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Benefits of active oxygenation during hypothermic machine perfusion of kidneys in a preclinical model of deceased after cardiac death donors

Raphaël Thuillier, PhD,[a,b,c,d] *Geraldine Allain, MD,*[e] *Olivier Celhay, MD,*[f] *William Hebrard, MSc,*[g] *Benoit Barrou, MD,*[a,b,d,h,i] *Lionel Badet, MD,*[a,d,j,k,l] *Henri Leuvenink, PhD,*[d,m] *and Thierry Hauet, MD, PhD*[a,b,c,d,g,*]

[a] *INSERM U1082, Poitiers, France*
[b] *Université Poitiers, Poitiers, France*
[c] *CHU Poitiers, Pole UBM, Service de Biochimie, Poitiers, France*
[d] *FLIRT: Fédération pour L'étude de l'Ischémie Reperfusion en Transplantation, Poitiers, France*
[e] *CHU Poitiers, Service de Chirurgie Cardio-Thoracique, Poitiers, France*
[f] *CHU Poitiers, Service d'Urologie, Poitiers, France*
[g] *GIS-IBISA, Domaine Expérimental du Magneraud, INRA, Surgères, France*
[h] *Service d'Urologie et Transplantation, Hôpital Pitié Salpétrière, Groupe Hospitalier Universitaire Est, Paris, France*
[i] *Université Pierre et Marie Curie, Paris, France*
[j] *Service d'Urologie et Chirurgie de la Transplantation, Pavillon V—Hôpital Edouard Herriot, Lyon, France*
[k] *Université Claude Bernard Lyon 1, Villeurbanne, France*
[l] *Réseau CENTAURE, Nantes, France*
[m] *Department of Surgery, University Medical Center Groningen, Groningen, The Netherlands*

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ABSTRACT

Background: Deceased after cardiac death donors (DCDs) represent a valuable source of organs; however, preventing poor outcome is difficult, even with the use of machine perfusion (MP). It is of paramount importance to improve this method. We proposed to evaluate the benefits of active oxygenation during kidney graft hypothermic MP using a novel perfusion machine: Kidney Assist (KA).

Methods: We used a pig model of DCD transplantation in Large White pigs. Cold preservation was performed by conventional non-oxygenated MP ($KAnoO_2$) or oxygenated MP (KA).

Results: In the first 2 wk post-transplant, KA grafts displayed a lower serum creatinine peak and a faster return to normal levels compared with $KAnoO_2$ animals, translating into a smaller area under the curve. Urinary neutrophil gelatinase-associated lipocalin levels and serum aspartate amino transferase levels were lower in KA animals compared with the non-oxygenated group. These correlated with better chronic function. Longer follow-up of the animals (3 mo) permitted evaluation of chronic outcome lesions. Interstitial fibrosis was reduced in the KA group, and these kidneys also displayed significantly lower levels of vimentin staining. Further histologic investigation also showed a trend toward decreased chronic inflammation in kidneys preserved with oxygen.

* *Corresponding author*. INSERM U 1082, CHU de Poitiers, Rue de la Milétrie, B.P. 577, 86021 Poitiers Cedex, France. Tel.: +33 5 49 44 46 70; fax: +33 5 49 44 38 34.
E-mail address: t.hauet@chu-poitiers.fr (T. Hauet).
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Conclusions: This new MP system is efficient in preserving DCD kidneys, greatly enhancing the capacity of the graft to withstand preservation stress and improving outcome. Oxygen delivery during preservation is thus valuable for highly damaged organs and offers an important therapeutic tool for transplant teams faced with decreased quality of donor organs.

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1. Introduction

Currently, kidney transplantation is the treatment of choice for patients with end-stage renal disease, with long-term benefits for patient survival and quality of life [1]. However, this therapy is now a victim of its own success, with an increasing shortage of organs: In 2010 in the United States, 15,429 kidney transplants were performed for 87,903 patients on the waiting list (OPTN, 2010). This leads to acceptance of donor organs of increased risks identified as marginal or extended criteria (ECD) donors: organs retrieved from donors presenting co-morbidity factors such as increased age, death from cardiovascular causes, cardiovascular risk factors, or a reduction in glomerular filtration [2]. These ECD donor organs tend to be more sensitive to the stresses of preservation [3]. Furthermore, there is renewed interest in deceased after cardiac death donors (DCDs) [4], which also display an increased rate of delayed graft function (DGF) and a higher incidence of primary nonfunction [5]. The increased proportion of these types of donors has led to rethinking of organ preservation methods.

