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Renal Transplantation: What Has Changed in Recent Years

Lead Guest Editor: Michele Santangelo
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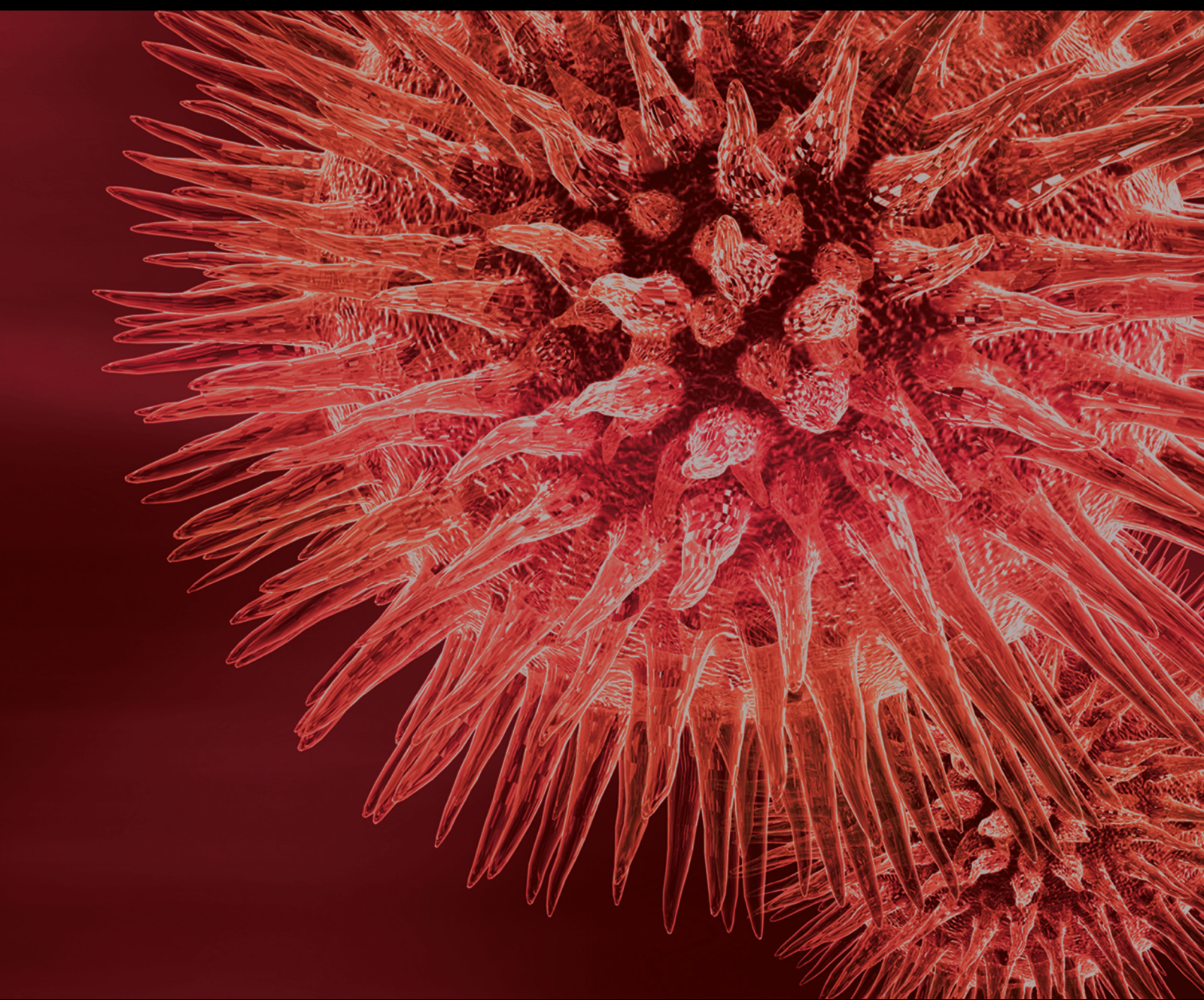

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Editorial

Renal Transplantation: What Has Changed in Recent Years

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Kidney transplantation is the best approved renal replacement therapy, although one of its biggest limitations remains a general organ donor shortage [1], not only in terms of absolute numbers, but particularly regarding preservation techniques.

The concept of static cold storage has been proved to damage marginal organs that are increasingly being accepted to match the waitlist demand. Modern dynamic preservation technologies have developed in recent years not only to actively prevent kidney damage before transplantation, but also to assess potential organ viability. In this special issue, containing 12 manuscripts, we focus on hypothermic machine perfusion, showing in a matched observational study by M. I. Bellini et al. a higher eGFR at one-year follow-up with the dynamic preservation compared to static cold storage. Moreover, during hypothermic machine perfusion, resistive index predicted delayed graft function (DGF) with accuracy of 0.78 and 0.87 for organs from donation after circulatory death (DCD) or after brain death (DBD), respectively, and significantly decreased incidence of DGF in DCD organs.

Another possible way to improve the standard preservation technique is described by A. Ostróżka-Cieślik et al., who found that the addition of ascorbic acid and prolactin to Biolasol solution affects the maintenance of the normal cytoskeleton of the stored graft. Furthermore, R. Thuillier and T. Hauet focused on the cytoskeleton integrity during cold preservation, highlighting the negative impact of hypothermia during static cold storage.

The existing barriers in transplantation are extensively reviewed by C. Steichen et al., in order to identify the development of novel strategies, such as machine perfusion and reliable biomarkers to monitor graft quality and predict short and long-term outcomes. To this end, it is noteworthy that severe injury of the renal microvasculature with relatively preserved tubular epithelium may be associated with some conditions of deceased kidney donors leading to early kidney graft nonfunction and loss as interestingly reported by N. Kojc et al., although not all the units rely on the use of histology preimplantation [2]. Long-term outcomes are in fact also related to other important conditions, and beyond rejection and delayed graft function, an accurate surveillance of immunosuppression should be constant to prevent side effects as malignancy, unfortunately more common in transplanted patients [3].

A. Reznik et al. describe the damage caused by ischemia reperfusion injury (IRI) in animal models. The group investigates the effects on AP-1 and Heat Shock Response in donor kidney parenchyma after warm ischemia, with the aim to map IRI related molecules as targets for reconditioning during machine perfusion.

In order to combat organ shortage and following the successful DCD programmes in the UK, Netherlands, and Spain, Poland has recently commenced its own DCD transplant

series. The article from H. Stadnik et al. reports the preliminary evaluation of the first 19 such cases in the country, focusing on clinical outcomes as well as public perception as an essential step for the initiation and maintenance of the programme.

Another possible way to increase the organ donor pool is the utilisation of HCV positive donors. The use of the new direct-acting antiviral agents, whose safety and efficacy in eradicating HCV infection without an increased risk of allograft rejection, is analysed in the report by A. Calogero et al. The effects of HCV eradication, in terms of quality of life, are further elucidated by Sabbatini's trial, showing a meaningful improvement as a patient reported outcome in kidney transplant recipients.

The special issue also explores, through a systematic review, the potential benefits of living donors having laparoscopic versus robot assisted donor nephrectomy. The rate of postoperative pain in the latter group was significantly lower, confirming the safety and feasibility of this technique.

Finally, as dialysis is still the only available treatment for those who do not get transplanted, particular attention should be devoted to preserve their dialysis access. G. Ietto et al. present a retrospective analysis of patients who underwent native polycystic kidney nephrectomy and whether they can go back to peritoneal dialysis after surgery. Their findings suggest that those patients who presented with postoperative complications [4], such as bleeding and therefore potentially requiring reintervention, did not recover their peritoneal dialysis routine.

In conclusion, we believe that this special issue provides a valuable update on the scientific progress of renal transplantation, notably by adding insight and giving future direction on scientific research and clinical practice.

Conflicts of Interest

The editors of this special issue and authors of this paper declare no conflicts of interest.

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Nicos Kessaris
Karine Hadaya
Diederik Kimenai
Maria Irene Bellini

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Research Article

Influence of Hypoxic Preservation Temperature on Endothelial Cells and Kidney Integrity

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Guest Editor: Nicos Kessaris

Ischemia-reperfusion (IR) injury is unavoidable during organ transplantation and impacts graft quality. New paradigms are emerging including preservation at higher temperature than "hypothermia" or "cold": although 4°C remains largely used for kidney preservation, recent studies challenged this choice. We and others hypothesized that a higher preservation temperature, closer to physiological regimen, could improve organ quality. For this purpose, we used an *in vitro* model of endothelial cells exposed to hypoxia-reoxygenation sequence (mimicking IR) and an *ex vivo* ischemic pig kidneys static storage model. *In vitro*, 19°C, 27°C, and 32°C provided protection against injuries versus 4°C, by reducing cell death, mitochondrial dysfunction, leukocyte adhesion, and inflammation. However, *ex vivo*, the benefits of 19°C or 32°C were limited, showing similar levels of tissue preservation damage. *Ex vivo* 4°C-preserved kidneys displayed a trend towards reduced damage, including apoptosis. Macrophage infiltration, tubulitis, and necrosis were increased in the 19°C and 32°C versus 4°C preserved kidneys. Thus, despite a trend for an advantage of subnormothermia as preservation temperature, our *in vitro* and *ex vivo* models bring different insights in terms of preservation temperature effect. This study suggests that temperature optimization for kidney preservation will require thorough investigation, combining the use of complementary relevant models and the design of elaborated preservation solution and new technologies.

1. Introduction

A significant fraction of kidney graft dysfunctions observed after transplantation is due to unavoidable ischemia-reperfusion (IR) injuries (IRI), which impact short- and long-term transplantation outcomes [1]. Indeed, despite extracorporeal "cold" preservation period which is used worldwide to overcome this issue, graft injuries are frequently observed and caused by pathophysiological mechanisms related to ischemia and severe hypothermia. Furthermore, because of the severe organ shortage, donor demography has changed and additional sources of organs are grafts recovered from extended criteria donors (ECD) and donation after circulatory death (DCD) [2, 3]. However, these organs are particularly exposed and sensitive to IRI and therefore more prone to develop dysfunctions. Although transplantation of marginal grafts demonstrates promising outcomes, increased rates of primary nonfunction, delayed graft function, and

reduced graft survival have been reported [3]. Cold ischemic injury, caused by static cold storage, is a significant risk factor for poor outcome. In this context, optimization of organ preservation protocols is a pivotal goal.

Currently, kidney preservation procedure is based on hypothermia: grafts are flushed and conditioned at 4°C in a preservation solution, such as University of Wisconsin solution (UW), Custodiol, Solution de Conservation des Organes et des Tissus (SCOT-15), and Institut Georges Lopez-1 (IGL-1). However, this preservation procedure is being questioned by scientific community and numerous articles highlighted that hypothermia indeed worsens ischemic injuries through (i) reduction of ATP synthesis and metabolic activity [4], (ii) reduced Na-K-ATPase activity, which induces osmotic perturbation [5, 6], (iii) mitochondrial perturbations, (iv) decreased cell survival [7, 8], and (v) endothelial activation [9, 10].

There are emerging concepts of alternative storage temperatures, which could limit IRI and recent studies advocate the use of normothermia, mild hypothermia, and subnormothermia [2, 11, 12]. Preclinical findings have ignited clinical organ preservation research that investigates dynamic preservation, its various modes (continuous, preimplantation), and temperatures (hypo-, sub-, or normothermic).

Applied to mouse livers, normothermic (37°C) ischemia reduced sinusoidal perfusion, increased leukocyte adhesion to the endothelium and altered tissue integrity. However, temperatures of 26°C, 15°C, and 4°C reduced these injuries equally [13]. Compared to normothermia, mild hypothermia (32°C) reduced infarct size on rabbit hearts undergoing IR [14]. Moderate hypothermia (30°C) decreased myocardial energy utilization during ischemia and subsequently promoted expression of proteins involved in cell survival, compared to 34°C [15]. In a mouse model of partial liver ischemia of 90 min performed at various temperature (4°C, 15°C, 26°C, 32°C, and 37°C), oxidative stress was reduced at 15°C to 32°C [16]. Rat liver perfusion temperature of 21°C showed an improvement of hepatic function recover, compared to 4°C or 12°C [17]. In addition, mild hypothermia (34 to 35°C) applied to organ donors significantly reduced the rate of delayed graft function [18].

Regarding normothermic preservation, different techniques are described. One of the proposed methods used an acellular normothermic perfusion system [19]. An alternative technique, developed by Nicholson and Hosgood, used an adapted pediatric system with a packed red cell based solution [20]. Novel strategies are emerging using machine perfusion at subnormothermia and normothermia levels [2]. Other approaches must be also taken into consideration such as hypothermic or normothermic abdominal regional perfusion in high-risk donors with extended warm ischemia times [21] or controlled rewarming after hypothermia [22, 23]. These are new principles and practices, which bear promising features and which ought to find a place in renal preservation. Collectively, these reports suggest that an intermediate temperature might be more efficient to protect organs against IRI, particularly from marginal donors.

We designed the present study to assess the effects of different temperature levels during preservation. We focused on targets demonstrated to be critical in the pathophysiology of cold ischemic and reperfusion injuries: endothelium, mitochondrial dysfunction, cell death, and activation of innate immunity and inflammation [24, 25]. Because our policy is to minimize the use of *in vivo* models, we designed alternative models in order to establish a *corpus* of consistent data, allowing clarifying specifications for organ preservation at different levels of temperature. First, we used an i*n vitro* model of IR (hypoxia-reoxygenation) applied to porcine primary endothelial cells, the endothelium being the first *in situ* histological component to be exposed during IRI. A second set of experiments was designed to evaluate the effect of preservation temperature *per se* in a preclinical, *ex vivo* porcine kidney preservation model. Indeed, the anatomic and physiologic similarities between pigs and humans, especially for the kidney, make this model a valuable and relevant translational model [26].

2. Materials and Methods

2.1. Porcine Endothelial Cells. Endothelial cells were prepared from cortex porcine renal tissue of a 3-month-old large white pig (MOPICT plateforme, INRA, Surgères). The remaining tissue was digested using 1 mg/mL Collagenase IA (Sigma Aldrich, France) + 200 μg/mL DNase I (Roche Diagnostics, France). Nonspecific binding was blocked with porcine IgG (Interchim, France). Cell suspension was incubated with anti-CD31 antibody conjugated to dynabeads (Fischer-Bioblock, France) and then isolated by immunomagnetic separation (Dynal, Fischer-Bioblock, France).

2.2. In Vitro Hypoxia-Reoxygenation Model of Ischemia-Reperfusion. Porcine renal endothelial cells were cultured on Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal bovine serum, 1% L-Glutamin, and 1% pyruvate (all from Fischer-Bioblock) (completed DMEM), at 37°C with 5% CO_2. Cells were used at passages 4 to 5. For preservation, cellular "ischemia" was achieved by incubating cells in a hypoxic atmosphere: 0% O_2, 5% CO_2, and 95% N2 (Bactal 2 gaz; Air Liquide, France) for 24 hours at 4°C, 19°C, 27°C, 32°C, or 37°C in UW solution. "Reperfusion" was performed either by replacing UW for 24 hours in completed DMEM or for 2 hours in anticoagulated porcine blood at 37°C in 20% O_2, 5% CO_2, and 75% N2. Control cells (CTL) were incubated in completed DMEM at 37°C in 20% O_2, 5% CO_2, and 75% N_2.

2.3. Oxygen Measurement. Confluent endothelial cells cultured in flask were incubated in a anoxic atmosphere: 0% O_2, 5% CO_2, and 95% N_2 (Bactal 2 gaz; Air Liquide, France) for 24 hours at 4°C, 19°C, 27°C, 32°C, or 37°C in UW solution. Partial pressure of oxygen was measured in real time with oxygen probes connected to the OXY-4 fiber optic oxygen transmitter (PreSens, Precision Sensing GmbH, Regensburg, Germany). At the different hypoxic incubation temperatures, measurements of oxygen were carried out in the atmosphere chamber and simultaneous in the atmosphere culture flask and in the UW preservation medium during the hypoxia period.

2.4. Lactate Dehydrogenase Release Quantification. At specific time points, supernatant and cells were collected to quantify lactate dehydrogenase (LDH) levels using an analyzer (Modular analytics P; Roche Diagnostics, Meylan, France).

2.5. Succinate Dehydrogenase Activity Assay by XTT. Following the different culture conditions, cells were incubated with XTT labeling mixture (XTT kit; Roche) following manufacturer's instructions and reaction was quantified by spectrophotometer (Victor3; Perkin-Elmer, France).

2.6. ATP Assay. We used ATP Bioluminescent Assay Kit (Sigma) following manufacturer's instructions. Briefly, ATP is consumed, and light is emitted when firefly luciferase catalyzes the oxidation of D-luciferin.

2.7. Reverse Transcription and Real-Time Reverse-Transcriptase Polymerase Chain Reaction. Total RNA isolation was performed with NucleoSpin® RNA-XS kit (Macherey Nagel, France). Genomic DNA was removed using DNA-free kit (Applied Biosystems) and first-strand reverse transcription (Applied Biosystems) was performed to obtained cDNA. Specifics porcine primers were designed using OligoPerfect™ (Invitrogen), sequences detailed in Table S1. Amplification was conducted on an automated analyzer ABI PRISM 7300 (PE Applied Biosystems, France). PCR amplification mix was 2 μL cDNA at 5 ng/μL + 2 μL forward and reverse primers (500 nmol/L final concentration) + 10μL SYBR green (SYBR® green PCR Master Mix, Applied Biosystems) + 6 μL ultrapure H_2O. PCR reaction was performed following manufacturer's instructions (hold: 10 min at 95°C, 40 cycles: 15 sec at 95°C, 1 min at 60°C, and finally melt). Finally, mRNA expression level in the sample was obtained with the comparative Ct method (and expressed relative to control, normalizing the target gene to an internal standard gene, L19 (RPL19)): 2-ΔΔCt, where $\Delta\Delta Ct = (Ct,T - Ct,R)_x - (Ct,T - Ct,R)_{cb}$, with Ct,T the threshold cycle of the target gene and Ct,R the threshold cycle of the standard gene and where *x* refers the experiment point and *cb* to the calibrator point.

2.8. Leukocyte Adhesion. After 24 h of hypoxia at various temperatures, ischemic porcine endothelial cells were reoxygenated 2 h with anticoagulated (Li-heparin) porcine blood at 37°C. Two hours after incubation, cell monolayer was washed 3 times in phosphate-buffered saline to eliminate blood red cells and no-adherent leukocytes, and refringent adherent leukocytes on monolayer endothelial cells were counted in culture wells, under microscopic evaluation.

2.9. Electron Microscopy. Cells samples were processed for transmission electron microscopy, as previously described [27]. Cells pellets were fixed in 3% glutaraldehyde, washed, and postfixed in 1% osmium tetroxide. Then pellets were dehydrated in acetone and embedded in araldite. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and were examined under an electron microscope (JEOL 1010, Tokyo, Japan). The degree of lesions was determined by using a semiquantitative graded score: 0 = no alteration, 1 = lesions between 1-25%, 2 = lesions between 26-50%, 3 = lesions between 51-75%, and 4 = lesions >76% (percentages of lesions by field).

2.10. Ex Vivo Experiments. The surgical and experimental protocols were performed in accordance with the ARRIVE guidelines, with EU Directive 2010/63/EU, and with institutional Poitou-Charentes Ethical Comity of animal experimentation (protocol number: CE2012-4). We used 3 months old large white pigs weighting 40 ± 4 kg (IBiSA plateforme, INRA, Surgères, France). After analgesia/anesthesia, surgical procedures were performed to remove the right kidney, which was flushed with UW solution (Bristol-Myers-Squibb, France) at various selected temperatures (4°C, 19°C or 32°C) and preserved during 24 hours under static conditions at the selected temperature.

2.11. Histological and Immunohistological Study. Biopsy samples from corticomedullary kidney sections were fixed with 4% formalin and paraffin-embedded. Renal histological injuries were quantified using Periodic Acid Schiff (PAS) coloration. The degree of histological lesions was determined in the cortex by using a semiquantitative graded scale. Brush border loss, tubular dilatation, and endoluminal detachment were assessed using a semiquantitative 6-points scale: 0 = no alteration, 0.5 = lesions <5%, 1 = 6-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, and 5 = >76% of lesions.

The number of infiltrates in the renal tissue was quantified using PAS staining. Quantitative determination of tubulitis was adapted from Banff classification: 0 = no mononuclear inflammatory cell in tubules; 1 = 1 to 4 mononuclear cells *per* tubular cross-section; 2 = 5–10 mononuclear cells *per* tubular cross-section, and 3 = >10 mononuclear cells *per* tubular cross-section.

For immunohistochemistry study of macrophage infiltration in the renal tissue, slides were deparaffinized, saturated with a specific buffer (Dako protein block X0909, from Agilent, Les Ulis, France), and incubated with primary antibody for 2 hours in a humid atmosphere (MAC-387 antibody ab80084, from Abcam, Cambridge, United-Kingdom, used at 1/100 in Dako antibody diluent S2022, from Agilent, Les Ulis, France). Secondary antibody was incubated at room temperature for 30 minutes (HRP anti-rabbit K4002 ready to use, from Agilent, Les Ulis, France).

For immunofluorescence study to quantify the number of cleaved caspase-3 positive cells in the renal tissue, slides were deparaffinized, saturated with a specific buffer (Dako protein block X0909, from Agilent, Les Ulis, France), and incubated with primary antibody for 2 hours in a humid atmosphere (Caspase 3 cleaved antibody MAB835, from R&D Systems, Lille, France, used at 1/200 in Dako antibody diluent S2022, from Agilent, Les Ulis, France). Secondary antibody was incubated for 1 hour at room temperature (Anti-rabbit 488, A11034, from Thermofisher, Illkirch, France, used at 1/1000).

Quantification of the number of apoptotic cells in the renal tissue was performed using the DeadEnd™ Fluorometric TUNEL System kit (staining of DNA fragmentation), according to the manufacturer's instructions (Promega, Charbonnières-les-Bains, France).

All evaluations were performed and scored under blinded conditions by an anatomopathologist.

2.12. Statistical Analysis. Results were expressed as percentage of level obtained in "IR simulated" cells over control living cells. Results are expressed as mean ± SEM. For multigroup comparisons we used the Kruskal-Wallis test and Dunn's posttest; differences were considered significant if $p < 0.05$.

3. Results

3.1. Oxygen Measurement. First, oxygen was reduced to 0% in the chamber atmosphere within 6.5 min after induction of hypoxia by the use of Bactal 2 gas. Independently of the incubation temperature, oxygen pressure level as measured both in the flask atmosphere and in the cell culture medium were reduced simultaneously and below 1% after 130 min of incubation with Bactal 2 (Figure 1; n = 3 *per* group).

3.2. Necrosis at the End of Hypoxic Preservation. To test the influence of temperature on cell survival after 24 h preservation in hypoxia, we measured LDH release (an indicator of necrotic cell death) from cells preserved in UW at 4°C, 19°C, 27°C, 32°C, or 37°C. Results showed that while basal LDH release level in controls was 17.1 ± 0.8%, it increased significantly at 4°C (37 ± 3%) and at 19°C (40.2 ± 3.3%) groups, indicating increased cell mortality. Conversely, intermediate temperatures significantly decreased cellular necrosis: 27°C (12.2 ± 1%), 32°C (12.9 ± 1%), and 37°C (13.8 ± 1.2 %) (Figure 2(a); n = 8 per group).

3.3. Mitochondrial Activity after IR. At the end of 24 h preservation in UW at either 4°C, 19°C, 27°C, 32°C, or 37°C in hypoxia, followed by 24 h reoxygenation at 37°C in completed DMEM, we measured mitochondrial dehydrogenase activity (complex II of the mitochondrial respiratory chain). We observed that while preservation at 4°C and 37°C induced important decrease in complex II activity (45.9 ± 1.1% and 33.7 ± 0.6% of control, respectively), intermediate temperatures induced a significantly higher activity, 19°C (63.8 ± 0.9%) and 27°C (63 ± 0.5%), the highest level of protection being brought about by 32°C (78.5 ± 0.5%; Figure 2(b); n = 16 *per* group). Finally, using the same experimental protocol, we showed that while 4°C hypoxic preservation severely decreased ATP levels (33 ± 4% of control cells), (i) 37°C preserved ATP production (81 ± 2%) and 19°C, 27°C and (ii) 32°C yielded higher ATP production, compared to control (respectively, 144 ± 3%, 130 ± 2%, and 206 ± 7% of control cells; Figure 2(c); n = 16 *per* group).

3.4. Innate Immunity Analysis after In Vitro Blood Reoxygenation at 37°C. To investigate the innate immunity signaling at the different preservation temperatures, cells were preserved for 24 h in UW 0% O_2 at 4°C, 19°C, 27°C, 32°C, or 37°C, followed by 2 h reoxygenation at 37°C, performed with porcine blood, and then submitted to transcriptional analysis. Endothelial activation marker ICAM-1 was significantly overexpressed in cells preserved at 4°C (12.3 ± 2.4 folds) while at 19°C, 27°C, 32°C, and 37°C preservation the increase was significantly less (2.1 ± 0.3, 3.1 ± 0.3, 4.2 ± 0.8, and 5.6 ± 1.9 folds, respectively; Figure 3(a); n = 3 *per* group). Danger signal receptor TLR4 was significantly overexpressed in the 4°C (7.7 ± 4.1 folds) and 37°C (9.9 ± 3.1 folds) groups, while it was unchanged at 19 and 27°C and significantly reduced at 32°C (0.3 ± 0.02 folds) (Figure 3(b); n = 3 *per* group). Finally, the innate immune system activator MCP-1 was significantly overexpressed in cells preserved at 4°C (220 ± 49.6 folds) and 37°C (27.1 ± 8 folds), while its expression remained similar to the control level in all other groups (Figure 3(c); n = 3 *per* group).

3.5. Leukocytes Adhesion after In Vitro Blood Reoxygenation. We further quantified leukocyte adhesion on endothelial cells subjected to 24 h hypoxic preservation in UW at different temperatures, followed by 2 h reoxygenation at 37°C with porcine blood. We found a higher number of adherent leukocytes in 4°C (59 ± 4), 19°C (52 ± 7), and 37°C (59 ± 6) groups, compared to 27°C (30 ± 4) and 32°C (38 ± 4 cells) groups (Figure 3(d); n = 3 *per* group).

3.6. Cellular Morphological Integrity. By electron microscopy analysis, performed at the end of 24 h hypoxia preservation in UW (4°C, 19°C, 27°C, 32°C or 37°C) followed by 24 h reoxygenation at 37°C in completed DMEM, we showed that, as compared to normal control cells, cells preserved at 4°C had smaller mitochondria, with fewer visible crests. These lesions were also observed in the 37°C group, exhibiting with small and lysed mitochondria, cytosolic vacuolization, and plasma membrane alteration. However, fewer mitochondria were lysed at 19°C, and 27°C and 32°C temperatures positively impacted mitochondria number and aspect with maintenance of longer mitochondria with visible crests and cytosolic integrity (Figure 4) (n = 3 per group).

3.7. Evaluation of Morphology, Leukocyte Infiltration, and Apoptosis after Ex Vivo Kidney Preservation. In addition to *in vitro* experiments we performed *ex vivo* determinations by submitting porcine kidneys to UW flush, followed by preservation in UW during 24 hours under static conditions at 4°C, 19°C, and 32°C. Histological analysis showed that, first, tubular dilatation was similar at the end of the UW flush and after 1 h of conservation for all temperature groups, but then gradually increased after 6 h and 24 h of conservation, indicating a degradation of the tissue structure along preservation time. Of note, tubular dilatation areas number was significantly higher in the 19 and 32°C groups versus the 4°C group after 24 h of preservation. Similarly, brush border loss and cell detachment (cellular necrotic injuries) increased significantly from 6 h to 24 h after conservation in the 19°C and 32°C groups while damage remained limited in the 4°C group (Figure 5; n = 3 per group).

After 24 h UW preservation at 19°C, the number of infiltrated monocytes/macrophages (MAC387 staining), cellular infiltrates, and the score of cortical tubulitis increased significantly compared to 4°C ($p<0.05$). These indicators were further increased at 32°C ($p<0.05$ versus 4°C and versus 19°C) (Figures 6(a) and 6(b)) (n = 3 per group).

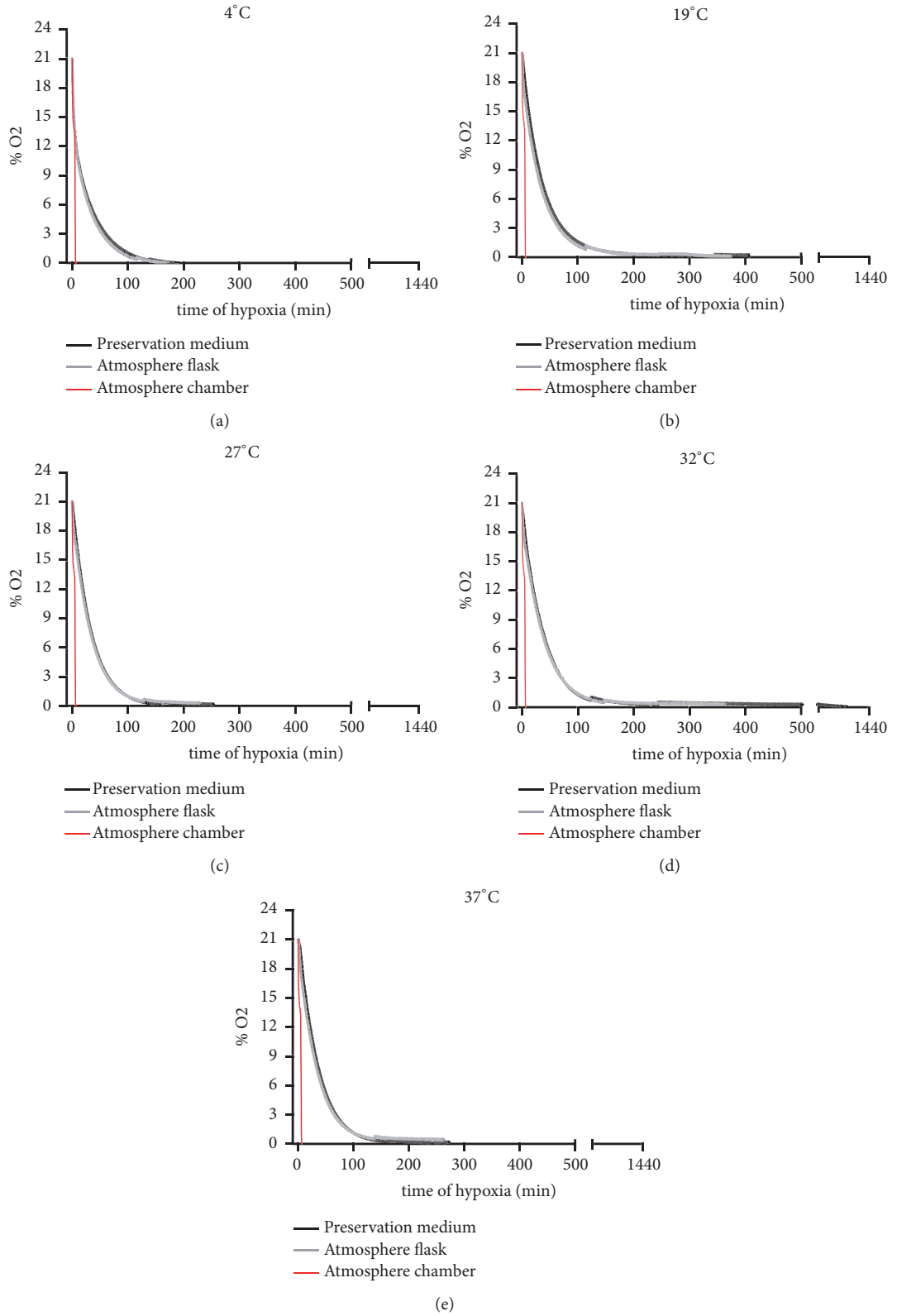


Figure 1: Effects of various preservation temperatures on oxygen pressure level during induction of hypoxia by Bactal 2: partial percentage of oxygen were measured in real time in the chamber atmosphere, in the cell culture flask atmosphere, and in the UW preservation medium during the hypoxia period, at 4°C (a), 19°C (b), 27°C (c), 32°C (d), and 37°C (e). Measured values of oxygen (atmospheric%) are expressed as the mean value of n = 3 determinations per group.

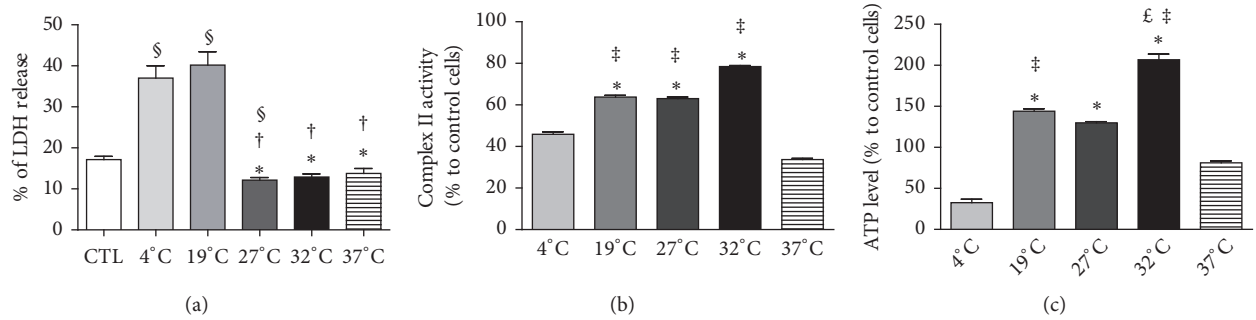


Figure 2: Effects of various preservation temperatures on endothelial cells viability and on the mitochondria. (a) LDH release, measured at the end of the hypoxic preservation, is expressed as LDH release in supernatant/ intracellular LDH (U/L). Results are expressed in mean ± SEM (n = 8 per group). CTL: control cells not subjected to any manipulation. The integrity and activity levels of mitochondrial respiratory chain complexes were measured after normothermic reoxygenation: (b) complex II (n = 16 per group); (c) ATP production by Complex V (n = 16 per group). Results are expressed as mean ± SEM (% of control cells). Statistical significance ($p<0.05$) were calculated using nonparametric Kruskal-Wallis test, followed by multiple comparison evaluation by Dunn's posttest (§ $p<0.05$ to control cells, ∗$p<0.05$ versus 4°C, † $p<0.05$ versus 19°C, £ $p<0.05$ versus 27°C, ‡ $p<0.05$ versus 37°C).

Furthermore, we observed a trend for a higher number of cleaved caspase 3 positive cells in the kidneys preserved for 6 h and 24 h at 19°C or 32°C compared to 4°C (Figure 7) (n = 3 *per* group).

In addition, we observed a trend for a higher number of apoptotic cells (DNA fragmentation detected by TUNEL staining) in the kidneys preserved during 6 h and 24 h at 19°C compared to 4°C (Figure 8; n = 3 *per* group).

4. Discussion

In preservation-transplantation, ischemia-reperfusion injury (IRI) is an inevitable event, caused primarily, although not exclusively, by hypothermia and hypoxia during the ischemic phase and by return to normal temperature and reoxygenation during the reperfusion phase. Circumventing the deleterious consequences of hypothermia is one pivotal objective to improve graft outcome. In the current study, we tested different preservation temperatures in an *in vitro* model of IR using endothelial cells, because this is the first cell type to be affected during the IR syndrome, with major consequences on vasculature integrity and function [25–28].

Of note, the temperature exerted a weak influence on O_2 level decrease in the culture flask atmosphere or in the UW preservation medium.

In vitro analyses of cell survival (Figure S1) showed that cell preservation at intermediate temperatures (19°C, 27°C and 32°C) was able to improve cell survival, compared to both extremes, i.e., 4°C/hypothermia (the farthest condition compared to physiological one, despite strongly reducing cell metabolism) and 37°C/normothermia (the condition linked with the higher metabolic demand). In order to characterize the effect of normothermic reoxygenation, we evaluated mitochondrial function and observed that both 4°C and 37°C hypoxic preservations severely impaired complex II activity and ATP production. Mitochondrial complex II oxidizes succinate to fumarate and reduces ubiquinone, thereby creating a direct link between the tricarboxylic acid (TCA) cycle (also known as citric acid or Krebs cycle) and the respiratory chain, the main ATP provider in eukaryotic cells [29]. In this model, we also noted that intermediate temperatures preserved ATP production better than 4°C. Within the range of tested temperatures, 32°C was the most efficient to improve cellular ability to produce ATP. Several studies have confirmed the importance of a relative preserved ATP level for cells exposed to hypoxia and hypothermia, followed by rewarming and reoxygenation, to maintain cell viability and optimal graft function [30, 31]. Indeed, we showed that ATP level is negatively correlated to LDH release, itself associated with level of cell integrity failure [30]. Use of intermediate temperature was also associated with weaker mitochondria and cytosolic alterations, as shown by electron microscopy. Such data suggest that antagonistic processes that predominate under different conditions were able to adapt mitochondrial morphology and dynamics to the bioenergetic requirements of the cell [32].

