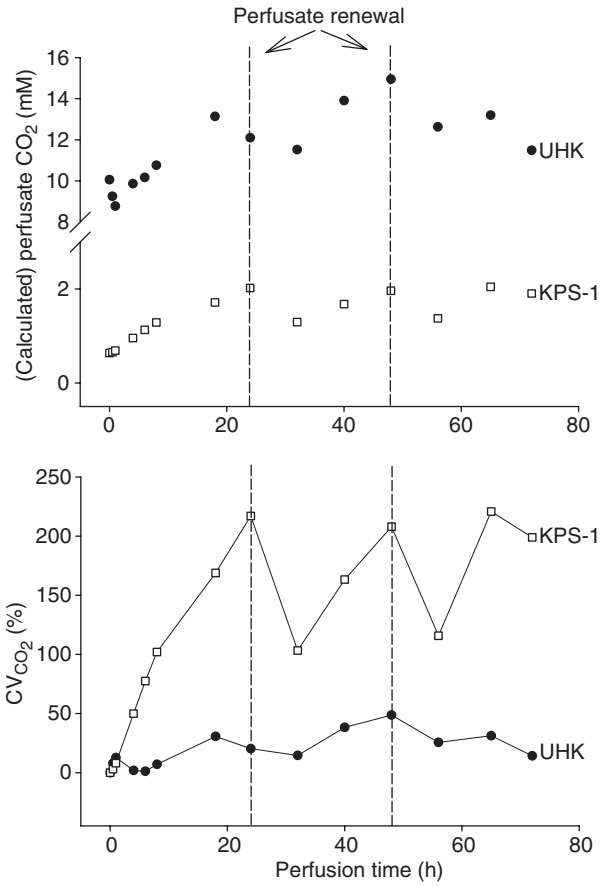


*Fig. 4*. Time dependency of perfusate $CO_2$ and $CO_2$ coefficient of variation. The amount of $CO_2$ released by the kidneys in the perfusate during hypothermic perfusion was calculated using the Henderson–Hasselbach equation and the pH–$HCO_3^-$/$pCO_2$ relationships. The $CO_2$ coefficient of variation was calculated as $CV_{CO_2} = abs(CO_{2base} - CO_2)/CO_{2base}$ (%). In each group data was averaged for seven kidneys and is presented as mean ± standard error of the mean (SEM).

acids (lactic acid, ketones) removal by the kidneys]. Under physiological conditions, pH is controlled through the alteration of levels of chemical components that determine $H^+$ production/consumption such as $pCO_2$ and $HCO_3^-$ (27, 28, 33). The $CO_2$ partial pressure is regulated by the lungs, tissue $CO_2$ production being directly correlated with $pCO_2$ and its excretion rate by the lungs. Kidneys control both bicarbonate recovery and proton excretion (27, 33). The bicarbonate buffer accounts for 36% and 86% of the total buffer content of the intracellular and extracellular fluids, respectively.

A description of renal physiology is beyond the purpose of this paper, however the salient events of renal mechanisms of acid–base regulation will be outlined relative to the interpretation of present study results. Briefly, the sodium proton antiporter located on the proximal tubule allows sodium to move into the cells and further into the interstitial fluid, in exchange for protons to be sent into the tubule lumen. The protons combine with filtered bicarbonate ions and form carbonic acid. In the distal convoluted tubule, with the help of the luminal carbonic acid anhydrase, carbonic acid dissociates into $H_2O$ and $CO_2$, and the latter enters the cells. Inside the cells $H_2O$ and $CO_2$ are converted back, by a second anhydrase, to carbonic acid that in turn dissociates to $H^+$ and bicarbonate, the protons being generated by a sodium-independent electrogenic ATP-ase. The bicarbonate is reabsorbed if filtered $HCO_3^-$ ions combine with protons. If the protons and non-carbonic ions (phosphate and ammonia) combine, acids are excreted in the urine and new $HCO_3^-$ is generated.

The use of hypothermia for isolated organ preservation, during transportation, static storage or continuous perfusion, has been proven an effective method for the protection of cells and tissues (14–17, 21). Low temperatures diminish the rate of biophysical processes (19) and the rate of chemical reactions, including metabolic activities. During hypothermic kidney preservation oxygen tension controls the balance between glycolysis and fatty acids oxidation (34), while glycolysis is the principal source of energy. Nevertheless, the effect of cooling on renal metabolism is complex and it cannot be reduced to a simple slowing-down of biochemical activities.