Machine perfusion (MP) is based on the controlled circulation of a preservation solution. Machine perfusion improves the quality of kidneys in the clinic [6] and is particularly efficient in decreasing both primary nonfunction and DGF in ECD [7], as well as DGF in DCD transplantation [8]. These benefits are also fuelling debate about the cost-effectiveness of the method and the subgroups of patients on which to use it [9]. However, despite its significant efficiency in reducing DGF and increasing survival, complication rates remain high, warranting additional research to improve this method.

The principle of cold hypothermic preservation is based on temperature reduction to reduce metabolism and the requirement for oxygen. However, the need for oxygen persists because the metabolic rate remains at levels estimated around 10%; therefore hypoxia, remains a principal source of lesions induced in the context of preservation. Hence, the concept of supplying O_2 during storage is growing, with the development of different approaches, such as the two-layer method using perfluorocarbons [10] or the persuffl ation technique consisting of retrograde venous application of gaseous O_2 [11]. A recent review on the different strategies for oxygenated preservation [12] highlighted the potential benefits of these techniques, particularly the possible advantages of oxygenated MP, because MP would combine active circulation of dissolved oxygen with the perfusion fluid.

A portable device for oxygenated perfusion has been developed [13] and tested in a pig model of kidney autotransplantation with a short follow-up (7 d). The results showed that active oxygenation could improve preservation quality compared with static preservation. On the other hand, a study on the same model comparing oxygenated *versus* non-oxygenated perfusion failed to provide a clear distinction between the two preservation methods [14]. There is thus a need for an in-depth study of the impact of oxygenation on MP.

Here, we tested the same MP as previously described [13] using two different settings (perfusion with oxygen *versus* perfusion without oxygen) in a pig model of kidney autotransplantation, reproducing conditions of DCD and permitting the study of ischemia-reperfusion injury without possible bias from the use of immune suppression, to determine the effect of oxygenation in hypothermic MP.

2. Materials and methods

2.1. Animals

Male pigs of the Large White lineage were bred onsite at the IBiSA Platform Experimental Surgery and Transplantation, located at the Institut National de la Recherche Agronomique campus of Le Magneraud (Surgères, France). Procedures were performed in accordance with the guidelines of the French Ministry of Agriculture for the use and care of laboratories animals, as previously described. We selected 30- to 35-kg animals for this study.

2.2. Experimental design

We used the well-described porcine model of DCD autotransplantation [15,16]. Briefly, we collected left kidneys after vascular clamping of the renal vessels for 60 min (warm ischemia). Then, we removed the organ and immediately cold-flushed it with preservation solution (KPS-1, Organ Recovery Systems, Brussels). Kidneys were preserved for 22 h at 4°C.

We studied two groups. The first was KAnoO_2, preservation using MP in the Kidney Assist device (Organ Assist BV, Groningen, The Netherlands) without active oxygenation, at a constant pressure of 25 mm Hg ($n = 4$). The second was Kidney Assist (KA), renal perfusion with the Kidney Assist machine, with active oxygenation provided by 100% oxygen through a hollow fiber membrane oxygenator built in the sterile disposable tubing set [16], according to the manufacturer's recommendations ($n = 4$).

Afterward, we reimplanted the kidney and nephrectomized the contralateral kidney. In all cases, the duration of rewarming ischemia during implantation until the end of anastomosis was 30 ± 5 min.

2.3. Renal function evaluation

After transplantation, pigs were placed in metabolic cages for 24-h urine collection. Blood and urinary samples were collected. Plasma creatinine, aspartate amino transferase (ASAT), and proteinuria were measured with Modular automates (Roche Diagnostics, France).

We evaluated urinary neutrophil gelatinase-associated lipocalin (NGAL) levels using the BIOPORTO Elisa kit (Eurobio, Courtaboeuf, France) following the manufacturer's recommendations.