IRI are associated with vascular dysfunction, increased vascular permeability, endothelial cell inflammation, and macrophage infiltration in the damaged tissue, such features representing the prototypical response of the macro- and microvasculature to a stress or an injury [33]. Thus, preservation of the endothelial wall integrity is crucial to improve function recovery and limit inflammation [9]. Furthermore, expression of immune cell receptors and chemokines by activated endothelial cells induces monocyte and neutrophil adhesion, triggers inflammation and is partially responsible for the "no-reflow" phenomenon through endothelial cell detachment [34, 35]. We sought to further characterize the effects of the different preservation temperatures on ICAM-1, TLR4, and MCP-1 transcriptional levels after endothelial cells reoxygenation with whole blood. ICAM-1 is a membrane receptor which favors leukocyte adhesion before infiltration. In the current study, expression of this molecule was significantly increased after preservation at 4°C, confirming the link between severe hypothermic preservation and leukocyte infiltration. On the other hand, preservation using intermediate temperatures (19°C, 27°C, and 32°C) was able to significantly reduce this expression

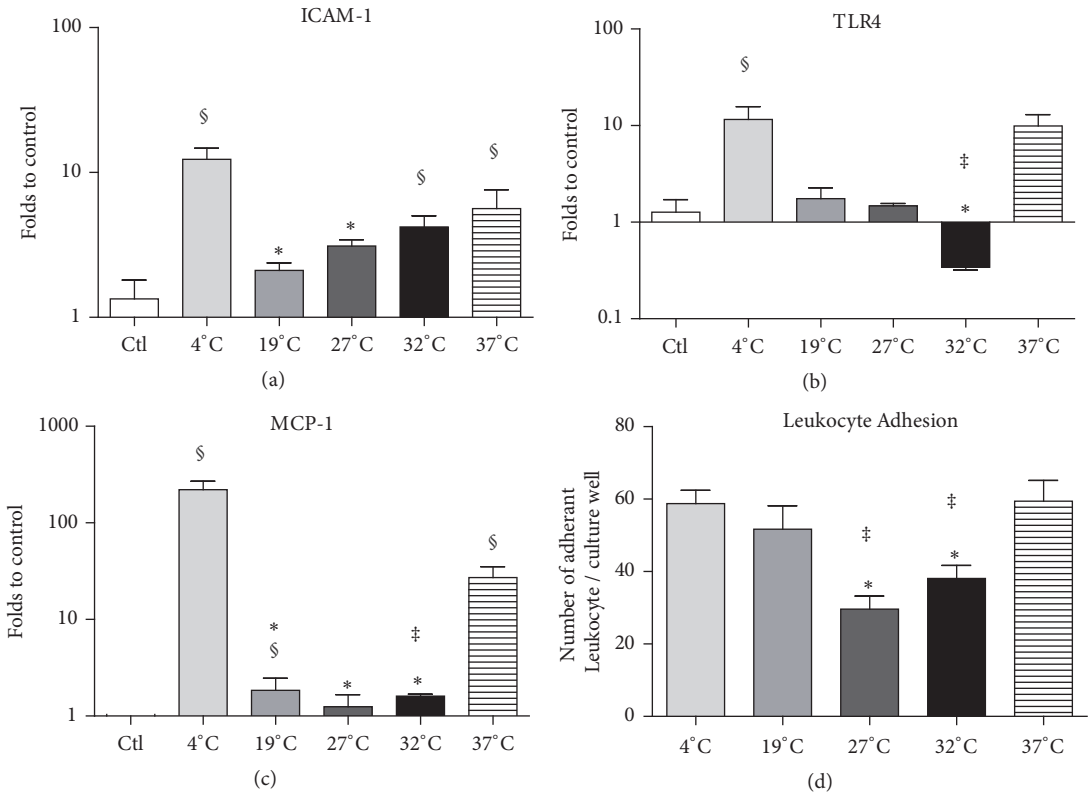
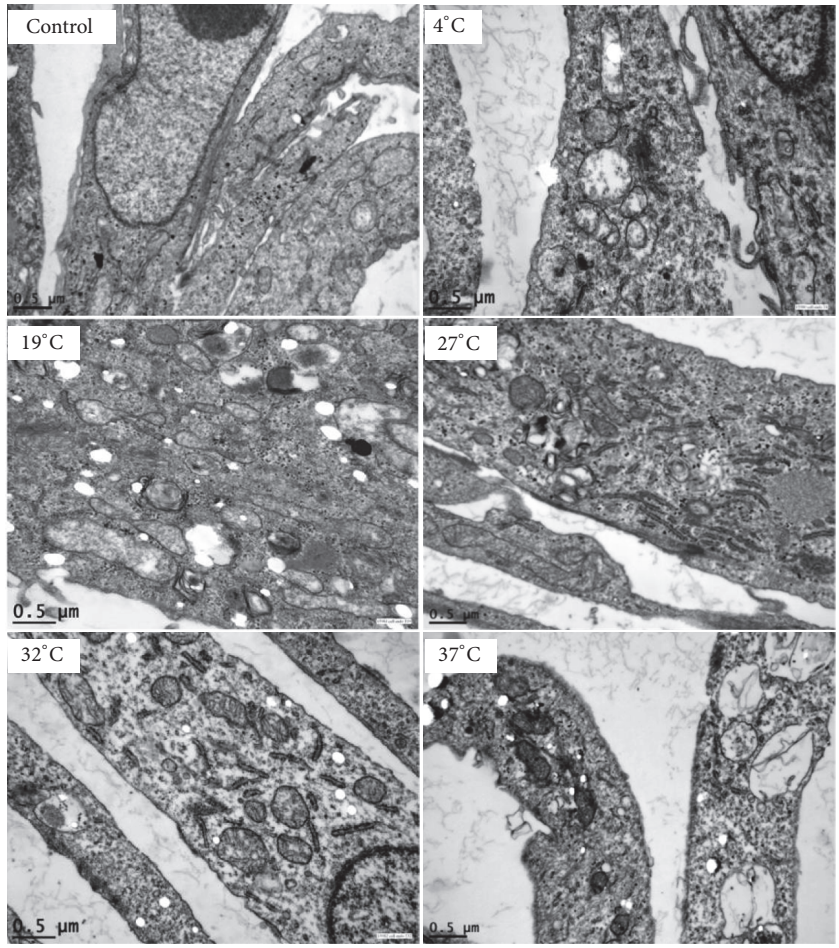


Figure 3: Effects of preservation temperature on endothelial cells phenotype: the influence of preservation at various temperatures was evaluated at the transcriptomic level by RTqPCR, measuring the expression of endothelial activation markers ICAM-1 (a), TLR4 (b), and MCP-1 (c). Results are expressed as fold-changes versus control cells (n = 3 per group). Endothelial activation was also evaluated by measuring the number of leukocytes adherent to the cell layer after 2 h reoxygenation with whole blood (d). Results are expressed in mean ± SEM of number of adherent leukocyte/culture well (n = 3 per group). Significant statistical data ($p<0.05$) were calculated using nonparametric Kruskal-Wallis test + multiple comparison evaluation by Dunn's posttest (§ $p<0.05$ to control cells, ∗$p<0.05$ versus 4°C, † $p<0.05$ versus 19°C, £ $p<0.05$ versus 27°C, ‡ $p<0.05$ versus 37°C).

[36]. The TLR4 exerts a crucial role during IRI. TLR4 is an established danger signal receptor, the increase of which is associated with an important proinflammatory response in IRI [24, 37, 38] and characterized by increased production of cytokines such as MCP-1. The activation of TLR4 leads to an increased production of proinflammatory cytokines and adhesion molecules. In our hands, TLR4 expression was increased at both 4°C and 37°C, while it remained at control levels for intermediate temperatures of 19°C and 27°C and was even decreased below control levels at 32°C. This result suggests that endothelial cells preserved at these temperatures are less exposed to danger signals and/or to activation of the innate immune system. To confirm our results, we measured MCP-1 mRNA expression. MCP-1 is implicated early after IR and contributes mainly to leukocyte attraction [39]. In the current study, increased expression of MCP-1 was detected in the 4°C and 37°C conditions, compared with control and intermediate temperature (19°C, 27°C, and 32°C). Collectively, our results suggest that endothelial cells preserved at temperatures ranging from 19°C to 32°C are less likely to foster the leukocyte adhesion and inflammation and consequently innate immunity activation.

We sought to confirm these observations by the quantification of adherent leukocytes after 2h reoxygenation with blood. In accordance with previous results, 4°C and 37°C preserved cell monolayers displayed an important number of adherent leukocytes. Surprisingly, preservation at 19°C did not reduce leukocyte adhesion, but a lower number of adherent leukocytes was observed at both 27°C and 32°C preserved cells compared to other conditions.

Our *in vitro* study suggests that intermediate preservation temperatures between 19°C and 32°C are more conducive to both the preservation of endothelial cell integrity and to the limitation of innate immunity during IRI. Our results confirm Post and coworkers' work, reporting the effect of different temperatures and preservation solution on endothelial cells viability and function [40]. In this study, authors showed that UW was more protective at 20°C than at lower temperatures. However, the authors were not able to show superiority of temperatures above 20°C, except in cell culture medium. Furthermore, Post and coworkers compared preservation temperatures (4°C to 37°C) and observed no influence of low-potassium (extracellular-like medium) or high potassium (intracellular-like medium) preservation solution in terms of cell injuries [40]. However, careful comparison of the study reveals conceptual differences. While our study used primary endothelial cells extracted from the microvasculature of the kidney and measured the effects of

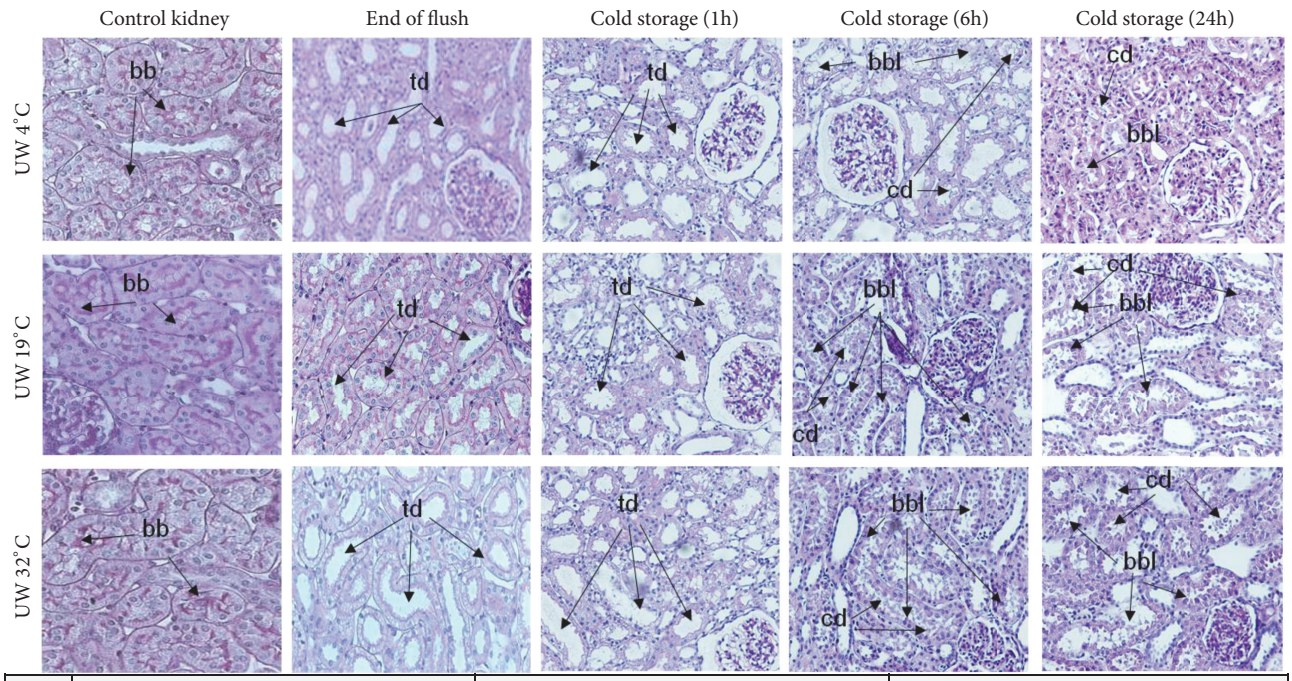


	Control	4°C	19°C	27°C	32°C	37°C
Mitochondrial lesions	0 ± 0	3.7 ± 0.3 *(p<0.05 vs Ctl vs 32°C)*	3.3 ± 0.3 *(p<0.05 vs Ctl)*	2.3 ± 0.3	1.7 ± 0.3 *(p<0.05 vs 4°C)*	3.3 ± 0.3 *(p<0.05 vs Ctl)*
Cytosolic vacuolisation	0 ± 0	3.3 ± 0.3 *(p<0.05 vs Ctl vs 32°C)*	2.0 ± 0.0	1.7 ± 0.3	1.3 ± 0.3 *(p<0.05 vs 4°C vs 37°C)*	3.3 ± 0.3 *(p<0.05 vs Ctl vs 32°C)*
Cellular membrane alteration	0 ± 0	2.7 ± 0.3 *(p<0.05 vs Ctl)*	1.3 ± 0.3	1 ± 0 *(p<0.05 vs 37°C)*	0.7 ± 0.3 *(p<0.05 vs 37°C)*	3.7 ± 0.3 *(p<0.05 vs Ctl vs 27°C vs 32°C)*

Figure 4: Effects of preservation temperature on endothelial cells morphological integrity. Electronic microscopy analysis of endothelial cells after preservation in UW at various temperatures followed by 24 h of reoxygenation 37°C in culture medium, (n = 3 per group). Results are expressed as mean ± SEM (n = 3 per condition) of percentages of lesions by field: scores: 0 = no alteration, 1 = lesions between 1-25%, 2 = lesions between 26-50%, 3= lesions between 51-75%, and 4 = lesions >76%. Significant statistical data (∗p<0.05) were calculated using nonparametric Kruskal-Wallis test + multiple comparison evaluation by Dunn's posttest.

both preservation and reperfusion, study from Post and his group used venous endothelial cells, considered less sensitive to hypoxia [40]. In addition, the authors incubated the cells in a hyperoxygenated environment (95% O_2, 5% CO_2), far from hypoxic the conditions generally prevailing during organ preservation.

Of note, we used the UW preservation solution displaying a high concentration of potassium and designed to be used at 4°C. Importantly, high concentrations of potassium are known to be toxic for cells. Lee et al. showed in a piglet *ex vivo* model that lower potassium concentration (25 versus 125 mEq/L) is superior for myocardial endothelial



	Tubular dilatation (td)					Brush border loss (bbl)					Cell detachment (cd)				
	Control kidney	End of flush	Cold storage (1h)	Cold storage (6h)	Cold storage (24h)	Control kidney	End of flush	Cold storage (1h)	Cold storage (6h)	Cold storage (24h)	Control kidney	End of flush	Cold storage (1h)	Cold storage (6h)	Cold storage (24h)
4°C	0	0.94 ± 0.1	1.37 ± 0.15	1.87 ± 0.12	2.12 ± 0.12	0	0.12 ± 0.07	0.25 ± 0.09	0.81 ± 0.08	2.56 ± 0.14	0	0.125 ± 0.08	0.19 ± 0.08	1.06 ± 0.11	2.31 ± 0.15
19°C	0	0.87 ± 0.12	1.6 ± 0.17	2.37 ± 0.17	2.87 ± 0.1 ∗versus 4°C	0	0.18 ± 0.12	0.44 ± 0.11	2.37 ± 0.23 ∗versus 4°C	3.19 ± 0.12	0	0.19 ± 0.08	0.31 ± 0.09	2.75 ± 0.125 ∗versus 4°C	3.31 ± 0.15 ∗versus 4°C
32°C	0	0.81 ± 0.21	1.62 ± 0.15	2.44 ± 0.21	3.31 ± 0.15 ∗versus 4°C	0	0.19 ± 0.1	0.5 ± 0.13	2.75 ± 0.13 ∗versus 4°C	3.625 ± 0.15 ∗versus 4°C	0	0.19 ± 0.08	0.5 ± 0.125	3 ± 0.09 ∗versus 4°C	3.81 ± 0.08 ∗versus 4°C

Figure 5: Effect of *ex vivo* kidney preservation at 4°C, 19°C or 32°C on renal histological injuries. Representative pictures of kidney histology for the evaluation of tubular dilatation (dt), brush border loss (bbl), and cell detachment (cd) injuries by histological analysis (PAS coloration) in renal tissue from control time to 1 h, 6 h, and 24 h of organ preservation at 4°C, 19°C, and 32°C (n = 3 per group). Results are expressed as mean ± SEM of percentage of lesions by field; scores: 0 = no alteration, 0.5 = lesions <5%, 1 = lesions 6-10%, 2 = lesions between 11-25%, 3 = lesions between 26-50%, 4 = lesions between 51 and 75%, and 5 = lesions >76%. Statistical significance (∗, $p<0.05$) was calculated using nonparametric Kruskal-Wallis test + multiple comparison correction by Dunn's posttest.

cell function at 37°C [41]. Similarly, in a human bypass saphenous vein model, De Caterina et al. showed that high potassium concentration in cardioplegic solutions inhibit prostacyclin production as a marker of endothelial function, without any observed endothelial detachment [42]. Indeed, potassium has been described to impact endothelial cell-derived hyperpolarizing factor mediated vasodilatation at 37°C [43, 44]. Similarly, we showed in our study that high potassium concentration is deleterious *in vitro* at 4 and 37°C (versus 19, 27, and 32°C) for cell integrity, which is in accordance with the literature. However, *ex vivo*, this hyperpotassic effect seems not worse at 4°C than at other tested temperatures (19 and 32°C) in terms of tissue integrity. This variation may be due to the fact that a part of the effects triggered by high potassium (such as vasodilatation) are dependent on the endothelial cell environment and interactions with other cell types within the tissue.

Nonetheless, both studies bring compelling arguments for the use of mild temperature to preserve organs. Our cell model was fruitful to mimic a particular IRI situation. However, *in vitro* results cannot be easily extrapolated to the patient level.

Thus, we moved forward with a preclinical pig model of kidney preservation for the second part of this study. This model is very close to the clinical situation and pig kidneys share strong anatomical and physiological similarities with human ones [26, 28]. In addition, the kidney is a multifunctional organ, not only eliminating metabolic waste, but also regulating the internal milieu *via* hydroelectrolytic balance. During its development toward adult kidney, different progenitor cells differentiate into more than 26 different cell types, exhibiting a variety of functions, metabolism levels and tolerance to IRI. Histological analysis of the first time points (immediate postflush or 1h preservation) suggested no

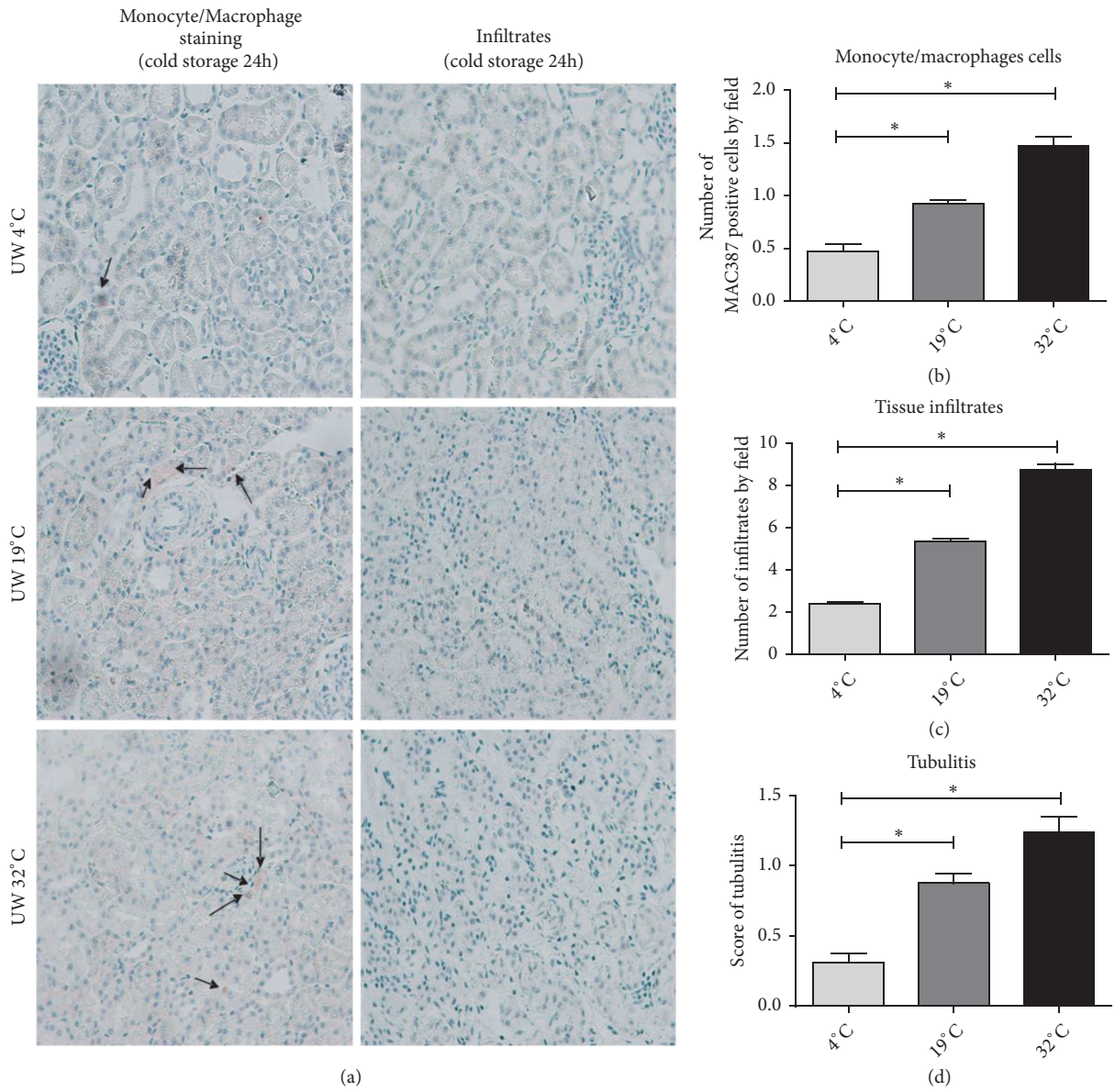


Figure 6: Effect of *ex vivo* kidney preservation at 4°C, 19°C, or 32°C on tissue leukocyte infiltration. (a) Representative pictures of monocyte/macrophage infiltration and leukocyte infiltrates in the tissue after 24 h of storage at 4°C, 19°C, and 32°C (n = 3 per group). (b) Number of monocyte/macrophage cells (MAC387 positive staining) by field. (c) Number of infiltrates by field. (d) Score of tubulitis. Statistical significance ($*p<0.05$) was calculated using nonparametric Kruskal-Wallis test, corrected by multiple comparison Dunn's posttest.

effect of the preservation temperature. Moreover, increasing the preservation time to 6 and 24 hours did not confirm the apparent superiority of intermediate temperatures. This observation is associated with increased necrosis (cell detachment), tubulitis, and monocytes/macrophages infiltration, in tissues preserved 6h and 24h at 19°C and 32°C versus 4°C storage. Nevertheless, we observed no statistical differences on apoptotic cells detection (cleaved caspase-3 positive cells and TUNEL staining) in the tissues preserved at 4°C, 19°C, or 32°C (despite an increase trend towards in the 19°C group).

This discrepancy between *in vitro* and *ex vivo* data highlights that (i) the choice of the experimental model is of critical importance in order to generate data relevant for translational research, (ii) *in vitro* data need to be thoroughly checked in complementary *ex vivo* or *in vivo* models, (iii) although moderate hypothermia yields interesting results regarding organ preservation, it will require the concomitant development of new solutions, specifically designed for maintaining the viability of an organ at the chosen temperatures. In addition, technological limitations will have to be surmounted. Overall, in order to enforce the static-to-dynamic/hypo-to-(sub)normothermic preservation paradigm shift, transplant community faces scientific, methodologic, and economic challenges.

A great deal of research is still needed to provide more rigorous information on pathophysiological mechanisms [12,

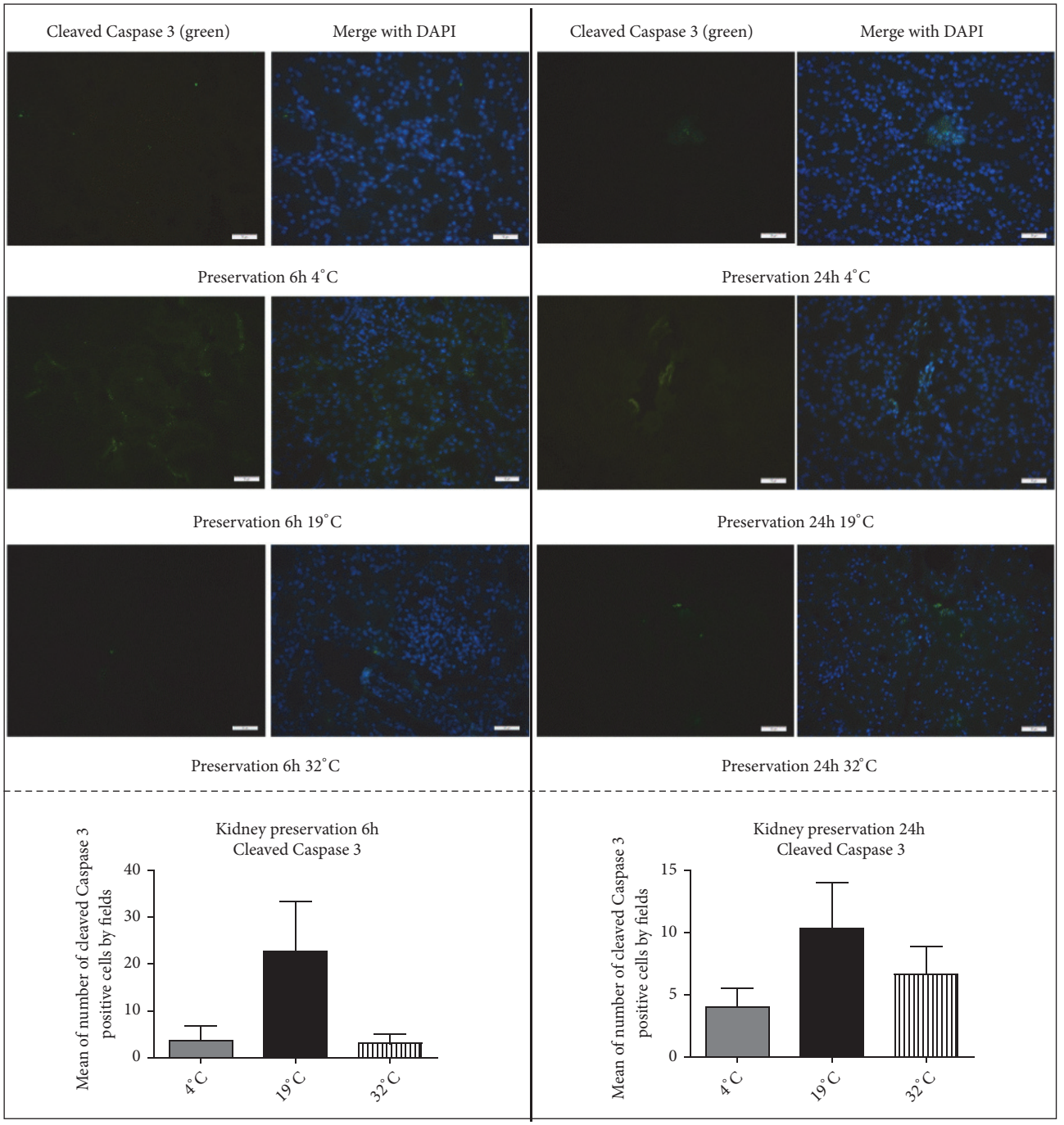


Figure 7: Effect of *ex vivo* kidney preservation at 4°C, 19°C, or 32°C on apoptosis. Left panel: representative pictures and histogram of the number of cleaved caspase 3 positive cells (mean of positive cells per field), as detected by immunohistofluorescence on kidney tissue after 6 h of preservation in UW at 4°C, 19°C and 32°C (n = 3). Right panel: representative pictures and histogram of number of cleaved caspase 3 positive cells (mean of positive cells per field), detected by immunohistofluorescence on kidney tissue after 24 h of preservation in UW at 4°C, 19°C, and 32°C (measure on 10 fields per slide. n = 3 per group).

25]. Promoting recovery by introducing an intervention during perfusion is a fascinating area of research. In addition, endothelium is likely a research area of interest.

In this vein, a pilot study demonstrated the superiority of Lifor Preservation Medium (an organ preservation medium containing sugars, amino acids, salts, buffers, colloids, fatty acids, antioxidants, vitamins, dextran, and an oxygen carrier) at room temperature perfusion compared to Belzer machine perfusion at both room temperature and 4°C, in a porcine model of uncontrolled donation after circulatory death [45]. It was also shown in a rat model that increasing the liver perfusion temperature to 21°C with HTK solution allowed

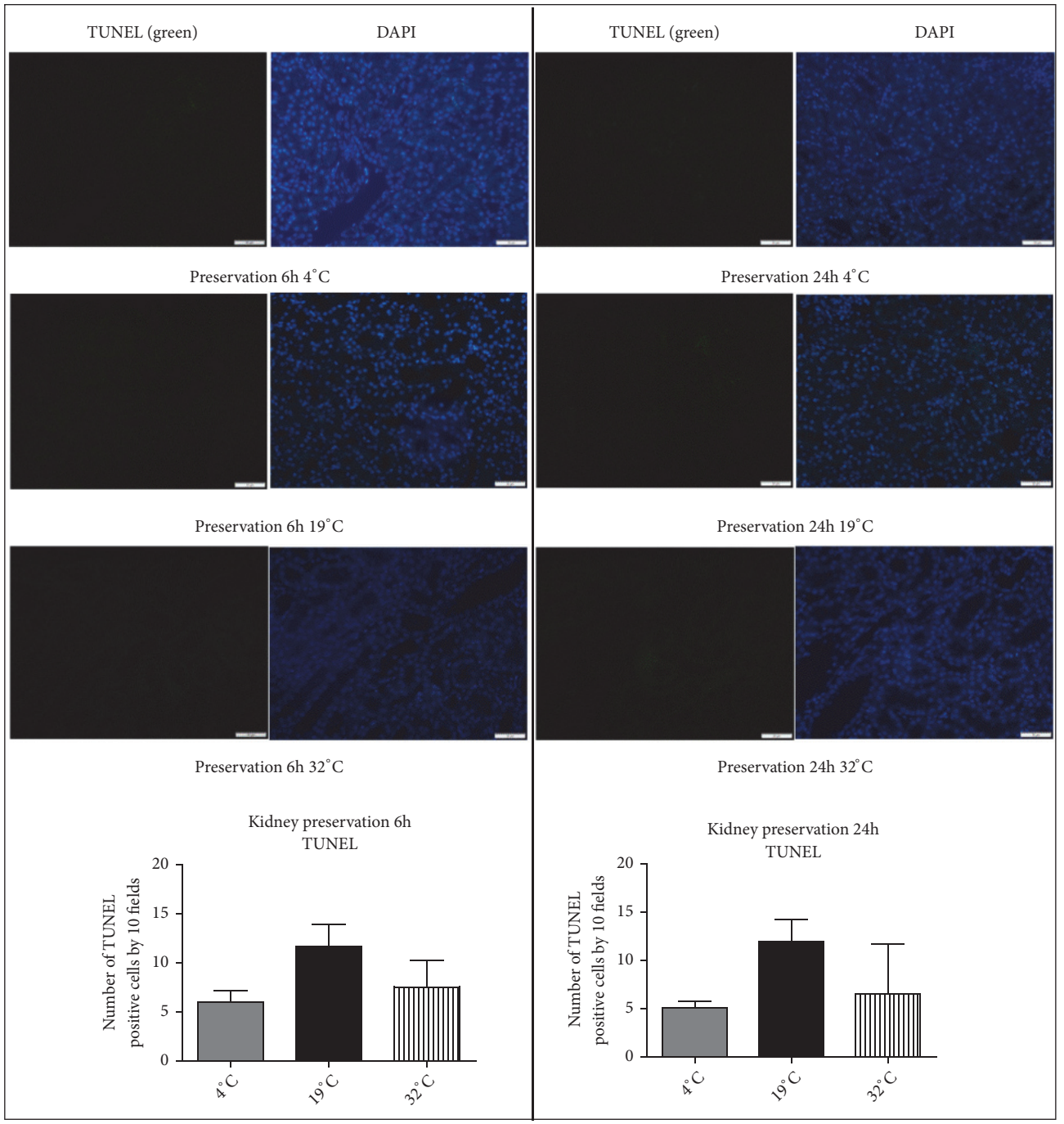


Figure 8: Effect of *ex vivo* kidney preservation at 4°C, 19°C, or 32°C on apoptosis (TUNEL evaluation). Representative pictures and histogram of number of TUNEL positive cells, detected by immunohistofluorescence on kidney tissue after 6 h (left panel) and 24 h (right panel) of preservation in UW at 4°C, 19°C, and 32°C (n = 3). Statistical significance (∗p<0.05) was calculated using nonparametric Kruskal-Wallis test and multiple comparison Dunn's posttest.

an hepatic functional capacity recovery, which was more efficient than perfusion at 4°C or 12°C [17].

Several reports support the notion that the preservation setting at other temperatures that 4°C is still not feasible during the whole ischemic period [11, 12, 46]. Alternatives using combination of short-term abdominal circulation, short *ex vivo* normothermic perfusion, and/or progressive organ rewarming, after cold storage may have to be considered [46]. For instance, in the rat liver, a short-term resuscitation with oxygenated machine perfusion (HTK, 12°C, 2 hours), with subsequent cold storage for 16 hours at 4°C, was shown to induce a better tissue ATP recovery compared to 4°C and 22°C [47]. Subnormothermic machine perfusion for preservation of porcine kidneys at 21°C [48] or human livers

at 20°C or 30°C seems to be a promising preservation method with the potential to improve functional parameters [49, 50]. Moreover, controlled oxygenated rewarming (COR) organ after cold storage is another alternative, with a prevention of mitochondrial initiation of cellular apoptosis, as evidenced by a reduced activation of caspase 9 [23]. Gradual rewarming at 10°C and 25°C during machine perfusion after static cold preservation reduced rat kidney IRI [51]. Subnormothermia or normothermia preservation condition could be an interesting strategy to prepare or help to condition the graft more physiologically. However this should be used with adequate technology and adapted conditions: (1) perfusion solution (e.g., whole blood versus leucocyte-free blood versus reconstituted washed red blood cells); (2) temperature (room temperature to normothermia); (3) judicious choice of time during the preservation phase (e.g., at the beginning of preservation process or few hours before transplantation).

One limit of our study was the use of the "intracellular-type" UW solution with high potassium content, designed and used for low temperature preservation, bringing difficulties to discriminate temperature and potassium effects in our models. However, at least under static condition, there is no preservation solution designed for temperature above 10°C. Thus, in order to investigate the effect of temperature *per se*, we chose to alter only one parameter of graft storage, leaving solution and oxygenation unchanged. This can limit the impact of our observations, while allowing for a clearer definition of temperature impact, in addition to the reduction in number of experimental groups and variables. Another limit is that we did not perform a kinetic investigation during storage *in vitro*. However, our foremost objective was to discriminate between the temperatures, so we did select an extended cold ischemia time (24 h), for which we knew [52, 53] that injuries would be severe enough to observe differences between the conditions. Nonetheless, this work allowed us to select the most pertinent temperatures to be investigated *ex vivo*, at different storage times. However, while cold storage is still widely used in clinics, alternative strategies to optimize organ quality during its preservation need to be developed. Furthermore, the suitable temperature does depend on the preservation solution composition; our study is facing the complexity of the organ preservation issue where all the parameters (temperature, preservation solution composition, oxygen, static or perfusion preservation, etc.) are dependent factors which have combined impacts on the tissue integrity and need to be optimized to fully answer to the targeted tissue metabolic demand.

5. Conclusion

The demography of organ donors is changing, pushing the transplant community to move towards new graft preservation paradigms. Based on our work and others, the versatile use of mild hypothermia, subnormothermia, or normothermia is a technological barrier that the transplant community has to overcome. Such conditions require sophisticated technologies and adapted preservation solutions since available technologies do not meet the relevant criteria. Our work also advocates that extrapolation of *in vitro* observations to *ex vivo* or *in vivo* settings must be carefully evaluated.

Critical in normothermia, oxygen is progressively understood as beneficial, if not required, even at the lowest preservation temperatures. On the other hand, normothermic preservation emerges as the next-to-come standard for higher-risk organs, while hypothermic preservation still prevails for standard and low-risk organs. From this viewpoint, the preservation temperature will have to be adapted to the organ and the donor, as well as to the perfusion regimen and conditions, including oxygen level, preservation solution, and its energetic metabolites. Research is being directed toward optimized graft-tailored preservation and repair is crucial and as important as management of acute rejection in the early days of transplantation. It deserves to be tackled with high priority and the same urgency.

Data Availability

All the data supporting our finding are included in the main manuscript and in the supplementary data.

Disclosure

Data of this manuscript were presented as a poster at the 17th Congress of the European Society for Organ Transplantation in 2015: "Influence of preservation temperature on endothelial cells and kidney phenotypes".

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: percentage of *in vitro* injuries induced by various tested preservation temperatures on endothelial cells. Table S1: primers used for real-time RT-PCR in porcine blood leukocytes and renal cortex tissue. *(Supplementary Materials)*

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Review Article

Donor and Recipient Outcomes following Robotic-Assisted Laparoscopic Living Donor Nephrectomy: A Systematic Review

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Aims. We aimed to summarize available lines of evidence about intraoperative and postoperative donor outcomes following robotic-assisted laparoscopic donor nephrectomy (RALDN) as well as outcomes of graft and recipients. *Methods.* A systematic review of PubMed/Medline, ISI Web of Knowledge, and Scopus databases was performed in May 2018. The following search terms were combined: nephrectomy, robotic, and living donor. We included full papers that met the following criteria: original research; English language; human studies; enrolling patients undergoing RALDN. *Results*. Eighteen studies involving 910 patients were included in the final analysis. Mean overall operative and warm ischemia times ranged from 139 to 306 minutes and from 1.5 to 5.8 minutes, respectively. Mean estimated blood loss varied from 30 to 146 mL and the incidence of intraoperative complications ranged from 0% to 6.7%. Conversion rate varied from 0% to 5%. The mean hospital length of stay varied from 1 to 5.8 days and incidence of early postoperative complications varied from 0% to 15.7%. No donor mortality was observed. The incidence of delayed graft function was reported in 7 cases. The one- and 10-year graft loss rates were 1% and 22%, respectively. *Conclusions.* Based on preliminary data, RALDN appears as a safe and effective procedure.

1. Introduction

Renal transplantation is the treatment of choice for suitable patients with end-stage renal disease (ESRD) as it provides better outcomes in terms of life expectancy and quality of life than dialysis [1]. Kidney transplants from living donors confer advantages in terms of graft function and survival if we compared transplants from deceased donors [2]. Indeed, the elective nature of living donor transplantation offers the opportunity to have good quality grafts and to perform the procedure when the recipient is in an optimal clinical status. The main obstacle to living donation is the exposure of a healthy subject to the risks of a major surgical intervention. Therefore, efforts have been made to reduce complications and postoperative pain, achieve faster recovery, and minimize the surgical incisions. Laparoscopic donor nephrectomy (LDN) was first introduced in 1995 and is currently accepted as the gold standard for kidney procurement from living donors. This minimally invasive procedure has greatly enhanced living donation rates and in 2001 the number of living donors exceeded the number of cadaver donors [3]. To date, living donors account for most of the kidney donor pool in Western countries [4]. However, deceased donors still represent about 67.6% of transplanted kidneys [4]. In 2000, the US Food and Drug Administration approved the da Vinci Surgical System (Intuitive Surgical Inc.), a system that combines robotic techniques and computer imaging to enable microsurgery in a laparoscopic environment [5]. Advantages of the da Vinci Surgical System include the precision and instinctive movements of open

surgery, an optimal ergonomic environment for the surgeon, and a 3-dimensional vision system that restores the hand-eye coordination lost in laparoscopic procedures [5–7]. The first worldwide robotic assisted laparoscopic donor nephrectomy (RALDN) was performed successfully at the University of Illinois at Chicago in 2000 by Horgan et al. [3]. Since then, the adoption of RALDN has increased worldwide and evidence about this procedure has slowly increased. The aim of the present review was to summarize available lines of evidence about intra- and postoperative donor outcomes following RALDN as well as outcomes of grafts and recipients.

2. Materials and Methods

We performed a systematic review using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement as a guideline in the development of the study protocol [19]. In May 2018 we used the National Library of Medicine PubMed search engine, the Scopus database, and the ISI Web of Knowledge official website to search for all published studies evaluating lines of evidence about donor and recipient outcomes following RALDN. The following search terms were combined: nephrectomy, robotic, and living donor. We included publications that met the following criteria: reporting original clinical studies; English language. Reference lists in relevant articles and reviews were also screened for additional studies. Abstracts (with no subsequent full-text publications) and unpublished studies were not considered. The quality of the randomized controlled trials was assessed using the Jadad score [20]. The following data were extracted from included studies: first author, year of publication, study design, sample size, study period, donors' age and sex, side of nephrectomy, surgical technique, control group, operative time (OT), console time (CT), warm ischemia time (WIT), estimated blood loss (EBL), conversion rate (CR), incidence, type and grade of intraoperative and postoperative complications, length of hospital stay (LOS), hemoglobin decrease, transfusion rate (TR), postoperative pain, duration of follow-up, last estimated glomerular filtration rate (eGFR), last creatinine, incidence of delayed graft function (DGF), duration of recipients' follow-up, recipients' last eGFR, recipients' last creatinine, graft survival, and recipients' complications.

3. Results

The search strategy revealed a total of 40 results. Screening of the titles and abstracts revealed 22 papers eligible for inclusion. Further assessment of eligibility, based on full-text papers, led to the exclusion of 4 papers. Finally, 18 studies involving a total of 910 patients who underwent RALDN from 2000 to 2018 were included in final analysis [2–18] (Figure 1). Specifically, 8 studies (44.4%) were retrospective observational, 4 (22.2%) were prospective observational, 1 (5.5%) was a randomized controlled trial, and 5 (27.7%) were case reports. The only randomized controlled trial was of low methodological quality (Jadad score =2). Eight studies (44.4%) had a control arm. The characteristics of the studies included are summarized in Table 1.

3.1. Intraoperative Outcomes. Table 2 summarizes RALDN-related intraoperative outcomes. Left kidneys were procured in 93.85% (n=854) of the donors.

3.2. Operative Times. Mean OT and mean CT ranged from 139 to 306 minutes and from 82 to 120 minutes, respectively. Three comparative studies found mean OT to be significantly longer in RALDN series with respect to LDN [4, 13, 16]. Mean OT of RALDN was also significantly longer than open donor nephrectomy (ODN) and hand-assisted retroperitoneoscopic donor nephrectomy (HARP) [4, 7, 13]. The relationship between vascular anatomy and OT is controversial. Gorodner et al. found that OT was significantly longer in patients with vascular anomalies with respect to those with normal vascular anatomy [8]. Similarly, Horgan et al. found OT to be significantly longer in patients with multiple renal arteries with respect to patients with normal anatomy [3]. Unlike previous authors, Janky et al. found no significant differences in terms of OT between donors with simple and complex vascular anatomy [13]. Interestingly, mean OT has been reported to significantly decrease with experience [3, 13]. In their study, Horgan et al. found a significant decrease in OT when their series was divided into three periods and the first 74 cases (201 minutes) were used for comparison with those in the second period (cases 75-144, 129 minutes) and third period (cases 145-214, 103 minutes) [3]. Similarly, Janki et al. found mean OT of RALDN procedures 1-19 to be significantly longer than RALDN procedures 40-59 (median OT of 240 and 172.5 minutes, respectively) [13]. In the study by Yang et al., the OT of RALDN approached that of LDN with each subsequent procedure over the course of the robotic cases and the standard OT of LDN was reached at the 22nd case [16].