Attempts have been made to identify the appropriate pH value for *ex vivo* hypothermic preservation of kidneys and to correlate the perfusate levels of pH measured during machine perfusion with ischemic renal injury and post-preservation function of kidneys (35–37). However, to date a definitive answer has not emerged. Lindel et al. (37) have shown that the pH of preservation solution plays a critical role in kidney functional recovery post-preservation. Thus, canine kidneys stored for 2 d at 5°C in UW solution with pH of 7.4 and 7.8 had 2.9 mg/dL serum creatinine on day two and normal creatinine levels on day 10 post-transplantation. In turn, kidneys stored in UW solution at pH 6.4 and 6.8 were not viable, either the recipients needed to be sacrificed 6 d post-transplantation or delayed graft function was observed. Canine kidneys hypothermically perfused for 3 d with Belzer-MP solution at pH 7.8 and 7.4 had excellent transplant results and minimal renal injury (37). Kidneys perfused with a solution of pH 6.4 led to the sacrifice of recipients 5 d post-transplantation, while perfusion with UW solution at pH 6.8 resulted in high serum creatinine levels and slow return to normal function of kidneys.

Numerous applications have addressed the implications of pH regulation in clinical hypothermia

(18, 21, 36, 38) and two principal pH management strategies have emerged (18,24). However, the consideration of these strategies in relation to the optimum preservation (21) of isolated organs has not been studied extensively. These two main pH regulatory strategies have been termed: α-stat and pH-stat regulation. The first one, named in recognition of the fact that intracellular pH buffering in all vertebrates is dominated by the degree of ionization (α) of the imidazole moieties (38), is characterized in ectotherms and warm blooded animals cooled in a closed system without gas exchange by a rise in pH with temperature at a rate of −0.0157 pH units per °C (Rosenthal slope). This pH increase with temperature reduction parallels the neutral point of water during cooling from 40 to 0°C. By contrast, the pH-stat regulation process is encountered in hibernating animals where an arterial pH of 7.4 is maintained regardless of systemic temperature and where metabolism continues even at a 5°C body temperature. Alpha-stat pH regulation helps to maintain a beneficial constant degree of alkalinity between the extracellular and intracellular compartments during cooling (36, 39).

We have recently reported that, in the absence of tissue/organs, a strong correlation exists between the solution pH regulating mechanisms and the solution chemical formulation, principally by the nature and concentration of its buffer components (22). The UW solution, the 'gold standard' for hypothermic preservation of abdominal organs, contains 25 mM of phosphate as its only effective buffer (22). Accordingly, at 5°C, the solution has a dissociation constant of 6.71 and a corresponding buffering capacity of about 10 Slykes (22). These relatively low values were corrected in the Belzer's modification of UW solution for machine perfusion, by the addition of 10 mM of Hepes. As a result, at 5°C, Belzer-MP solution is characterized [Table 1, (22)] by a dissociation constant of 6.99 (lower than the extracellular space fluid pH 7.4) and 12.7 Slykes buffer capacity and 13.4 Slykes physiological buffer capacity (the acid–base volume increment needed to change the pH within ± 0.4 units in the region of pH 7.4). Moreover, in comparison with the UW solution, Belzer-MPS has an increased $Na^+$ concentration (from 30 to 100 mM) and a reduced $K^+$ content (from 125 to 25 mM), and it has the lactobionate and raffinose replaced by 30 mM of mannitol and 85 mM of gluconate (Table 1), as principal impermeants (22). Based solely on the ionic content, Belzer-MPS is classified as an extracellular-type isotonic solution that mimics the normal extracellular environment of cells.

The UHK solution contains 35 mM of Hepes as the main buffer, at 5°C it has a dissociation constant of 7.66 and conventional and physiological buffer capacities of 14 and 15.5 Slykes, respectively (Table 1). Thus, by design, the UHK solution dissociation constant comes closer to the physiological value of pH 7.4 that corresponds to the extracellular fluid pH. In the absence of organs (22), it has been proven that solutions containing the ampholyte buffer Hepes have excellent buffering capabilities at 5°C, in the pH range of 7.0–7.8, that is regarded as the region of maximum importance for the control of pH for biological homeostasis. Accordingly, both UHK and Belzer-MP solutions are characterized by high buffer efficiencies of 0.937 and 0.897, respectively, in comparison to phosphate (EuroCollins, 0.641, and UW, 0.884) or histidine (Custodiol, 0.373, and Celsior, 0.397) buffer containing solutions (22).