2.4. Morphologic studies

We collected corticomedullar kidney samples at 3 mo and used a standard procedure to estimate the level of tubulointerstitial fibrosis using Picro-Sirius staining [17] on five to 10 fields (×100).

For immunohistochemistry, we used anti-Vimentin (Cell Marque, Rocklin, CA), anti-α-smooth muscle actin (αSMA) (DAKO, Trappes, France), anti-ED1 (Southerbiotech, Birmingham, AL), and anti-CD3 (Santa Cruz Biotechnologies, Santa Cruz, CA) antibodies. We performed quantitative evaluation *in silico* on 10 high-powered fields (×200) per animal [18].

2.5. Statistical methods

Results are shown as means ± standard deviation. For the statistical analysis among groups, we used NCSS software (Statistical Solutions, Saugus, MA) to perform Student's *t*-test in case of normality (evaluated by the Skewness, Kurtosis, and Omnibus tests) and equality of variance (evaluated by Modified-Levene Equal-Variance Test) and the Mann-Whitney U-test in case these parameters were not met. Correlation analyses were performed with NCSS software using the Spearman rank test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Perfusion parameters

We perfused kidneys for 22 h at a constant pressure (25 mm Hg) with or without oxygen. In each group, there was a slight increase in flow and lowering in resistance between the beginning and the end of preservation (Table). There was no statistical difference in terms of flow, resistance, or temperature between groups.

Table – Perfusion parameters during preservation.

Group	Flow (mL/min)	Resistance (mm Hg/mL/min)	Temperature (°C)
$KAnoO_2$			
Start	54.3 ± 9.3	0.48 ± 0.08	3.3 ± 0.6
End	65.5 ± 7.5	0.39 ± 0.04	3.0 ± 0.0
KA			
Start	54.3 ± 6.1	0.47 ± 0.05	3.5 ± 1.3
End	73.5 ± 9.0	0.34 ± 0.05	4.3 ± 1.9

3.2. Recovery of function

The contralateral nephrectomy on the day of the transplant enabled us to use posttransplant serum creatinine levels to measure the ability of the graft to resume its function. During the first 2 wk (Fig. 1A), animals transplanted with a non-oxygenated kidney presented a peak of creatinine at day 3 that reached approximately 1200 μmol/L and plateaued until day 5, and then decreased from day 7 on. Time to peak did not differ in the animal group transplanted with an oxygenated kidney (3 d); however, the level of the peak was lower (approximately 800 μmol/L) and the decrease started as early as day 5 ($P = 0.02$ for $KAnoO_2$), reaching consistently lower levels than $KAnoO_2$ animals on the next measurement times. This was also highlighted by the area under the curve comparison, with smaller areas found in the KA group ($P = 0.02$ for $KAnoO_2$).

To further assess acute kidney injury, we determined urinary levels of NGAL during the first week (Fig. 1B). The levels reached a similar peak at day 1, but in the $KAnoO_2$ group the NGAL levels slowly decreased whereas in the KA animals there was a swifter decrease, significantly inferior to $KAnoO_2$ as early as day 3 posttransplant.

We also performed serum analysis for markers of injury, measuring the activity of the ASAT in the first 3 d posttransplant (Fig. 1C). We observed that whereas the levels in the $KAnoO_2$ group increased rapidly posttransplant and peaked at day 2 at approximately 300 UI/L before recovery, KA group animals showed a more modest increase, peaking at 120 UI/L at day 2 ($P = 0.003$ for $KAnoO_2$).

3.3. Chronic function

To measure the effect of preservation quality on chronic graft outcome, we observed our animals for 3 mo. Measurement of serum creatinine at 3 mo follow-up indicated more complete recovery in the KA group, with levels approximating pretransplant measurements, whereas the $KAnoO_2$ group displayed higher concentrations of serum creatinine ($P = 0.0001$ *versus* KA) (Fig. 2A). Concomitantly, $KAnoO_2$ animals at 3 mo showed increased proteinuria (approximately 0.35 g/24 h) (Fig. 2B), which was significantly higher than the levels recorded in the KA animals (approximately 0.2 g/24 h; $P = 0.007$ *versus* $KAnoO_2$), reaching levels of the native proteinuria in Large White pigs.