3.3. Warm Ischemia Time. WIT ranged from <1.5 to 5.8 minutes. The impact of surgical technique on WIT is controversial. Two comparative studies found significantly longer WIT in patients undergoing RALDN with respect to those undergoing LDN [12, 16]. Two other comparative studies found WIT to be significantly longer in patients undergoing ODN and HARP [7, 13]. Unlike previous authors, Liu et al. and Janki et al. failed to find significant differences in terms of WIT between RALDN and LDN [1, 13]. The impact of vascular anatomy on WIT is controversial. Gorodner et al. found WIT to be significantly lower in patients with normal vascular anatomy with respect to patients with vascular anomalies [8]. Horgan et al. failed to find significant differences between patients with multiple renal arteries and patients with normal vascular anatomy in terms of WIT [3].

3.4. Intraoperative Complications. The incidence of intraoperative complications ranged from 0% to 6.7%. Bleeding was the most frequent complication and was reported in 11 patients (1.2%). Mean EBL varied from 30 mL to 146 mL. Janki et al. found EBL to be significantly lower in patients undergoing RALDN compared to HARP and LDN [13]. Serrano et al. found significantly lower EBL in patients undergoing RALDN compared to ODN, hand assisted LDN (HALDN) and LDN (80 mL, 296 mL, 91 mL, and 130 mL, respectively) with intraoperative transfusion rate of

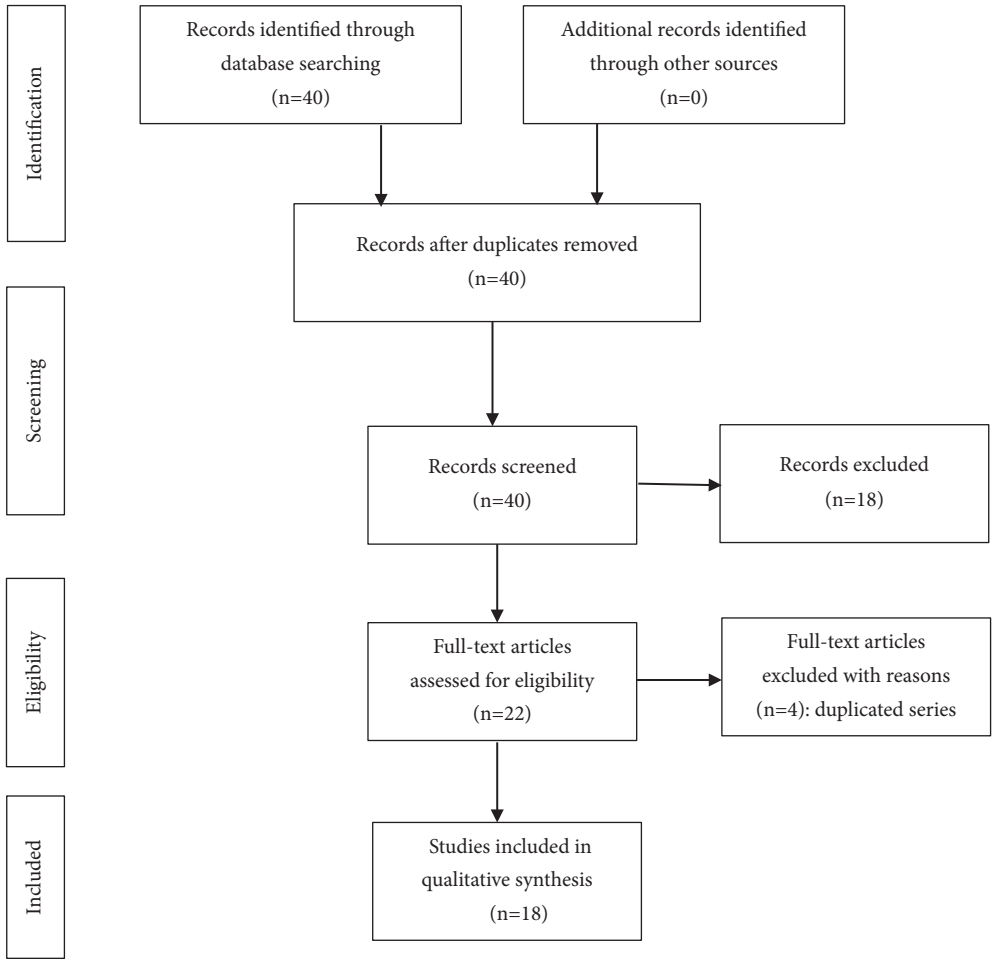

Figure 1: Flow diagram of the systematic review.

0%, 0.3%, 0.5%, and 3% in RALDN, HALDN, LDN, and ODN, respectively ($p<0.05$) [4]. Other authors failed to find statistically significant differences between RALDN and LDN in terms of EBL [10, 13, 16]. One study found EBL to be significantly higher in patients with vascular abnormalities (107 mL vs 72 mL, $p<0.05$) [3].

3.5. Conversion Rates. CR varied from 0% to 5%. Overall, 14 cases (1.5%) of open conversion were reported. The reasons for conversion included inability to control bleeding from lumbar veins, bleeding of the stump of the renal artery, failure of the stapling device on the renal artery stump, and bleeding from renal vein laceration [3–5, 8, 10, 13]. In the study by Gorodner et al., all conversions occurred during the initial 100 cases [10]. Similarly, all the 4 conversions reported by Horgan et al. occurred in the first 74 cases [3].

3.6. Early (< 30 Days) Postoperative Donors' Outcomes. RALDN-related early postoperative donors' outcomes are summarized in Table 2.

3.7. Hospital Length of Stay. The mean hospital LOS ranged from 1 to 5.8 days. One comparative study found mean hospital LOS to be significantly shorter after RALDN compared to LDN [12]. Two other comparative studies found mean hospital LOS to be significantly shorter after RALDN compared to ODN [4, 7]. Five studies failed to find statistically significant differences between RALDN and LDN [1, 4, 10, 13, 16]. Cohen et al. found that mean LOS decreased with increasing RALDN experience [5]. Indeed, mean hospital LOS was 1.5 days, 2.3 days, and 2.0 days in the last 80 RALDN procedures, in the initial 20 RALDN, and in the last HALDN, respectively, with 20% of donors undergoing HALDN still in the hospital on postoperative day 3 compared to only 3.7% of donors in the last 80 RALDN procedures [5].

3.8. Postoperative Complications. The incidence of early postoperative complications ranged from 0% to 15.7%. In their study, Horgan et al. found that the postoperative complication rate dropped from 24% in the first 74 cases to a steady rate of 7% in the last part of their cohort [3]. Only 4 studies graded complications according to the Clavien Classification system by showing a high prevalence of Grades I and II complications [12, 13, 15, 16]. Comparative studies failed to find statistically significant differences between RALDN, ODN, LDN, and HARP in terms of postoperative complications [12, 13, 16]. No donor mortality was observed.

Table 1: Study characteristics.

Author	Year of publication	Study design	Sample size (n)	Study period	Age, years (mean)	Male: Female	Left: Right	Technique	Control group(s)
Gorodner (n.v.a.) [8]	2006	Retrospective	148	2000-2005	35	74:74	148:0	RALDN	-
Gorodner (v.a.) [8]	2006	Retrospective	61	2000-2005	35	27:34	61:0	RALDN	-
Renoult [7]	2006	Retrospective	13	2002-2004	39.3	3:10	12:1	RALDN	ODN
Hubert [6]	2007	Prospective	38	2002-2006	43	15:23	37:1	RALDN	-
Horgan [3]	2007	Prospective	214	2000-2006	36	110:104	214:0	Hand Assisted RALDN	-
Pietrabissa [9]	2010	Case report	1	2010	48	0:1	1:0	RALDN with transvaginal extraction of the kidney	-
Geffner [10]	2011	Retrospective	35	2007-2008	44.5	14:21	35:0	RALDN	LDN
Kaouk [11]	2012	Case report	1	2012	61	0:1	1:0	Transvaginal hybrid natural orifice transluminal robotic donor nephrectomy	-
Galvani [2]	2012	Case report	1	2012	21	0:1	1:0	SIRA	-
Liu [1]	2012	Retrospective	5	2005-2011	34.8	n.r.	0:5	RALDN	LDN
Cohen: Initial 20 cases [5]	2015	Retrospective	20	2009-2010	36.1	n.r.	20:0	RALDN	HA-LDN
Cohen: Last 80 cases [5]	2015	Retrospective	80	2010-2012	37.7	n.r.	59:21	RALDN	HA-LDN
Bhattu [12]	2015	Randomized controlled	15	2014-2015	46.4	2:13	6:9	RALDN	LDN
Serrano [4]	2016	Retrospective	94	2004-2014	40	36:58	88:6	RALDN	ODN, HA-LDN, LDN
Janki [13]	2016	Prospective	59	2009-2014	53.0	23:36	59:0	RALDN	LDN, HARP
Heredia [14]	2016	Case report	1	2016	34	0:1	0:1	Hand assisted RALDN	-
Giacomoni [15]	2017	Retrospective	98	2009-2016	53	29:69	88:10	RALDN	-
Yang [16]	2017	Retrospective	22	2011-2016	38.2	12:10	20:2	RALDN	LDN
Lamattina [17]	2018	Prospective	3	2015	52	0:3	3:0	Single-site robotic surgery	-
Barman [18]	2018	Case report	1	2018	30	1:0	1:0		-

HA-LDN: Hand Assisted Laparoscopic Donor Nephrectomy, HARP: Hand Assisted Retroperitoneoscopic Donor Nephrectomy, LDN: Laparoscopic Donor Nephrectomy, ODN: Open Donor Nephrectomy, n.r.: not reported, n.v.a.: normal vascular anatomy, SIRA: Single incision robotic assisted donor nephrectomy, and v.a.: vascular anomalies.

Table 2: RALDN associated intraoperative and postoperative outcomes.

	Intraoperative data							Early (< 30 days) postoperative data						Late (> 30 days) postoperative data			
Author	OT, min (mean)	CT, min (mean)	WIT, min (mean)	EBL mL (mean)	CR n (%)	Complications n (%)	Complications Type (n)	LOS, days (mean)	Hb decrease (g/dL)	TR n (%)	Pain (VAS) mean	Complications n (%)	Complications type (CDC) (n)	FU (mo)	Complication type (CDC) (n)	Last eGFR (mL/min/ 1.73m^2)	Last creatinine (mg/dL)
Gorodner (n.v.a.) [8]	146	n.r.	1.5	76	3 (2.0)	1 (0,6)	Bleeding	2	n.r.	0 (0)	n.r.	11 (7.4)	Pneumonia (n=2) Pancreatitis (n=1)	n.r.	n.r.	n.r.	n.r.
Gorodner (v.a.) [8]	158	n.r.	1.7	107	1 (1.6)			2	n.r.	0 (0)	n.r.		Superficial wound infection (n=8)	n.r.	n.r.	n.r.	n.r.
Renoult [7]	185.5*	n.r.	7.1*	n.r.	0 (0)	0 (0)		5.8**	-0,9	0 (0)	n.r.	1 (7.6)	Deep vein thrombosis (n=1)	n.r.	n.r.	n.r.	n.r.
Hubert [6]	181	n.r.	5.8	n.r.	0 (0)	0 (0)		5.5	-0.8	0 (0)	n.r.	6 (15.7)	Acute pyelonephritis (n=2) Deep vein thrombosis (n=1) Pulmonary embolism (n=1) Wound infection (n=1) Urinary tract infection (n=1)	n.r.	n.r.	n.r.	n.r.
Horgan [3]	150	n.r	1.6	82	4 (1.8)	4 (1.8)	Bleeding	2	n.r.	n.r.	n.r.	24 (11.2)	Pneumonia (n=1) Pancreatitis (n=1) Wound infection (n=10) Ileus (n=11) Ventral hernia (n=1)	n.r.	n.r.	n.r.	n.r.
Pietrabissa [9]	215	95	3,15	<50	0 (0)	0 (0)		1	n.r.	0 (0)	n.r.	0 (0)	0	1.5	n.r.	65	n.r.
Geffner [10]	149	n.r.	n.r.	146	1 (2.9)	1 (2.8)	Bleeding	3.2			n.r.	1 (2.8)	Acute urinary retention	n.r.	n.r.	n.r.	n.r.
Kaouk [11]	240	120	5,46	n.r.	0 (0)	0 (0)		2	n.r.	0 (0)	24h:3/10	0 (0)	0	n.r.	n.r.	n.r.	n.r.
Galvani [2]	150	n.r.	3,3	75	0 (0)	0 (0)		3	n.r.	0 (0)	24h: 6/10 72 h: 2/10	0 (0)	0	n.r.	n.r.	n.r.	n.r.
Liu [1]	218	n.r.	3.8	30	0 (0)	0 (0)		3.6	n.r	0 (0)	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Cohen: Initial 20 cases [5]	149	n.r.	n.r.	n.r.	1 (5)	1 (5)	Bleeding	2.3	n.r.	0 (0)	n.r.	2 (10)	Deep vein thrombosis (n=1) Bleeding (n=1)	n.r.	n.r.	n.r.	n.r.

Table 2: Continued.

Author	Intraoperative data							Early (< 30 days) postoperative data						Late (> 30 days) postoperative data			
	OT, min (mean)	CT, min (mean)	WIT, min (mean)	EBL mL (mean)	CR n (%)	Complications n (%)	Complications Type (n)	LOS, days (mean)	Hb decrease (g/dL)	TR n (%)	Pain (VAS) mean	Complications n (%)	Complications type (CDC) (n)	FU (mo)	Complication type (CDC) (n)	Last eGFR (mL/min/ 1.73m^2)	Last creatinine (mg/dL)
Cohen: Last 80 cases [5]	139	n.r.	n.r.	n.r.	0 (0)	1 (1.2)	Tear in the mesocolon	1.5	n.r.	1 (1.2)	n.r.	2 (2.5)	Nausea (n=1) Bleeding (n=1)	n.r.	n.r.	n.r.	n.r.
Bhattu [12]	156.6	n.r.	5.3∗	n.r.	0 (0)	0 (0)		3∗∗	-0.8		6h:4.3/10∗∗ 24h:2.7/10∗∗ 48h:1.4/10∗∗	2 (13.3)	n.r. (I) (n=2)	n.r.	n.r.	n.r.	n.r.
Serrano [4]	306∗	n.r	n.r.	80∗∗	3 (3.1)	3 (3.1)	n.r.	3∗∗	n.r	0 (0)	n.r.	8 (8.5)	Infection (n=1) Ileus (n=3) Urinary tract infection (n=4)	120	Incisional hernia (n=1)	n.r.	1.01
Janki [13]	205.0∗	n.r.	4.0∗	100∗∗	1 (1.6)	4 (6.7)	Kidney torsion (n=1), Stapler malfunction (n=1), bleeding (n=2)	2.0	n.r.	0 (0)	n.r.	5 (8.4)	Wound infection (I) (n=1) Subcutaneous hematoma (I) (n=1) Seroma of the trocar incision (I) (n=1) Urinary tract infection (II) (n=1) Chylous leakage (IIIA) (n=1)	3	Nausea-dizziness (I) (n=1)	54.0	1.07
Heredia [14]	170	n.r.	<1.5	n.r.	0 (0)	0 (0)		3	n.r.	n.r.	n.r.	0 (0)	0	n.r.	n.r.	n.r.	n.r.
Giacomoni [15]	239	111	n.r.	n.r.	n.r.	3 (3.0)	Bleeding (n=2) Pneumothorax (n=1)	4,5	n.r.	2 (2.0)	n.r.	13 (13.2)	Seroma (I) (n=5) Rhabdomyolysis (II) (n=1) Subocclusion (II) (n=1) Paralytic ileus (II) (n=1) Hypertensive crisis (II) (n=1) Oxygen desaturation (II) (n=1) Postoperative anemia (II) (n=1) Chylous collection (III) (n=2)	n.r.	n.r.	n.r.	n.r.
Yang [16]	192.3∗	n.r	3.4∗	55.9	n.r.	n.r.		2.5	-1.8	0 (0)	n.r.	1 (4.5)	Chylous leakage (II) (n=1)	12	Nausea (I) (n=1)	60.6	1.2
Lamattina [17]	262	82	n.r.	77	0 (0)	0 (0)		2	n.r	n.r	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Barman [18]	215	n.r.	5	50	n.r.	n.r.		n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.

∗: significantly higher with respect to control group, ∗∗: significantly lower with respect to control group, CDC: Clavien Dindo classification, CR: conversion rate, CT: console time, EBL: estimated blood loss, eGFR: estimated glomerular filtration rate, FU: follow-up, LOS: length of stay, mo: months, n.r.: not reported, n.v.a.: normal vascular anatomy, OT: operative time, TR: transfusion rate, v.a.: vascular anomalies, VAS: Visual Analog Scale, and WIT: warm ischemia time.

3.9. Postoperative Pain. Postoperative pain was investigated by three studies [2, 11, 12]. In the study by Bhattu et al., visual analogue scale scores for postoperative pain at 6, 24, and 48 hours as well as analgesic requirement were significantly lower after RALDN compared to LDN [12].

3.10. Late (>30 Days) Postoperative Donor Outcomes. Late postoperative donor outcomes were investigated by four studies [4, 9, 13, 16]. Mean follow-up ranged from 1.5 to 120 months (Table 2). Janky et al. failed to find statistically significant differences in terms of serum creatinine and eGFR at 3 months' follow-up after RALDN or LDN [13]. Yang et al. found similar results at one-year follow-up [16]. Serrano et al. found the incidence of ESRD to be close to 0% for all donors at 10 years' follow-up and this outcome was not influenced by surgical procedure (ODN, LDN, HA-LDN, and RALDN) [4]. The incidence of late complications was similar between RALDN and LND at one-year follow-up [16].

3.11. Recipient and Graft Outcomes. Table 3 summarizes results from studies evaluating recipient and graft outcomes relative to donors undergoing RALDN. Most of grafts functioned immediately after transplantation and DGF was reported in 7 cases. The incidence of DGF was not influenced by the surgical procedure adopted to procure the kidney (RALDN or LDN) [1]. Janki et al. failed to find significant differences in graft or recipient survival between RALDN and LDN as well as between RALDN and HARP at three-month follow-up [13]. In the study by Renoult et al. no significant differences emerged between recipients of donors undergoing RALDN and ODN [7]. Similarly, Yang et al. found no significant differences in terms of recipients' and grafts' outcomes at one-year follow-up regardless of the donor procedure [16]. In detail, there were no cases of graft failure or DGF in recipients of donors undergoing RALDN while there were three cases of graft failure and one case of DGF in patients who received kidneys from donors undergoing LDN [16]. eGFR was similar between recipients of donors undergoing RALDN and LDN at one-year follow-up [16]. In the study by Bhattu et al., eGFR at 7 days and at 1, 3, 6, and 9 months were similar among recipients of donors undergoing RALDN and LDN and no graft loss was observed in the two groups [12]. Indeed, each of the transplanted kidneys functioned correctly following surgery and none of the recipients required posttransplant dialysis [7]. Based on the measurement of the creatinine reduction ratio from posttransplantation day 1 to day 2, graft function improved more rapidly in the first two days after transplantation in the RALDN group with respect to ODN (42.6% vs 32.6%, $p=0.01$) and the mean estimated creatinine clearances at day 5 showed no differences between the two groups [7]. In the study by Serrano et al. the one-year graft loss rate for recipients whose living donor was in the ODN cohort was 9% versus the 2% in HA-LDN, 2% in P-LND, and 1% in RALDN cohorts [4]. The 10-year graft loss rate for recipients whose living donor was in the ODN, HA-LDN, P-LDN, and RALDN cohort was 27%, 23%, 20%, and 22%, respectively [4].

4. Discussion

Kidney transplant represents a common surgical intervention, with many cases performed yearly around the world [21, 22]. The procedure offers advantages over chronic dialysis in terms of quality of life and life expectancy. The number of patients requiring kidney transplantation increases with time and an improvement of the donation experience is strongly advocated [5, 16]. Unlike most surgical procedures, live donor nephrectomy is a unique, elective procedure, where a subject undergoes surgery for the sole benefit of another [16]. Therefore, it is of great importance to keep the morbidity and mortality of the procedure as low as possible [23]. Moreover, efforts should be made to procure the kidneys in optimal conditions for transplantation [23]. For many years, live donor nephrectomy was performed only with an open surgical approach and thereby many potential donors were reluctant to donate due to the morbidity associated with the procedure [23]. In 1995, Ratner et al. performed the first LDN at Johns Hopkins University of Baltimore [24]. LDN demonstrated several improvements over ODN such as decreased postoperative pain, decreased hospital LOS, faster recovery, and reduced perioperative blood loss [16]. Due to these advantages, LDN has become the standard of care and several modifications have been made to improve the technique [16]. The introduction of precise surgical robotic systems, like the da Vinci system, has expanded surgeons' ability to complete complex surgical tasks in a minimally invasive fashion. Some authors hypothesized that robotic assistance could result in a shorter and simpler learning curve for the procurement of kidneys from living donors and that it could enable an easier and more efficient management of complications [15]. By decreasing the learning curve for difficult surgical tasks, surgical robots may also expand the number of available surgeons for complex interventions as well as allow newer surgeons to quickly master these procedures [16]. In 2000, Horgan et al. performed for the first time a RALDN [25]. Since then, it has been adopted by several Institutions worldwide and the amount of evidence has progressively increased. To date, RALDN represents an evolving field. A new surgical technique should be compared against the gold standard. OT, EBL, incidence of complications, and conversion to open surgery are relevant intraoperative outcomes for most laparoscopic and robotic surgical procedures. The OT of LDN has been reported to range from 183 to 340 minutes [23]. Some authors have reported significantly longer OT with RALDN compared to LDN. Yang et al. hypothesized that the longer OT could be a result of their cautious, slower approach with RALDN due to their initial unfamiliarity with the procedure [16]. Interestingly, it has been demonstrated that OT associated with RALDN significantly decreases with experience and approaches the mean OT of LDN after few cases. Bleeding represents the most frequent intraoperative complication. However, two of the most recent comparative studies found EBL to be significantly lower during RALDN compared to LDN [4, 13]. Other studies found no significant difference between the two techniques in terms of EBL. It has been hypothesized that robot assistance may allow surgeons to dissect rapidly and efficiently and to control problematic

Table 3: Graft and recipient outcomes.

Author	DGF n (%)	Follow-Up (mo)	Serum creatine, mg/dL, mean (Follow-up)	eGFR mL/min/1.73m^2 (Follow-up)	Graft survival, % (Follow-up)	Complications, type (n)
Gorodner (n.v.a.) [8]	0 (0)	12	1.4 (6 mo)	n.r.	96.6 (12 mo)	Vascular thrombosis (2) Pyeloureteral junction stricture (1)
Gorodner (v.a.) [8]	0 (0)	12	1.4 (6 mo)	n.r.	96 (12 mo)	0
Renoult [7]	0 (0)	1	n.r.	n.r.	100 (1mo)	0
Hubert [6]	0 (0)	1	n.r.	n.r.	100 (1 mo)	Temporary pyeloureteral dilatation (1)
Horgan [3]	0 (0)	12	1.4 (6 mo)	n.r.	98 (12 mo)	Renal artery thrombosis (1) Acute rejection (1) Abnormal kidney anatomy (1)
Pietrabissa [9]	0 (0)	2	n.r.	n.r.	100 (2 mo)	0
Geffner [10]	n.r	120	n.r.	n.r.	97.1 (12 mo)	Graft rejection due to lymphoma (1)
Kaouk [11]	1 (100)	1	1.1 (1 mo)	67 (1 mo)	100 (1 mo)	Acute rejection (1)
Galvani [2]	0 (0)	3	0.8 (3 mo)	n.r.	100 (3 mo)	0
Liu [1]	1 (20)	12	1.4 (12 mo)	60.8 (12 mo) $*$	n.r.	n.r.
Cohen (Initial 20 cases) [5]	n.r.	1	2	n.r.	n.r.	n.r.
Cohen (last 80 cases) [5]	n.r.	1	1.6	n.r.	n.r.	n.r.
Bhattu [12]	0 (0)	9	n.r.	68.7 (9 mo)	100 (9 mo)	Acute tubular necrosis (1) Recurrence of focal segmental glomerulosclerosis (1)
Serrano [4]	4 (4.2)	120	1.4 (12 mo)	n.r.	99 (12 mo) 89 (60 mo) 78 (120 mo)	Acute rejection (28)
Janki [13]	n.r.	3	n.r.	n.r.	94.9 (3 mo)	n.r.
Heredia [14]	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Giacomoni [15]	1 (1.0)	n.r.	n.r.	n.r.	n.r.	Intraoperative venous thrombosis (1)
Yang [16]	0	120	1.2 (12 mo)	70.6 (12 mo)	0 (120 mo)	n.r.

$*$: statistically significant higher if compared to control group, DGF: delayed graft function, eGFR: estimated glomerular filtration rate, mo: months, and n.r.: not reported.

bleedings more easily [7]. The reported frequency of open conversion during LDN ranges from 0% to 13% [4, 23]. The CR reported in the overall RALDN population analyzed in the present review is within the published ranges for LDN. Interestingly, most conversions occurred during the early phase of the learning curve. Giacomoni et al. hypothesized that the use of the robotic assistance can help to avoid the open conversion in cases of acute bleeding as it may facilitate the repair of vascular lesions [15]. WIT has traditionally represented a major concern during donor nephrectomy as it has been thought that any increase in this parameter would have translated into a poor graft function [23]. However, this notion has been disproved by various studies. WIT during LDN ranges between 95 and 300 seconds. Globally, WIT reported during RALDN is within published ranges for LDN. Some authors reported significantly longer WIT with RALDN compared to LDN probably related to the extraction that is performed by a second attending surgeon [16]. As RALDN is a relatively new procedure, the learning curve is a possible cause of differences observed in some intraoperative outcomes including OT and WIT [16]. Major advantages of RALDN over LDN are in the early postoperative period. Indeed, RALDN is characterized by lower postoperative pain and shorter hospital LOS. Bhattu et al. hypothesized that one of the possible reasons for less pain following robotic surgery is robotic arms, which are pivoted around the port site and moved at fixed remote center [12]. Consequently, there is less leverage and lesser pressure at port sites with subsequent lesser trauma to abdominal wall tissues around the port [12]. Some authors attributed the short LOS in the robotic-assisted program to reduced manipulation of the peritoneum, better identification of dissection planes, and limited energy use from cauterization leading to minimal inflammation and pain [5, 12]. Shorter LOS makes the RALDN procedure more convenient for the donor by allowing him to return as soon as possible to routine activities [5]. In a systematic review and meta-analysis of published work relative to 32,038 nephrectomies following different surgical techniques, Kortram et al. reported a global complication rate of 9.3% with complication rate after LDN being 23% [15, 26]. The present review demonstrates that the incidence of early postoperative complications is similar between RALDN and LDN. Interestingly, most of the complications occurred at the beginning of the learning curve. Unfortunately, the incidence of late postoperative complications was largely underinvestigated. Graft function and survival as well as recipient outcomes are of great importance when considering kidney donor procedures. Available lines of evidence demonstrate that RALDN does not adversely affect allograft function and survival. Although OT and WIT are longer in many RALDN experiences with respect to LDN, this seems not to translate into poorer graft function or recipient outcomes [16]. The incidences of DGF, graft function, and survival were similar between recipients whose living donor was in the RALDN and LDN cohorts in many studies. These data are coherent with the evidence that WIT up to 720 seconds do not correlate with graft function [16, 27]. Major limitations of current robotic systems are high costs and lack of haptic feedback [15, 28, 29]. However, daily use of the robot may reduce robotic costs mainly in a high-volume institute if the system is made available to multidisciplinary surgical teams [15]. The adoption of single-site robotic platforms has been described by some authors [17]. Early experience showed the safety of this approach but found that the technology added cost and complexity without tangible benefits [17]. Although the single port technology may decrease surgical invasiveness, its widespread adoption in the clinical practice will require the development of dedicated articulating instruments, energy, and stapling devices [17]. Potential limits of available literature must be acknowledged: available studies are few, often of low methodological quality, and with short follow-up. They enroll a small number of patients and often populations and surgical technique employed are different. Moreover, the quality of life after RALDN remains largely underinvestigated. Finally, the outcomes in specific populations, such as older living donors, need to be addressed [30, 31].

5. Conclusions

Available studies point out the feasibility and safety of RALDN. Although OT and WIT have been reported to be longer with respect to LDN in some studies, a progressive improvement with experience is evident. The procedure can provide potential advantages in terms of EBL, hospital LOS, and postoperative pain with respect to LDN. Graft and recipient outcomes are comparable to LDN. However, the technique is still in its infancy in many Institutions and available lines of evidence are still of poor quality. Consequently, these results should be interpreted with caution and role of RALDN needs further investigations.

Conflicts of Interest

Authors have no conflicts of interest to declare.

Authors' Contributions

Massimiliano Creta and Armando Calogero contributed equally to this work.

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Review Article

Eradication of HCV Infection with the Direct-Acting Antiviral Therapy in Renal Allograft Recipients

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Hepatitis C virus (HCV) infection unfavorably affects the survival of both renal patients undergoing hemodialysis and renal transplant recipients. In this subset of patients, the effectiveness and safety of different combinations of interferon-free direct-acting antiviral agents (DAAs) have been analyzed in several small studies. Despite fragmentary, the available data demonstrate that DAA treatment is safe and effective in eradicating HCV infection, with a sustained virologic response (SVR) rates nearly 95% and without an increased risk of allograft rejection. This review article analyzes the results of most published studies on this topic to favor more in-depth knowledge of the readers on the subject. We suggest, however, perseverating in this update as the optimal DAA regimen may not be proposed yet, because of the expected arrival of newer DAAs and of the lack of data from large multicenter randomized controlled trials.

1. Introduction

Hepatitis C virus (HCV) infection affects nearly 150 million people worldwide and more than 350,000 people die each year of HCV-related diseases [1–7]. In the adult population, HCV infection is associated with an increased risk of developing chronic kidney disease [8] and the prevalence of HCV infection is greater among patients with chronic kidney disease (1.8% -8%) than in the general population (0.5-4%), especially in those undergoing hemodialysis and in renal allograft recipients [9–22]. In these patients, HCV infection is a risk factor for other comorbidities [9, 23, 24]. In fact, in renal allograft recipients, HCV has been found to be associated with posttransplant proteinuria, with an increased risk of new-onset diabetes possibly leading to cardiovascular diseases and malignancies, and with allograft loss, infections, and death [9, 12, 25–40]. Therefore, it is highly desirable to treat HCV infection before or after renal transplant to eliminate at the same time both HCV infection and the risk of complications [40–50].

Interferon-based regimens had been the backbone of HCV treatment for kidney transplant recipients until 2014, a therapeutic approach limited by the relatively low efficacy in achieving viral eradication, poor tolerability [51, 52], and the obligation to use it only before transplant because of the high risk of inducing immune stimulation and allograft rejection [53–55].

The first-generation direct-acting antivirals (DAAs) protease inhibitors telaprevir and boceprevir present a similar

limitation because their efficacy depends on the coadministration of interferon [56–63]. Instead, the currently available new generation DAAs, associated with a greater sustained viral response (SVR) and with a good safety profiles, have strongly improved the treatment of chronic HCV infection even in renal patients [64–68]. HCV eradication is testified by the achievement of a sustained virologic response 12 (SVR12), highlighted by the undetectability of serum HCV RNA within the 12^{th} week of treatment and throughout a subsequent follow-up of 12 weeks.

A clear example of the efficacy of DAAs in eradicating HCV infection in patients with chronic kidney diseases (CKD) is offered by the C-SURFFER study, a multicenter, double-blind, randomized study where 224 patients with HCV GT1 infection and CKD stages 4 or 5 were randomly assigned to an "immediate treatment group" receiving grazoprevir and elbasvir (n=111) for 12 weeks or to a "deferred treatment" group treated with the same schedule (n=113) 16 weeks later. Most of patients were haemodialysis dependent; half of them had HCV genotype 1a infection, 80% were HCV treatment naïve, and 6% had liver cirrhosis. SVR rates in the "immediate treatment" and "deferred treatment" groups were 99% and 98, respectively [69].

There are four classes of direct-acting antiviral drugs that combine the attack on more than one HCV life cycle goal: (1) NS3/4A protease inhibitors (galexos, grazoprevir, sunvepra, glecaprevir, paritaprevir, and voxilaprevir); (2) the nucleoside and nucleotide NS5B polymerase inhibitors (sofosbuvir); (3) NS5A inhibitors (ombitasvir, pibrentasvir, daclatasvir, elbasvir, ledipasvir, ombitasvir, and velpatasvir); and (4) nonnucleoside NS5B polymerase inhibitors (dasabuvir).

General symptoms (adverse events, fatigue, nausea, dizziness, or headache) associated with DAA treatment in kidney transplant recipients have been reported in nearly 40% of treated patients, whereas severe adverse events (i.e., anemia, portal vein thrombosis, or pneumonia) are infrequent events (around 1% of treated patients) [70].

Glomerular disease after DAAs has been also described. Lubetzky et al. [71] reported no significant change in glomerular filtration rate (GFR) before or after therapy, but 3 patients showed a decrease of GFR to less than 20 and 19.3% of 31 patients had worsening proteinuria during or shortly after therapy. Besides, patients with a proteinuria over 500 mg/g before treatment showed an increased value during treatment more frequently than those with initial values lower than 500 mg/g ($P < 0.001$) [71].

In this review article we analyze the data from several real-life studies on the feasibility, effectiveness, and tolerability of DAA therapies for kidney transplant recipients. The experience of most single studies is limited, prevalently by the low number of patients examined, but a comprehensive analysis of the available data can give useful indications to the readers [68–75].

Episodes of reactivation of an indolent (open or occult) Hepatitis B virus (HBV) infection in patients with HBV/HCV-related chronic hepatitis during or after a successful DAA therapy have been documented [76]. HBV reactivation of open or occult HBV infection has been observed even in kidney-transplant recipients with HBV/HCV-related chronic hepatitis after a SVR had been achieved with DAA treatment. [77].

2. DAA Agents for Treatment of HCV Infection in Patients with Chronic Kidney Disease

In HCV-related liver diseases, the combined use of two or more second-generation DAAs allows the eradication of HCV infection in nearly 95% of treated patients [78–83], a percentage obtained even in liver transplant recipients [64, 66].

The knowledge on HCV eradication with DAA therapy in HCV-positive renal recipients or in those on a waiting list for renal transplantation is fragmentary, but the published data are highly promising. For HCV-positive patients in the wait list for kidney transplantation the first decision is whether to treat them before or after transplantation. For HCV patients with mild or moderate hepatic fibrosis who have a living kidney donor, treatment with DAA has been suggested prior to kidney transplantation [55, 67]. For HCV-positive patients who do not have a living donor and are at risk of progression to a more severe liver disease it has been suggested to perform kidney transplantation and to treat them with DAAs soon after, a decision that allows receiving a kidney from an HCV-positive donor [68] and reducing wait time for transplantation dramatically. Indeed, it has been recently reported that patients who accepted to be transplanted with an HCV-positive kidney, compared with those who have reclaimed an HCV-negative donor kidney transplant, have lower average waiting (469 vs 856 days) [67]. For an exhaustive analysis, it should be also considered that, due to the shortage of transplantable kidneys, in 2014, of 98, 956 patients on waiting list for kidney transplantation in the USA, more than 8,000 (8%) had died or were removed from the list due to worsening in clinical conditions [69, 75, 78].

All this considered, it is commonly accepted that HCV-infected transplant candidates may receive a kidney from an HCV-positive donor. This eventuality had occurred in about 2500 donations in the USA between 2005 and 2014 [73]; otherwise these kidneys would have been discarded. The transplantation of HCV-infected kidneys in HCV-infected patients, in addition to reducing waiting times [82], expands the pool of usable kidneys. The largest series [83] of renal transplants performed under these conditions demonstrated a 5-year and 10-year equivalent patient survival ($p= 0.25$) compared to those who received a kidney from an HCV-negative donor. Considering the persistent shortage of kidneys that can be transplanted, it has been also proposed to transplant HCV- positive kidneys into HCV-negative recipients and to initiate treatment with DAA immediately after transplantation and before a liver disease might develop.

At present, no valid recommendation comes from prospective trials, but several studies, prevalently small and from single clinical centers, provide useful information on the effectiveness and tolerability of different DAAs combination regimens in HCV-positive transplant recipients.

In a retrospective, multicentric study Fernández et al [84] observed a SVR12 in 98% of 103 kidney transplant recipients treated with DAAs (sofosbuvir plus ledipasvir in 59 and sofosbuvir plus daclatasvir in 18). Ribavirin was used in 1% of cases. Of these 103 patients, therapy was administered for 12 weeks in 72, for 24 weeks in 29, and for 8 weeks in two. There were three episodes of acute humoral graft rejection. No patient discontinued therapy due to adverse events, but 57 patients required immunosuppression dose adjustment. SVR was achieved by 98% of patients receiving DAAs alone and 97.6% of those receiving DAAs plus RBV, by 97% of those receiving 12 weeks of therapy, and 100% of those treated for 24 weeks. The presence of cirrhosis did not influence the response to treatment [84].

Lin et al. [85] treated 6 HCV-positive kidney recipients with a 12-week sofosbuvir/daclatasvir treatment (4 of 6 with HCV genotype 1b). HCV RNA was undetectable at week 4 and a SVR12 was obtained in all cases. Half dose of sofosbuvir was used in two patients with creatinine elevation. Treatment was well tolerated, and no adverse reaction had occurred.

Eisenberger et al. [83] assessed the efficacy and safety of 8- or 12-week combination of sofosbuvir plus ledipasvir in 15 kidney recipients with HCV infection (genotypes 1a, 1b, or 4). All patients achieved a SVR12, had a stable renal transplant function, and did not develop serious adverse events. A dose adjustment for tacrolimus was necessary in some cases. The authors concluded that the treatment was safe and effective.

Kamar et al. [66] treated 25 kidney transplant recipients with HCV infection with different sofosbuvir-based regimens. A rapid SVR, defined by undetectable viremia after 4 weeks of DAA therapy, was obtained in 22 (88%) cases and SVR12 in all. Treatments were well tolerated, and no adverse event had occurred, apart from a decrease in calcineurin inhibitor levels after HCV clearance. Lubetzky et al. [71] performed a retrospective cohort analysis of 31 HCV-positive patients who underwent kidney transplantation and received DAA therapy. The combination of sofosbuvir/ledipasvir was the most commonly used and 30 out of the 31 (97%) achieved SVR12; all patients showed a satisfactory allograft function. Six (19.3%) out of the 31 showed an increased proteinuria during or shortly after DAA therapy. The authors concluded that DAA therapy is safe and effective in HCV-positive kidney recipients and that patients with proteinuria should be monitored closely [71].

Bhamidimarri et al. [86] described 25 HCV patients in the end-stage kidney disease, transplanted with a kidney from an anti-HCV-positive deceased donor and treated with DAAs in the early post-transplant period (median 125 days). Of these 25, 24 (96%) achieved a SVR12. Tacrolimus dose adjustments were required in 13 patients.

Sawinski et al. [87] used 4 different DAA regimens to treat 43 renal transplant recipients; all of them obtained SVR 12 independently from the origin of the allograft (from an HCV-positive or -negative donor) and none of them showed a serious adverse reaction. A calcineurin inhibitor dose adjustment during treatment was applied in nearly half treated patients. Noteworthy, the waiting time to transplantation was longer in patients transplanted with a kidney from an HCV-negative donor than in those transplanted with an HCV-positive organ (969 versus 485 days) [84].

Colombo et al. [88] carried out a randomized, phase 2, open-label multicenter study including 114 kidney transplant recipients with HCV infection (HCV genotypes 1 or 4) infection and with a filtration rate (eGFR) of 40 mL/min or greater. These 144 were randomly assigned 1:1 to receive ledipasvir 90 mg and sofosbuvir 400 mg either for 12 or for 24 weeks; 91% had HCV genotype 1 infection and 15% compensated cirrhosis. A SVR12 was achieved by all treated patients, with no regard to the duration of treatment. Treatment was well tolerated in most cases, but serious adverse events occurred in 13 (11%) patients. The authors concluded that treatment with ledipasvir/sofosbuvir is effective ad shows an acceptable safety profile in HCV-positive kidney transplant recipients.