The temperature coefficients (Table 1) were calculated considering a linear relationship of p$K$a′ with temperature between 20 and 5°C, $\Delta p Ka/\,^{\circ}C = (pKa'_{20} - pKa'_{5})/(20 - 5)$ (22). The UHK temperature coefficient (−0.0126) is closely aligned with the Rosenthal slope of pH −0.015 units per °C, considered optimal for α-stat pH regulation, while Belzer-MP temperature coefficient is very low. These values, which are directly related to the solutions buffer content, emphasize that Belzer-MP solution composition, with phosphate as its main buffer, is sub-optimal for regulating pH within physiological limits. On the contrary, the UHK solution, with 35 mM Hepes as its main buffer offers good buffering. Hepes, a zwitterionic sulfonic acid buffer, has by itself a temperature coefficient of −0.0106 pH units per °C (22).

Considering the aforementioned solution's physico-chemical features (buffering efficiency, temperature coefficient) (22), kidneys perfused with UHK would be expected to experience a more efficient control of their pH variation during hypothermic perfusion that in turn, would result in minor changes of the perfusate pH relative to the baseline pH. Indeed, this study confirms that the pH of renal perfusate of UHK perfused kidneys was kept in a tight range for the entire duration of perfusion (Fig. 2). On the contrary, in the KPS-1 kidney group, pH had an immediate drastic departure from baseline that could not be rectified by perfusate replacement (fresh solution with unexploited buffering potency) and solution buffers, indicating that Belzer-MP solution buffering strength and efficiency were not sufficient to adequately regulate $H^+$ protons production/release in response to low temperature kidney metabolism. In the KPS-1 group a wider range of pH values was recorded in the renal effluent, and a pronounced pH reduction was seen in response to the significant rises in $HCO_3^-$

and $pCO_2$ concentrations in the renal perfusate (Figs 1 and 2). In both experimental groups the levels of perfusate oxygenation maintained throughout perfusion by a constant infusion of 100% $O_2$ ($pO_2$ of 200–300 mmHg) helped to keep pH fluctuations within tolerable limits and avoid acidosis (pH $<7.0$). If $pO_2$ would not have been so closely regulated, or no oxygenation at all would have been used, lower pH and higher $pCO_2$ values would have been reached, especially in the Belzer-MPS perfused kidneys. Nonetheless, the same strong correlation between proton release and $pCO_2$ and $HCO_3^-$ increases in the perfusate would have been recorded, but at different levels.

The concentration of $CO_2$ in the perfusate was a direct indication of renal $CO_2$ production. At low temperatures, *ex vivo*, $CO_2$ accumulates in the tissue, its rate of production being influenced by the preservation method, temperature and solution chemical composition and by the functional status of the kidney at its procurement time. The two experimental groups differed only in perfusion solution chemistry. Therefore, chemical formulation of the perfusion solution was the main controller of renal $CO_2$ and $CV_{CO_2}$ dynamics (Fig. 4). A 200% increase in the perfusate $CO_2$ production relative to baseline was encountered during kidneys perfusion with Belzer-MPS. Metabolically, the experimental *ex vivo* hypothermic conditions stimulated glycolysis, which in turn led to proton generation and a decrease in tissue pH. As acidosis was not reached, it is possible that the increase in $CO_2$ production in response to pH reduction was caused by suboptimal metabolic support.

The difference in perfusion solution chemical formulations triggered various metabolic responses between the UHK and KPS-1 kidney groups (not shown). For example, stronger glucose, glutamine, glutamate, and ammonium concentration dynamics were recorded in the perfusate of UHK group relative to the corresponding baseline values and in comparison with the KPS-1 perfused kidneys. Higher fluid accumulation was documented for the UHK perfused kidneys while similar arterial flow rate and vascular resistance were recorded for all kidneys. The morphological changes induced by perfusion and/or cold ischemia were comparable between the two experimental kidney groups, yet minor. The validation of all these findings through the use of an animal kidney transplantation model and correlation of renal solution chemical composition with kidney *ex vivo* function and *in vivo* performance are the subject of further planned studies.

To conclude, the present study confirmed for kidney machine hypothermic perfusion the previously published (22) preservation solution physicochemical properties of pH regulation at low temperature. It was demonstrated that solutions containing Hepes as their main pH buffer do maintain tissue pH in the physiological range when employed for organ hypothermic preservation. Thus, solutions characterized by high buffering strength and efficiency proved to have the ability, under given preservation conditions, to exert a better regulatory control over the renal pH and to minimize acid–base shifts without eliminating changes in pH induced by kidneys metabolic activity. The Belzer-MP solution, containing 25 mM phosphate as its main buffer, and 10 mM Hepes, had a greater buffering requirement to attenuate larger pH changes. By contrast, UHK, a Hepes-based solution, 35 mM, with additional 5 mM of bicarbonate buffer, provided a tighter pH control, allowing significantly smaller pH changes to be induced in response to tissue $CO_2$ and $HCO_3^-$ production.

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