3.4. Histological analysis of chronic outcome

To determine the impact of the preservation method on chronic graft outcome at the tissue level, we evaluated fibrosis development through quantification of Sirius red staining on tissue sections collected at the end of the follow up (Fig. 2C). These analyses demonstrated an important development of interstitial fibrosis in the $KAnoO_2$ group (averaging 25% of the observed area), whereas animals transplanted with oxygenated kidneys showed reduced levels (averaging 10%; $P = 0.04$ for $KAnoO_2$).

To investigate the process further, we analyzed the expression level of epithelial to mesenchymal transition

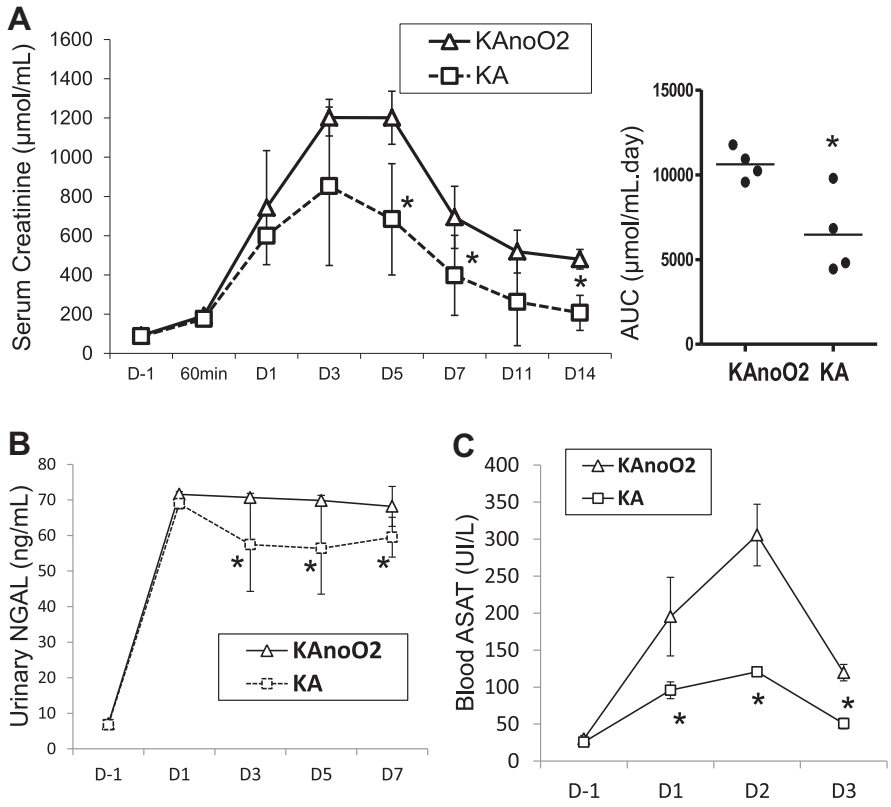

Fig. 1 – Function recovery post-transplantation. (A) Serum creatinine measurements during the first 2 wk post-transplant. Left: evolution of creatinine levels with time. Right: areas under the curve for the time period. (B) Neutrophil gelatinase-associated lipocalin levels detected in urines collected during the first week post-transplant. (C) Aspartate amino transferase activity levels in the blood during the first 3 d post-transplant. Results are shown as means ± standard deviation. *$P < 0.05$ compared with $KAnoO_2$.

markers, typically associated with fibrosis development [19]. Quantification of vimentin staining (Fig. 2C) showed that similar to fibrosis results, there was increased staining in the $KAnoO_2$ group (averaging 24% of the observed area), whereas staining in the KA group remained low (9%; $P = 0.02$ for $KAnoO_2$). These results were confirmed by αSMA measurements, which also showed a trend toward larger stained areas in the $KAnoO_2$ group (14.2%) than in the KA group (8.8%) (Fig. 2C).

Because chronic fibrosis is often linked with increased inflammation [20–23], we quantified the degree of inflammatory cell invasion within the tissue, using ED1+ staining for macrophages and monocytes as a marker of innate inflammation, and CD3+ staining for T lymphocytes as a marker of adaptative immunity (Supplementary Table 1). These quantifications showed that an elevated number of these invading cells were present in the $KAnoO_2$ group, whereas there was a trend for reduced levels in the KA group, particularly regarding CD3+ cells; however, the trend did not reach statistical significance.