Londono et al. [89] described 103 HCV-positive kidney transplant recipients, 15% with cirrhosis, treated with DAAs, prevalently sofosbuvir/ledipasvir (n=59, 57%) or sofosbuvir/daclatasvir (n=18, 17%). Ribavirin was associated with DAAs in nearly half patients. The SVR12 rate was 98%. A grade 2 or 3 anaemia developed in 14 (33%) patients treated with ribavirin and in 9 (15%) untreated (p=0.03). No patient discontinued therapy because of an adverse event, but an adjustment of the dose of immunosuppressive drugs was required in nearly half of them. A mild allograft dysfunction occurred only in cirrhotic patients. An acute humoral graft rejection occurred in 3 patients. The authors concluded that DAA therapy was highly efficacious and safe in kidney transplant recipients.

Gallegos-Orozco et al. [90] described 8 HCV-positive kidney recipients (7 with an HCV-positive kidney and one with an HCV-negative kidney), treated three to six months after transplantation with DDA therapy according to HCV genotype and their prior treatment experience: all of them had functioning kidney grafts and achieved SVR12. The authors concluded that HCV-positive patients can successfully receive an HCV-positive kidney from an HCV-positive donor, with a substantial reduction of time on the wait list.

Durand et al. [91] conducted a nonrandomized open label study to evaluate the tolerability and efficacy of DAA treatment given before and after kidney transplantation to 10 non-HCV infected kidney recipients receiving an HCV-infected organ. All recipients transplanted with an HCV-positive genotype 1 infected kidney were treated with grazoprevir, 100 mg, and elbasvir, 50 mg, immediately before transplantation and for 12 weeks after transplantation, while for the recipients of a kidney from a donor infected with HCV genotypes 2 or 3 sofosbuvir, 400 mg, was added to grazoprevir and elbasvir in a triple therapy during the 12-week treatment period after transplantation. All patients achieved a SVR12 and no treatment-related adverse events had occurred [91].

Goldberg et al. [92] evaluated safety and efficacy of kidney transplantation from HCV genotype 1 donors into HCV-negative recipients, followed by elbasvir/grazoprevir treatment in an open-label, single-group, pilot trial. Patients undergoing dialysis who had long awaited kidney transplant have been included in the study and transplanted. Ten patients received HCV-infected kidneys, became HCV

RNA positive beginning from the day 3 after transplantation, received a 12-week elbasvir/grazoprevir combination, and achieved SVR12. The authors concluded that the transplantation of HCV genotype 1 infected kidneys into HCV-negative recipients, followed by the administration of elbasvir/grazoprevir, ensures an excellent allograft and the healing of the provoked HCV infection [92].

3. Strategies, Future Expectation, and Conclusion on the DAA Treatment of Renal Recipients with HCV Infection

The DAA regimens for HCV-positive renal transplant recipients should be adequate to HCV genotype, the entity of liver disease, the quality of posttransplant renal function, and the immunosuppressive drugs administered. A close monitoring of renal function during the DAA administration, with adjustment of DAA doses to the patients' eGFR, and a careful observation of a possible onset of drug to drag interactions, with adjustment of doses of immunosuppressive drugs, were recommended. For example, sofosbuvir-based DAA regimens may induce renal adverse events in patients with reduced renal function, particularly in those in an end-stage renal disease (ESRD) [93, 94], while the administration of the combination therapy of dasabuvir, ombitasvir, paritaprevir, and ritonavir [95] may lead to drug to drug interactions in patients under a CNI-based immunosuppressive therapy.

Calcineurin inhibitors (tacrolimus and ciclosporin) and the mechanized target of rapamycin inhibitors (everolimus and sirolimus), used as immunosuppressants in patients undergoing renal transplantation, are substrates of cytochrome P450 and therefore the possible interactions between these drugs and DAAs should always be taken into consideration [58, 96–98]. During the DAA treatment of HCV-positive kidney recipients, the decrease in HCV load is frequently followed by a decrease in tacrolimus levels that often requires dose adjustment. This possibility has been well demonstrated by Kamar et al. [66] in renal transplant patients treated with sofosbuvir in various combinations with other antiviral drugs. Although no episodes of rejection or deterioration of the transplanted organ had occurred, the data highlight the need for careful monitoring of immunosuppressive drug levels and dose adjustment [66].

The optimal timing of HCV therapy (pretransplant versus posttransplant) in HCV-positive transplant recipients is topic of debate. Several studies have shown that the wait times for kidney transplantation from HCV-positive donors are significantly shorter than those for kidneys from uninfected donors. These considerations support the use of all kidneys available for transplantation in HCV-positive patients, both HCV-negative and HCV-positive.

The Kidney Disease Improving Global Outcomes guidelines currently recommend treating with DAAs the HCV RNA positive candidates for renal transplant while still in dialysis [41], since once SVR12 has been achieved, a relapse after renal transplant seems unlikely [99]. However, once SVR12 has been achieved with DAA treatment before transplantation, the possibility to receive a renal graft from an HCV-positive donor is lost, together with the benefit of shortening the length of dialysis and of wait time in the kidney transplantation list and with the possibility of reducing comorbidities (i.e., cardiovascular complications) [99, 100]. However, for HCV-positive patients with mild or moderate hepatic fibrosis on a waiting list the high effectiveness of the new DAAs has allowed starting treatment immediately after transplantation [99, 100], a benefit balanced in some cases by the risk of an acute renal injury due to the potential interaction between DAAs and immunosuppressants. In this regard it should also be considered that many of the current DAAs require a creatinine clearance level of 30 ml/min or greater because their main elimination route is renal [58].

Because of the persistent shortage of kidneys that can be transplanted, it has been proposed to transplant HCV-positive kidneys into HCV-negative recipients and to initiate treatment with DAA immediately after transplantation, before the liver disease develops. However, although a few preliminary studies have provided encouraging results, the ethical implications of this procedure should be carefully evaluated, since approximately 5% of patients may not obtain SVR12 with DAA treatment; in some cases the donor HCV genotype may be difficult to treat one (i.e., HCV genotype 3) and in very rare cases fibrosing cholestatic hepatitis may develop after kidney transplantation.

4. Final Remarks

A careful assessment of the stage of liver disease should be made including an accurate anamnesis, complete physical examination, serial determinations of serum liver function tests (serum levels of albumin, bilirubin and liver enzymes, prothrombin time, and platelet count), upper abdominal ultrasound examination, and endoscopic examination of the upper intestinal tract in cirrhotic patients. Patients with advanced fibrosis should be regularly revaluated.

The occurrence of reactivation of an indolent (open or occult) Hepatitis B virus (HBV) infection in HBV/HCV kidney-transplant recipients [77] during or after DAA [76] should be prevented by a nucleoside/nucleotide analogue prophylaxis in HBsAg-positive patients and early identified by an accurate monitoring (ALT serum levels and HBsAg) in HBsAg-negative/anti-HBc-positive patients [74]. In case of HBV reactivation in HBsAg-negative/anti-HBc-positive patients a treatment with nucleoside/nucleotide analogues should be started.

Most DAA therapeutic regimens are practically restricted to HCV genotypes 1 and 4, while HCV genotype 3 is quite frequent and equally dangerous. This need will be overcome by the newer pan-genotypic DAAs regimens (glecaprevir/pibrentasvir).

The optimal regimens to be applied either before or after kidney transplantation may not be proposed yet, because of the expected arrival of other newer DAAs and of results from multicenter randomized controlled trials. Newer DAA regimens requiring a shorter duration of treatment may also accelerate the access to transplantation.

The possibility to transplant HCV-positive kidneys into HCV-negative recipients has been recently analyzed by the American Society of Transplantation Consensus Conference on HCV-positive donors and kidney transplantation. It has been considered that the high level of safety and efficacy of DAAs in eradicating HCV infection provides the opportunity to explore their use in transplanting kidneys from HCV-viremic patients into non-HCV-viremic recipients, a practice that could save the life of numerous CKD patients with organ failure. Although more research is needed before making a final settlement on this topic, the consensus underlines the need to guarantee the access to DAA therapy to the HCV-negative recipients at the time they will receive a kidney from an HCV-positive donor.

It has been also underlined that a living donor who has cleared HCV infection after DAA treatment does not transmit HCV infection to the kidney recipients [101–105].

Treating HCV infection before kidney transplantation could be potentially advantageous, since eradicating HCV would lead to better allograft and patient survival.

Ideally, patients with ESRD and HCV infection should be comanaged by surgeons, nephrologists, and hepatologists before and after transplantation.

Conflicts of Interest

All the authors of the manuscript declare they have no conflicts of interest regarding this paper.

Authors' Contributions

Calogero Armando, Sagnelli Evangelista, and Sagnelli Caterina equally contributed to this work, designed the study, and wrote the manuscript.

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Research Article

Pretransplant Nephrectomy for Large Polycystic Kidneys in ADPKD (Autosomal Dominant Polycystic Kidney Disease) Patients: Is Peritoneal Dialysis Recovery Possible after Surgery?

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The choice of modality for renal replacement therapy in patients with ADPKD varies, often based on patient choice, physician-related factors, and resource availability. For a long time peritoneal dialysis (PD) was considered as relative contraindication due to the possible limited intraperitoneal space. In recent years, some studies suggested it is a valid option also in patients with ADPKD to be considered as a first line treatment in potentially fit patients. Diuresis volume lowering and potential permanent damage of peritoneal integrity, both leading to a necessary switch to haemodialysis, are the two most important dangers after nephrectomy, especially if bilateral, in PD patients. We performed a retrospective analysis of patient underwent native polycystic kidney nephrectomy in order to state the possibility to recover peritoneal dialysis after surgery.

1. Introduction

The term polycystic kidney disease should be reserved for two hereditary diseases: autosomal recessive polycystic kidney disease (ADPKD) and, most commonly, autosomal dominant polycystic kidney disease (ADPKD) [1, 2]. ADPKD is characterized by relentless development and growth of cysts causing progressive kidney enlargement associated with hypertension, abdominal fullness and pain, episodes of cyst haemorrhage giving rise to gross haematuria, nephrolithiasis, cyst infections, and progressive renal failure (Figure 1) [3]. ADPKD is one of the most common human hereditary diseases and leads to end stage renal disease requiring dialysis or renal transplantation in about 50% of the patients [4, 5]. Diuresis and fluid volume control is often preserved also during the dialytic period [6, 7].

Before transplantation native kidneys are not routinely removed considering significant morbidity and mortality associated [8, 9]. Indications for nephrectomy include recurrent and/or severe infection, symptomatic nephrolithiasis, recurrent and/or severe bleeding, intractable pain, suspicion of renal cancer, and space restrictions prior to transplantation (Figure 2), taking into account that kidney size typically declines after transplantation [10–13].

In recent years, some studies suggested peritoneal dialysis (PD) as valid option also in patients with ADPKD [14], which was considered a relative contraindication previously due to the possible limited intraperitoneal space to accommodate the dialytic fluid, as well as the risk of hernia [15]. The optimum time for native polycystic kidney nephrectomy is still a topic of debate in the scientific literature. Many factors have to be considered to make the right choice for each patient: dialysis or pre-emptive transplantation, complication severity, anuria, easy access to transplantation, and potential living donor. For those patients waiting for transplantation, requiring renal replacement therapy, either haemodialysis

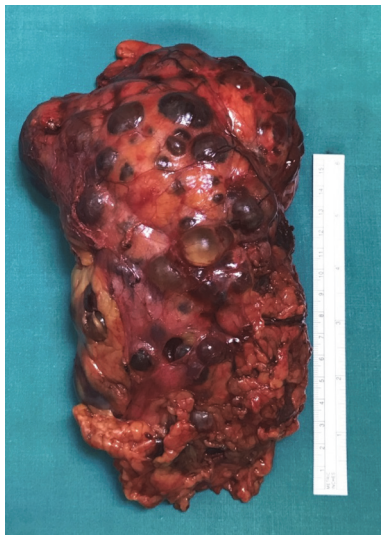

Figure 1: Renal parenchima is largely replaced by cysts that may become haemorrhagic or infected.

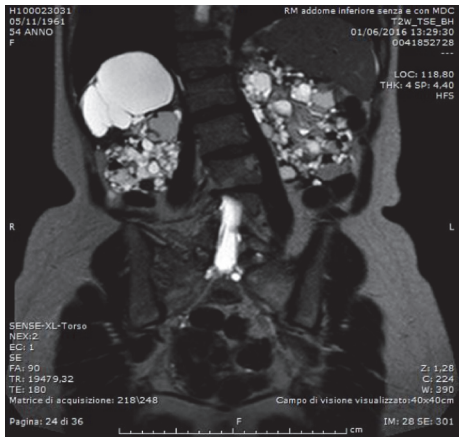

Figure 2: Preoperative MR scan shows size of the polycystic kidneys and eventually haemorrhagic or infected cysts and suspicious areas of malignancy [18].

(HD) or peritoneal dialysis (PD) is suitable modalities. The choice of modality for renal replacement therapy in patients with ADPKD varies, often based on patient choice, physician-related factors, and resource availability. Although intra-abdominal space restrictions, increased risk for abdominal wall hernias, and increased prevalence of colonic diverticula may pose challenges, ADPKD is not a contraindication for PD. For patients whom started PD as replacement therapy native polycystic nephrectomy may be harmful considering the manipulation and possible damage of peritoneum required by the procedure. After surgery, it may be no more efficient in supporting peritoneal dialysis. Therefore, the choice for nephrectomy is more difficult for ADPKD patient in PD.

We performed a retrospective analysis of patient underwent native polycystic kidney nephrectomy in order to state the possibility to recover peritoneal dialysis after surgery.

2. Materials and Methods

2.1. Patients and Surgical Technique. A retrospective analysis of ADPKD patients in waiting list for kidney transplant, which underwent nephrectomy in our transplant surgery department between December 2012 and December 2017, was carried out. All patients were included, male and female, and the indications for nephrectomy were symptoms (pain, urinary tract infections, or hematuria) or kidney size responsible of subocclusive symptoms or difficulty for future graft positioning.

In all cases, nephrectomy was performed with laparotomic transperitoneal surgical technique through subcostal incision. The whole procedure was carried out using the harmonic scalpel Ultracision® (Ethicon US, LLC) in order to limit postsurgical bleeding and lymph spread. After completing nephrectomy, the posterior peritoneal flap was always reconstructed to preserve peritoneal cavity avoiding visceral adhesions for future peritoneal dialysis recovery.

2.2. Data Collection. Clinical data, including demographic parameters, biochemical data, and copathology during the surgery, were collected from our database based on a review of patient medical notes. Demographic including the gender, age, cause of ESRD, haemodialysis (HD) or peritoneal dialysis (PD), and comorbid conditions. We considered pre and post-surgery residual diuresis, pre and post nephrectomy creatinine value, the time from the beginning of dialysis to nephrectomy. Indications for nephrectomy include recurrent and/or severe infection, symptomatic nephrolithiasis, recurrent and/or severe bleeding, intractable pain, suspicion of renal cancer, and space restrictions prior to transplantation. The data concerning the intervention include side of procedure, operator surgeon, and any peri- and postoperative complications. The renal volume was defined as the total volume of both kidneys and calculated after the nephrectomy on the specimen. After surgery we considered if residual diuresis was present or not and if peritoneal dialysis was resumed.

3. Results

During the last five years thirty-three patients, all affected by ADPKD related ESRD and underwent nephrectomy in our transplant surgery department from December 2012 to December 2017. Among the patients 20 (60%) were males and 13 (40%) were females, with a mean age of 54 ± 8.

Thirty patient were in waiting list for kidney transplant from cadaveric donor (30/33), two of them also were scheduled for living donor transplantation as signaled previously, and three patients were already transplanted (3/33).

The nephrectomy was bilateral in 2 patients (2/33) (6%), and unilateral in 31 (31/33) patients (94%) (20 right - 64% in 11 left -36%). The mean surgical specimen weight was 1975.58 Kg.

Nine patients (9/30) (27.3%) underwent peritoneal dialysis as replacement therapy, twenty-two patients (22/30) (66.7%) underwent hemodialysis, and two (2/30) (6%) patients had a residual renal function yet useful to avoid

Table 1

Dialysis	Number of patients
HD	22 (73.3%)
PD	6 (20%)
Pre-emptive transplantation	2 (6.6%)

Table 2

Indications for nephrectomy	Percentage
Kidney volume	42.3%
Symptoms	57.7%
(i) Urinary Tract Infections (UTIs) recurrence	52.9%
(ii) Persistent hematuria	35.3%
(iii) Chronic pain	11.7%

Table 3

Dialysis after nephrectomy in PD patients	Percentage
PD patients	6
(i) HD beginning	33.3% - 2 patients
(ii) PD recovery after surgery	66.6% - 4 patients

replacement therapy and were scheduled for living donor transplantation: pre-emptive transplantation (Table 1). Three of nine patients (3.3%) in PD underwent the nephrectomy after the kidney transplant; the indications for the surgery were in all three cases recurrent urinary tract infections.

Simultaneous the nephrectomy two patients underwent the cholecystectomy for cholelithiasis, two underwent prophylactic appendectomy, and one repaired umbilical hernia and two inguinal hernia.

Nephrectomy became necessary in 19/33 (57.7%) of patients considering their symptoms. Among them 52,9% had recurrent UTIs and 11.7% chronic pain and persistent hematuria was the symptom at admission in 35.4% of cases. Quite half of our patients required nephrectomy considering kidney size to improve renal graft positioning avoiding compression (Table 2).

Considered only pretransplanted patients (30/33) the mean value of pre nephrectomy residual dieresis was 1540 ml/die (min 0 ml-max 3500 ml) while, after nephrectomy, the residual diuresis value decreased to 860 ml/die (min 0 ml-max 1500 ml). Prenephrectomy mean creatinine value was 7.86 mg/dl (min 3.14 mg/dl-max 12.78 mg/dl) and post-nephrectomy this value increase 8.74 mg/dl (min 1.23 mg/dl-max 13.84 mg/dl). The mean waiting time from begin of dialysis and native nephrectomy was 22.78 months (min 1 month-max 60 months).

All six PD patients underwent positioning a central line during surgery to allow a bridge period of hemodialysis as replacement therapy after nephrectomy in order to obtain a complete healing of peritoneal surgical incisions to avoid extra-peritoneal spread of dialytic solution and to reduce the risk of incisional hernia.

Two patients never recovered peritoneal dialysis after the nephrectomy because of perioperative complications occurred.

Four of six patients started again peritoneal dialysis after a mean time of 35±5 days after surgery. All had preservation of a sufficient diuresis and no difficulty in loading dialytic fluid into the peritoneum or to evacuate it after dialysis. Purifying efficacy of the treatment was satisfactory. No incisional hernia was reported (Table 3).

4. Discussion

In many Western countries, peritoneal dialysis (PD) is not often the treatment of choice for ADPKD patients because of the possible limited intraperitoneal space to accommodate the dialysis fluid, as well as the risk of hernia [15]. Recently some studies suggested that PD may be a valid option for dialysis in ADPKD related ESRD [16].

Peritoneal dialysis ensures a better survival during the first two years of therapy and should be therefore employed as a first line treatment in potentially fit patients [17].

Polycystic Kidneys should not be routinely removed prior to transplantation since nephrectomy in ADPKD patients is associated with significant morbidity and mortality. Indications for nephrectomy include recurrent and/or severe infection, symptomatic nephrolithiasis, recurrent and/or severe bleeding, intractable pain, suspicion of renal cancer, and space restrictions prior to transplantation (even considering that kidney size typically declines after transplantation) [18]. Brazda et al. [19, 20] in their series reported a higher rate of native nephrectomy (35.4%) and advocated that if native nephrectomy were needed, it would be better to perform it before transplantation rather than after. In our experience only three patients underwent nephrectomy after kidney transplant for recurrent and worsening UTIs, apparently related to the immunosuppressive regimen.

Nephrectomy in PD patients, especially if bilateral, carries a twofold risk: a decline of diuresis volume and a potential permanent damage of peritoneal integrity, both leading to a necessary switch to hemodialysis. Data retrieved from our series shows that 66.6% of patients who underwent monolateral native nephrectomy prior to transplantation successfully recovered PD within 35 ± 5 days after the surgical operation avoiding, thus, the problems linked to the hemodialytic routine: a worse quality of life and a progressive vascular impairment potentially harmful in view of future transplantation. Differently from what is reported in Hsu et al. [21] case series which demonstrates that concerning the PD recovering, nephrectomy by retroperitoneal route is strongly recommend, in our population it seems that even a transperitoneal approach for the nephrectomy may be a feasible technique and does not interfere with the peritoneal dialysis recovery.

In our experience only those patients who presented postoperative complications, both haemorrhagic, requiring in one of the two cases reintervention, and open abdomen treatment, did not recovered their PD routine.

Obviously there are many limitations in our analysis in particular those linked to the small sample size which do not allows a clear identification of clinically meaningful

differences between groups. Furthermore, because of the retrospective design, we lack data concerning liver size or other surrogate measures for internal organ enlargement (e.g., waist circumference). As a result, it is not possible to evaluate correctly the correlation between kidney volume and the clinical outcome (especially the risk of hernia).

5. Conclusions

Native polycystic kidney nephrectomy is still a topic of debate. The optimum time is not well established especially for patients undergoing PD as replacement therapy considering the risk of peritoneal damage and HD as the only one possible choice before transplant.

Our experience suggests monolatheral nephrectomy with laparotomic approach as a safe and feasible procedure also for very large kidneys. Performance before transplant with meticulous surgical technique allows peritoneal preservation avoiding common complication and limiting adherence syndrome in order to rapid peritoneal dialysis recovery.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Introducing of the First DCD Kidney Transplantation Program in Poland

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In many countries, including Poland, the main problem with transplantation is the insufficiency of organ donors in relation to the demand for organs. Hence, the common aim globally is to increase the pool of donors. The prolonged survival of patients after transplantation, with respect to the survival time of patients on dialysis, makes the search much more intense. After the recourse of expanded criteria donors (ECD), the next step was obtaining kidneys from donors after irreversible cardiac death (DCD). Therefore, based on Dutch, British, and Spanish experience, it can be hypothesized that the introduction of DCD procedures in countries that have not launched these programs and the improvement of DCD procedures may shorten the waiting time for organ transplantation globally. The legal basis for the procurement of organs after irreversible cardiac arrest came into existence in Poland in 2010. Previously, such organ procurements were not in practice. Since 1984, when Poland published irreversible cardiac arrest as a criterion of brain death, it became the only way to determine death prior to the procurement of organs. The aim of this report was to evaluate the results of the first 19 transplantation cases involving harvested kidneys from donors after cardiac arrest, which was irreversible and clinically confirmed, without any doubt as per the ethical protocol of DCD. Understanding, support, and public perception are essential for this program's initiation and maintenance.

1. Introduction

A recent global problem is the persistent shortage of the kidney pool for transplantation. Despite increases in kidney transplantation from expanded criteria donors (ECD) and living related donors (LRD) in the last decades, the supply of donor kidneys remains insufficient [1]. Shortage of organs for transplantation connected with the promising results of organs transplanted from donors after cardiac death has evolved the use of donors after irreversible cardiac death (DCD) into routine practice in many western countries and accounts for 16.5% of organ transplantations in the US (based on OPTN Data of March 2016) and up to 52% in Netherlands [2–5]. In some Asian countries and in Japan, DCD constitutes the main source of organs [6]. The First International Workshop on DCD held in Maastricht in 1995 described four categories of DCD, depending on the irreversible cessation of circulatory and respiratory functions [5]. Based on the Maastricht categories, uncontrolled DCDs are Types I (dead on arrival) and II (unsuccessful resuscitation), while controlled DCDs are Types III (awaiting cardiac arrest) and IV (cardiac arrest occurring with brain death). The

European countries that actively perform DCD differ in their protocols. The highest rate of controlled DCD is recorded in the United Kingdom, Belgium, and the Netherlands while uncontrolled DCD mainly is described in France, Latvia, and Spain [7]. In recent years, in the Council of Europe, 10 out of 27 participating countries confirmed DCD activity. For a long time, Poland and 9 other countries such as Cyprus, Estonia, Luxembourg, Norway, Portugal, Romania, Slovak Republic, Slovenia, and Sweden have been planning to start the DCD program [8, 9]. Successful European uncontrolled donation after circulatory determination of death (UDCDD) programs rely on legislation permitting organ procurement without consent. The legal basis for the procurement of organs from donors after irreversible cardiac arrest has existed in Poland since 2010, but it was in May 2015 when the first cases occurred in the Department of General and Transplant Surgery, Clinical Hospital of Poznan University of Medical Sciences [10, 11].

The procurement was initiated according to a standardized protocol, designated to select and manage kidney DCD. Based on this, the implemented DCD program describes as eligible for organ retrieval, uncontrolled Maastricht Types I and II–the UDCDD protocol. In Poland, Maastricht III donors must be excluded based on the law.

2. Materials and Methods

Between May 2015 and April 2017, in the Clinical Hospital of Poznan University of Medical Sciences, 10 non-heart-beating donors (DCD) Maastricht Types I/II were accepted for organ retrieval.

The acceptable criteria for DCD were as follows: (i) known identity; (ii) age less than 60 years; (iii) time from cardiac arrest to cardiopulmonary resuscitation (CPR) less than 30 min; (iv) donors' warm ischemia time less than 180 min; (v) prosecutor's consent; (vi) negative history of diabetes mellitus, uncontrolled hypertension, malignancy, renal disease, extensive trauma, or systemic sepsis.

After retrieval the kidneys were put into the WAVES system which provides controlled pulsatile kidney perfusion using oxygenated hypothermic physiologic solutions and monitors, displays, trends, and saves important perfusion parameters, including perfusate flow, temperature, pressure, and renal resistance. The temperature and resistance to flow were constantly recorded. One of 20 kidneys was not accepted for transplantation because of significantly high renal resistance (RI>0,4).

During the 2-year period from May 10, 2015, to April 21, 2017, a total of 19 renal transplantations of DCD kidneys involving 11 male and 8 female recipients were performed. The ages of the recipients ranged from 32 to 69 years (median, 50.9 years). All the patients were dialyzed before the transplantation for 9 to 120 months (median, 30 months). The causes of kidney failure are shown in Table 1. For 4 patients, it was the second kidney transplantation. In the previous transplantation procedure kidneys derived from DBD donors.

Patients were informed that the kidneys were from deceased donors after irreversible cardiac arrest and they signed a specially prepared consent form. HLA typing and cross-matching were routinely performed before the DCD kidney transplantation.

Table 1: Causes of renal failure.

Cause of renal failure	Number of recipients
Glomerulonephritis	3
Diabetic nephropathy	3
Hypertensive nephropathy	2
Polycystic kidney disease	2
Focal segmental glomerulosclerosis	2
Interstitial nephritis	2
Vasculitis	1
Others	4

2.1. Ethics Committee Approval. Renal transplantation of DCD kidneys is a standard procedure and does not need the ethics committee approval.

2.2. Immunosuppressive Protocols. Recipients were immunosuppressed using a triple-therapy regimen at the beginning of the DCD transplantation program. Patients were given Simulect (Basiliximab 20 mg) intravenously as induction therapy prior to the transplantation and on the 4th day after kidney transplantation. Methylprednisolone (intravenous) 500 mg, 250 mg, and 125 mg were administered for 3 days after the transplantation, consecutively. Prednisone was introduced on the 3rd day after transplantation (20 mg/day). Tacrolimus (for 11 patients) was started at a dose of 0.1 mg/kg/day with mycophenolate mofetil (1.0-1.5 g/day) on day 0 and was adjusted to maintain whole blood levels in the range of 8 to 14 ng/mL, in the first 3 months after transplantation, and 5 to 12 ng/mL, thereafter. Cyclosporine (for 5 patients) was started at a dose of 10 mg/kg/day with mycophenolate mofetil (2.0-3.0 g/day) and the dose was adjusted to maintain whole blood levels in the range of 250 to 350 ng/mL. Subsequently, this regimen, according to the practice of more experienced centers, was based on two drugs without mycophenolate mofetil. Everolimus was started with tacrolimus and steroids in the remaining 3 cases. These protocols differed due to comorbidities, PRA level, and coexisting precancerous state.

2.3. Follow-Up. The follow-up period was 10 to 28 months. The results were obtained directly from the transplant outpatient clinic visited by the patients and via a telephone survey.

3. Results

There were 6 male and 4 female cadavers among the donors. The age of the donors ranged from 28 to 62 years (median 50 years).

CPR lasted for 35 to 125 min, with a 5-min "no touch time" period. External cardiac massage was performed using

Table 2: Donors' characteristics.

Sex	Age (in years)	Cause of death	CPR on arrival (min)	CPR in the hospital (min)	No touch time (min)	Chest compression	WIT
M	45	Myocardial infarction	35	57	5	Manual	169
K	28	Asphyxia	20	76	5	Mechanical Lucas II	164
M	62	Myocardial infarction	30	75	5	Mechanical Lucas II	175
M	53	Myocardial infarction	5	86	5	Mechanical Lucas II	145
M	49	Myocardial infarction	66	30	5	Mechanical Lucas II	160
K	58	Myocardial infarction	84	41	5	Mechanical Lucas II	203
K	45	Myocardial infarction	46	47	5	Mechanical Lucas II	158
K	41	Myocardial infarction	53	46	5	Mechanical Lucas II	177
K	54	Myocardial infarction	35	37	5	Mechanical Lucas II	160
M	47	Myocardial infarction	12	23	5	Mechanical Lucas II	160

mechanical chest compression (Lucas II) in 9 cases and manually in the first case. In 3 of the 10 procurements, normothermic extracorporeal membrane oxygenation (nECMO) was used. The average total warm ischemic time (WIT) was 167.1 min. Seventeen of 19 transplanted DCD kidneys were preserved using cold continuous pulsatile preservation perfusion with an IGL-1 liquid. (Table 2)

3.1. Early Graft Function and Rejection Rates. Two deaths were recorded: 1 female patient, due to severe pneumonia in the 54th postoperative day, and a male patient, due to a large retroperitoneal hematoma in the 23th postoperative day. The deaths were not related to the type of donation.

Three NHBD kidney transplants did not function satisfactorily, giving an overall primary nonfunction rate of 15.8%. In the 2 patients who died, it was not known whether the allografts were viable at the time of death. The acute rejection rate was 5.3%. This single case was successfully treated with solu-medrol pulses.

Delayed graft function was observed in all the recipients. The number of necessary hemodialysis after transplantation ranged between 2 and 18 (average, 8.4). The number of transfused units of RBC concentrate ranged between 2 and 18 (median, 6).

The urological complication rate was 5.3%. Urological complication was observed in a male patient who was operated on several times because of a large retroperitoneal hematoma. After one of the surgical procedures, urine leak from the ureterovesical anastomosis appeared.

3.2. Graft Survival. One-year graft survival rate was 68.4%. Excluding the early deaths, the rate is 78.9%. Four graftectomies were performed: 3 because of unsatisfactory graft function and 1 because of surgical postoperative complications leading to graft infection. 3 patients required chronic dialysis. No rejection was found in performed graft biopsies. In addition, urinary tract infections recurred.

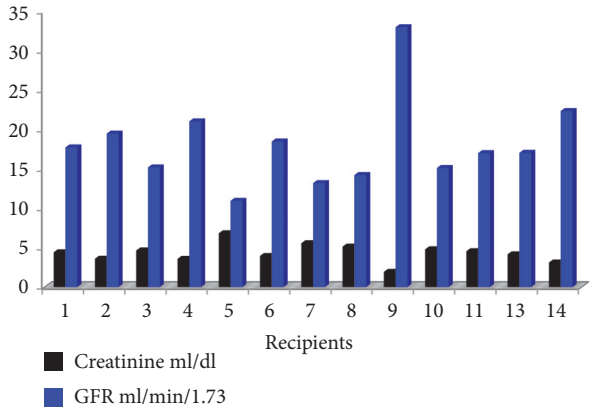

Figure 1: Creatinine and GRF level at the end of posttransplant hospitalization.

3.3. Late Graft Function. The baseline serum creatinine levels (at the end of posttransplant hospitalization) are shown in Figure 1. They ranged between 1.93 and 5.57 mg/dL (median, 4.12 mg/dL). GFR levels ranged between 11 and 36 mL/min/1.73 m^2 (median, 17)

All the DCD kidneys improved functionally in 1 to 3 months after the recipients' discharge from the hospital

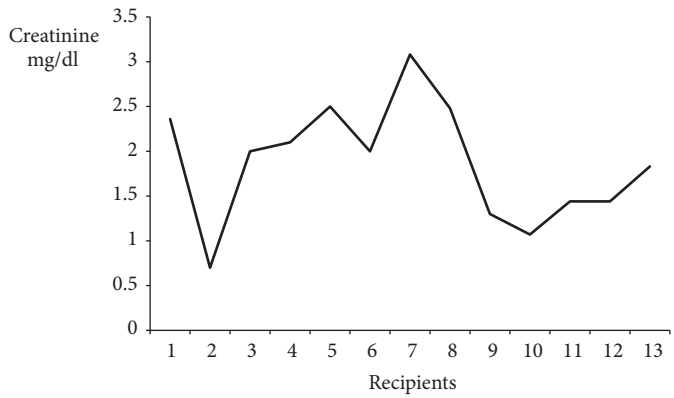

Figure 2: Minimal creatinine level.

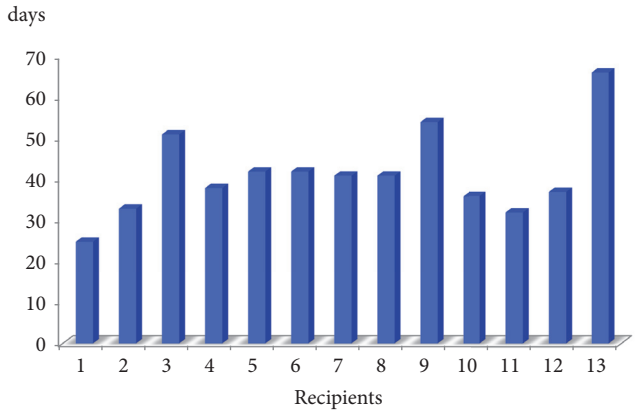

Figure 3: Hospitalization.

and remained stable in the follow-up period. The minimal creatinine levels ranged between 0.7 and 3.04 mg/dL (median 2) (Figure 2).

GFR levels ranged between 24 and 98 mL/min/1.73 m^2 (median, 38).

None of the patients required reoperation. All of them are very satisfied with the transplanted kidneys, although they admitted that the prolonged hospitalization (Figure 3) and waiting for the transplanted kidney to exhibit good renal function were difficult.

3.4. Contribution of DCD Kidneys. Figure 4 shows the contribution made by DCD kidneys to the transplant rate in Poland in 2016. In 1 year, 13 DCD kidney transplants were performed, leading to 1.32% of a total of 978 kidney transplants.

4. Discussion

One-year graft survival rate was 68.4%. The results were understated due to 2 deaths not related to the type of donation. All the DCD kidneys improved in functionality in the 1 to 3 months following the recipients' discharge from the hospital and remained stable during the follow-up period. Moreover, none of the patients needed to be reoperated on; instead, they were satisfied with the outcome of the procedure. Although the results are not good enough comparing with other experienced centers, they show honestly initial difficulties in the development of a non-heart-beating donor program. The troubles with kidneys derived from UDCD were common in transplantation teams starting this program [12, 13].

For dialysis patients with end-stage renal failure, transplantation may provide additional years of life and an improvement of quality of life. The decrease in the number of organ procurements from donors after brain deaths observed in the previous year in Poland is associated with a reduction in mortality from traffic accidents and cerebrovascular diseases. In some other countries, it has remained at a similar level as in the UK, for example, or has decreased as in Spain, for example [13, 14].

The median waiting time before transplant for adult patients was about 9 months. Meeting the ever-growing difference between the demand for organs and their harvesting involves the use of ECD, considered less than optimal, involving older donors, donors with comorbidities who show deteriorated long-term graft survival, and donation after cardiac death [15].

Early graft loss is more frequent among DCD and ECD kidney recipients than among DBD kidney, and DCD kidneys are more susceptible to cold ischemic injury and have a higher incidence of delayed graft function than DBD ones. Short and medium transplant outcomes were similar in the DBD and DCD groups [16–19]. It is also emphasized that kidney transplant patients following uncontrolled DCD recover renal function at a slower rate than recipients of controlled DCD [20]. Moreover, several papers indicate similar long-term outcomes between expanded-criteria DCD (ECDCD) and DBD (ECDBD) donors. Early graft loss in DCD recipients is associated with a higher incidence of primary nonfunction and acute vascular occlusion because DCD kidneys are more vulnerable to ischemia-reperfusion injury [21]. A higher percentage of early graft loss due to acute thrombosis may be linked to poor quality vessels and endothelial activation in DCD donation [22]. It is therefore important to seek a procedure that improves the quality of procured organs and shortens the time of warm

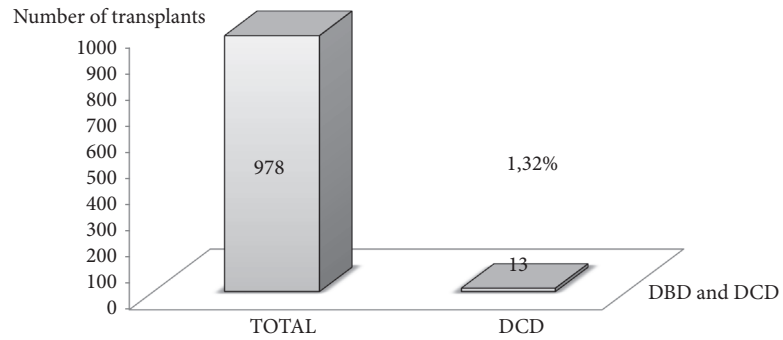

Figure 4: The contribution made by DCD kidneys to the transplant rate in Poland in 2016.

ischemia. Successful UDCDD programs exhibited optimal results when warm ischemic time was up to 120 min [23], but the success was achieved at time up to 180 min [24]; thus, experts suggest considering only witnessed cardiac arrests or patients with recent cardiac activity (any rhythm other than asystole) during resuscitation. To prevent microvascular clotting, initiation of preservation with heparin infusion, chest compression, and assisted ventilation should occur soon after death determination. In addition, nECMO is selected as the preservation method due to its protective and reparative properties [25–28]. Based on many studies, the currently implemented procedure in our hospital involves the use of nECMO perfusion of organs, which can cause minor injury owing to ischemia-reperfusion injury and improve the quality of procured organs [28, 29]. Additionally, the cold continuous pulsatile preservation perfusion will use WAVES and an IGL-1 liquid. The results should be better by ensuring lower renal resistance RI<0,4.

The UDCDD program has qualified donors who meet the following criteria: age 18-60, known personal data, excluded potentially reversible causes of cardiac arrest, known CPR time <30 min or documented electrical activity of the heart leading to a cardiac arrest (suggesting a recent cardiac arrest), and without exclusive factors: CPR performed over 30 minutes after the arrest, massive hemorrhage, renal failure (e.g. dialysis access), liver disease (e.g. jaundice, ascites), drug addiction, homelessness, cancer and infections, severe trauma, amputations in the course of vascular disease, or period of severe hemodynamic insufficiency longer than 1 hour (e.g. peripheral edema) before death.

The DCD procedure has raised ethical controversies not only in Poland, which is important in the protocol of organ recovery and the procedure followed by the transplantation team. The standard way to avoid conflicts of interest, loyalty, and obligation is that members of the transplant team, acting on behalf of potential recipients, are not involved in CPR or diagnosis of patient death. This so-called "hands-off" period with separation of roles for teams is indispensable for public trust and the development of the organ donation program [30, 31].

Every year in Poland, approximately 600 kidney procurements from donors after brain death are performed. The number of transplants from living donors is small, approximately 55 per year [30]. The program of organ donation after cardiac arrest is a strategy to increase the pool of donors while shortening the waiting time for transplant patients on waiting lists. However, this group of donors is not fully utilized worldwide, especially in Poland. The protocol established in the Department of General and Transplant Surgery of Poznan University of Medical Sciences might be helpful for other transplant teams in Poland to start a program.