3.5. *Correlation analysis between ischemia-reperfusion injury markers and functional parameters*

To assess the level of correlation between the intensity of ischemia-reperfusion injury withstood by the kidney and its functional outcome, we used Spearman's rank test to measure the degree of association between both NGAL and ASAT levels and serum creatinine levels at 14 d and 3 mo (Fig. 3). We demonstrated that NGAL levels in the urine at day 5 was highly correlated with both acute and chronic outcomes, with ranks of 0.83 each ($P = 0.01$), and that early serum analysis, measuring ASAT levels as early as day 1, was also highly correlated with acute (rank of 0.9; $P = 0.002$) as well as chronic (rank of 0.98; $P = 0.0001$) outcomes. These indicate that the level of injury in the preserved organ and, by extension, the adequacy of the preservation strategy, is intimately correlated with outcome, both acute and chronic.

4. Discussion

In this study, we evaluated the benefits of active oxygenation during MP of DCD kidney grafts using a new perfusion machine designed to permit oxygenation without impeding the transportability of the device. Previous studies with this machine [13] showed superiority of the device over standard static storage in University of Wisconsin solution, and furthermore demonstrated that a perfusion pressure of 30/20 mm Hg provided a better degree of protection compared with a higher pressure of 60/40 mm Hg. We thus used the lower pressure setting for the present work, and compared

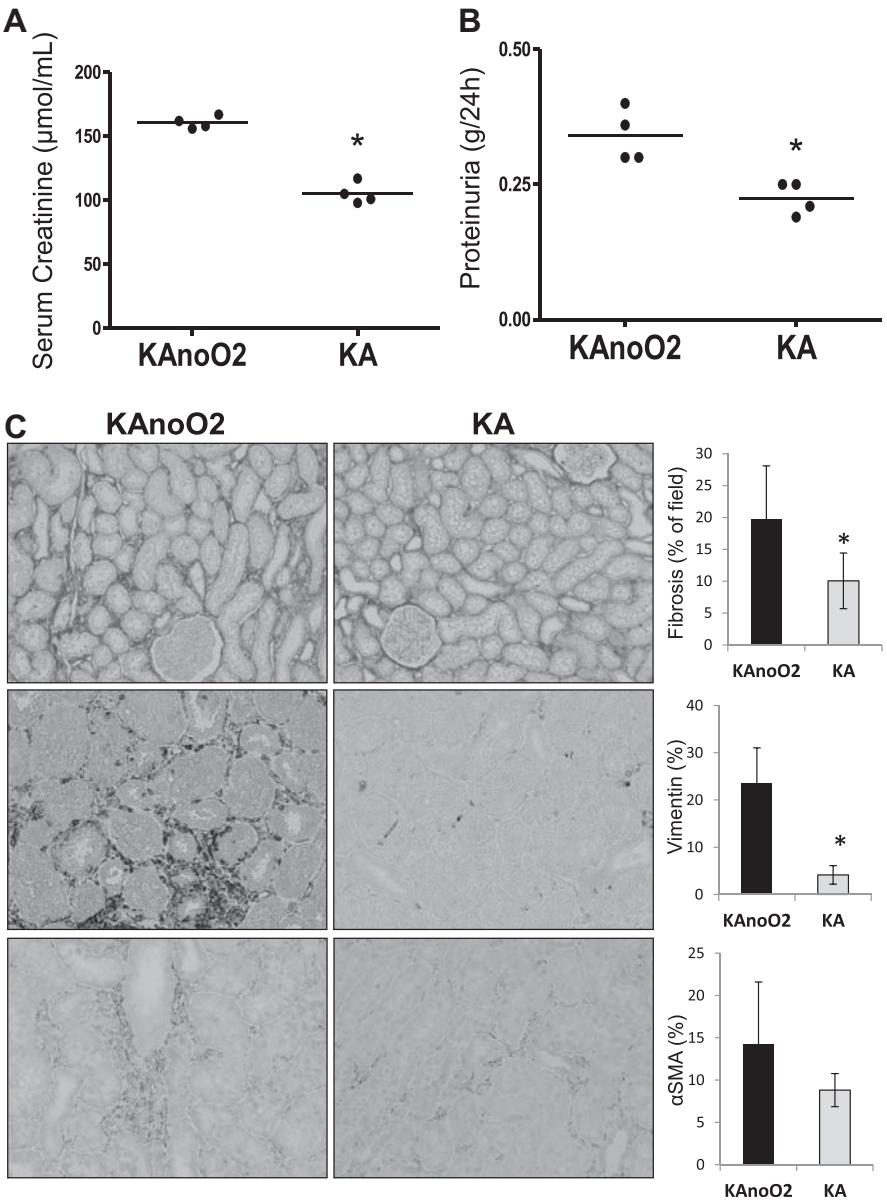

Fig. 2 – Graft chronic outcome. Typical functional and histologic end points were recorded at 3 mo post-transplant. (A) Serum creatinine. (B) Proteinuria. (C) Chronic kidney fibrosis and epithelial to mesenchymal transition markers. Left: representative staining obtained in each group: line 1: Sirius Red (magnification, ×100); line 2: vimentin (magnification, ×200); line 3: αSMA (magnification, ×200). Right: *In silico* quantification of staining in 10 representative fields for each animal. Results are shown as means ± standard deviation. *P < 0.05 compared with $KAnoO_2$.

oxygenation *versus* non-oxygenation in a pig model of DCD donor, in surgical conditions close to that of the clinic, allowing for a high degree of translatability for our results. In this auto-transplantation setting, without the confounding bias from the use of immunosuppressors [24], we can accurately measure the effect of preservation conditions [22]. In the present study, the only parameter varying between groups was the oxygen content of the solution, because both the machine and the preservation solution used were identical.

In terms of early outcome, we observed the benefits of oxygen as early as day 3; animals transplanted with oxygenated kidneys showed lower serum creatinine peak, and furthermore displayed a swifter return to normal creatinine levels. In the non-oxygenated group, the peak was higher and the subsequent decrease started later and was slow, never reaching pre-transplant levels.

Furthermore, we measured the presence of NGAL in the urine of transplanted animals during the first week after transplantation. Neutrophil gelatinase-associated lipocalin is expressed in epithelial cells of the kidney tubules [25], and its presence in the urine is likely related to the level of cellular destruction taking place at reperfusion, which makes it a valuable marker in the diagnosis of acute kidney injury [16,26]. We determined that in both animal groups, the levels of urinary NGAL increased dramatically 1 d post-transplant, consistent with the washout of cellular debris as animals recovered

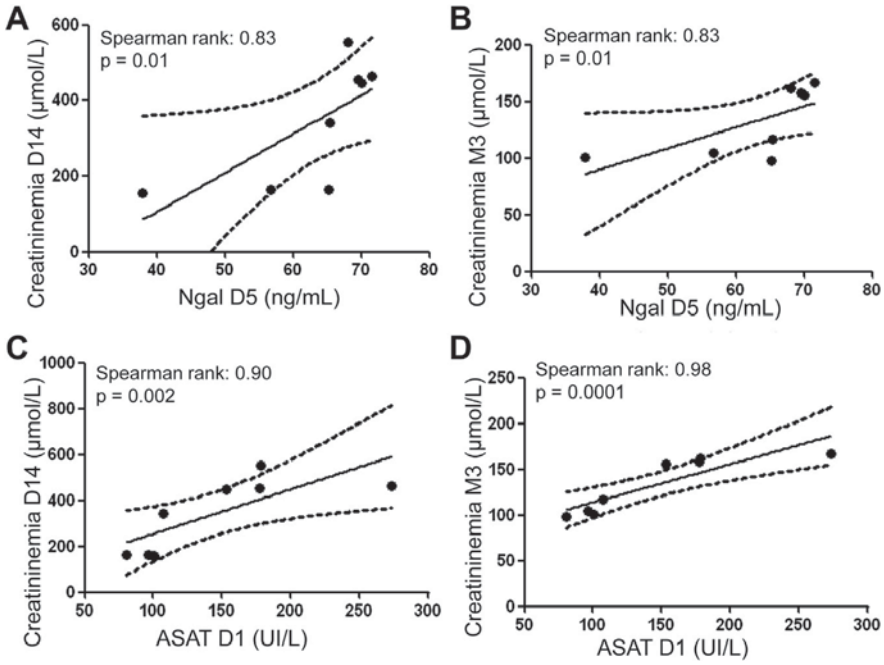

Fig. 3 – Correlation analysis between acute kidney injury markers and outcome. Correlation analysis was performed with NCSS software. Spearman's rank and *P* value are provided for each test. Urinary NGAL (A and B) as well as blood ASAT (C and D) levels were correlated with serum creatinine levels recorded at day 14 (A and C) and 3 mo (B and D).