5. Conclusion

Our data show convincing results concerning the first 19 cases of UDCDD in Poland. The DCD program involving uncontrolled donors is challenging for transplant coordinators, hospital transplant teams, and out-of-hospital emergency services. Continuous effort by many people, with nationwide information campaigns for the acceptance of the donation of organs after cardiac death by the Polish population, is crucial for future success in increasing the pool of organs donors. It seems that only the establishment of a program of donor renal transplantation from DCD Maastricht Types I and II is a promising option to reduce the number of potential recipients on waiting lists in Poland. These campaigns may also influence Polish law and change the legislation to allow for DCD Maastricht Type III.

Data Availability

Data supporting the conclusion of the study are available in patient's medical records and Polish transplantation records.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Cold Pulsatile Machine Perfusion versus Static Cold Storage in Kidney Transplantation: A Single Centre Experience

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Introduction. We present our experience with hypothermic machine perfusion (HMP) versus cold storage (CS) in relation to kidney transplant outcomes. *Methods*. Retrospective analysis of 33 consecutive HMP kidney transplant outcomes matched with those of 33 cold stored: delayed graft function (DGF), length of hospital stay (LOS), estimated glomerular filtration rate (eGFR), and patient and graft survival were compared. Renal Resistive Indexes (RIs) during HMP in relation to DGF were also analysed. *Results*. In the HMP group, mean HMP time was 5.7 ± 3.9 hours with a mean cold ischaemic time (CIT) of 15 ± 5.6 versus 15.1 ± 5.3 hours in the CS group. DGF was lower in the HMP group ($p=0.041$), and donation after Circulatory Death (DCD) was a predictor for DGF ($p<0.01$). HMP decreased DGF in DCD grafts ($p=0.036$). Patient and graft survival were similar, but eGFR at 365 days was higher in the HMP cohort ($p<0.001$). RIs decreased during HMP ($p<0.01$); 2-hours RI ≥ 0.45 mmHg/mL/min predicted DGF in DCD kidneys (75% sensitivity, 80% specificity; area under the curve 0.78); 2-hours RI ≥ 0.2 mmHg/ml/min predicted DGF in DBD grafts (sensitivity 100%, specificity 91%; area under the curve 0.87). *Conclusion*. HMP decreased DGF compared to CS, offering viability assessment pretransplant and improving one-year renal function of the grafts.

1. Introduction

Worldwide, the increasing demand for renal allografts and growing waiting lists has led to the utilization of organs through donation after Circulatory Death (DCD), although these organs are associated with higher rates of discard, retrieval associated injury [1], and up to 50% delayed graft function (DGF) in comparison to transplanted organs from donors after Brainsteam Death (DBD) [2].

In order to achieve the optimum outcome from each donated kidney and increase the survival benefit compared to the dialysis population [3], optimal organ preservation remains one of the major challenges to reduce current DGF rates [4] and the relevant detrimental long-term impact [5]. DGF is a well-established risk factor associated with reduced long-term graft and patient survival [6]; furthermore, recipients transplanted with kidney grafts that develop DGF face prolonged hospitalization and the overall relevant increased costs [7]. Promisingly, the use of machine perfusion technology has been associated with improved DGF rates, particularly for DCD organs [4].

The process underlying DGF include several pathophysiologic mechanisms derived from the donor ischemic injury and inflammatory signalling; it is the clinical manifestation of the acute kidney injury which affects the transplant parenchyma and, subsequently, renal function [8]. Animal studies have demonstrated that one of the main benefits of using cold pulsatile perfusion for preservation, is attributed to the improved endothelial release of nitric oxide and reduced secretion of endothelin-1 [9], resulting in a renoprotective effect [10], not achievable with standard static cold storage. This effect in the renal microvasculature provides a unique platform for active organ reconditioning during HMP, expressed in significantly lower number of pathological lesions on kidney biopsies [10].

One of the flow parameters in HMP, the Renal Resistance Index (RI), has been previously identified as a marker of the whole-organ microcirculatory damage after the retrieval ischaemic injury [11]. Monitoring RI could also provide a real time evaluation of the organ recovery during HMP [12].

The aim of the present study was to evaluate the effect of HMP during kidney transplant preservation in comparison to static cold storage based on a single centre experience.

2. Patients and Methods

The study is a single centre retrospective cohort analysis of hypothermic machine perfused kidneys (RM3® Waters Medical System, US) transplanted from March 2012 to April 2018 versus cold storage only. It was conducted in accordance with institutional ethics regulations; since it was a retrospective chart analysis, no informed consent was required.

The case controls were matched on 1:1 basis according to graft type (DBD or DCD), donor age, cold ischaemic time (CIT), and number of Human Leukocyte Antigen (HLA) mismatches between donor and recipients.

As soon as the kidneys were delivered into our centre and there was an impediment to proceed immediately with the transplant, HMP was chosen as the preservation method.

The University of Wisconsin solution was used for HMP, at a temperature between 4-5°C and at an initial peak systolic pressure of 45 mmHg. After 30 minutes of cold perfusion, the pressure was held constant ≥ 40 mmHg. RIs were recorded to monitor kidney parenchymal recovery.

CIT was defined the time from the start of cold perfusion during organ retrieval to the time of reperfusion during the transplant, including the HMP time. DGF was defined as the need for dialysis within 1 week of transplantation with a perfused graft. Furthermore, we compared the mean Modification of Diet in Renal Disease estimated glomerular filtration rate (eGFR) [13] until day 365 from transplantation and the length of hospital stay (LOS) between the two groups. Graft failure, censored for death, was defined as permanent return to dialysis.

All the patients received a steroid sparing immunosuppressive regimen (7-day course of steroids) with alemtuzumab induction and long-term Tacrolimus (TAC) monotherapy (trough level, 5-8 ng/mL) and neither the renal replacement therapy nor the immunosuppression protocol of our centre changed over the last 10 years.

3. Statistical Analysis

Continuous variables are presented as mean ± standard deviation and were compared using one way ANOVA. Independent t-test was used to analyse RI trend during machine perfusion. The confidence interval was set to 95%, and p was considered significant at less than 0.05. We used a linear regression model with stepwise procedure to test which parameters were acting as independent predictors for DGF. A generalised linear model of univariate repeated ANOVA with post hoc Bonferroni correction was used to determine whether mean eGFR differed statistically significantly during follow up. A receiver operator characteristic (ROC) curve was constructed to investigate the predictive accuracy of RI for DGF.

Analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 20.0; IBM Corp, Armonk, NY).

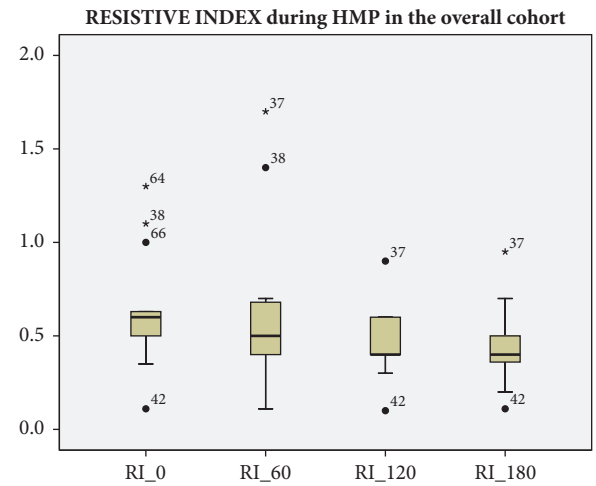

Figure 1: RI 0: Renal Resistive Index at the beginning of HMP. RI 60: Renal Resistive Index at 60 minutes of HMP. RI 120: Renal Resistive Index at 120 minutes of HMP. RI 180: Renal Resistive Index at 180 minutes of HMP. RIs are measured in mmHg/ml/min. HMP: Hypothermic machine perfusion.

4. Results

Sixty-six transplanted kidney outcomes were analysed. Donor and recipient demographics are shown in Table 1. No statistical difference was observed between the HMP and CS group baseline characteristics: mean recipient's age, cause of kidney failure, numbers of grafts from DCD and DBD donors, number of HLA mismatches between donor and recipient, donor's age and CIT.

The mean HMP time was 5.7 ± 3.9 hours; in the HMP group, the mean CIT of 15 ± 5.6 hours and 15.1 ± 5.3 hours in the CS group (p=ns).

In a linear regression model with stepwise procedure, DCD was an independent predictor for DGF ($p<0.01$) in the whole cohort, occurring in 24/66 kidneys (36%): 8/33 (24%) machine perfused and 16/33 (48%) cold stored ($p=0.041$). The DCD kidneys that developed DGF in the HMP group were 5/12 versus 10/12 in the CS control cohort ($p=0.036$), confirming a protective effect of the HMP preservation for the grafts retrieved from DCD donors.

The patients receiving grafts with subsequent DGF had higher length of hospital stay (LOS): 11.6 ± 5.8 days versus 29.1 ± 18.1 days ($p<0.001$).

The RI decreased statistically significantly during HMP: mean RI at baseline (R0) was 0.65 ± 0.25 mmHg/ml/min ($p<0.01$); after 60 minutes (RI60) was 0.62 ± 0.33 mmHg/ml/min ($p<0.01$); after 120 minutes (RI 120) was 0.46 ± 0.16 mmHg/ml/min ($p<0.01$); after 180 minutes (RI180) was 0.44 ± 0.22 mmHg/ml/min ($p<0.01$). The higher impact in decreasing the original RI value was observed between the first and the second hour of HMP ($p< 0.01$). Figures 1 and 2

Table 1: Baseline recipients and donors characteristics. No significant difference between the two groups with ANOVA.

		Preservation Modality			
		Cold Storage		Hypothermic Machine Perfusion	
		Mean ± St. Dev	Total	Mean ± St. Dev.	Total
Recipient Age (years)		57 ± 10		55 ± 11	
Cause of kidney failure	Adult Polycystic Kidney Disease		3		1
	Diabetes Mellitus		9		11
	Glomerulosclerosis		3		4
	Hereditary Nephritis		1		1
	HIV Nephropathy		1		2
	Hypertension		3		4
	IgA Nephropathy		1		3
	Ischaemic Nephropathy		1		0
	Lithium toxicity		1		1
	Membranous Nephropathy		1		1
	Myeloma derived Nephritis		1		0
	Obstructive nephropathy after Rhabdomyolysis		1		0
	Systemic Lupus Erythematosus nephritis		1		0
	Tubulo-Interstitial Nephritis		1		0
	Unknown		5		5
Donor after Circulatory Death	N		21		21
	Y		12		12
HLA Mismatch	0		1		1
	1		1		2
	2		8		6
	3		12		12
	4		11		3
	5		0		2
Donor age (years)		58 ± 14		58 ± 14	
Cold Ischaemic Time (hours)		15.1 ± 5.3		15.0 ± 5.6	

HLA= human leukocyte antigen, N= no, and Y= yes.

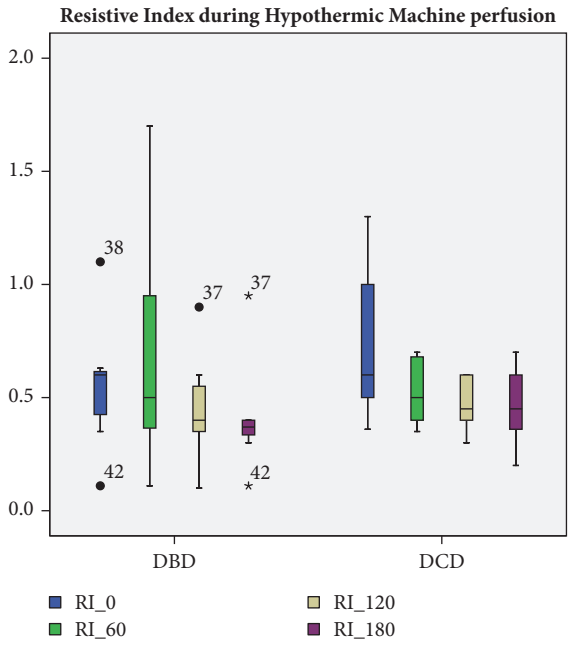

Figure 2: RI 0: Renal Resistive Index at the beginning of HMP. RI 60: Renal Resistive Index at 60 minutes of HMP. RI 120: Renal Resistive Index at 120 minutes of HMP. RI 180: Renal Resistive Index at 180 minutes of HMP. DBD: Donation after Brain Death. DCD: Donation after Circulatory Death. HMP: hypothermic machine perfusion. RIs are measured in mmHg/ml/min.

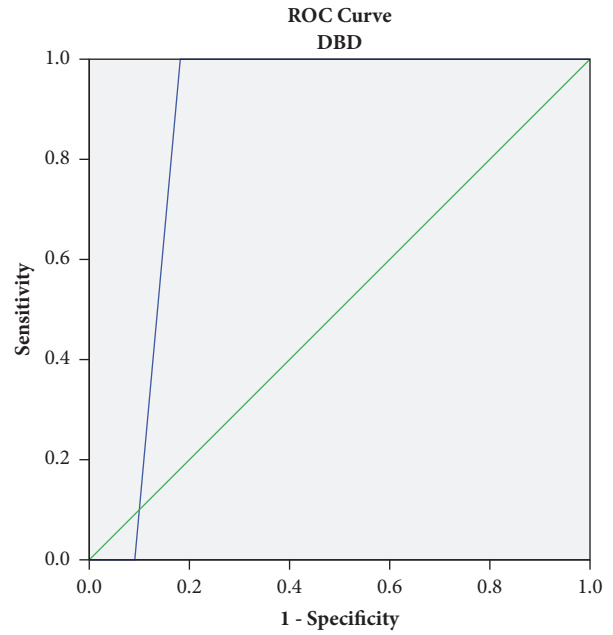

Figure 3: Receiving Operator Curve (ROC) for Resistive Index at 120 minutes (RI120) ≥ 0.2 mmHg/ml/min in DBD grafts: sensitivity 100%, specificity 91% in DGF prediction. Area under the curve 0.87.

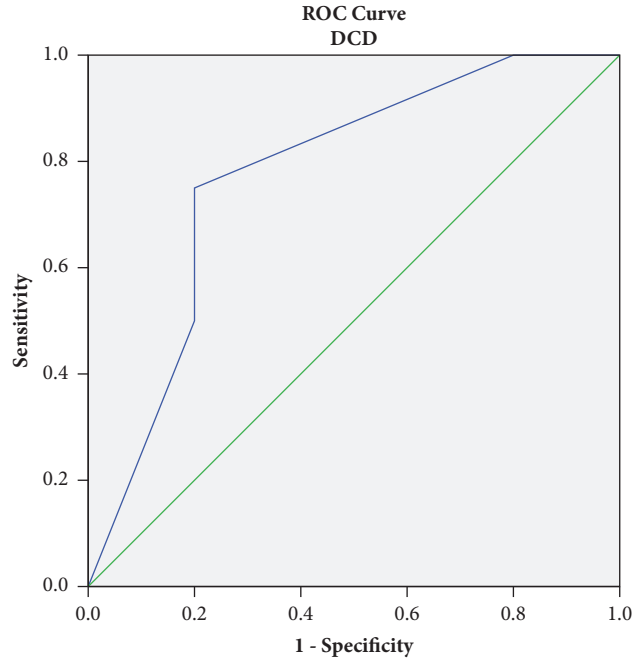

Figure 4: Receiving Operator Curve (ROC) for Resistive Index at 120 minutes (RI120) ≥ 0.45 mmHg/ml/min in DCD grafts: sensitivity 75%, specificity 80% in DGF prediction. Area under the curve 0.78.

represent mean RI during HMP for the whole cohort, and the DBD and DCD subgroups.

Furthermore, we subanalysed the difference in RIs between DCD and DBD grafts. A 2-hours RI value ≥ 0.2 mmHg/ml/min was associated with 100% sensitivity and 91% specificity in DGF prediction for DBD grafts. The area under the curve was 0.87 (Figure 3).

A 2-hours RI value ≥ 0.45 mmHg/ml/min was associated with 75% sensitivity and 80% specificity in DGF prediction for DCD grafts. The area under the curve was 0.78 (Figure 4).

Forty-seven patients had a transplant follow up longer than 365 days: 20/33 and 27/33 in the HMP and CS cohorts respectively. Multivariate analysis of univariate repeated eGFRs measures showed a statistically significant difference between the HMP and the CS groups (p=0.039), with the eGFRs for the HMP transplanted kidneys being persistently higher (Table 2). Post hoc tests using the Bonferroni correction revealed that higher values of eGFRs at day 365 were associated with HMP perfusion (p<0.001), Figure 5.

One graft loss occurred in the HMP group at 180 days due to acute rejection; 3 grafts were lost in the CS cohort: venous thrombosis (n=1) at 120 days posttransplant and acute rejection (n=2), at 180 days posttransplant (p=0.31). One patient died in the HMP group with a nonfunctioning graft after 270 days posttransplant due to myocardial infarction; no patient died in the CS (p=0.32). Results are summarised in Table 3.

5. Discussion

DGF is the Achille's heel of kidney transplantation from DCD donors, affecting more than half of the subsequently

Table 2

Effect of Preservation modality in eGFR during follow up					
Measure: eGFR ml/min/1.73m^2					
Preservation Modality	Time	Mean eGFR	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Cold Storage n = 27	Day 1	10.481	.978	8.511	12.452
	Day 2	12.000	1.350	9.281	14.719
	Day 3	14.000	2.274	9.421	18.579
	Day 4	16.296	3.257	9.737	22.856
	Day 5	19.593	3.687	12.168	27.018
	Day 6	21.407	3.982	13.386	29.428
	Day 7	24.667	4.100	16.409	32.925
	Day 14	28.000	3.555	20.840	35.160
	Day 90	34.370	3.379	27.564	41.176
	Day 180	32.926	3.347	26.185	39.667
	Day 365	36.630	3.443	29.695	43.565
Hypothermic Machine Perfusion n=20	Day 1	9.750	1.137	7.461	12.039
	Day 2	11.500	1.569	8.340	14.660
	Day 3	16.050	2.642	10.729	21.371
	Day 4	20.150	3.784	12.529	27.771
	Day 5	22.750	4.283	14.123	31.377
	Day 6	26.150	4.627	16.831	35.469
	Day 7	27.350	4.764	17.755	36.945
	Day 14	34.600	4.130	26.281	42.919
	Day 90	41.100	3.926	33.192	49.008
	Day 180	41.900	3.889	34.067	49.733
	Day 365	40.600	4.001	32.542	48.658

Table 3: Results. DGF was statistically significantly higher in the Cold Storage group and in DCD grafts. Length of hospital stay was longer in kidney that developed DGF (ANOVA).

				Preservation modality				*p value*
				Cold Storage		Hypothermic Machine Perfusion		
				Total	Mean ± St. Dev.	Total	Mean ± St. Dev.	
DGF		N		17/33		25/33		**0.041**
		Y		16/33		8/33		
DGF	N	LOS (days)		17/33	12.8 ± 6.4	25/33	9.9 ± 4.1	0.69
	Y	LOS (days)		16/33	36.4 ± 20.6	8/33	25.3 ± 16.1	
	N	LOS (days)		42/66		11.6 ± 5.7		**<0.01**
	Y	LOS (days)		24/66		29.1 ± 18.2		
DGF	N	DCD	N	15/21		18/21		0.27
			Y	2/21		7/21		
	Y	DCD	N	6/12		3/12		**0.036**
			Y	10/12		5/12		
Graft loss		N		30/33		32/33		0.31
		Y		3/33		1/33		
Pt survival		N		0/33		1/33		0.32
		Y		33/33		32/33		

DCD= donation after circulatory death; DGF=delayed graft function; LOS= length of hospital stay; N= no; Y=yes.

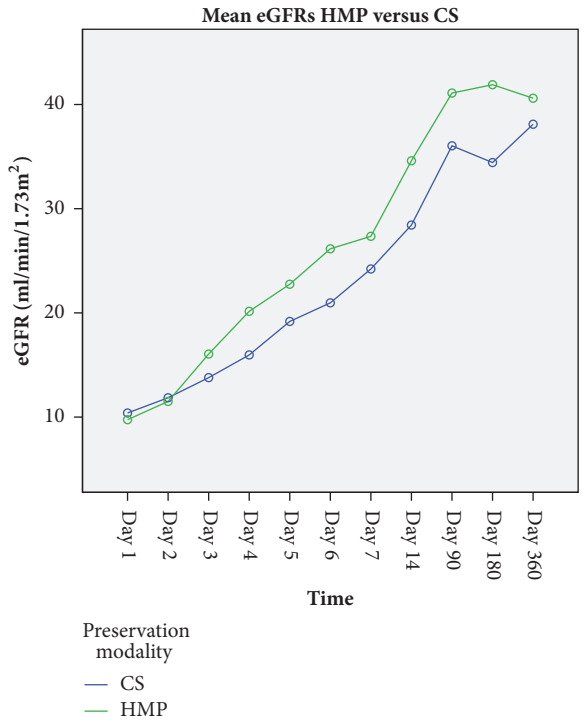

Figure 5: Generalised linear model of univariate repeated measures ANOVA. A total of 47 kidney recipients completed 365 days follow up and were analysed (20/33 HMP and 27/33 CS). Mean eGFRs were statistically different during follow up in HMP preserved when compared to CS kidneys (p= 0.039). Post hoc tests using the Bonferroni correction revealed that at day 365 mean eGFRs are higher in the HMP group (p<0.001).

transplanted grafts [14]. Our findings demonstrated the protective role of HMP in this particular subcohort, related to reduced DGF rate. In our study, the incidence of DGF was higher in the CS compared to the HMP cohort (p=0.041). For transplants from DCD kidneys the incidence of DGF was lower in the HMP compared to the CS cohort (p=0.036).

The use of cold pulsatile technology is a long-established alternative to static cold storage and it has been shown to be a better preservation method [15]. There are different types currently available for clinical kidney preservation; our policy is to use cold pulsatile perfusion devices, like the RM3® because of its potential renoprotective effect [10]. This particular technology results in better preservation of the endothelial integrity and recovery, with improved endothelial release of nitric oxide and reduced secretion of endothelin-1 in ex-vivo models [9]. In this way, the underlying mechanisms of DGF are actively repaired, with a substantial difference from static cold preservation. In our study, the use of HMP resulted in the lower incidence of DGF, especially in the challenging DCD group, and higher eGFRs observed for HMP kidneys consistently during the 365 days of follow-up; this demonstrates the protective short- and long-term effect of HMP. Another advantage that the pulsatile technology provides is a platform during which the graft could be actively reconditioned, making it particularly attractive for higher-risk kidneys [16], as it delivers oxygenation, or any other nutrients or reconditioning agents, and creates a window of opportunity during which to assess the viability and quality of the graft before transplantation [17]. It has been previously shown that the RI is an independent predictor during HMP for the later development of DGF; however, it cannot be a stand-alone tool in predicting DGF, especially when considering the heterogeneity of the factors that can affect the transplant outcome [12].

Nevertheless, an important advantage of RI monitoring could be the ability to estimate the risk of a particular kidney to develop DGF [12]. RIs are known to rise in parallel to the development of parenchymal injury [18] and increased RIs are associated with donation after Circulatory Death and donor age [19]. In our study, the prevalence of the parenchymal damage in kidneys from DCD donors was demonstrated by the higher renal Resistive Index at 2 hours post HMP: 0.2 mmHg/ml/min for DBD versus 0.45 mmHg/ml/min for DCD. The relative ROC curves, associated with DGF incidence, had an accuracy of 87% and 78%, respectively (Figures 3-4). As previously reported, RIs are expression of the microcirculatory damage occurring within the parenchyma [11]; therefore the stress induced by the circulatory arrest is unsurprisingly linked to a worse profile.

Knowing the risk profile of a particular kidney earlier in the preservation process would be of great benefit for the postoperative management and it would provide objective information for selecting a particular recipient for a particular kidney, thus tailoring the offered renal replacement therapy to the patient who would benefit most. In the era of patient tailored consent [20] and patient centred outcomes, it is mandatory to involve the transplant recipient and allow him/her to consider the risks related to increased chances of DGF if transplanted with kidney for which there is evidence that it is in such risk. The present study showed that the HMP cohort had significantly higher eGFRs at 365 days of follow up when compared to the CS group. Thus, the impact of DGF in the long-term outcome is elicited by the difference in the preservation techniques, particularly for kidneys from high risk donors, like DCDs. We have also shown that DGF is associated with prolonged LOS, thus significantly impacting on patient morbidity and hospital cost; the advantages demonstrated in our study by the use of HMP are associated with better outcomes related to those important social and economic aspects [20].

6. Conclusions

Within the limitations of the size of the HMP and CS groups, our study demonstrated that hypothermic machine perfusion offers an advantage in deceased donor renal transplantation of high risk kidneys, since it reduces significantly DGF rates and is associated with higher posttransplant eGFRs. This preservation modality has a positive impact in kidney transplant outcomes from DCD donors and offers an early viability assessment that allows prediction of short- and long-term posttransplant graft function. It represents a real time opportunity to recondition the retrieval ischaemic injury, plan the postoperative recovery, and enhance the

decision-making process by offering the patients evidence that allow them to make an informed decision.

Abbreviations

CIT: Cold ischaemic time
CS: Cold storage
DBD: Donation after Brainsteam Death
DCD: Donation after Circulatory Death
DGF: Delayed graft function
eGFR: estimated glomerular filtration rate
HLA: Human Leukocyte Antigen
HMP: Hypothermic machine perfusion
LOS: Length of hospital stay
RI: Renal Resistive Index
RI 60: Renal Resistive Index at 60 minutes of hypothermic machine perfusion
RI 120: Renal Resistive Index at 120 minutes of hypothermic machine perfusion
RI 180: Renal Resistive Index at 180 minutes of hypothermic machine perfusion.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The preliminary results of this study were presented at the Associations of Surgeons of Great Britain and Ireland annual meeting in Liverpool, UK, on the 9th of May, 2018 [21].

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Maria Irene Bellini and Paul Elliot Herbert participated in research design, performance of the work, data analysis, and writing the article. Sotiris Charalampidis and Vasileios Bonatsos collected the data and participated in performance of the work. Frank J. M. F. Dor, Jeremy Crane, and Anand Muthusamy participated in performance of the work and writing the article. Vassilios Papalois participated in performance of work, research design, data analysis, and writing the article.

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Review Article

Barriers and Advances in Kidney Preservation

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Despite the fact that a significant fraction of kidney graft dysfunctions observed after transplantation is due to ischemia-reperfusion injuries, there is still no clear consensus regarding optimal kidney preservation strategy. This stems directly from the fact that as of yet, the mechanisms underlying ischemia-reperfusion injury are poorly defined, and the role of each preservation parameter is not clearly outlined. In the meantime, as donor demography changes, organ quality is decreasing which directly increases the rate of poor outcome. This situation has an impact on clinical guidelines and impedes their possible harmonization in the transplant community, which has to move towards changing organ preservation paradigms: new concepts must emerge and the definition of a new range of adapted preservation method is of paramount importance. This review presents existing barriers in transplantation (e.g., temperature adjustment and adequate protocol, interest for oxygen addition during preservation, and clear procedure for organ perfusion during machine preservation), discusses the development of novel strategies to overcome them, and exposes the importance of identifying reliable biomarkers to monitor graft quality and predict short and long-term outcomes. Finally, perspectives in therapeutic strategies will also be presented, such as those based on stem cells and their derivatives and innovative models on which they would need to be properly tested.

1. Introduction

Kidney transplantation remains the treatment of choice for many patients with end stage renal disease and is a superior long-term therapy compared to dialysis in terms of quality of life and life expectancy. During the transplantation process and particularly the preservation step, a certain degree of ischemia-reperfusion injury (IRI) inevitably occurs in the immediate posttransplant setting. Ischemia-reperfusion (IR) process plays a significant role in the pathogenesis of both delayed graft function (DGF) in allografts and hemodynamic mediated acute kidney injury (AKI) of native kidneys. [1]. This clinical problem is exacerbated by the current situation, which is characterized by a shortage of organs driving to the use of marginal donors. Indeed, despite the extracorporeal cold preservation protocol used worldwide to overcome this issue, graft injuries related to IR are frequently observed and caused by pathophysiological mechanisms directly related to nonoptimal preservation strategies.

The main issue is that there is no clear consensus regarding optimal conservation solution composition, oxygenation, hypo- or normothermic conservation, and perfusion method [2], stemming directly from the fact that as of yet, the mechanisms underlying IRI are not entirely defined and the role of each of these parameter not clearly outlined. In the meantime, as donor demography changes, organ quality is

decreasing which directly increases the rate of poor outcome. This situation has an impact on clinical guidelines and protocols and impedes their possible harmonization in the transplant community, which has to move towards changing organ preservation paradigms: new concepts must emerge and the definition of a new range of adapted preservation method is of paramount importance.

This review presents existing barriers in transplantation (e.g., temperature adjustment and adequate protocol, interest for oxygen addition during preservation, and clear procedure for organ perfusion during machine preservation), discusses the development of novel strategies to overcome them, and exposes the importance of identifying reliable biomarkers to monitor graft quality and anticipate short and long-term outcomes. Finally, perspectives in therapeutic strategies will also be presented, such as those based on stem cells and their derivatives and innovative models on which they would need to be properly tested.

2. Kidney Preservation: Where Are We Starting from?

Organ preservation contributes to the induction of injuries induced by decreased ATP production, acidosis, cellular edema, and mitochondria alterations [3, 4]. The choice of preservation solutions is thus critical. Experimental models showed that (i) the ionic composition needs to be close the plasma's potassium (K^+) concentration (≈5mM) and sodium concentration (Na^+) (≈140 mM), in order to limit hyperpotassic effects (membrane depolarization, vasoconstriction, and consequently low perfusion) [5], and that (ii) the presence of molecules exerting an osmotic and/or oncotic pressure to prevent edema is essential to optimize graft quality [6]. Many solutions of different compositions are commercialized, such as University of Wisconsin solution (UW), Custodiol (HTK), Celsior, and fourth generation solutions such as Solution de Conservation des Organes et des Tissus (SCOT-15) and Institut Georges Lopez-1 (IGL-1), all with various colloids and ionic composition. A strong corpus of experimental results in preclinical models is available for SCOT-15 and IGL-1. These solutions include polyethylene glycols as colloids, a suitable option to protect organ integrity/functionality [5–9], and their characteristics are presented in Table 1 (adapted from Bon D et al. [1]).

Ten years ago, a multicenter analysis of kidney preservation drew several conclusions: (1) kidneys from deceased donors should ideally be transplanted within 18 hours; (2) within the 18-hour window, the time of ischemia has no significant influence on graft survival and (3) UW solution should be used if longer preservation is envisioned [10]. HLA matching improves graft survival regardless of length of ischemia [10]. This meta-analysis included 5 different conservation solutions and 91,674 patients, mostly brain dead donors. Unfortunately, these observations are not adapted to the current donor demographics which includes a growing number of suboptimal donors, such as Extended Criteria Donors (ECD) and Donation after Circulatory Death donors (DCD). Indeed more recently, a clinical study studying brain death donors (including ECD, 3939 patients) showed that each additional hour of cold ischemia time beyond 6 h significantly increased the risk of graft failure and mortality [11]. In addition, keeping the cold ischemia time as short as possible has also been shown to be crucial during machine perfusion [12]. However, the true impact of ischemia time is still debated with diverging conclusions, especially for donors displaying AKI [13, 14]. A need for wisely triaged donors is absolutely mandatory.

Organs from DCD donors or ECD are more susceptible to preservation injury and have a higher risk of unfavorable outcomes, and there is thus growing need for new potential and standardized protocols for organ preservation. Concepts such as machine perfusion (MP), temperature, and other technical advances need to be assessed through rigorous common networks and research programs, with a complete characterization and rationalization of solution composition, preservation temperature, the exact role of oxygen, and the most adapted perfusion protocol.

3. Adding Oxygen during Preservation: Is It Time to Take a Breath?

One of the hallmarks of current organ preservation methods is hypoxia/anoxia. Indeed, unpublished data from our laboratory show that the oxygen dissolved in the preservation solution is consumed within the first two hours of kidney preservation.

In the absence of oxygen, mitochondria are able to maintain some protonic gradient and produce ATP as long as supplies last, accumulating succinate [15]. However, when oxygen is reintroduced in the system at the reperfusion stage, it is captation of a single electron which produces superoxide anion, the first reactive oxygen species (ROS), and source of oxidative stress. If not controlled, the production of ROS and subsequent destruction of structures is fatal to the cell. ROS production is also mainly responsible for the destruction of the glycocalyx at the cell surface and consequence lesions, among which coagulation and sterile inflammation [16].

Oxygen thus appears to be a two-edged sword which should be wielded carefully. However, experimental evidence tends to show a majorly beneficial use of oxygenation. In the current context of unavoidable donor pools expansion, oxygen supplementation during hypothermic preservation is the focus of numerous preclinical and clinical studies, including nonheart-beating, heart-beating, and higher-risk donors [17–19]. Naturally called for, the use of oxygen in normothermic preservation is considered elsewhere [2, 18, 20].

Several methods have been used in animal models to provide oxygen during storage: oxygenated perfusate or perflurocarbon emulsion, hyperbaric oxygenation by the delivery of oxygen under increased atmospheric pressure, or retrograde persufflation of gaseous oxygen bubbled through the renal vasculature [21]. Several studies have investigated hyperbaric chambers as a mean to oxygen delivery and demonstrated that perfusion was necessary to improve function, rather than static storage, hinting towards the need for

Table 1: Characteristics of current kidney preservation solutions and machine perfusion.

Solutions	K^+ (mM)	Na^+ (mM)	Buffer	pH	Impermeant	Adenosine (mM)	Anti-oxidant	Colloid (g/L)
Flush and Static cold storage								
Blood	4.25	139	HCO3-	7.4	+	0	+	Albumine (50 g/L)
HTK (Custodiol®)	10	15	Histidine	7.2	+	5	-	-
UW (Viaspan®) (Bridge to life®)	100	28.5	(K)H2PO4 HEPES	7.4	+	5	Glutathion	HES (50 g/L)
Celsior®	15	100	HEPES	7.3	+	0	Glutathion	-
IGL-1®	30	125	(K)H2PO4	7.3	+	5	Glutathion Allopurinol	PEG 35kDa (1g/L)
Lifor®	16	98	Phosphates	7.07	+	?	?	?
Polysol®	15	120	(K)H2PO4 HEPES Histidine	7.4	+	5	Glutathione Acid ascorbic	PEG 35kDa (20g/L)
SCOT 15®	5	118	HCO3-	7.4	+	0	-	PEG 20 kDa (15g/L)
Flush solutions								
Carolina RS®	5	115	(K)H2PO4	6,5	+	1	Glutathion Allopurinol	HES (50 g/L)
Dynamic preservation solution (for hypothermic perfusion machine)								
KPS-1®	25	97.5	(K)H2PO4 HEPES	7.4	+	5	Glutathion	HES (50 g/L)
PERF-GEN®	25	100	(K)H2PO4 HEPES	7.4	+	5	Glutathion	HES (50 g/L)
MPS®	25	100	(K)H2PO4 HEPES	7.4	+	5	Glutathion	HES (50 g/L)

Kidney hypothermic perfusion machines				
Machine	**Solution type**	**Pulsatile perfusion**	**Temperature**	**Oxygen supply (100%)**
LifePort®	KPS-1® MPS®	-	4°C	No
WAVES®	PERF-GEN®	+	4°C	100%
Kidney Assist-Transport®	KPS-1® MPS®	-	4°C	100%

HTK (Custodiol®, Dr Franz Köhler Chemie GMBH, Alsbach-Hähnlein, Germany); UW (University of Wisconsin, Alumni Research Foundation, Madison, WI, USA); Celsior® (Genzyme Corporation, Cambridge, MA, USA); IGL-1® (Institut Georges Lopez, Civrieux d'Azergues, France); Lifor™ (Lifeblood Medical, Freehold, NJ, USA); Polysol® (Doorzand Medical Innovations B.V., Amsterdam, The Netherlands); SCOT15® (MacoPharma, Tourcoing, France); Carolina RS® (Carolina Rinse Solution, University of North Carolina, Chapel, USA); KPS-1® (Organ Recovery Sytems, Chicago, USA and Brussels, Belgium); MPS® (Belzer MPS® UW Machine Perfusion Solution, Bridge to life; Columbia, USA); PERF-GEN® (Institut Georges Lopez, Civrieux d'Azergues, France); LifePort® (Organ Recovery Sytems, Chicago, USA and Brussels, Belgium); WAVES® (Institut Georges Lopez, Civrieux d'Azergues, France); Kidney Assist-Transport® (Organ Assist B.V, Groningen, The Netherlands); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HES (hydroxyethyl starch); PEG (Polyethyleneglycol).

active delivery within the organ rather than changing the outside conditions [22, 23]. In a pig kidney model, retrograde oxygenation also showed beneficial compared to static storage [24]. However, both techniques of hyperbaric preservation or retrograde persufflations are difficult to envision within a clinical setting. Therefore, efforts have been deployed to use perfusion in order to deliver oxygen. Interestingly, oxygenation pressure was again shown to alter outcome, with the benefits being lost at higher pressure (60/40mmHg) [25, 26]. These studies however did not compare oxygenated perfusion to standard perfusion.

In canine, comparison of standard machine perfusion with the Lifeport to oxygenated perfusion on the RM3 did not demonstrate a difference in outcome, albeit with a short follow up and with light IR lesions (45 min warm ischemia) [27]. This study is an example of the animal models limits: study animals are healthy, and machine perfusion is already a good preservation method; therefore measuring the impact of optimization may be difficult without a necessary increase in the level of IR lesion (either through lengthened warm ischemia or marginal donor modeling). Superiority of oxygen addition was also demonstrated when using an

oxygen emulsion in machine perfusion, also in terms of function recovery in canine [28].

Addition of oxygen to the perfusion circuit was tested. Our own group studied the use of oxygen in a machine intentionally designed to deliver it: the Kidney Assist. In a pig model of deceased after circulatory death donor (60 min warm ischemia), we demonstrated that oxygenation improved early function recovery as well as long-term outcome (in terms of function and fibrosis development) [19]. When compared to historical data using either the Lifeport or the RM3 in a similar model, we showed that oxygenation indeed permitted better early recovery, but long-term outcome was within comparable range (Unpublished data).

It thus appears clear that oxygenation is a very promising avenue of optimization for organ preservation, particularly if coupled with machine perfusion. Numerous mechanisms are involved in the benefits of active oxygenation at 4-8°C, mostly the ability to produce some ATP and maintain cellular and repair processes [29]. However, in both static and dynamic hypothermic preservation protocols, actual levels of oxygen within the kidney remain largely undetermined, as well as oxygen consumption. Unpublished data from our laboratory assessed oxygen and ATP in static and machine-preserved kidneys (20 hours; n = 5 *per* group) using our established preclinical porcine model of severe warm ischemic injury (WI, 1 h), mimicking donation after circulatory death. WI reduced tissue ATP by 90% (control: 2.6 ± 0.5 mM). In both preservation protocols, PO_2 decreased rapidly ($t_{1/2}$ ~1h) from atmospheric levels to 51.8 ± 0.2 mmHg and 7.6 ± 0.2 mmHg, respectively. During machine perfusion, arterio-venous (av) oxygen consumption was calculated (QO_2, μmol/min *per* kidney) and was 3.5 ± 0.1 *versus* 1.6 ± 0.6 μmol/min *per* kidney in static preservation. Post-preservation, tissue ATP amounted to 5.4 ± 0.6 and 0.1 ± 0.01 mmol/L in machine and static, respectively. Despite profuse assertions and hypotheses in the field, this is the first comparison and quantification of renal oxygen levels, oxygen consumption and associated ATP levels in standard, non-oxygenated static and machine preservation. This type of study of effective renal graft oxygen levels (and consumption) should translate into a better understanding of the graft's requirements and open the way to improvements of organ preservation devices and conditions.

4. Preservation Temperature: Should We Really Keep It Cool?

The drive to use hypothermia for organ preservation naturally stems from fact that, on a biochemical point of view, lowering temperature slows cells metabolism, through two relations:

(i) The van't Hoff equation: At 4°C, average temperature of hypothermic organ storage in transplantation, a chemical reaction will only be 40% as effective as the same reaction taking place at 37°C.

(ii) The Arrhenius relation, quantifying the impact of temperature on the speed of a chemical reaction, highlighting that a reaction taking place at 4°C is 90% slower than the same reaction at 37°C.

However, as organs are not test tubes in which run single chemical reactions, but complex structures deploying a plethora of reaction and interactions, the validity of hypothermia may be put into question. Indeed, molecular structures, such as hydrogen and hydrophobic bonds, are deeply affected by hypothermia. Thus, both proteins and lipids structure and therefore function are altered when lowering temperature. For instance, it been demonstrated that the ability of transcription factors to bind DNA is affected by temperature [30].