dieresis. However, KA animals showed reduced levels of NGAL faster than in the $KAnoO_2$ group, which suggests a lower level of injury withstood during MP with oxygen.

Furthermore, because urine production is not a discriminating marker with regard to graft quality evaluation, we investigated the potential for another marker of injury in the blood. Hence, we measured serum ASAT levels, an enzyme generally used to estimate liver function, but also expressed in kidney cells. Its increased presence in the blood is a marker of kidney injury. Indeed, in our hands, we observed an important increase in circulating ASAT in animals after reperfusion and for the first few days post-transplant. Interestingly, we noted lower levels of ASAT in the blood of animals transplanted with kidneys preserved with oxygen.

These results indicate a better preservation quality with active oxygenation, allowing the kidneys to better withstand the stresses of preservation and recover faster from ischemia-reperfusion injury and resume an appropriate level of function.

In our hands, the pig autotransplantation model showed a high degree of correlation between preservation quality and chronic outcome [27]. To evaluate whether active oxygenation during preservation could have long-lasting consequences for kidney grafts, we investigated the histologic features of the kidneys at the end of the follow-up (3 mo).

We first measured the extent of interstitial fibrosis, the unavoidable feature of chronic graft loss [28]. Using anatomopathologic techniques similar to those of the clinic, we showed a significant reduction in the extent of fibrosis in kidneys preserved by oxygenated MP compared with kidneys preserved without oxygen. This demonstrates that oxygenation during preservation prevents the development of tissue fibrosis in our model.

In an attempt to further understand the protective effect of oxygen, we investigated markers for chronic damage mechanisms linked to the development of fibrosis: epithelial to mesenchymal transition and chronic inflammation.

Epithelial to mesenchymal transition regroups the series of steps leading epithelial cells to differentiate toward mesenchymal cell phenotypes, in which they lose their polarized, anchored, and quiescent features cell for an unpolarized, mobile, and fast-proliferating phenotype, intended to repopulate an injury tubule. If deregulated, for instance in the context of chronic graft injury, this process is believed to lead to the differentiation of fibroblasts and promote fibrosis [19]. In the present study, we investigated the expression at the tissue level of two typical markers of EMT: vimentin, a structural component of intermediate filaments in mesenchymal cells cytoskeleton, and αSMA, element of the mesenchymal actin skeleton. Using immunohistochemistry, we detected a high amount of vimentin staining in the kidneys preserved without oxygenation, whereas levels in oxygenated kidneys were significantly reduced; further analysis revealed a similar trend in αSMA staining. These results indicate that the EMT process started in both groups; however, it was more advanced in animals transplanted with a kidney preserved without oxygen, a sign of inferior preservation quality.

We then investigated the extent of chronic inflammation in these grafts, a process well correlated with fibrosis and chronic graft loss. Quantification of invading cells, of both the innate and adaptative immunity subfamilies, revealed no statistical differences between groups. However, there was a trend toward a lower degree of adaptative inflammation in the kidneys preserved with oxygen, which is in accordance with the other results of this study.

Previous studies on the benefits of MP in our model of DCD transplantation showed that it was difficult to obtain discriminating data when attempting to measure the benefits of MP improvement through optimization of the solution [18,29]. However, in this study, we demonstrated a notable improvement with the addition of oxygen, highlighted by improvement of classical end points such as serum creatinine and histopathologic measurement, and also by early markers of injury such as urinary NGAL and serum levels of ASAT. Interestingly, correlation analysis between these later markers and serum creatinine at both 14 d and 3 mo post-transplant show a high Spearman rank. This highlights the value of measuring early markers of injury for the accurate evaluation of organ quality, and furthermore confirms the critical role of ischemia-reperfusion injury in determining the fate of the organ, in terms of both acute and chronic outcomes.