In this context, hypothermia conservation is being questioned by the scientific community, and numerous articles highlighted that hypothermia indeed worsens ischemic injuries through; (i) reduction of ATP synthesis and metabolic activity [31], (ii) reduced Na-K-ATPase activity, which induces osmotic perturbation [32], (iii) mitochondrial perturbations, (iv) decreased cell survival [33] and (v) endothelial activation [34, 35]. Optimization of organ preservation temperature is thus a pivotal goal [36].

Among emerging concepts of alternative storage temperatures, recent studies advocate the use of normothermia (35-38°C), subnormothermia (25-34°C) [36–38] or mild-hypothermia (12-24°C). The use of normothermia may be considered for the whole preservation or combined with periods of hypothermia [36] and aims to restore normal cellular processes while facilitating viability assessment or to prepare organs to reperfusion. Data from our laboratory focusing on endothelial cells submitted to different temperatures *in vitro* during hypoxia show that subnormothermic temperatures provided protection against injuries *versus* 4°C, by reducing cell death, mitochondrial dysfunction, leukocyte adhesion and inflammation. However, *ex vivo* pig kidney evaluation on a perfusion apparatus showed that the benefits of 19°C or 32°C were limited, with similar levels of tissue preservation damages (submitted manuscript). This study suggests that temperature optimization for kidney preservation will require thorough investigation, combining the use of complementary relevant models and the design of elaborated preservation solution and new technologies.

Additional data from our laboratory studying the impact of temperature on the cytoskeleton showed using *in vitro* model of renal endothelial cells submitted to cold ischemia (4°C) that, while intermediary filaments were unaffected, cells microfilaments showed radical changes with disappearance of the structure replaced by a disorganized array of nodules; moreover, microtubules almost completely disappeared with time [39]. Furthermore, temperature, and not oxygen deprivation or the solution, was the determining factor of the cytoskeleton's loss of integrity during preservation.

Obviously, the specifications for normothermic preservation may require an oxygenated perfusate with an oxygen carrier or blood itself and use of MP. In addition, perfusate for normothermic perfusion will mandate elaborate compositions including nutrients, anti-oxidant and metabolic substrates. Subnormothermic dynamic preservation aims to avoid cold-induced injury without increasing metabolism to a level at which intense oxygenation requires an oxygen carrier. These elements will be further discussed below since

most of subnormothermia or nomothermia protocols are performed on dynamic preservation.

5. Kidney Perfusion: Pump It up!

Preservation time with cold storage (CS) is limited as prolonged CS increases the risk of delayed graft function (DGF) that contributes to chronic complications. Furthermore, the growing demand for the use of marginal donor organs requires methods for organ assessment and repair. Machine perfusion has resurfaced and dominates current research on organ preservation. Since 2009, compared to cold storage, MP benefits are demonstrated in terms of reduced risk of DGF, risk of graft failure, and improved graft survival [37, 38]. However, the donor populations in which MP should be applied have not yet been resolved but it seems that there is no reason to limit MP to marginal kidneys. Indeed, extracted from UNOS database from 2005 to 2011, a review showed that, similarly to marginal kidneys, MP is beneficial in reducing DGF even when standard donors are considered [40]. Our laboratory identified the benefits of kidney MP as being mediated by endothelium releases of the vasodilator nitric oxide (NO), due to shear stress activating the endothelial NO synthase (eNOS) by phosphorylation, resulting in improvement of cortical microcirculation (measured by laser Doppler) [41].

Another non negligible advantage of using MP during conservation is the possibility to assess organ quality. Organ resistance during MP has been described as predictive value for graft survival (initial resistance) and DGF (resistance measured after 2 hours of MP) [42], although this is still debated. A more valuable approach is offered by the machine giving access to the organ perfusate throughout the preservation period, allowing the measurement of biomarkers predictive of transplantation outcome. Indeed, an international study showed that GST, NAG and H-FABP were independent predictors of DGF but not of primary nonfunction and graft survival [43]. In addition, rapid metabolomic analysis in the perfusate by nuclear magnetic resonance showed, in a preclinical model, that the levels of several metabolites during MP are associated with function recovery [44].

Regarding temperature, MP is optimal to test alternative temperatures such as normothermia or subnormothermia. Hypothermic dynamic preservation aims to slow down cellular metabolism and counteract undesirable and detrimental effects of ischemia. It combines low temperature (4–10°C) with an acellular colloid-containing preservation solution using, in the majority of cases, the Na-gluconate/hydroxyethyl starch MP solution developed by Belzer et al. [45]. Subnormothermic machine perfusion at temperatures of 20 – 25°C potentially allows elimination of cold-induced injury without increasing metabolism too high, since normothermic preservation mandates an oxygenated perfusate with an oxygen carrier (usually red blood cells) [18] complicating the process. A pilot study demonstrated the superiority of Lifor Preservation Medium (a complex organ preservation medium containing sugars, amino acids, buffers, colloids, fatty acids, antioxidants, vitamins, dextran and an oxygen carrier) at room temperature perfusion compared to Belzer machine perfusion both at room temperature and 4°C, in a porcine model of uncontrolled donation after circulatory death [46]. In an acellular normothermic perfusion system, the use of Oxygent (a complex fluid supplemented with an oxygen carrying perfluorocarbon emulsion) was able to preserve canine kidney autografts using pulsatile preservation at 32°C and static storage at 25°C [28]. Such data underline the evidence for a technological evolution of cold storage concepts.

A published economic evaluation, using a Markov model with a 10-year time horizon, showed that life-years and quality-adjusted life-year can be gained while reducing costs at the same time, when kidneys are preserved by MP instead of CS [47]. However, several questions regarding the optimal machine perfusion system still remain unanswered. Future research needs to explore optimal perfusion modalities such as oxygen used and concentration, pressure, pulsatility, temperature. In addition, optimal perfusion solution (enriched perfusion medium, whole blood leucocyte-free blood etc.) need to be carefully investigated (machine vs. solution effects) [48]. Finally, the question of timing is of utmost importance [37]; at present it is unknown whether brief hypo- sub- or normothermic MP following CS is sufficient to renal reconditioning or if CS should be completely replaced by MP.

6. Donor - Organ – Recipient Conditioning

Machine Perfusion associated or not wit extracorporeal circulation procedures could also be used as a tool to condition the donor before organ procurement. Abdominal regional in-situ perfusion (ARP) has been applied clinically at hypothermic and normothermic temperatures in organ donors. These methods have been found to improve kidney graft function, to replenish ATP and to reduce injury in a number of large animal models [38]. The first alternative is called In Situ Cooling and consists in performing organ cooling by using diluted blood solution previously cooled at 0 to 20°C. The second option called NRP for Normothermic Regional Perfusion, consists in using the donor blood to perfuse the abdominal organs before collection. NRP is the preferred form of donor management in uncontrolled / unexpected donation such as DCD donors [49, 50], compared to *in situ* cold perfusion and total body cooling [51]. Reports from different groups in Europe, the USA, and Asia have described the use of NRP in both uncontrolled DCD and controlled DCD kidney transplantation, with rates of delayed graft function approximating 50% and 30–40%, respectively; negligible (if any) primary no function; and excellent one-year graft survival [25, 52–55].

Perfusion could also be used to condition the organ itself, such as at the end of a static preservation phase, in order to "wake up" the organ before its transplantation. Several reports reported that abrupt change in temperature from hypothermic preservation to normothermic reperfusion at the time of transplantation produces detrimental effects on renal graft quality [18, 37]. Recently, Controlled Oxygenated Rewarming (COR) of grafts immediately before transplantation has been described as a modification of MP, bringing

a new approach for organ conditioning and strengthening the concept of a pretransplantation organ preservation and evaluation unit. COR following CS demonstrated superior results over MP for liver and kidney [20, 56], avoiding "heat shock" and possibly the side effects (including mitochondrial dysfunction) of quick rewarming [57]. Subsequent studies showed that COR improves renal function after reperfusion (better renal creatinine clearance) and protect mitonchondria integrity [58, 59].

Another alternative technique is to recondition a kidney preserved by hypothermic preservation (either CS or MP) using 2 h of normothermic perfusion with blood. This short period of *ex vivo* normothermic perfusion (EVNP) immediately before transplantation, has a positive conditioning effect on the graft [60]. A first clinical case published in 2015, demonstrates the feasibility and safety of this technique [61]. A proof of concept clinical trial is currently being carried out in the UK to validate this technique. Alternatively, a slow and controlled increase in temperature up to normothermia using a combination of acellular medium and autologous erythrocytes addition is also currently under evaluation [56, 62].

Finally, at the end of the chain, recipient conditioning could also be applied: recent reports suggested potential therapeutics to protect organs from reperfusion injury, such as remote ischemic conditioning [63]. Other approaches are also interesting with the use of molecules such as statin (HMG-CoA reductase inhibitors).

7. Additives in Preservation Solution: Improve the Now While We Wait for the New

Improving preservation may not necessarily require revisiting the composition of the solution or its temperature, as indeed several compounds have shown the ability to significantly improve quality when added to existing technology. We previously published an extensive review of molecules which could thus be used [1], and are proposing an update below. Some targets such as mitochondria integrity and/or permability, innate immunity, anoxia and O2 transport, endothelial cell integrity and coagulation pathways are outlined in this section [64].

Firstly, several studies have shown that coagulation pathway was one of the key to counteract IRI. Coagulation inhibition takes multiple forms, uncovering the complexity of this pathway. As a first example, in a mouse model of liver IRI, the Protease activated receptor (PAR)-4 pathway was targeted [65], while a clinical study showed that PAR-1 is expressed by DCs in DGF grafts and its activation may induce complement production and a Th1 bias [66]. Secondly, in a mouse model of hepatic IRI, recombinant human thrombomoduling was protective, and specifically this activity was brought through the N-terminal lectin-like domain 1 (D1) subunit, involving TLR4 signaling [67]. Moreover, anticoagulants have demonstrated efficacy, such as an anti-Xa molecule protecting against preservation injury in a pig autotransplantation model [68]; a novel multi-arm heparin PEG conjugate adsorbing the endothelium and protecting against hypoxia *in vitro* [69]; a mast cell heparin proteoglycan mimetic (APAC), which was shown to be more effective than heparin in protecting against renal IRI in rats [70]; and finally a dual anti-Xa/IIa compound which was successful in limiting reperfusion injury in a pig kidney autotransplantation model [71].

The involvement of complement in IRI has been extensively demonstrated in a variety of mouse models [72] and prompted the testing of complement-targeted therapies against IRI [73] and the initiation of clinical trials to test the benefits of an anti-C5 antibody (Eculizumab) to prevent DGF (Delayed Graft Function) (NCT01403389; NCT01919346), which are still ongoing. Eculizumab treatment in pediatric kidney transplantation permitted better early graft function and improved graft morphology, however there was an unacceptably high number of early graft losses [74]. Inhibition of C1 protease using a recombinant human inhibitor (RhC1INH) inhibited complement deposition in a large animal model of kidney warm ischemia [75] and reduced fibrosis in a mouse model of warm IR [76]. Moreover, this treatment was able to protect kidney grafts, when used only during the reperfusion phase, against acute and chronic IRI in a pig model [77]. Finally, this inhibitor was used in a Phase I/II clinical trial to measure the impact on need for hemodialysis during the first week post-transplant, with significant reductions in need for dialysis and improvements in long-term allograft function observed with C1INH treatment [78]. C3 also appear a viable target, either in vascularized composite allograft model with a targeted inhibitor [79] or at the donor level, when the inhibitor was given as a nebulized solution priori to lung transplantation in a mouse model [80]. Moreover, targeting the alternative pathway also appears beneficial, through for instance the administration of anti-factorB antibody in a mouse kidney transplantation model [81]. Finally, a novel membrane-localized complement inhibitor based on a recombinant fragment of soluble CR1 (APT070, Mirococept) is currently tested in patients (EMPIRIKAL trial, REC 12/LO/1334), offering the possibility to treat the donated kidney before transplantation [82].

Additionally, a natural oxygen carrier extracted from Arenicola marina with high oxygen affinity developed as an additive to standard organ preservation solutions showed a protective effect in a variety of experimental conditions [83, 84]. A novel non-steroidal mineralocorticoid receptor antagonist was recently studied in kidney IRI models and its protective effect was well established [85, 86].

Enhanced understanding of cell and mitochondrial behavior during preservation is paramount to improve outcome. Several promising avenues of research are emerging from the study of hibernating species [87], such as the use of H_2S [88]. Other concepts include: the replacement of damaged mitochondria with healthy mitochondria at the onset of reperfusion by auto-transplantation in the heart [89]; the control of pH regulation through inhibition of carbonic anhydrase in lung transplantation, which impacts both pCO_2 levels, and Na-K-ATPase expression [90]; the sensitization of calcium channels in human hepatocytes for liver transplantation [91]; or the control of systemic iron load to protect against renal ischemia-reperfusion injury-associated

sterile inflammation [92]. Finally, other drugs were recently studied: pharmacologic targeting of DHPS by N1-guanyl-1,7-diaminoheptane (GC7) or RNA interference–mediated inhibition of DHPS or DOHH induced tolerance to anoxia in immortalized mouse renal proximal cells [93].

8. Studying Organ Preservation: Where Are the Top Models?

The quest for innovative strategies relies on the availability of predictable models recapitulating as physiologically as possible transplantation-induced IR injury. The models currently available are of 3 types: *in vitro* cultures of renal cells, *ex vivo* perfusion of isolated renal structures/organs and *in vivo* models of kidney ischemia reperfusion and/or transplantation.

In vitro models include culture of renal primary cells or renal cell lines. Both of these cellular systems are cheap, flexible and compatible with high-throughput screening. Indeed, primary tubular cells for instance are able to temporarily keep the architecture, function and polarity of renal epithelial cells [94]. However their proliferation is limited and a de-differentiation rapidly occurs in culture [95]. This is why immortalized cell lines are widely used but their physiological relevance is questioned based on the modification they harbor to proliferate extensively.

In classical cell culture conditions, the Petri dish is composed of one major cell type with cells spread in two dimensions (2D), whereas the adult kidney is a complex organ composed of 26 cell types and displaying highly complex cell-cell and cell-environment interactions. Thus, experiments performed on *ex vivo* isolated kidneys are of high interest to predict the organ answer to complex stimuli. Rodents or pig kidneys can be collected and perfused on complex apparatus with buffered solutions or whole blood, in an attempt to maintain "normal" physiological/biochemical conditions in a closely monitored perfusion system. In our laboratory and others, pig kidneys are often chosen for their high similarity with human's and placed in a home-made perfusion apparatus in hypothermic or normothermic conditions, mimicking organ conservation or its reperfusion during transplantation into the recipient. Of note, perfusion systems are disconnected from extrinsic regulatory control mechanisms allowing targeted evaluation of the kidney, real time assessments of various parameters reflecting its state and function, as well as its response to different situations without confounding systemic responses that are present in *in vivo* studies [96]. The main limitation of these systems are (i) the lifespan of the organ (a few hours with regular system, however using specific perfusion systems 24 hours of conservation may be possible [97]), (ii) the necessity to have access to animal kidneys.

Finally, only *in vivo* animal experiments allow long-term follow-up of the organ function, and therefore represent the most predictive model especially when performed in large animals. In our laboratory, we have developed a pig preclinical model of kidney auto-transplantation which is invaluable to analyze various mechanisms and treatments in relation to ischemia reperfusion during transplantation [68, 93, 98, 99].

However, mainly for obvious ethical reasons, it is of crucial importance to avoid or limit the use of animals for experimentation whenever possible. In this light, cutting edge technologies can be applied to the field of IR research. Indeed, since 2006 and the first publication describing induced pluripotent stem cell (iPSCs) technology, it is possible to cultivate in the laboratory human iPSCs able to differentiate into all cell types of the human body. Recently, this technology has been combined with 3 dimensions (3D) culture systems to differentiate those cells into complex structures, highly resembling a tiny organ, called organoids [100]. They can be obtained in a 2 step protocol: firstly, cells are differentiated in a 2D monolayer using growth factors and cytokines which mimic first steps of *in situ* kidney embryonic development. During differentiation, cells are detached and placed on a suspension culture system enabling the further differentiation/maturation and auto-assembly of the cells in 3D. This protocol lead to the formation of spherical structures of a few millimeters in diameter (after ≈25 days of differentiation) intricately organized, vascularized and presenting 8 types of renal cells; authors note the presence of nephron-like structures with evidences of cells from distal tubules, loop of Henlé, Bownman's capsule, parietal cells, podocytes, epithelium from the collecting duct connected to the nephrons as well as a stromal population and endothelial capillaries.

Thus, working *in vitro* with human kidney-like miniaturized structures becomes possible. Additionally, use of iPSCs allows choosing the kidney organoid's genotype. This is possible either by selecting patients affected with one disease of interest to generate iPSCs and further differentiate them into kidney organoids to study renal disease mechanism and treatment [101], or by using genome editing technologies (CRISPR/Cas9) [102] to target specific genes of interest and study how they impact kidney organoid's response to various stimuli including hypoxia/reoxygenation protocols and resistance/sensitivity to conservation.

9. Improving Kidney Transplantation Outcome: What Else?

Recent advances in regenerative medicine brought new potential strategies in the field of organ transplantation. Among them, cell therapy (i.e injection of cells, usually stem cells or their derivatives) to repair or replace tissues is at the forefront of personalized medicine. Controlling or reducing IR injuries with cell therapy is a tempting approach. Most cell types that have been tested in the context of renal IR are mesenchymal stem cells (MSCs) from various origin, despite some studies describing the use of endothelial progenitor cells (EPCs) [103, 104] or cells differentiated from pluripotent stem cells [99, 100]. Importantly, most MSC-based cell therapy approaches have been tested on rodent models of IR-induced AKI, but not on models involving kidney transplantation. Overall, these studies show that administration of stem cell therapy improve global renal function, decreasing fibrosis

and tissue damage and augmenting animal survival [105–108].

Cell injection timing has been revealed important. One study highlighted that pre-treatment with MSCs (7 days before IR induction) is more efficient than post-treatment (1 day after IR induction) to reduce lesions, this being probably due to a protective effect triggered by lipid metabolism modulation [109]. In our laboratory, using a pig preclinical model of kidney auto-transplantation, we choose the inject MSCs 7 days after kidney transplantation and observed significant improvement of kidney structural integrity and function [98]. Yet, optimal cell injection timing is far from consensual and this issue will have to be carefully studied in relevant preclinical model. Indeed, cell administration route and dosage are two critical factors which may be crucial for cell therapy efficacy: a comparative study observed that 1×10^5 MSCs injected through the renal artery produces a dramatic improvement in renal function and morphology in rat model of renal I/R injury [110].

However, regarding MSCs at least (since the issue can be different for iPS-derived cells for example), there is no strong evidence that the cells are indeed able to graft or even remain in the kidney after their injection, and their protective effects does not appear to rely on their ability to differentiate and replace damaged tissues, but are primarily mediated by paracrine mechanisms. Thus, most approaches under development focus on the use of cell's secretome, instead of cells themselves. This is possible either by the use of conditioned medium (medium that was placed in contact with the cells for a period of time allowing cell secretion of paracrine factors and cytokines) or microvesicules (MV) directly isolated from the conditioned medium. These are extracellular vesicules important for cell-cell communication and containing miRNAs, mRNAs and proteins. Among paracrine factors identified as important for repair after IR are VEGF [111], Ang-1, and Ang-2. [103] and Glial-derived neurotrophic factor (GDNF) [112]. In the case of acute renal IR injury, the literature shows that MSCs contribute to the recovery of mice with IRI-induced AKI primarily through the release of MV [113]. Another study shows that MV from adult rat renal tubular cells significantly improved renal function in rats through a large transcriptomic shift [114]. Of note, exosomes can be injected alone or in combination with MSCs [115], hence an appealing option would be to combine MSC-derived exosomes with cells that are indeed able to graft and differentiate into kidney tissue such as iPS-derived kidney progenitors.

Among MV components, miRNAs are also a potential therapeutic target *per se*. The role of miRNA in IR was uncovered through a mouse model with genetic deletion of Dicer, enzyme involved in miRNA maturation [116]. This deletion lowered miRNA expression by approximately 80% and was shown to be protective against kidney bilateral I/R. While this approach was highly unspecific, the demonstration was made that miRNA were involved in I/R injury development. The same study showed that IR profoundly affected the miRNome after 12 and 48 hours of reperfusion, with at least 14 targets demonstrating a more than 2 fold change. Another study on mice subjected to 30 min kidney IR confirmed miRNome dysregulation [117]. Other studies in small animals have confirmed the alteration of miR-21 after IR [118]. Interestingly, this target was shown to play an important role in Ischemic Preconditioning (IPC), an efficient technique to ameliorate damage by IRI in different organs like heart, brain, liver, and kidney in several animal models [119–121]. miR-21 has several pro-apoptotic targets, hence the hypothesis that its overexpression could protect against cell death during IR. Indeed, in a rat model it was demonstrated that IPC induced miR-21 expression and subsequently protected against kidney IR, an effect that was negated by treated IPC animals with anti-miR-21 [122].

Likewise, long noncoding RNAs (lncRNAs) constitute a new class of noncoding RNAs that interfere with gene expression and are also involved in the progression of I/R injury such as myocardial, cerebral, hepatic, renal and mesenteric I/R injury [123]. For example, hypoxia-induced long non-coding RNA Malat1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) has been described to be upregulated in renal I/R injury [124].

Additionaly, preconditionning or pre-treatment of MSCs is also a valuable option: IL-17A-pretreated MSCs resulted in significantly lower acute tubular necrosis scores, serum creatinine and BUN of mice with IRI-AKI [125]. Additionally, hypoxia-treated MSCs attenuate AKI through enhanced angiogenic and antioxidative capacities [126], mimicking organ preconditioning. Thus, such approaches can be combined and renal IR in rats was modulated by combination of ischemic preconditioning and adipose-derived mesenchymal stem cells (ADMSCs) [127].

Finally, in recent years, gene therapy has been developing, both in terms of targeting and efficacy. In transplantation, several studies have shown the feasibility of such an approach to improve IRI. As an example, in the liver, hepatic stimulator substance (HSS), a protein demonstrated to improve mitochondrial function, was overexpressed (through adenoviral transfer) and conferred resistance to IRI. siRNA can also be used intravenously, for instance to silence the expression of TNF-α: in a lethal kidney ischemia model, this was effective in protecting against IRI [128]. Finally, the stability of siRNA can also permit it to be used during preservation, improving outcome [129].

All these strategies will have to be carefully tested for their safety and short and long-term efficacy in predictive and pertinent models, as we discussed in the last paragraph.

10. Predicting the Future: The Importance of Biomarkers

Detection of chronic allograft injury remains a challenge after kidney transplantation. The objective is to define non-invasive biomarkers, both for graft quality evaluation during machine perfusion and graft function in the recipient. Mixed advances have been made to search for biomarker at the earlier step of the transplantation process, during machine perfusion. A clinical metabolomic study of machine perfusion perfusates showed differences in the metabolomic profiles for kidneys with immediate graft function (IGF) and delayed graft function (DGF) [130].

At the recipient stage, transplantation success is determined with measures of biochemical parameters such as serum creatinine or histopathological biopsy analysis, an invasive method. But the best biomarker of early graft function remains undetermined. Most of the time, scoring systems lacks sensitivity and specificity to achieve unanimity at an international level. The efficiency of creatinine as the best measure of kidney state remains questionable, since its inexpensive and easily implemented measurement is biased by certain physiological parameters such as tubular secretion, the influence of muscular mass or protein intake *via* nutrition [131]. And most importantly, it is a late marker.

Urinary NGAL, KIM, L FABP-1, Cystatin C, and IL18 were proposed as tools for early detection of acute kidney injury (AKI) but the determination of their validities and clinical utility is still in progress [132]. Concerning short-term outcomes, the presence of urinary IL-18 and NGAL immediately after transplant was associated with increased risk of delayed graft function [133]. Long-term outcomes may also be predicted with associated risk of graft failure and death correlating with elevated urinary tubular injury biomarkers such as IL-18, NGAL, NAG, and KIM. Some of these markers also have particular physiological importance and their presence can be related to structures alterations in specific part of the nephrons such as in proximal tubule structure alteration (KIM1, IL18, and FABP-1) or distal tubule (NGAL, FABP) [134, 135]. Recently, the cytokine IL-33 has been identified on rats as an alarmin contributing to kidney IRI by promoting iNKT cell recruitment and cytokine production, resulting in neutrophil infiltration and activation at the injury site [136]. However, IL-33 potential as a biomarker for kidney transplantation outcome has not been properly tested yet.

Besides "classical" biomarkers, different investigation paths are followed; some of them are listed below:

(i) Chemokines. Two multicentric studies highlighted chemokines as an early predictive tool for kidney rejection, CXCL9 and CXCL10 mRNA especially [137]. CXCL9 mRNA and protein levels showed a negative predictive power [138]. CXCL10, CD3ε, and 18S RNAs allowed the distinction between antibody-mediated and borderline rejection [139].

(ii) Exosomal Urinary NGAL. It has been suggested that NGAL in the exosomes fraction could be more specific to evaluate renal damage because exosomes should be a representation of the physiological sate of the organ while whole urinary NGAL is not only specific for kidney damages [140].

(iii) Serum Uromodulin. Lower level of this kidney-derived glycoprotein was associated with risk of kidney allograft failure [141].

(iv) Epigenetics and miRNA Regulation. Aberrant DNA methylation patterns are already used as biomarkers in cancer, but only a few studies evaluated their role in transplantation. In kidney transplant recipients with subclinical rejection, long-term allograft outcome was better when FOXP3+Treg cells were present, a subtype characterized by unmethylated locus near the FOXP3 gene [142]. Similarly, the Klotho promoter is hypermethylated in renal tissue and in peripheral blood mononuclear cells of patients with CKD, with the degree of hypermethylation correlating with the clinical and histological severity of CKD [143]. Another promising tool is circulating and urinary miRNA: numerous miRNA associated with kidney disorders has been reported and some of them in the case of transplantation [144]. A panel of 22 urine miRNA measured 3 months after transplantation allowed prediction of chronic allograft dysfunction (CAD) [145].

(v) Epithelial-to-Mesenchymal Transition (EMT) or Endothelial-to-Mesenchymal Transition (EndMT). Both these processes are of high interest since they generate fibrosis and are induced by several molecular signatures among them TGF beta, EGF, and FGF2. A noninvasive approach has been developed for predicting fibrosis *via* assessment of the mRNA expression levels of genes implicated in EMT fibrogenesis such as Vimentin and CD45 [146]. Retrospective evaluation of EMT markers (Fascin1, Vimentin, and Heat Shock Protein 47) by immunochemistry in biopsy samples showed that they are a sensitive and reliable diagnostic tool for detecting endothelial activation during antibody-mediated rejection and predicting late loss of allograft function [147].

While the relationship between recipient kidney injury biomarkers and outcomes is relatively clear, the relationship between donor kidney injury biomarkers and recipient outcomes is more complex [134, 148]. In order to lift the hurdles preventing the discovery of new early and effective biomarkers, the generation of biobanks [149] and the use of laboratory for reconditioning the organ [150] (machine perfusion, ex vivo circulation, for instance) are important perspectives to set up projects looking for "ideal" biomarker.

11. Conclusion

While static cold storage is still widely used, and alternative means and solutions to optimize organ quality during its preservation exist in multiple and of various forms (Figure 1). The identification of the donor population which will most benefit from these strategies or combination of these strategies is also a critical question. The complexity also relies on the fact that all the preservation parameters (temperature, oxygen, static or perfusion, etc.) are dependent factors which need to be scientifically evaluated in independent experiments using models which all have their own limits.

There is an urgent need to promote translational research programs for the development of new clinical protocols. Members of the transplant community (academia, the biotechnology and pharmaceutical industries, and funding agencies) need to engage in an active dialogue and collective effort to find and advance therapies for organ preservation, but we need to assemble the evidences and target key questions in one unified effort.

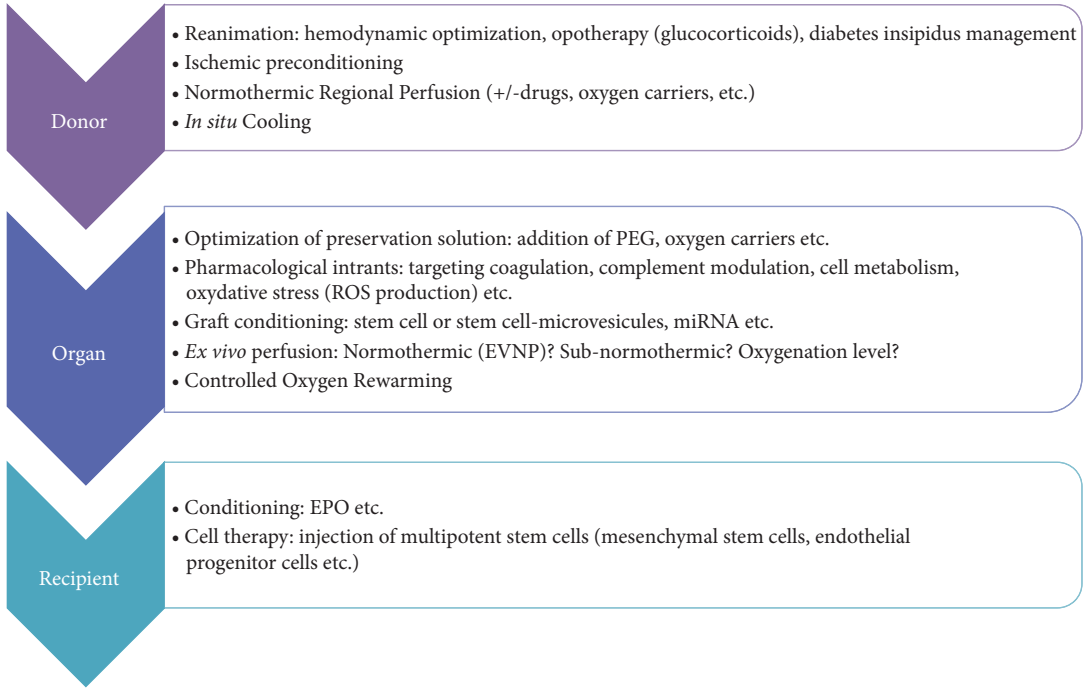

Figure 1: Strategies to overcome existing barriers in kidney preservation during transplantation.

Disclosure

This state-of-the-art review has been written taking into account most important or innovative studies and reviews in the field of kidney preservation (based on the authors' point of view) with a specific interested for highly translational or clinical studies.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

C. Steichen, S. Giraud, T. Hauet, and R. Thuillier have equal contributions.

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Research Article

The Effect of Modified Biolasol Solution on the Efficacy of Storing Isolated Porcine Kidneys

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Biolasol is a newly developed solution for storing the liver, pancreas, kidneys, and heart by simple hypothermia. It exhibits high efficacy in maintaining structural and functional integrity of the graft prior to its transplantation. The solution was modified by the addition of ascorbic acid (0.088g/l) and ascorbic acid with prolactin (1 μg/l PRL + 0.088g/l vitamin C). The effectiveness of the obtained solutions in the protection of nephrons of isolated porcine kidneys was assessed based on the analysis of the activity of ALT (alanine aminotransferase), AST (aspartate aminotransferase), and LDH (lactate dehydrogenase) as well as lactate concentration determined in perfundates collected after 2 h (0′ and 30′ preservation) and 48 h (0′ and 30′ preservation) of graft storage. It has been found that the synergistic action of Biolasol components determines the integrity and stability of cell membranes, which in turn affects the proper functioning of the organ after transplantation. The addition of ascorbic acid and prolactin to Biolasol affects the maintenance of the normal cytoskeleton of the stored graft.

1. Introduction

Biolasol is a newly developed solution for storing the liver, pancreas, kidneys, and heart by simple hypothermia. It exhibits high efficacy in maintaining structural and functional integrity of the graft prior to its transplantation. Biolasol is an extracellular fluid with a sodium concentration of 105 mmol/l and potassium concentration of 10 mmol/l. Dextran70 (colloid osmotic) affects the maintenance of the correct volume of fluids in the intravascular space. Disodium edetate (EDTA) complexes multivalent metal cations. By chelating Ca^{2+} ions, it blocks the activation of zymogens involved in the coagulation process. In complex with Fe^{2+} ions, it reduces the risk of damage caused by the activity of the hydroxyl radical formed in the presence of iron with H_2O_2 in the Fenton reaction. Iron chelators reduce the release of lipid peroxidation products, which minimizes the inflammatory response and the influx of neutrophils into the graft. Magnesium fumarate minimizes cell damage during ischemia and reperfusion. Sodium bicarbonate functions as a buffer system and helps maintain the proper acid-base balance. Glucose is involved in the renewal of ATP [1–4]. Table 1 compares the composition of Biolasol with other fluids available on the world market [5].

Biolasol limits the effects of organ ischaemia and prevents its dysfunctions resulting from rapid cooling. Oxygen deficiency and the switch of cells to anaerobic metabolism reduce ATP reserves and impair the sodium-potassium pump. There occurs an uncontrolled inflow of sodium and calcium to the cell. Ca^{2+}-dependent proteases and phospholipases are activated causing lysis of the cell membrane and damage to ion channels. A decrease in pH, lactate accumulation, and inhibition of oxidative phosphorylation are also observed. Free oxygen radicals are generated, including the superoxide radical, which is toxic to the lipid membranes of the cell and damages the structure of proteins and enzymes. As a consequence, it can lead to severe damage to organs. Biolasol enables restoring their proper functioning after transplantation [1–6].

Table 1: Composition of preservation solutions.

Component	Biolasol	Viaspan	IGL-1	HTK	Celsior
IC/EX	EX	IC	EX	EX	EX
Electrolytes (mmol/l)					
Potassium	10	125	25	10	15
Sodium	105	29	120	15	100
Calcium	0.5	-	-	0.015	0.25
Magnesium	5	5	5	4	13
Chloride	10.5	20	-	32	42
Colloids (g/L)					
HES	-	50	-	-	-
PEG-35	-	-	1	-	-
Dextran 70	0.7	-	-	-	-
ROS scavengers (mmol/l)					
Allopurinol	-	1	1	-	-
Glutathione	-	3	3	-	3
Mannitol	-	-	-	30	60
Tryptophan	-	-	-	2	-
Buffers (mmol/l)					
Histidine	-	-	-	198	30
KH_2PO_4	-	25	25	-	-
$NaHCO_3$	5	-	-	-	-
Impermeants (mmol/l)					
Citrate	30	-	-	-	-
Glucose	167	-	-	-	-
Lactobionate	-	100	100	-	80
Raffinose	-	30	30	-	-
Additives (mmol/l)					
Adenosine	-	5	5	-	-
EDTA	5	-	-	-	-
Fumarate	5	-	-	-	-
Glutamic acid	-	-	-	-	20
Ketoglutarate	-	-	-	1	-
Insulin (U/l)	-	40	-	-	-
Dexamethasone (mg/l)	-	16	-	-	-
Penicillin G (UI/l)	-	2-00	-	-	-
pH	7.4	7.4	7.4	7.20	7.3
Viscosity	Low	High	High	Low	Low
Osmolality mOsm/kg H_2O	330	320	290	310	320-360

IC: intracellular, EX: extracellular.

A number of clinical trials were carried out to assess the effectiveness of Biolasol, also in relation to commonly used perfusion and organ preservation fluids. Its effectiveness was not worse than that of HTK, UW, and Viaspan fluids. Biolasol protects grafts against ischemic damage in a similar way as the aforementioned solutions. It has been found that Biolasol provides better homeostasis of isolated porcine kidneys during storage compared to the HTK solution [2]. Jóźwik et al. [7] transplanted into patients 42 kidneys which had previously been rinsed and stored in Biolasol and UW solutions. They demonstrated comparable effectiveness of both fluids [7]. Cierpka et al. performed comparative

studies of the effectiveness of Biolasol and Viaspan in the procedure of kidney autotransplantation in 12 pigs. They have shown that the used solutions protect the kidneys from ischemia-reperfusion injury in a similar way [3]. Based on our histopathological examinations, we have found that adding prolactin to the HTK preservation fluid minimizes hepatocyte damage in the model using an isolated rabbit liver [8]. Cierpka et al. confirmed by means of histopathological examination that the structure of the isolated porcine kidney cortex was not damaged after using Biolasol [1].

Biolasol was modified by the addition of ascorbic acid and ascorbic acid with prolactin. Prolactin, a hormone secreted by pituitary cells, and an exogenous antioxidant, vitamin C, were used in the modification. Ascorbic acid plays an important role in maintaining the appropriate oxidation-reduction potential in cells and neutralizes the reactive forms of oxygen and nitrogen resulting from cellular metabolism. It occurs both outside and inside the cells. The normal concentration of ascorbic acid in the plasma is over 17 μmol/l, usually 45-80 μmol/l, whereas in leukocytes and platelets it is approximately 1480 μmol/l. In turn, prolactin (PRL) is a protein hormone and a strong cytokine with a broad spectrum of biological activities. It acts as an immunoregulator in cell proliferation and differentiation and is an apoptosis inhibiting factor. It enhances the expression of IL-2 receptors on lymphocytes and stimulates the production of antibodies by B-lymphocytes. It affects the production of lysozyme and lowers the high ceruloplasmin level induced by inflammatory reactions [9, 10]. Ryszka et al. administered prolactin subcutaneously at a dose of 25 μg/kg of body weight in rats. They have found that the distribution of prolactin in selected organs and tissues decreases in the following order: milk gland> blood> pituitary> ovaries> lungs> liver> cranial bone> spleen> heart> kidneys> muscular tissue> adenose> adipose tissue> brain [11]. Prolactin acts by means of specific PRLRs, belonging to type I cytokine transmembrane receptors. Specific PRLRs are located at various places in cells and tissues [12]. The presence of PRL receptors was found in the proximal renal tubules and in the nephron, in the thick section of the ascending arm of the Henle loop, and in the distal tubule and the collecting duct [13]. Ibarra et al. have found that PRL is a natriuretic hormone that interacts with the renal dopaminergic system in inhibiting Na+, K+, and ATP-ase in the proximal renal tubules [14]. Prolactin may affect the filtration rate in the renal glomerulus and the renal plasma flow [15]. It has also been found to affect the proliferation of renal tubular epithelium [16]. It is suggested that PRL receptors are also located in the three zones (cytoplasm of cells, zona glomerulosa, and zona fasciculata) of the adrenal cortex [17].

An important consequence of renal ischaemia is the disorder of apoptosis and repair processes within the renal tubules. The loss of integrity of the cytoskeleton of cells, detachment of the brush border of the proximal tubules, and disturbance of expression of adhesion particles are observed. The C3 segment of the proximal tubule of the nephron and the thick ascending limb of Henle's loop are the most sensitive to ischemia. Damaged cells of the tubules peel off and clog the lumen of the tubules, which causes leakage of filtrate into the lumen of the capillary vessels and a decrease in glomerular filtration [18].

The effectiveness of the modified Biolasol fluid in the protection of nephrons from the effects of ischemia and hypoxia was assessed based on the study of aminotransferase activity, LDH activity, and lactate concentration in the perfusates taken from the renal vein. AST and ALT belong to cellular enzymes, whose increased activity correlates with the increased permeability of cell membranes and/or indicates the breakdown of cells. Aspartate aminotransferase is in 30% present in the tissues of the body as a cytoplasmic isoform (AST1) and in 70% as a mitochondrial isoform (AST2). The increase in its activity is mainly related to the damage of mitochondrial membranes. In turn, alanine aminotransferase is produced in the renal tubular epithelium, and its increased activity indicates damage to the cytoplasmic membranes. Lactate dehydrogenase is located in the cytoplasm of the cell, and its activity increases when cell/tissue necrosis occurs [19]. Lactates are produced in the tissues of the whole body in the process of anaerobic glycolysis. The amount of released LDH and lactates indicates the degree of acidification of the intracellular environment. The determined values of the abovementioned markers in perfusate samples may be helpful in determining the extent of kidney damage during storage [20, 21].

The aim of the study was to evaluate the modified Biolasol solution in terms of the protection of nephrons of isolated porcine kidneys based on the analysis of the activity of ALT (alanine aminotransferase), AST (aspartate aminotransferase), and LDH (lactate dehydrogenase) as well as lactate concentration determined in perfundates collected after 2 h and 48 h of graft storage.

2. Materials and Methods

The study used Biolasol solution (FZNP "Biochefa", Poland) and Biolasol modified by the addition of porcine prolactin - 1 μg/l (FZNP "Biochefa", Poland) and/or ascorbic acid – 0.088g/l (PLIVA Pharmaceutical Company, Cracow, Poland). The study used 30 kidneys from 15 adult Great White Poland pigs weighing 90-110 kg, aged 175-180 days. The kidneys were collected in the slaughterhouse of the Meat Plant H.A.M in Radzionków. After collection, the kidneys were cannulated and stored in a suitable preservation solution (Biolasol, Biolasol+vit.C, or Biolasol+vit.C+PRL) at 4°C for 2 hours (it was the time necessary to transport the organ from the slaughterhouse of H.A.M Meat Plant in Radzionków to the laboratory). The kidneys were then rinsed under the pressure of 73,5 mmHg H_2O with the following solutions: Biolasol, Biolasol + vit.C, and Biolasol + PRL + vit.C. The perfusate samples were collected from the kidney vein at 0 and 30 minutes of perfusion. After 30 minutes, the kidneys were cooled and placed in a sterile bag filled with 500 ml of appropriate preservation solution (Biolasol, Biolasol+vit.C, or Biolasol+vit.C+PRL) for 48 hours (maximum time of organ storage in Biolasol). After this time, activities related to renal perfusion were repeated. In the perfusate samples, the activity of the released indicator enzymes, namely, aspartate aminotransferase (AST), alanine aminotransferase (ALT),

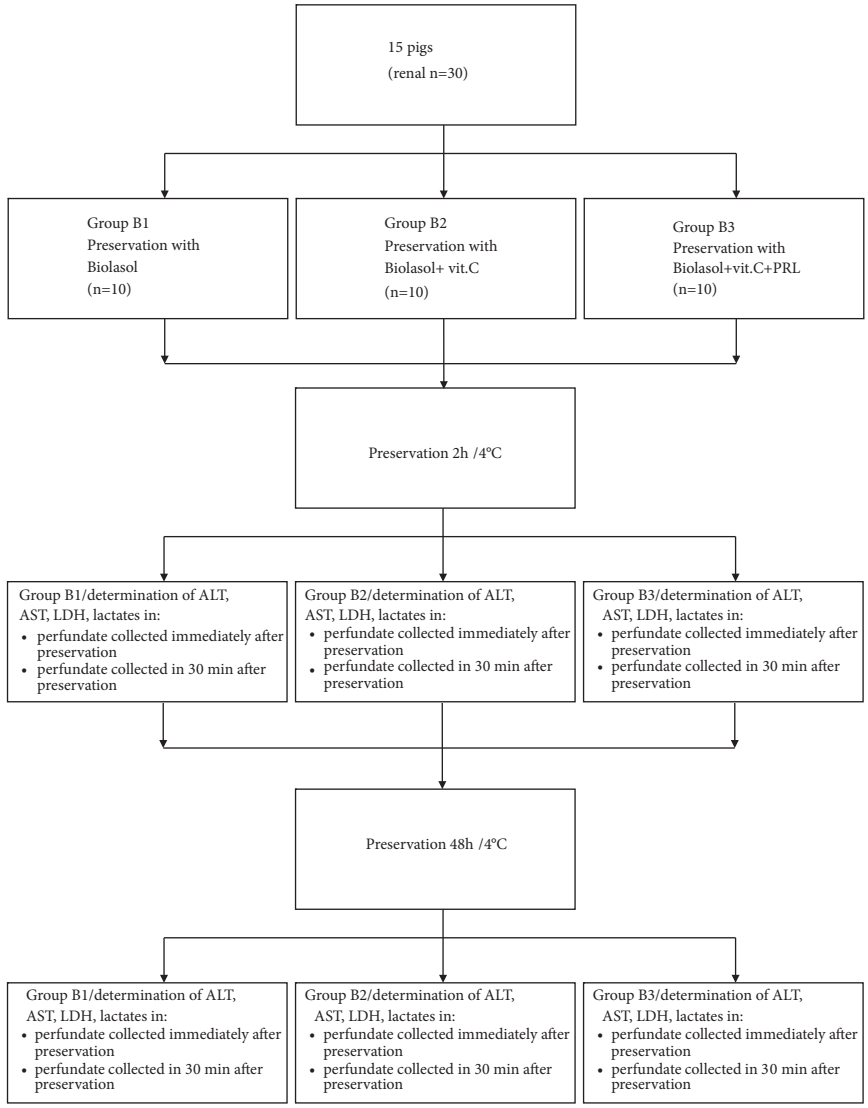

Figure 1: Study design.

and lactate dehydrogenase (LDH), as well as lactate concentration was determined by spectrophotometric methods using the bioMérieux diagnostic kit, Lyon, France (Figure 1).

The normality of the distribution of variables was checked using the Shapiro-Wilk criteria. Comparison among groups was performed using the Kruskal-Wallis test for nonparametric continuous variables, or variance (ANOVA) for parametric continuous variables. The calculations were made using Statistica version 8.0 software (StatSoft, Poland).

3. Results

On the basis of the conducted tests (Table 2), it has been found that the increase in AST activity in the modified solution perfusates is accompanied by a marked lower increase in ALT activity, which translates into the ratio of the activity of these enzymes in porcine serum under physiological conditions (AST - 32-84 U/l, ALT - 31-58 U/l) [2]. It has also been observed that, after 2 hours of storage, both in perfusates of Biolasol modified with the addition of vitamin C and Biolasol modified with the addition of vit.C and PRL, ALT activity remained at the physiological level: 34.7 U/l vs. 43.7 U/l. After 48 h of storage, there was a slight decrease in ALT activity in both cases: 29.6 U/l (~14.7%) vs. 43.2 U/l (~1.1%). The difference is not significant. The obtained results of alanine aminotransferase activity are lower compared to its activity in Biolasol solution perfusates (70.6 U/l-2h, 68.6 U/l-48h). The difference is not significant.

Aspartate aminotransferase (AST) activity remained at the physiological level in the perfusates of all analysed solutions after 48 hours of renal storage. An increase in this parameter was observed after 2 h of storing the graft in Biolasol modified with the addition of vit.C and PRL - 103.4 U/l (~18.8% vs. norm 84 U/l). This may indicate that nephrons were damaged in the early period of kidney storage, presumably as a result of increased expression of prolactin receptors. After 2 hours of 30′ preservation using Biolasol modified with the addition of ascorbic acid and prolactin,

Table 2: Biochemical parameters of the efficacy of storing kidneys in Biolasol and Biolasol –modified solutions with the addition of ascorbic acid and prolactin ± SD.

Time [min]	Biolasol (control group)	Biolasol +vit.C (experimental group)	Biolasol +PRL+vit.C (experimental group)	Significance
		ALT [U/l]		
2h preservation 0′	70.6±19.0	34.7±4.9	43.7±16.6	NS
2h preservation 30′	58.8±15.5	21.0±4.6	24.3±9.1	NS
48h preservation 0′	68.6±16.9	29.6±7.3	43.2±11.2	NS
48h preservation 30′	60.1±17.9	12.8±4.9	24.1±9.0	P<0.05
		AST [U/l]		
2h preservation 0′	60.5±16.4	54.3±14.0	103.4±34.6	NS
2h preservation 30′	32.6±8.9	34.9±13.6	63.9±16.8	NS
48h preservation 0′	60.3±11.1	48.0±18.7	63.8±17.5	NS
48h preservation 30′	35.8±9.4	18.0±6.0	36.0±12.3	NS
		LDH [U/l]		
2h preservation 0′	720.8±164.6	444.8±195.1	602.0±171.0	NS
2h preservation 30′	168.1±41.4	313.2±112.1	305.7±161.0	NS
48h preservation 0′	416.0±59.9	475.0±113.0	473.0±95.4	NS
48h preservation 30′	216.5±135.5	129.5±54.9	145.7±71.0	NS
		Lactates [mmol/l]		
2h preservation 0′	0.9±0.3	1.2±0.2	1.2±0.4	NS
2h preservation 30′	0.5±0.1	0.5±0.2	0.2±0.1	NS
48h preservation 0′	1.0±0.4	0.9±0.3	1.5±0.7	NS
48h preservation 30′	0.6±0.2	0.5±0.3	0.2±0.1	NS

Comparisons between the three groups were performed by analysis of variance (ANOVA) or the Kruskal-Wallis test.

the AST activity decreased by 38%. Rinsing the blood off the organ might have resulted in the restoration of intracellular calcium homeostasis and improvement of mitochondrial cell activity [22, 23].

The physiological norm of lactate dehydrogenase activity in porcine serum is 380-634 U/l [2]. After 48 hours of storage, LDH activity determined in the perfusates of all solutions oscillates within the normal range. A slight increase in LDH activity (~12%) was reported after 2 hours of storing the kidneys in Biolasol solution.

Metabolic acidosis is caused by an increase in serum lactate level above 2 mmol/l. In all the analysed perfusate samples, the lactate concentration was within the normal range. The lowest concentration of this parameter was determined in the perfusates of Biolasol modified with the addition of vit.C and PRL at 30 minutes of perfusion after 2 h and 48 h of storage (0.2 mmol/l).

4. Discussion

High aminotransferase activity, LDH activity, and an increase in lactate concentration may indicate renal ischemic damage and may correlate with the loss of secretory function after transplantation [2, 21, 24]. A similar relationship has been observed by Li et al. [25]. Hypoxia of renal tubule cells during cold ischaemia results in a significant LDH release [26]. Renal damage also causes the release of AST and ALT located in the proximal tubule [27, 28]. The abovementioned parameters were significantly reduced during preservation in Biolasol, Biolasol + vit.C, and Biolasol + vit.C + PRL. Biolasol solution and its modifications were used in maintaining the structural and functional integrity of kidneys under hypoxic conditions. One of the possible protective mechanisms may be an antioxidant effect.

Prolactin, besides fulfilling many biological functions, has a pleiotropic effect. On the basis of the conducted research, it is supposed that PRL participates in the removal of free oxygen radicals (ROS) generated in the cellular space. At present, the mechanism of its operation in this aspect is unknown [29]. It is suggested that PRL may act as an antioxidant in enhancing endogenous antioxidants [29]. It has been found that prolactin indirectly influences the increase of glutathione (GSH) concentration in the cell by stimulation of, e.g., transcription factors, including those regulating insulin secretion. This increase may result from increased glutamate-cysteine ligase (GCL) activity, which catalyses the key step of GSH synthesis [30, 31]. The results of our research suggest that PRL may also exhibit a synergistic effect with exogenous antioxidants, i.e., vitamin C.

The obtained parameters suggest a positive effect of ascorbic acid on the integrity of the cytoskeleton of the stored graft. During ischemia, the formation of reactive oxygen

species (ROS) is activated, which reduces the effectiveness of antioxidative systems. With a large amount of ROS, lipid peroxidation reactions, which are one of the causes of cellular damage, are triggered. Polyunsaturated fatty acids included in phospholipids, which are the building blocks of cell membranes, undergo peroxidation [32]. Supplementing the liquid with an exogenous antioxidant in the form of ascorbic acid supports the weakened graft antioxidant system. Consequently, vitamin C reduces the formation of ROS and has a protective effect on cell integrity. Lloberas et al. carried out research which has shown that the administration of vitamin C during kidney transplantation in a rabbit reduces the concentration of lipids and myeloperoxidase and improves organ function [33]. It has been noted that the administration of vitamin C during kidney transplantation in humans significantly reduces damage caused during reperfusion [34]. In addition, it has been found that preservation solutions modified with vitamin C are more durable [35].

Prolactin may indirectly affect the dilation of renal vessels and, as a consequence, increase the glomerular filtration rate [36]. It is suggested that PRL blocks the inflow of Ca^{2+} to the inside of the cells. Increased calcium concentration in the cell results in the activation of intracellular enzymes that cause the degradation of phospholipids and increase the permeability of the cell membrane.

A number of our studies indicate the hepatoprotective and nephroprotective effects of prolactin [8, 20, 21]. PRL protects the structure and function of cells against the negative effects of ischemia and hypoxia [20, 37]. The addition of this hormone to preservation solutions affects the regeneration of cells after hepatectomy and nephrectomy. It also ensures the integrity of the cell membrane and contributes to the maintenance of normal balance of ions and normal morphological parameters of the liver and kidneys [20, 37]. The addition of 1 μg/l rh-PRL to Biolasol solution reduces ALT and AST activity during reperfusion [20].

5. Conclusions

The synergistic action of Biolasol components determines, inter alia, the integrity and stability of cell membranes, which in turn affects the proper functioning of the organ after transplantation. The addition of ascorbic acid and prolactin to Biolasol solution affects the maintenance of the normal cytoskeleton of the stored graft.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Clinical Study

Eradication of HCV in Renal Transplant Recipients and Its Effects on Quality of Life

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Background. The use of direct antiviral agents (DAA) has radically modified the course of HCV hepatitis in renal patients. Aim of this study was to assess the effects of HCV eradication on quality of life (QOL) in renal transplant recipients (RTR), measured by CLDQ and SF-36. *Methods*. Sixteen RTR with well preserved GFR (mean: 60.3±19.3 ml/min) and chronic HCV infection with moderate liver stiffness (9.3±1.7 kPa) were given a sofosbuvir-based regimen for 12 weeks and had a 1 year follow-up. *Results*. At end of treatment (EOT) a complete viral clearance was observed in all the patients, with normalization of most laboratory data and a consistent reduction in liver stiffness. All these parameters remained stable after 1 year, as well as renal function and proteinuria. Questionnaire data showed consistent amelioration in different "emotional" domains at EOT, which persisted after 1 year and were associated with a globally improved QOL, although there was no change in most of the "physical" domains in both questionnaires. One patient under ribavirin developed an acute anemia and withdrew from the study, but no further adverse episode was observed throughout the study. *Conclusions*. Our data, while confirming the efficacy of oral DAA, show that HCV infection represents a heavy psychological burden in renal transplant recipients, greatly alleviated by viral eradication, which determines a significant improvement in QOL that represents an important outcome in management of all transplant recipients. This trial is registered with ISRCTN97560076.

1. Background

Chronic hepatitis by C virus infection (HCV) is a significant public health problem with a worldwide estimated prevalence of 3% [1], consistently higher in patients with chronic kidney disease (CKD), either in conservative and substitutive treatment [2]. In renal transplant recipients (RTR), HCV infection is associated with a significantly higher risk for all-cause mortality and graft loss compared to the uninfected counterpart [1, 3] and represents a serious trouble for these patients, due to the negative impact of immunosuppression on disease progression and its effect on the outcome of their graft, as a consequence of increased incidence of diabetes, enhanced onset of cardiovascular diseases, easier recurrence of glomerulonephritis, and faster progression of chronic allograft nephropathy [4–8].

The recent introduction of second-generation direct antiviral agents (DAA) has dramatically changed the therapeutic scenario of HCV infection in general population, as well as in renal patients [9, 10], and mostly in RTR, in whom interferon-based regimens are contraindicated because of the risk of acute graft rejection [8]; indeed, these new regimens have evidenced an optimal response in terms of either viral clearance or patients' tolerability [2, 11–18].

Given the high impact of HCV infection on patients' quality of life (QOL) in general population [19], the aim of the present study was to evaluate the effects of HCV eradication on health-related QOL in RTR, commonly affected by a heavy

clinical and psychological burden. Today, improvements in QOL represent a widely accepted measure of treatment outcome in any chronic disease, since patients require greater attention to their physical and emotional well-being in everyday life.

To this extent a small cohort of RTR underwent a 12-week sofosbuvir-based regimen and was prospectively followed up for one year, with repeated evaluation of QOL questionnaires. Our working hypothesis was that eradication of HCV may determine consistent improvements in QOL.

2. Materials and Methods

2.1. Patients. This case series consisted of 16 HCV-infected kidney transplant recipients in regular follow-up at our Nephrology and Kidney Transplant Unit, referred to our hepatologists (NC, FM), who selected the proper DAA combination on the basis of HCV genotype and estimated glomerular filtration rate (eGFR) and drug availability in Italy at time of starting treatment. Our transplant recipients were white-Caucasian, all being first transplant from cadaver donors (two with simultaneous renal and liver transplantation).

The first 10 patients enrolled in the study were selected among RTR in regular follow-up at the Day Hospital of Renal Transplantation of University Federico II of Naples, on the basis of specific criteria suggested by Italian Ministry of Health in September 2015. They were as follows: age ≥18 years; presence of HCV-antibodies and of HCV-RNA replication, independently of liver enzymes alteration; a liver stiffness value ≥7 kPa at transient elastography (TE); presence of HCV genotype 1, 2, 3, or 4; and stable renal function in the last 6 months, with an estimated glomerular filtration rate (eGFR) >35 ml/min and no graft rejection in the preceding 12 months. From September 2016 DAA treatment could be started also in patients with lower TE values, and 6 further patients were enrolled. Exclusion criteria were as follows: decompensated liver cirrhosis, chronic B-hepatitis or human immunodeficiency virus infection, and presence of specific intercurrent clinical problems, like infections or ESA-resistant anemia.

2.2. Treatment. Antiviral therapy consisted of sofosbuvir (400 mg/day) in all patients, associated with daclatasvir (60 mg/day; n=9), ledipasvir (90 mg/day; n = 2), ribavirin (weight-based dosage; n=2), or velpatasvir (100 mg, n=2); one patient was under sofosbuvir + ledipasvir + ribavirin. The addition of ribavirin and of its discontinuation was at the discretion of hepatologist. Treatment lasted 12 weeks, like in general population, and its efficacy was evaluated by monitoring viral load at baseline, after 4, 8, and 12 weeks of treatment (end of treatment, EOT), and 12 and 48 weeks after EOT. Liver stiffness was evaluated by TE [FibroScan; Touch 5.02; Echosens; France] at baseline, at EOT, and 48 weeks after EOT by a skilled operator (SC), on the basis of current guidelines. Maintenance immunosuppression therapy consisted of a calcineurin inhibitor (CNI) in 12 patients (5 on tacrolimus and 7 on cyclosporine) in conjunction with steroids (n=9), mycophenolic acid derivatives (n=6), and sirolimus (n=2). Four patients were treated with sirolimus in association with steroids (n=4) and mycophenolic acid derivatives (n=2). In all the patients, induction therapy consisted of Basiliximab (20 mg, during surgery and at the 4th postoperative day).

2.3. Quality of Life Measurement. All the patients were administered the Chronic Liver Disease questionnaire (CLDQ) and the Short Form Health Survey (SF-36) questionnaire during their clinical visits, i.e., before starting the therapy (Basal), at the end of therapy (EOT), and 48 weeks later (1 year). CLDQ is the first disease specific instrument developed to evaluate health-related quality of life (QOL) in patients with liver disease [20] which includes 29 items in the following domains: fatigue, activity, emotional function, abdominal symptoms, systemic symptoms, and worry. The answers to each question are graded with scores ranging from 0 (best option) to 6 (worst option), according to its Italian version [21]: high scores denote a worse liver-related quality of life.

Health-related QOL was evaluated using the Italian version of SF-36. This tool contains one item that evaluates the perceived changes in health status, while the remaining 35 items are used to generate eight subscales, which are the weighted sums of the questions in their section. Each scale is directly transformed into a 0-100 scale on the assumption that each question carries equal weight; the lower scores reflect greater disability. The eight sections are general health perception, physical functioning, role limitation due to poor physical health, role limitation due to poor emotional health, social role functioning, bodily pain, emotional well-being, and vitality. Both questionnaires were given and explained to patients in the early morning of the scheduled visit by their trained caregiver (IC) and were completed at her presence after the visit.

2.4. Laboratory Data. Trough levels of tacrolimus, cyclosporine, and sirolimus were monitored every week during DAA administration and every 4 weeks later on by commercial immunoassays. Estimated-GFR (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Collaboration Equation (CKD-EPI). Urinary protein excretion was measured on 24-hour urine samples.

All laboratory values were determined by standard methods. Plasma HCV mRNA levels were measured by a real-time PCR-based method (Abbott Real Time, Lower Limit of Quantification: 12 IU/ml).

2.5. Statistical Analysis. Data were first analyzed with Shapiro's test to determine their distribution. Parametric data were presented as mean and standard deviation; nonparametric data were presented as median and range. Parametric data were analyzed with repeated measures ANOVA or Student's t test for paired data, as appropriate; nonparametric data analysis was performed by Friedman's test. Post hoc analysis was conducted by Bonferroni's test for parametric data and Dunn's test for nonparametric data. Differences were considered statistically significant if $p<0.05$. Data were analyzed using statistical software R (R version 3.3.3).

Table 1: Patient characteristics at baseline (n=16).

Demographic characteristics	
Age, y	64 (26-71)[a]
Sex (M/F)	11/5
Time from RTX to HCV therapy, mo	150.9 (84.4)[b]
Cause of end-stage renal disease	
Vesicoureteral reflux	1
Chronic glomerulonephritis	6
Others or unknown	9
HCV genotype:	
1a	1
1b	10
2	4
4	1
Baseline laboratory values	
Creatinine, mg/dL	1.29 (0.80-1.80) [a]
eGFR, mL/min/1.73 m^2	60.3 (19.3) [b]
Proteinuria (g/24 hours)	0.582 (0,768) [b]
Bilirubin: total, mg/dL	0.58 (0.50-0.80) [a]
GGT, U/L	47.5 (16-306) [a]
ALT, U/L	30.2 (11 -137) [a]
AST, U/L	31.4 (17 -85) [a]
ALP, U/L	95.1 (41.3) [b]
Total serum protein, g/dL	6.91 (0.50) [b]
Albumin, g/dL	4.15 (0.37) [b]
AFP, IU/mL	2.93 (2.62) [b]
Hemoglobin, g/dL	12.87 (1.13) [b]
WBC count, /μL	7.40 (1.57) [b]
Platelet count, /μL	218.43 (62.16) [b]
FibroScan value (kPa)	9.31 (1.75) [b]
Viral load (log_{10} IU/mL)	$2.1E^6$ ($4.1E^4$-$1.3E^7$) [a]

Data are expressed as median (range)[a] or mean (standard deviation)[b]. Abbreviations. eGFR: estimated glomerular filtration rate; GGT: γ-glutamyl-transpeptidase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; AFP: alpha fetoprotein (0-4 IU/ml).

The study was approved by Ethical Committee of "Federico II" University (#290/15). At time of starting treatment, all the patients were adequately informed about the potential adverse effects of DAAs and their possible interactions with immunosuppressive drugs; all the patients gave their informed written consent. Data were collected from October 2015 to February 2018.

3. Results

Baseline patients' demographic and clinical data are summarized in Table 1. Six patients had a FibroScan value >10 kPa at time of starting therapy. All the patients had documented HCV infection prior to transplantation and received HCV-negative organs. Their median HCV-RNA concentration was $2.1E^6$ ($4.1E^4$-$1.3E^7$) log 10 IU/mL, and a viral load greater than 800,000 IU/mL was present in 14 patients. Mean plasma concentrations of liver enzymes were in the upper zone of normal ranges.

The etiology of renal disease was undetermined in 57% of patients; six patients had biopsy proven glomerular diseases (2 focal and segmental glomerular sclerosis, 1 IgA-nephropathy, 1 postinfectious, and 2 not defined), and proteinuria exceeded 1 g/24 hours in 2 patients; 3 patients were affected by posttransplant diabetes (one insulin-dependent). One patient withdrew from the study during the 4th week of treatment because of acute anemia and his data are not considered in statistics; the remaining 15 patients completed at least 1-year follow-up. At baseline, no correlation was detected between FibroScan values, viral load, and liver enzymes (P=0.14).

3.1. Effects of DAA on HCV Infection . A complete viral clearance, i.e., undetectable HCV-RNA replication, was observed in all the patients at EOT. It was associated with a significant decrease of GGT, AST, and ALT values (-60%, -49%, and -57%, respectively, versus Basal), with no change in alkaline phosphatase nor in total bilirubin (Figure 1); these parameters remained stable throughout the follow-up. Interestingly, also FibroScan values were consistently reduced at EOT (-33%) and were not modified thereafter (Figure 2), implying that no further improvement was obtained in the medium term

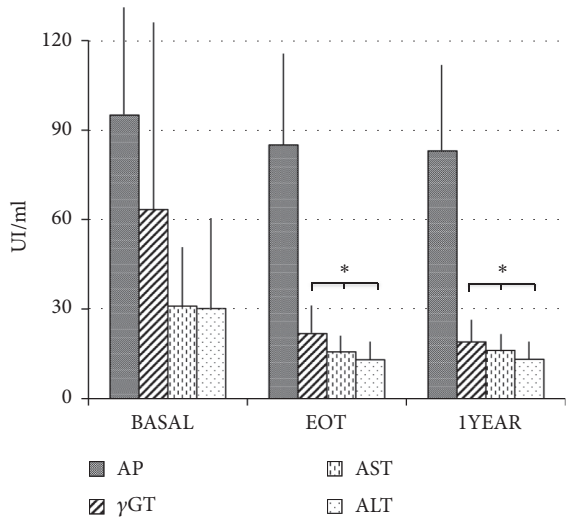

Figure 1: Main liver enzymes plasma concentrations in Basal condition, at end of therapy (EOT) and 1 year after EOT (n=15). Abbreviations. AP: alkaline phosphatase; γ GT: γ-glutamyl-transpeptidase; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Data are expressed as mean values ± SD. $*$ means $p<0.05$ AST/ALT/GGT versus respective Basal value ($p<0.05$, minimum value).

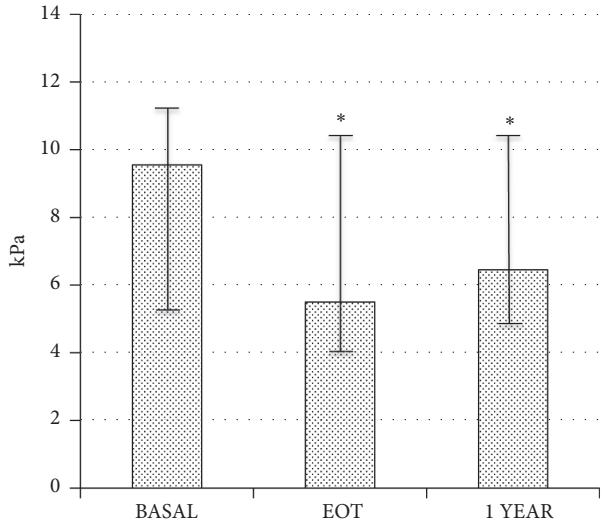

Figure 2: Modifications of transient elastography data (FibroScan) at end of antiviral therapy (EOT) and after 1 year compared to Basal (n=15). Data are expressed as median values and range (error bars). $*$ means $p<0.0001$ versus Basal.

despite a better functioning liver. No change was detected in hemoglobin, albumin, and glucose plasma levels throughout the study (data not shown).

3.2. Effects of DAA on Renal Function. DAA administration did not affect renal function. No change, in fact, was observed in mean eGFR at EOT (59.9±16.8 versus 60.3±19.3 ml/min in Basal, NS) nor after the 1-year follow-up (56.0±19.9 ml/min, NS), although two patients had developed an important impairment of eGFR (-31% and -49%, versus respective Basal). Similarly, proteinuria values remained quite stable throughout the observation period, averaging 0.58±0.76 g/24hrs at Basal, 0.55±0.77 at EOT, and 0.47±0.88 after 1 year (NS). Indeed, we observed an improvement in urinary protein excretion in 7/15 patients (with complete disappearance of proteinuria in 3 patients) and a clear worsening in the 2 patients who developed the renal impairment.

Trough levels of CNI were slightly reduced (tacrolimus, -24%; cyclosporine, -11%), and mild adjustments of drug doses were necessary in 3 patients. Sirolimus plasma concentrations, conversely, remained stable. Daily doses of mycophenolic acid and steroids were not changed during the study. The need for recombinant erythropoietin support or treatment of established diabetes also remained unchanged during therapy.

3.3. Quality of Life. HCV eradication was associated with a better QOL. At baseline, median value of CLDQ was 2.89 (range: 1.18-5.36), which denotes a relatively preserved liver-related quality of life. All the domains tended to be lower at EOT, although not significantly, with the exception of "activity". At completion of the 1-year observation period (Figure 3), a consistent and significant decrease was recorded in global score, whose median value decreased by 36% ($p<0.01$ versus Basal), following the significant improvements observed in "worry", "emotional function", and perceived "abdominal symptoms". Conversely, the domains of "fatigue", "systemic symptoms", and "activity" were not affected by HCV infection recovery.

The results of SF-36 questionnaire are reported in Table 2. It is interesting to note that both the scores of the "emotional well-being" and of the "role limitation due to emotional problems" were already significantly higher at EOT and further improved after 1 year, where also the perception of a beneficial "health change" was described, associated with a better perception of patient's social role. Interestingly, like in CLDQ, physical functioning and role limitation due to physical problems or vitality were marginally affected by DAA treatment.

3.4. DAA Side Effects. One patient developed a symptomatic episode of acute anemia during the 4th week of treatment with sofosbuvir + ribavirin (nadir of hemoglobin: 6.6 g/dl), which required hospitalization, transfusion of 2 blood units, and DAA withdrawal; the patient refused a new treatment with different drugs. Throughout the entire study, tolerance to treatment was excellent: mild headache (n=2) and fatigue (n=3) were the most common reported side effects, requiring no therapy. No acute graft rejection or infectious episode occurred in any patient, and after the initial decline, trough levels of immunosuppressive drugs remained stable during the follow-up period.

4. Discussion

In this paper we describe our single-center experience with a 12-week course of DAAs on 16 renal transplant recipients affected by long lasting HCV infection, with moderate liver

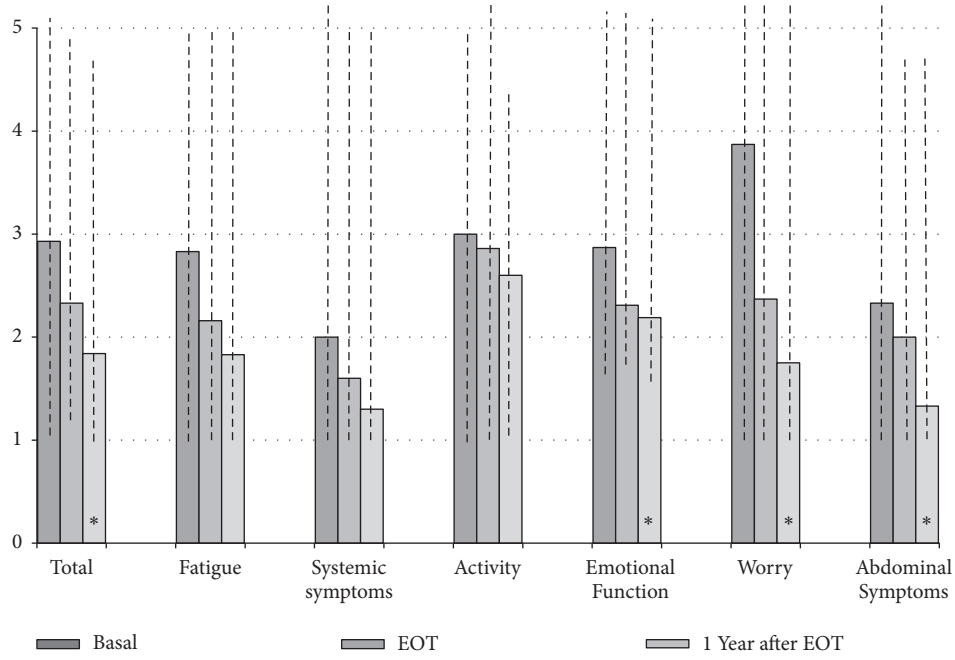

Figure 3: Scores of Chronic Liver Disease questionnaire (CLDQ) before starting treatment (Basal), at the end of therapy (EOT), and 1 year after EOT. Data are expressed as median values (range, dashed lines). ∗ means $p<0.05$ versus Basal (Friedman's test followed by Dunn's post hoc analysis).

Table 2: Scores of SF-36 before starting treatment (BASAL), at the end of therapy (EOT), and 1 year after EOT (n=15).

	BASAL	EOT	1 YEAR	*p value*
Health Change [a]	50 (25-100)	50 (25-100)	75 (25-100) [†]	*0.01129*
General Health	42 (28.59)	50.3 (26.69)	47.3 (23.82)	*0.1018*
Physical Functioning [a]	70 (20-100)	75 (0-100)	90 (0-100)	*0.3359*
Role limitation – Physical [a]	50 (0-100)	100 (25-100)	100 (0-100)	*0.006152*
Role limitation – Emotional [a]	33.3 (0-100)	100 (0-100) [†]	100 (0-100) [†]	*0.0145*
Social role functioning [a]	62.50 (25-100)	75 (25-100)	87.50 (25-100) [†]	*0.08548*
Bodily Pain [a]	77.5 (22.5-100)	90 (22.5-100)	90 (0-100)	*0.09072*
Emotional well-being [b]	57.6 (18.57)	70.13 (12.99) [§]	73.87 (12.82) [§]	$8e^{-04}$
Vitality [b]	56.3 (22.16)	68 (10.82)	66.67 (20.41)	*0.0807*

Data are expressed as [a] median values (range) or [b] mean (standard deviation). [†] $p<0.05$ vs BASAL (Friedman's test followed by Bonferroni's correction); [§] $p<0.05$ vs BASAL (ANOVA followed by Tukey post hoc test).

stiffness and a well preserved renal function. Our data confirm the prolonged efficacy of DAAs in clearing viral infection and show that HCV eradication is associated with an early and persistent amelioration of self-perceived QOL.

4.1. Liver and Renal Data. All the modifications observed in liver function occurred during the 12 week treatment, and no further change was observed within the first year; similarly, hepatic stiffness greatly decreased at EOT (-33%) and remained quite stable thereafter: this huge reduction probably reflects the early improvement in liver necroinflammation induced by antiviral treatment [22].

HCV eradication did not modify mean eGFR nor proteinuria; it is noteworthy, however, that an important loss of filtrate was observed in 2 patients: the first one (combined liver/kidney transplantation) had a low baseline GFR and a high proteinuria (1.9 g/24hrs) at time of starting sofosbuvir + daclatasvir, which further increased to the nephrotic range despite the worsening of GFR (-49.5% at 1 year); the second one, conversely, presented a well preserved renal function and a proteinuria of 0.91 g/24hrs before starting sofosbuvir + ledipasvir. While, generally, our data provide confidence that DAA do not substantially affect renal function, it remains to be elucidated whether a preexisting, consistent proteinuria may predict the worsening of renal function after treatment. Our data, however, recall those of previous studies describing an unexplained early fall of eGFR in a small number of patients [11, 13, 23].

4.2. QOL Data. Undoubtedly, the most interesting aspect of the present study is the positive effect of HCV eradication on patients' QOL. Both questionnaires, in fact, evidenced

interesting ameliorations in several domains, mostly relevant to the psychological sphere of our patients. At EOT, in fact, SF-36 data showed that both the well-being and the feeling of role limitation due to emotional problems were significantly better than those in Basal, and such improvements persisted after one year, associated with a healthier functioning role and a reduction in worry and emotional functioning observed with the CLDQ: taken together, all contributed to the positive perception of a beneficial modification in their QOL. Physical domains, conversely, were less affected by viral eradication; this result was in part expected, considering the long duration of HCV infection, which had probably reduced the intensity and the perception of symptoms, and the mild clinical expression of liver disease (no patient was cirrhotic).

The changes in QOL scores and, mostly, the rapidity of their improvement after DAA treatment clearly witness the heavy psychological burden that HCV infection determines in transplant recipients, who are obviously aware of the negative impact of immunosuppression on disease progression and fear the negative effects of a decompensated liver disease on the outcome of their graft; the entity of such improvement is not negligible considering that among solid organ transplant patients, kidney recipients show the lowest improvement in SF-36 after transplantation [24]. Although the questionnaires were not anonymous, but were proposed by their physician, we believe that patients did not overemphasize their feelings: all had a long follow-up in our unit and a complete confidence with their caregiver.

These data deserve attention since today QOL represents a crucial point in management of transplant recipients. In fact, traditional graft outcomes like organ survival, rejection rates, or transplant complications represent just a part of patients' concerns, and increasing attention is devoted to QOL: patients are more and more interested in how well they feel in "real life", considering how much the disease has modified their lives. Therefore, although QOL measurements are based on patients' subjective sensations, they become a significant clinical measure, and patient's perspective becomes as important as that of the clinician [25]. Unfortunately, many physicians are still reluctant to consider improvements of QOL as a main outcome of transplantation.

In general population, two different reports describe improvements in QOL after DAA treatment [19, 26]. These data, quite surprisingly, were not confirmed by Ichikawa et al. in a cohort of cirrhotic patients that showed significant ameliorations in many cirrhosis-related symptoms after treatment [27]. Probably, the life expectancy of cirrhotic patients (compared to transplant recipients) and, mostly, the degree of hepatic impairment have conditioned this result: in fact, it may require several years after relief from liver-related symptoms for an effect on QOL to become apparent [27].

Tolerability of our sofosbuvir-based regimen was excellent, as also indirectly confirmed by the improved QOL. The only serious adverse effect was an acute episode of anemia probably related to the use of ribavirin; unfortunately, the patient refused a new therapeutic trial with different drugs. The good safety profile of DAA should alert physicians to treat potential candidates as soon as possible, while on dialysis or in the transplant waiting list, to prevent further liver deterioration and improve a depressed QOL.

The main limit of our prospective study is the small sample size, due to difficulties in obtaining the drugs from our Health National System, and the inclusion of only patients with moderate liver dysfunction. Our data, however, suggest that greater improvements of QOL could be expected in patients with more pronounced symptoms and greater psychological involvement.

5. Conclusions

In conclusion, our study demonstrates that, beyond the clinical results on liver disease progression, HCV eradication by DAA determines a consistent improvement in QOL of transplant recipients. Although new studies are necessary to evaluate whether the early HCV eradication will result in improved graft outcomes, decreased recurrence of glomerulopathies, or reduced incidence of posttransplant diabetes, the possibility of improving patients' QOL represents a further incentive to early treat all HCV-infected patients.

Abbreviations

CLDQ: Chronic Liver Disease questionnaire
DAA: Direct antiviral agents
eGFR: Estimated glomerular filtration rate
EOT: End of treatment
ESA: Erythropoiesis stimulating agents
QOL: Quality of life
SVR: Sustained virological response
TE: Transient elastography.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The study was approved by the Ethical Committee of "Federico II" University (#290/15). All procedures were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Massimo Sabbatini, Nicola Caporaso, and Filomena Morisco participated in research design and in the paper writing; Ivana Capuano, Silvia Camera, Lucia Ferreri, and Laura Donnarumma participated in the performance of research; Pasquale Buonanno participated in data analysis.

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Research Article

Reperfusion Activates AP-1 and Heat Shock Response in Donor Kidney Parenchyma after Warm Ischemia

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Utilization of kidneys from extended criteria donors leads to an increase in average warm ischemia time (WIT), which is associated with larger degrees of ischemia-reperfusion injury (IRI). Kidney resuscitation by extracorporeal perfusion in situ allows up to 60 minutes of asystole after the circulatory death. Molecular studies of kidney grafts from human donors with critically expanded WIT are warranted. Transcriptomes of two human kidneys from two different donors were profiled after 35-45 minutes of WIT and after 120 minutes of normothermic perfusion and compared. Baseline gene expression patterns in ischemic grafts display substantial intrinsic differences. IRI does not lead to substantial change in overall transcription landscape but activates a highly connected protein network with hubs centered on Jun/Fos/ATF transcription factors and HSP1A/HSPA5 heat shock proteins. This response is regulated by positive feedback. IRI networks are enriched in soluble proteins and biofluids assayable substances, thus, indicating feasibility of the longitudinal, minimally invasive assessment *in vivo*. Mapping of IRI related molecules in ischemic and reperfused kidneys provides a rationale for possible organ conditioning during machine assisted ex vivo normothermic perfusion. A study of natural diversity of the transcriptional landscapes in presumably normal, transplantation-suitable human organs is warranted.

1. Introduction

Across a variety of transplanted organs, short-term patient and graft outcomes continue to improve [1], with 1-year survival rates for kidney recipients being well over 90% [2, 3]. However, improving longer-term outcomes remains a challenge [3].