These results are at odds with a previous study on oxygenated MP [14] in which the results did not discriminate between the groups as clearly as in the present study. However, that earlier study used porcine kidney auto-transplantation in which the kidney was not subjected to warm ischemia, a setting in which cold storage induces only a limited amount of injury that could easily be reduced by MP alone, and not profit from the addition of oxygen. Considering the already beneficial effects of MP over cold storage, it is possible that demonstration of further improvement of MP by oxygenation can be done only in a model with a high level of injury, such as the one used here. We use 60 min warm ischemia in this study to mimic as closely as possible the conditions of DCD classes I and II according to the Maastricht classification. As opposed to class III, the controlled DCD found in most current MP trials, classes I and II are subjected to extended warm ischemia time. Because these donors represent a possible 40% increase in the donor pool, we focused our efforts in defining the best possible mean of organ preservation in this particular setting.

Furthermore, the addition of a period of warm ischemia before MP induces severe hypoxia in the organ, which may not have been encountered in the model used by Gallinat *et al*. [14], in which the kidney was set on the machine immediately after collection, and thus was submitted to only a mild degree of hypoxia. In this instance, oxygenated MP could be of particular value for organs from DCD, which are subjected to such a period of severe warm hypoxia.

More studies are needed to determine which of these possibilities is the most accurate: for instance, using conditions mimicking marginal donors, in which kidneys show a high degree of lesion but remain oxygenated.

In conclusion, organ preservation remains an open area of research, because little is known about the mechanisms taking place during the period of transport that is unavoidable in most transplant settings. It is has become increasingly clear that metabolic reactions take place and that they need oxygen to function properly, because without it the organ will fair poorly. Previous work from our team showed similar results when delivering oxygen during static storage, using a dedicated carrier [30]. Other studies of oxygen during preservation also showed improved organ quality, notably through increased production of adenosine triphosphate during the hypothermic phase [12]. Although it is likely that a similar mechanism took place during our study, we could not verify it. Such analyses were not compatible with our setting, because iterated tissue sample collection during MP would alter the perfusion conditions. Moreover, we voluntarily limited the scope of this study to obtain proof of concept, a required element in the setting of extended large animal studies. There is a strong need for further investigation into the intricacies of hypothermic preservation and oxygen use, which would lead to the development of new tools to improve preservation quality, and thus permit the use of more marginal organs.

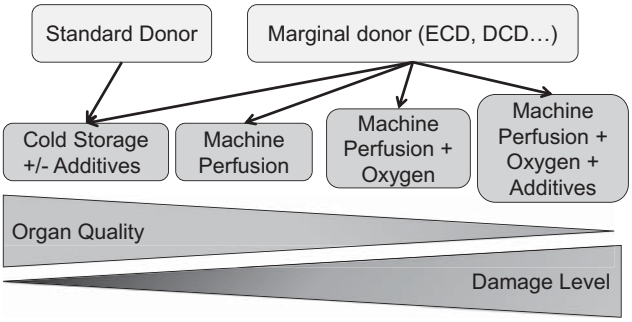

Fig. 4 – Decisional diagram for adaptation of preservation conditions to donor organ quality.

In the present study, we determined in a preclinical model of DCD kidney transplantation, allowing for a high degree of translatability to the clinic, the benefits of active oxygenation during hypothermic MP. Kidneys provided with oxygen during preservation will display the signs of higher quality, resume function faster in the first 2 weeks with lower serum creatinine peaks, and return to levels close to pre-transplant. Furthermore, in terms of outcome, the typical lesions of chronic graft loss are reduced, which confirms better preservation of the tissue and improved ability to recover from the damage of ischemia-reperfusion. Oxygen delivery during preservation thus appears to be valuable for highly damaged organs and offers an important therapeutic tool for transplant teams faced with decreased quality of donor organs. The combined development of more accurate tools to measure graft quality early and advances in organ storage strategies such as the use of additives and active oxygenation could lead to customization of graft storage, providing the most adaptative conditions depending on the need of the organ, as proposed in Figure 4.

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Supplementary data

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