In kidney transplantation, ischemia-reperfusion injury (IRI) is unavoidable. IRI contributes to both immunologically mediated chronic rejection [4] and so-called chronic allograft dysfunction (CAD) [5]. IRI, which is proportional to donor warm ischemia time (WIT), is one of the main factors influencing kidney graft survival [6]. Severity of renal IR is strongly associated with the circumstances of kidney donation [7]. Recent dramatic increase in the utilization of kidneys from donors after circulatory death and extended criteria donors lead to an increase in average WIT [8, 9]. It is widely accepted that the prevention or the reduction of IRI is imperative to improve graft survival and decrease posttransplant morbidity.

In transition between the donor and the recipient, renal allograft is typically preserved by static cold storage. Recently, there has been considerably increased interest in machine perfusion for preservation of kidneys, with meta-analysis showing that machine perfusion improves outcomes through the better preservation of tubular, glomerular, and endothelial function and integrity [10]. Recently, kidney resuscitation by extracorporeal perfusion in situ was added to the list of

options, with up to 60 minutes of asystole allowed after the circulatory death [11].

High-throughput profiling technologies have enabled systemic investigation of the pathophysiological processes on the "omics" landscapes and subsequent molecular dissection of observed functional changes [12]. In previous studies, a number of mRNAs, miRNA, and proteins playing role in tubular or vascular damage to the donor organ were associated with the incidence and severity of IRI [13, 14]. However, no molecular studies so far were performed in kidney grafts from human donors with critically expanded WIT.

In this study, we analyzed the transcriptomes of two kidney grafts from two different donors. Grafts were biopsied after 40 and 49 minutes of WIT, respectively, and then after 120 minutes of normothermic perfusion. We showed that reperfusion does not lead to substantial change in overall landscape of kidney transcription but rather activates a specific program resulting in overexpression of highly connected protein network with hubs centered on Jun/Fos/ATF transcription factors and HSP1A/ HSPA5 heat shock proteins.

2. Materials and Methods

This study design, protocols of perfusion and samples preparation, organ procurement, and transplant procedures were approved by the Scientific Board and Ethics Committee of the Saint-Petersburg State Research Institute for Emergency (Decision 7/0615/09) and authorized for clinical application by the Federal Advisory Service of the Ministry of Healthcare of the Russian Federation (Resolution N2010/299). Both donors had unexpected irreversible asystole and circulatory death in course of their stay in the hospital. After unsuccessful attempts of advanced cardiopulmonary resuscitation, the entry to donor program was activated by "in-house" hospital transplant coordinator as described previously [11]. After the permission was obtained from hospital administration, donors were transferred to an operating room for femoral vessels catheterization and perfusion procedure setup. Simultaneously kidney parenchyma biopsies were taken using 20G needles (SuperCore II, Angiotech, USA) under ultrasound control. According to current legislation in Russia, informed consent from the relatives and next-of-kin for femoral access and for nonlaparotomic biospecimen collection is not required.

For both donors, in situ extracorporeal perfusion of an isolated abdominal region with membrane oxygenation and leukocyte depletion was performed. To prime the circuit, we used up to 2L of Custodiol™ (HTK, histidine-tryptophan-ketoglutarate solution, Dr. F. Kohler Chemie GmbH, Bensheim, Germany). Controlled organ reperfusion procedure consists of the following obligatory subprocedures:

(1) Abdominal in situ thrombolysis and heparinization through perfusion circuit

(2) Elimination of leuko- and thrombocyte clots from the vascular bed of abdominal organs using the hemodilution and leukofilter incorporated into perfusion circuit

(3) Subnormothermic extracorporeal membrane oxygenation of the perfusate.

Organs were perfused with the modified donor blood augmented with the following: 1.5 million units of Streptokinase (Belmedpreparaty AO, Minsk, Belarus) and 25,000 IU of Heparin (Gedeon Richter, Hamburg, Germany). During the first 30 minutes of perfusion, the perfusate flow was gradually increased from 500 ml/min to 3500 ml/min. The oxygen supply was maintained at 150–350 ml/min which corresponds to an average pO2 of 300.1±9.38 mm Hg. All procedures were performed under mild normothermic or subnormothermic conditions (27–32°C). Blood samples were collected and assayed for leukocyte counts, pH levels, oxygen, and CO_2. A count of 1×10^9 or lower was empirically considered as a satisfactory perfusion outcome. On average, elimination of leukocytes from the abdominal perfusion circuit required no more than 120 minutes, the time that is sufficient to complete legal paperwork and obtain the next-of-kin consent.

Although the perfusion procedures were initiated prior to the arrival of the forensic pathologist, the organ procurement procedures started only after completion of legal documentation, including the consent of next-of-kin. Laparotomy and kidney mobilization were performed and organ recovery commenced while the donor was still on the continuous extracorporeal perfusion. The perfusion procedure was terminated just before the surgical kidney explantation, performed immediately after second needle biopsy. Each kidney graft was placed in a separate plastic bag for subsequent static cold preservation in HTK solution.

Four kidney grafts were subsequently transplanted into 4 patients according to approved protocol in Saint-Petersburg [11]. Prior to transplantation, all recipients signed informed consent to ensure their awareness of the study procedures performed with donor kidneys. In three of these patients, an immediate graft function was observed, while one had delayed graft function, which was restored after 6 sessions of hemodialysis. All four patients were routinely discharged after 21-day hospital stay. 3-year patients and grafts survival rates were at 100%. To date, one patient died of a stroke in the fifth year after transplant with functioning graft; one returned to kidney replacement therapy due to noncompliance in the fourth year after transplant. Two remaining patients are alive with functioning grafts and the most recent serum creatinine monitoring results at 98.7 and 101.0 mmol/L, respectively (test date: June 1st, 2018).

2.1. Kidney Donor Procedures. From 2011, Saint-Petersburg's Organ Procurement Center prospectively collects samples from brain dead donors and deceased donors enrolled throughout donation program. In this study, four kidney parenchyma specimens were collected from two different donors; one kidney in each pair was biopsied after 40 and 49 minutes of WIT, respectively, and after 120 minutes of normothermic perfusion. Biopsies were performed using 20G needles (SuperCore II, Angiotech, USA). Each parenchyma specimen was divided into 7 equal pieces, snap frozen into liquid nitrogen, and placed at -70°C for storage.

This study design, protocols of perfusion, organ procurement, and transplant procedures were approved by

the Scientific Board and Ethics Committee of the Saint-Petersburg State Research Institute for Emergency (Decision 7/0615/09) and authorized for clinical application by the Federal Advisory Service of the Ministry of Healthcare of the Russian Federation (Resolution N2010/299). Both donors had unexpected irreversible asystole and circulatory death in course of their stay in the hospital. After unsuccessful attempts of advanced cardiopulmonary resuscitation, the entry to donor program was activated by "in-house" hospital transplant coordinator as described previously [11]. For both donors, in situ extracorporeal perfusion of an isolated abdominal region with membrane oxygenation and leukocyte depletion was performed. To prime the circuit, we used up to 2L of Custodiol (HTK, histidine-tryptophan-ketoglutarate solution, Dr. F. Kohler Chemie GmbH, Bensheim, Germany). Controlled organ reperfusion procedure consists of the following obligatory subprocedures:

(4) Abdominal in situ thrombolysis and heparinization through perfusion circuit

(5) Elimination of leuko- and thrombocyte clots from the vascular bed of abdominal organs using the hemodilution and leukofilter incorporated into perfusion circuit

(6) Subnormothermic extracorporeal membrane oxygenation of the perfusate.

Organs were perfused with the modified donor blood augmented with following: 1.5 million units of Streptokinase (Belmedpreparaty AO, Minsk, Belarus) and 25,000 IU of Heparin (Gedeon Richter, Hamburg, Germany). During the first 30 minutes of perfusion, the perfusate flow was gradually increased from 500 ml/min to 3500 ml/min. The oxygen supply was maintained at 150–350 ml/min which corresponds to an average pO2 of 300.1±9.38 mm Hg. All procedures were performed under mild normothermic or subnormothermic conditions (27–32°C). Blood samples were collected and assayed for leukocyte counts, pH levels, oxygen, and CO_2. The decrease of leukocyte count in the perfusion circuit was used as an indirect indication to start the surgical recovery procedure. A count of 1×10^9 or lower was empirically considered as a satisfactory perfusion outcome. On average, elimination of leukocytes from the abdominal perfusion circuit required no more than 120 minutes, the time that is sufficient to complete legal paperwork and obtain the next-of-kin consent.

Although the perfusion procedures were initiated prior to the arrival of the forensic pathologist, the organ procurement procedures started only after completion of legal documentation, including the consent of next-of-kin. Laparotomy and kidney mobilization were performed and organ recovery commenced while the donor was still on the continuous extracorporeal perfusion. The perfusion procedure was terminated just before the surgical kidney explantation. Each kidney graft was placed in a separate plastic bag for subsequent static cold preservation in histidine-tryptophan-ketoglutarate (HTK) solution (Essential Pharmaceuticals, LLC, Durham, USA).

2.2. Kidney Biopsy Procedures. From 2011, Saint-Petersburg's Organ Procurement Center prospectively collects samples from brain dead donors and deceased donors enrolled throughout donation program. In this study, four kidney parenchyma specimens were collected: two after 35-45 minutes of WIT and two after 120 minutes of normothermic perfusion. Each parenchyma specimen was divided into 7 equal pieces, snap frozen, and placed at -70°C for storage.

2.3. RNA Extraction and Library Construction. Total RNA was extracted from tissue specimens using Trizol reagent (Fisher Scientific, Hampton, USA) according to manufacture instruction. RNA quality was confirmed with BioAnalyser and RNA 6000 Nano Kit (Agilent, Santa Clara, USA). PolyA fraction of RNA was purified with Dynabeads® mRNA Purification Kit (Fisher Scientific, Hampton, USA). Illumina library was made from polyA RNA with NEBNext® mRNA Library Prep Reagent Set (NEB, Ipswich, USA) according to manual. Sequencing was performed on HiSeq1500 with average 50 bp read length for 10 million reads generated for each sample.

2.4. Transcriptome Bioinformatic Analysis. Initial quality control of sequencing outputs was performed using FastQC. The raw reads were mapped to the hg19 using the CLC Genomics Workbench 6.0.64 with a mismatch cost of 2 and controlled through generating Mapping Reports for each sample. For all the genes both Reads Per Kilobase of transcript per Million (RPKM) and total reads count were calculated. For each of the four libraries, RNAseq procedures generated about 8 mln reads, approximately 85% of which were effectively mapped to hg19. For each sample, a total of 32 860 genes were annotated.

The correlation analysis of gene expression values in two kidneys was performed by Pearson's tests executed separately for comparisons of the donor-specific profiles collected before and after reperfusion. Only genes with the expression level of at least 0.01RPKM were taken into account. Person's correlation test was also used for comparing expression landscapes before and after reperfusion across two kidneys. p value and the correlation were calculated by python scipy package.

To identify differentially regulated genes in reperfusion cases compared with nonreperfusion samples, the test of Baggerly implemented in CLC Genomic Workbench was applied to the data [15]. To determine significantly expressed genes, t-tests on weighted expression proportions were used. Transcripts with fold change > 1.5 or < -1.5 (p value <0.05) were considered for further analysis as up- or downregulated between sampling conditions, respectively.

Genes detected as differentially expressed in both of studied kidneys were further explored using heatmap analysis; gene functions were interpreted using PANTHER toolkit Version 12.0 (http://www.pantherdb.org/tools).

Pathway Studio software (Elsevier, Rockville, MD) that is able to dynamically create and draw protein interaction networks and pathways was employed for building various networks and performing Gene Set Enrichment Analysis.

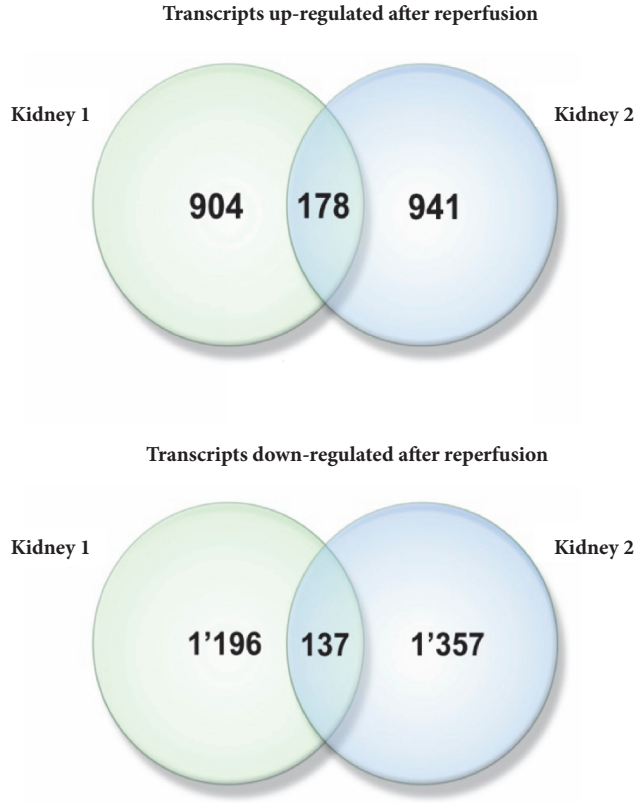


Figure 1: **Venn diagram of differentially expressed gene sets.** Venn diagram of gene sets differentially expressed after reperfusion in Kidney 1 and Kidney 2 and an intersection of gene sets unidirectionally overexpressed and downregulated after reperfusion in Kidney 1 and Kidney 2.

3. Results

3.1. Reperfusion Does Not Lead to Substantial Change in Overall Landscape of Kidney Transcription. For each pair of samples, correlation analyses were performed by taking into consideration all genes expressed at levels of at least 0.01 RPKM in each specimen. When paired statistical tests of the differential gene expression were performed for ischemic samples taken from two different kidneys, the Pearson's correlation of expression profiles between two samples was at R= 0.89 (p value < 2.2e-16). A substantial upregulation of the genes involved in detection of chemical stimuli, olfactory sensing, and ion binding, probably reflecting intrinsic differences in the functioning of Organic Anion Transporters (OATs) [16], was detected in Kidney 2. For two reperfused specimens, similarly calculated correlation was at R = 0.91 (p value < 2.2e-16). When ischemic and reperfused specimens collected from each of the kidneys were compared to each other, same-kidney samples correlated at R = 0.98, indicating that reperfusion does not lead to substantial change in overall transcription landscape.

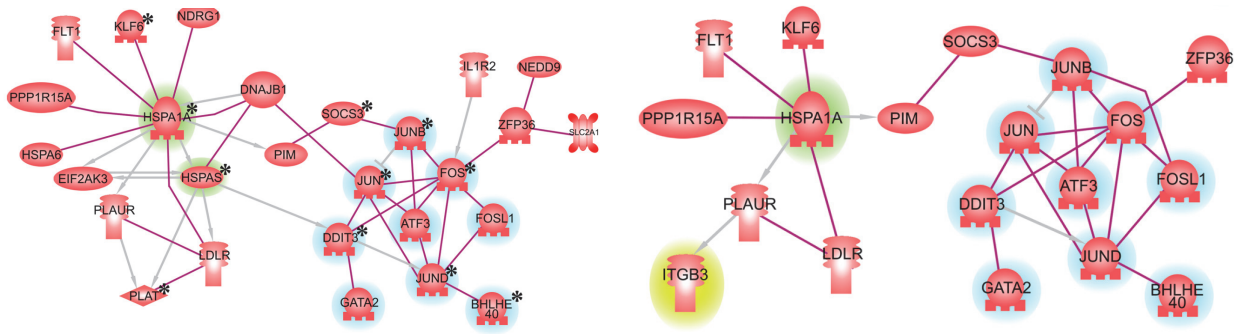
3.2. Reperfusion-Specific Transcription Program in Kidney Parenchyma. A comparison of ischemic and reperfused specimens collected from each of two kidneys yielded comparably sized sets of differentially expressed mRNAs. For Kidney 1, changes in expression levels with cut-off of 1.5 folds were detected for 2,415 genes (upregulated: N=1,082 genes; downregulated: N=1,333). For Kidney 2 at same cut-off, the list of differentially expressed transcripts included 2,613 mRNAs (upregulated: N=1,119 genes; downregulated: N=1,494). Venn diagrams reflecting the genes unidirectionally and significantly changing their expression levels in two sets of specimens are presented at Figure 1, with 178 commonly upregulated and 137 downregulated genes observed.

Common up- and downregulated genes were analyzed for relative representation of Gene Ontology (GO) terms by PANTHER. The distribution of GO functions revealed that most upregulated genes were predominantly involved in protein binding (92 genes, P< 0.0373), followed by fifteen different functional categories of genes encoding products with various types of DNA-binding activities (group p-values ranging from P < 0.00000147 to 0.0249). When all genes encoding DNA-binding proteins (N=24) were combined together, enrichment for this generalized category was detected at p< 0.000424. Remarkably, among the transcripts downregulated after the reperfusion, no enrichments for any functional category were detected, despite successful Ensemble ID-guided recognition of 75 out of 137 differentially expressed transcripts.

Reperfusion-upregulated genes encoding proteins known to bind other proteins were further explored with Pathway

Table 1: **Analysis of enrichment of the lists of differentially expressed genes with known targets of transcription factors present within the same list.** Columns correspond to four independent performed runs of enrichment analysis. In each instance, relative enrichments were calculated separately for each list of differentially expressed genes: upregulated ("UP"), downregulated ("DOWN"), or merged ("UP+DOWN").

Kidney 1		Kidney 2		Intersection of Kidney 1 and Kidney 2 datasets		Kidney 1 and Kidney 2 pooled together	
UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
N=62	N=41	N=87	N=13	N=40	N=3	N=15	N=0
P < 9.73e-06	NS	P < 1.65e-13	P <6.39e-10	P < 3.86e-22	NS	P < 2.99e-12	NS
UP+DOWN		UP+DOWN		UP+DOWN		UP+DOWN	
N=103		N=100		N=43		N=15	
P < 1.17e-3		P <1.15e-2		P < 3.12e-15		P < 7.66e-10	



(a) (b)

Figure 2: **Highly connected networks built using reperfusion-upregulated mRNAs coding for proteins known to interact with human proteins.** (a) Protein-Protein Interaction Network (PPIN) generated using the intersection of mRNAs sets unidirectionally overexpressed after reperfusion in Kidney 1 and Kidney 2. (b) PPIN generated using set of mRNAs detected to be overexpressed after comparing combined amounts of reads from both kidneys before and after reperfusion. Network was generated by Pathway Studio Network Building. Each node represents a protein. Network hubs are highlighted in blue (Jun/Fos/ATF hub) or green (HSP1A/ HSPA5 hub). In Figure 2(a), stars indicate the targets of transcription factors overexpressed in reperfusion. In Figure 2(b), protein ITGB3 is highlighted in yellow as the only entity added to the network as a result of read combining.

Studio Network Building tool set to display only direct binding connectivity. This analysis revealed that 27 out of 92 (29.3%) proteins form tightly knit network (Figure 2); three proteins (GADD45B, GADD45G, and CDKN2A) form an interacting triplet, and HES1/HES6 and S100A8/S100A9 form interacting duplets, while the rest of the upregulated genes (N = 58) remained unconnected. Remarkably, highly connected protein network (Figure 2) included two prominent hubs, which were centered on Jun/Fos/ATF transcription factors and HSP1A/ HSPA5 heat shock proteins.

The cut-off for enrichments analysis of GO Biological Processes associated with genes upregulated in reperfusion was selected at 9.99e-05. This analysis highlighted 24 protein entries broadly belonging to following categories: regulation of cell death (3 entries with p values ranging from 8.46E-07 to 4.75e-05); response to various external stimuli including bacteria and bacterial components and lipids and oxygen-containing compounds (8 entries with p values ranging from 2.41e-06 to 6.85e-05); and the regulation of metabolism in a broad sense, with a total of 13 entries, with positive regulation of nitrogen compound metabolic processes being highlighted by lowest detected p value of 7.15E-07.

As an additional control, same set of analyses were performed after joining two sets of samples. In this comparison, reads obtained for Kidney 1 and Kidney 2 were combined and compared to similarly combined gene expression values at reperfusion. This experiment affirmed the confidence in detection of genes upregulated after reperfusion, while providing a different set of downregulated transcripts (not shown). Thirty out of 43 reperfusion-upregulated mRNAs were also detected as mRNAs with unidirectionally and significantly changed expression levels in two sets of specimens analyzed separately (Table 1). Remarkably, 11 out of 30 most confidently detected genes were also present in the network formed by proteins directly binding each other (Figure 2(b)), with the most central nodes being preserved to larger degree than the dangling nodes. Only one additional protein, ITGB3, was added to the network as a result of combining the reads obtained from both kidneys.

3.3. Reperfusion-Specific Proinflammatory Expression Program Is a Subject of Positive Feedback Regulation. Separately, an analysis of mRNA targets for 24 DNA-binding proteins commonly upregulated after reperfusion was performed.

Known validated targets of these transcription factors, including 18 protein complexes and 685 individual proteins or miRNAs, were pulled from the database hosted by Pathway Studio. Further downstream functional analysis of these targets showed substantial enrichments in the following Pathway Studio Ontologies: inflammatory cytokines (P<7.08E-19), extracellular matrix degradation proteins in general (7.66e-15), and matrix metalloproteinases in particular (2.66e-14), oncogenes (2.68e-14), adipokines (3.12e-10), lymphokines (5.18e-10), extracellular matrix polymerization proteins (6.52e-10), transcription factors of C/EBP family (1.31e-09), tumor suppressors (1.16e-08), and CSF-1/PDGF receptor family signals (1.89-e08). Importantly, target genes encoding proteins BHLHE40, DDIT3, FOS, HSPA1A, HSPA5, JUN, JUNB, JUND, KLF6, SOCS3, and PLAT were also mapped to highly connected networks built using reperfusion-upregulated mRNAs (Figure 2(a)). This observation possibly indicates positive feedback regulation of reperfusion program. Notably, a majority of these proteins (9 out of 10) were also preserved in highly connected network independently generated using the list reperfusion overexpressed genes detected after combining the read counts obtained for both kidneys.

Further functional analysis of 43 downstream targets, which, indeed, changed their expression levels in both kidneys (Table 1) revealed an enrichment in the following Pathway Studio Ontologies: soluble protein (P<1.66E-22), biofluids assayable substances (1.06e-11), a range of Jun/Fos related subnetworks (p values from 2.99e-10 to 1.14 e-4), Hairy/E(SPL)/Orange domain proteins (1.67e-8), and calprotectin (4.58e-7). A summary analysis of biological functions associated with these molecules showed an abundance of inflammation-related regulatory subnetworks, including cortisol in resolving inflammation ($p < 4.08$ e-4), AXL receptor inhibiting macrophages and dendritic cell function ($p < 5.89$ e-4), mast cell activation without degranulation through CXCR4 signaling ($p < 1.11$ e-3), ER stress (unfolded protein response) ($p < 1.78$e-3), and others.

3.4. Reperfusion-Specific Changes Expression of Noncoding RNAs in Kidney Parenchyma. A number of noncoding RNA transcripts unidirectionally and significantly changed their expression levels in both reperfused kidneys. Among upregulated and downregulated transcripts, there were 23.6% (42/178) and 40.1% (55/137) RNAs classified as noncoding, respectively. Additionally, in both kidneys, five noncoding transcripts were completely suppressed, while one coding and one noncoding transcript were awakened after reperfusion (Figure 3).

These transcripts were analyzed for correlations of their expression patterns in 30 human tissues with various functional groups of coding transcripts as described in [17]. For "switched-on" lncRNA RN7SL32P, top coexpression units were related to inflammatory and interferon responses and IL6/JAK/STAT3 signaling as well as allograft rejection ($p <$ 4e-07 for each functional category). Another "switched-on" lncRNA, RP11-297M9.1, showed coexpression with various sets of genes associated with ciliary or bacterial-type flagellar motility (p values ranging from 2.5e-6 to 0.03). Importantly, lncRNA RP11-297M9.1 locates in the 3' area of protein-coding gene *GRIN2A*, whose expression increases immediately after onset of stroke [18] and being highlighted in many other publications on ischemia/reperfusion models [19–21].

Among five commonly "switched-off" RNAs, three (AC073321.3, RP11-525J21.1, and MIR1302-9) were predominantly coexpressed with genes involved in various aspects of spermatogenesis, one (RP11-659G9.3) with olfactory genes, and one (RP11-142L4.2 pseudogene) with a variety of cellular programs, including unfolded protein response and MTORC1 signaling as well as the cell cycle and its checkpoints.

4. Discussion

Ischemia-reperfusion injury (IRI) in transplanted organs has been a subject of many studies performed both in animal models [22–24] and in human grafts [25], with numerous biomarkers of IRI identified in blood, serum, plasma, urine, and kidney biopsies [26, 27]. From early studies of single transcripts by RT-PCR to the emergence of microarrays and, recently, RNAseq assessments of RNA profiles, the studies of expression landscapes provided a window into overall understanding of IRI in the transplanted kidneys [28–31]. In this study, we present the results of RNAseq profiling of human kidneys undergoing circulatory death-related warm ischemia (35-45 min) with subsequent extracorporeal perfusion in situ for 120 min [11].

We showed that reperfusion does not lead to substantial change in overall landscape of kidney transcription but rather activates specific program resulting in overexpression of highly connected protein network with two hubs centered on Jun/Fos/ATF transcription factors and stress response/heat shock HSP1A/HSPA5 proteins. These results align well with early observations on the role of the AP-1 dependent stress response in propagation of reperfusion injury [32, 33]. The induction of HSP proteins is a highly conserved response that protects human tissues, including renal parenchyma, from diverse physiological and environmental stressors by assisting in the refolding of denatured proteins and degradation of irreparably damaged proteins [33]. Our study indicates that the heat shock response is tightly linked to activation of the transcription factors binding to AP-1 sites. Importantly, two similarly looking HSP/AP-1 centered networks have been built independently (Figure 2(b)), indicating robustness of this finding.

We also performed an analysis of mRNA targets for 24 DNA-binding proteins commonly upregulated after reperfusion. As many validated targets of the reperfusion-upregulated transcription factors are also present in the general list of upregulated genes, we conclude that reperfusion-specific, proinflammatory expression program may be regulated by a positive feedback loop. Importantly, evidences of positive feedback regulation are seen for both nodes, namely, the heat shock response, which is generally protective against injury [33, 34], and stress-induced AP-1-dependent transcription program which may, depending on context, either contribute to injury or help in alleviation by modulating the

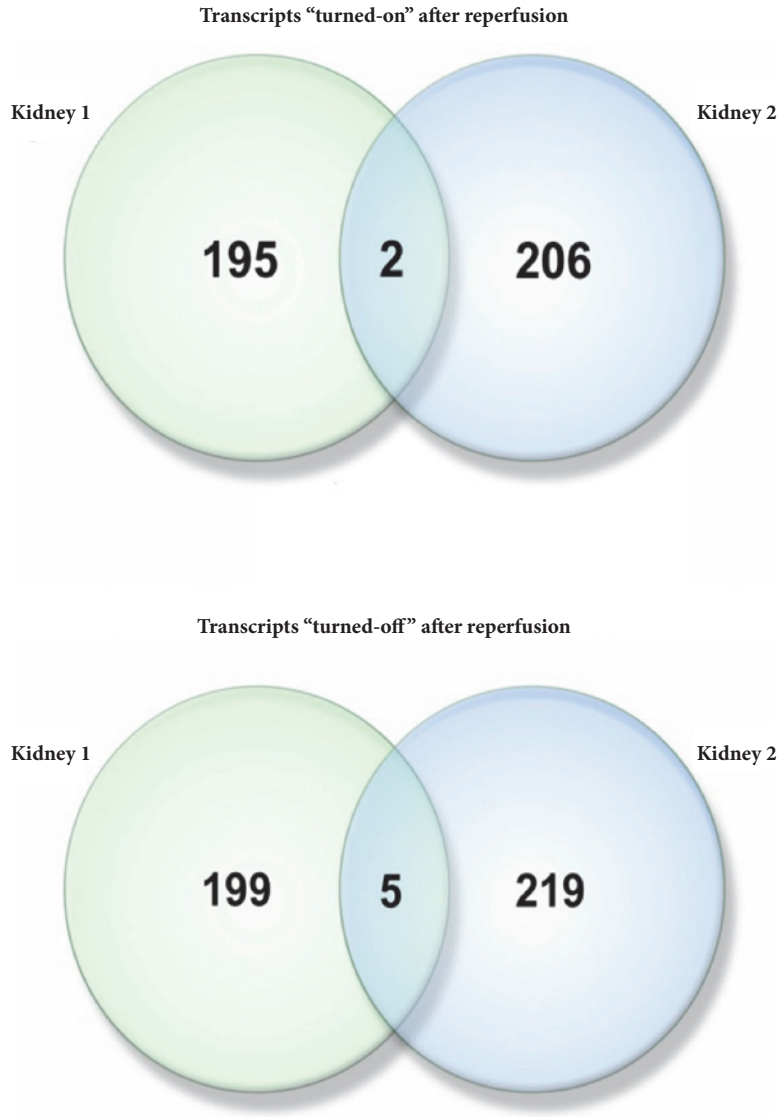


Figure 3: Venn diagram of gene sets awakened or silenced after reperfusion in Kidney 1 and Kidney 2 and an intersection of gene sets which changed their expression after reperfusion from nondetectable or to nondetectable.

activation of different immune cells and control cytokine expression at multiple levels [35].

In particular case of transplanted kidneys of study, the damage alleviating properties of JUN/JUNB/FOS/ATF3 network seem to prevail. The network presented at Figure 2 includes both RNA binding anti-inflammatory protein tristetraprolin, encoded by the ZFP36 gene [36] and IL-1R2, the decoy regulator of the IL-1 signaling [37]. Importantly, in context of reperfusion, the outcomes of AP-1 signaling are tied to relative expression of heat shock proteins, one of key contributors to long-term transplantation outcomes (Figure 2).

Mapping of injury-promoting and stress-protecting molecules changing their expression levels in ischemic and reperfused kidneys provides a rationale for possible organ conditioning during machine assisted ex vivo normothermic perfusion. For example, the levels of protection provided by Hsp70-like proteins could be augmented by several well-tolerated pharmacologic agents, including aspirin and geranylgeranylacetone [38], while the genes encoding proinflammatory molecules may by suppressed by siRNA-based gene-targeting approaches [39]. All types of small therapeutic molecules may be added directly to the organ preservation solution, thereby circumventing the need for injection into the bloodstream. Moreover, as the graft may be thoroughly rinsed before transplantation, the remaining cell-free siRNA and other therapeutic molecules could be removed, thus avoiding any off-target effects or systemic toxicity.

Another interesting observation made during this study was that baseline gene expression patterns in ischemic grafts may display substantial intrinsic differences. For example, baseline ischemic induction of the genes involved in detection of chemical stimuli, olfactory sensing, and ion binding

was detected in Kidney 2, probably reflecting natural history of particular donor and/or intrinsic differences in the functioning of Organic Anion Transporters (OATs) [16, 40]. In Kidney 2, expression levels of OATs-encoding genes *SLC22A6* and *SLC22A8* were much higher than in Kidney 1. These OATs mediate the renal absorption and excretion of a wide range of metabolites and xenobiotics and involve elimination of uremic toxins, in particular, indoxyl sulfate, the molecular circumstance which may be relevant to subsequent functioning of the organ in the body of the recipient. Molecular subtyping of donor organs may possibly lead to the development of personalized approaches to the therapy of isolated organs within normothermic perfusion contours with individualized graft-conditioning cocktails.

5. Conclusion

This is the first study to profile gene expression and resultant molecular networks in kidney grafts from human donors with critically expanded warm ischemia time (WIT) before and after being reperfused in situ. Albeight very small, this study opens up a number of important lines for follow-on investigation. In particular, a study of natural diversity of the transcriptional landscapes in presumably normal, transplantation-suitable human organs is warranted. Additionally, as transplantation outcomes may be influenced by summarily outputs of the networks formed both by protective and by injury-promoting molecules, larger transcriptome-based studies of donors organs should be performed, and the resultant networks correlated with short- and long-term clinical outcomes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The data described in this manuscript would be also presented at BGRS\SB-2018 biannual meeting which will take place in Novosibirsk, Russia, on August 21-27, 2018.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Alexandr Reznik and Olga Plotnikova contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplementary 1. Supplementary Table 1. RNA sequencing data: results of the read mapping.

Supplementary 2. Supplementary Table 2. The lists of genes identified as upregulated in reperfused samples.

Supplementary 3. Supplementary Table 3. The lists of genes identified as downregulated in reperfused samples.

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Research Article

Early Graft Loss after Kidney Transplantation: Endothelial Dysfunction of Renal Microvasculature

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Decision process about the acceptance of the deceased donor kidney for transplantation might be challenging. Although histological evaluation of pretransplant donor kidney biopsy provides reliable information regarding cortical necrosis, vascular thrombosis, extensive global glomerulosclerosis, and interstitial fibrosis/tubular atrophy, only electron microscopy enables thorough and reliable insights into microvasculature changes of kidney graft. The aim of the present paper is to briefly present two cases of early kidney graft loss. In one case, the donor was exposed to long-term extracorporeal membrane oxygenation (ECMO); in the other case, the donor experienced Takotsubo cardiomyopathy. In both cases, light microscopy of pretransplant biopsy found no pathology or significant discrepancy in morphology of kidney graft, while electron microscopy revealed severe endothelial dysfunction of renal microvasculature. Our results suggest that severe injury of renal microvasculature with relatively preserved tubular epithelium may be associated with some conditions of deceased kidney donors leading to early kidney graft nonfunction and loss. Further studies are needed to determine prognostic significance of severe ultrastructural microvasculature lesions and to evaluate disease states and conditions that could be associated with severe endothelial dysfunction of kidney graft.

1. Introduction

Since 1950s, when the first renal transplantation was performed, continuous progress in the replacement therapy has resulted in improved kidney transplant outcomes. Long waiting lists for renal transplants and shortage of living and standard criteria deceased donors have led to the expansion of donor pool, involving an increasing number of suboptimal and marginal donors [1]. Studies have shown that the use of kidneys from expanded criteria deceased donors can be associated with worse long-term graft outcomes in some cases and may increase the risk of early graft loss [2, 3]. A few retrospective and prospective studies have examined various clinical and histological parameters for their usability to predict the performance of kidneys after transplantation, but none of them are standardized or widely applied [1, 4–8]. Therefore, the decision process to accept or reject suboptimal kidney is complex and challenging.

To help judge the quality and to avoid unacceptably high discard rates of deceased donor organs, pretransplant kidney biopsy is recommended [9]. In our center, pretransplant kidney biopsies are routinely performed. However, due to the time consuming procedures needed for preparation of permanent tissue samples for light and electron microscopy, results are usually available after transplantation. In urgent cases, when clinicians are faced with difficult decision and biopsy evaluation is needed before a graft is accepted for transplantation, histologic analysis is performed on frozen donor kidney sections.

According to our experience, both light and electron microscopies of pretransplant biopsy offer valuable information on graft quality. Light microscopy offers an insight into organ pathology, including acute cortical necrosis, acute tubular injury, arterial/ arteriolar thrombosis, and chronic lesions (like percentage of glomerulosclerosis, interstitial fibrosis and tubular atrophy, and arteriolosclerosis), while

electron microscopy provides insights into the ultrastructural changes that cannot be appropriately evaluated by light microscopy, such as basement membranes, microvasculature endothelium (endothelial cells in glomeruli, peritubular capillaries, and arteriole/small arteries), or cellular structures of renal epithelium. Such assessment (morphological and ultrastructural) of a kidney graft enables precise evaluation of the quality of the kidney graft and accurate differentiation of the preexisting changes from those arising after transplantation. In addition, it enables critical evaluation of observed ultrastructural alterations of the kidney graft in cases of early graft loss.

Recently, we were faced with two cases of early graft loss. Although histologic evaluations of pretransplant biopsies were promising in both cases, subsequent electron microscopy revealed severe endothelial dysfunction of renal microvasculature. It is tempting to speculate that the injury of renal microvasculature on the pretransplant renal biopsy might be associated with early kidney graft nonfunction and loss. Therefore, the aim of the present paper is to demonstrate our recent findings and to briefly discuss potential risk factors associated with endothelial dysfunction of exposed kidney grafts.

2. Endothelial Dysfunction of Renal Microvasculature and Early Graft Loss

Endothelial dysfunction, more appropriately considered as endothelial activation, represents a switch from a quiescent phenotype toward phenotype showing host defense response. Endothelial cells can lose integrity, progress to senescence, and detach into the circulation [10].

On transmission electron microscopy, activated glomerular endothelial cells are swollen, with extensive microvillus transformation of endothelial cell membrane, and loss of fenestration. In severe injury, endothelial cells may rupture leading to loss of intracellular content/debris into capillary lumen followed by detaching and loss of endothelial cells (personal observation).

To get an insight into the endothelial cell alterations a blinded light and electron microscopy analysis was performed on 50 consecutive pretransplant biopsies obtained from deceased donors after brain death. Tubular injury was assessed according to preservation of tubular epithelial cells, preservation of apical brush border evaluated in periodic acid Schiff (PAS) stain, and estimated percent of vacuolization in tubular epithelial cells.

By light microscopy, glomeruli in all biopsies appeared unremarkable (Figure 1(a)-PAS). Chloroacetate esterase (CAE) stain revealed up to one neutrophil (0-1, average 0.4) in glomeruli of 48/50 biopsies (Figure 1(a)-CAE). None of pretransplant biopsies showed glomerular or vascular thrombotic microangiopathy. In two biopsies, light microscopy showed unremarkable glomeruli with increased intraglomerular neutrophils, up to 6 per glomerulus (1-6, average 3) in the first (Figure 1(b)-PAS, CAE) and up to 2 per glomerulus (0-2, average 0.8) in the second case (Figure 1(c)-PAS, CAE). All examined biopsies revealed similar mild to moderate acute tubular injury without significant deviations (Figures 1(a), 1(b), and 1(c)-PAS).

Interestingly, ultrastructural examination showed similar features in 48/50 cases. Glomerular endothelial cells showed preserved fenestration with scattered endothelial projections (microvillus transformation of cell membrane). Focal mild endothelial cell swelling was observed in some cases with no or little intracellular debris in glomerular capillary lumens and no detached endothelial cells (Figure 2(a)). Endothelial cells in arterioles/small arteries appeared unremarkable or slightly swollen (Figure 2(b)). Peritubular capillaries had intact monolayer of basement membrane and were covered with normal delicate endothelial cells without swelling or detachment (Figure 2(c)). Tubular epithelial cells were attenuated with thinning of apical brush border, but showing intact desmosomes (Figure 2(d)). In all 48 cases there was no graft loss in 6-month follow-up.

In contrast, 2/50 cases with increased intraglomerular neutrophil granulocytes showed significant and severe ultrastructural changes of renal microvasculature without significant changes in tubular epithelium (Figures 3(a) and 4(c)).

Changes in glomerular endothelial cells looked similar in both cases (Figures 3(b) and 4(a)). There were loss of fenestration and extensive swelling of glomerular endothelial cells, which filled the lumen of glomerular capillaries. A lot of endothelial cells were detached from the glomerular basal membrane and peeled off in the lumens. Extracellular debris in the glomerular capillary lumens probably originated from ruptured endothelial cells. In both cases endothelial cells of the peritubular capillaries were swollen and formed endothelial projections (microvilli) (Figures 3(c) and 2(b)). In the first case (deceased donor exposed to ECMO), small arteries/arterioles showed completely detached and shrunken endothelial cells (Figure 3(d)), whereas in the second case (deceased donor exposed to Takotsubo cardiomyopathy) endothelial cells in arterioles were swollen and only partly detached (Figure 4(d)).

Interestingly, tubular epithelium showed only minimal changes with slightly decreased brush border similar to other 48 cases (changes very likely related to cold ischemia). Desmosomes in tubular epithelium were preserved (Figures 3(a) and 4(c)). Nevertheless, in both described cases serious (post)operative complications followed. To better understand the whole situation, brief history of both cases is presented.

2.1. Clinical History from Deceased Donors to Early Graft Loss Outcome. In the first case (Figures 1(b) and 3), the donor of both kidneys experienced cardiac arrest with successful resuscitation; the underlying condition was acute myocardial infarction. Due to cardiorespiratory failure, the donor was supported with ECMO as a life bridge for 4 days. After brain death establishment kidneys were offered for donation. Importantly, the donor had normal urine output and unremarkable markers of kidney function and morphology. There were 3 mismatches (1, 1, 1). Recipient (who had no recognized coagulation abnormalities) experienced diffuse subcapsular hemorrhage with multifocal blood leakage through the capsule immediately after completion of

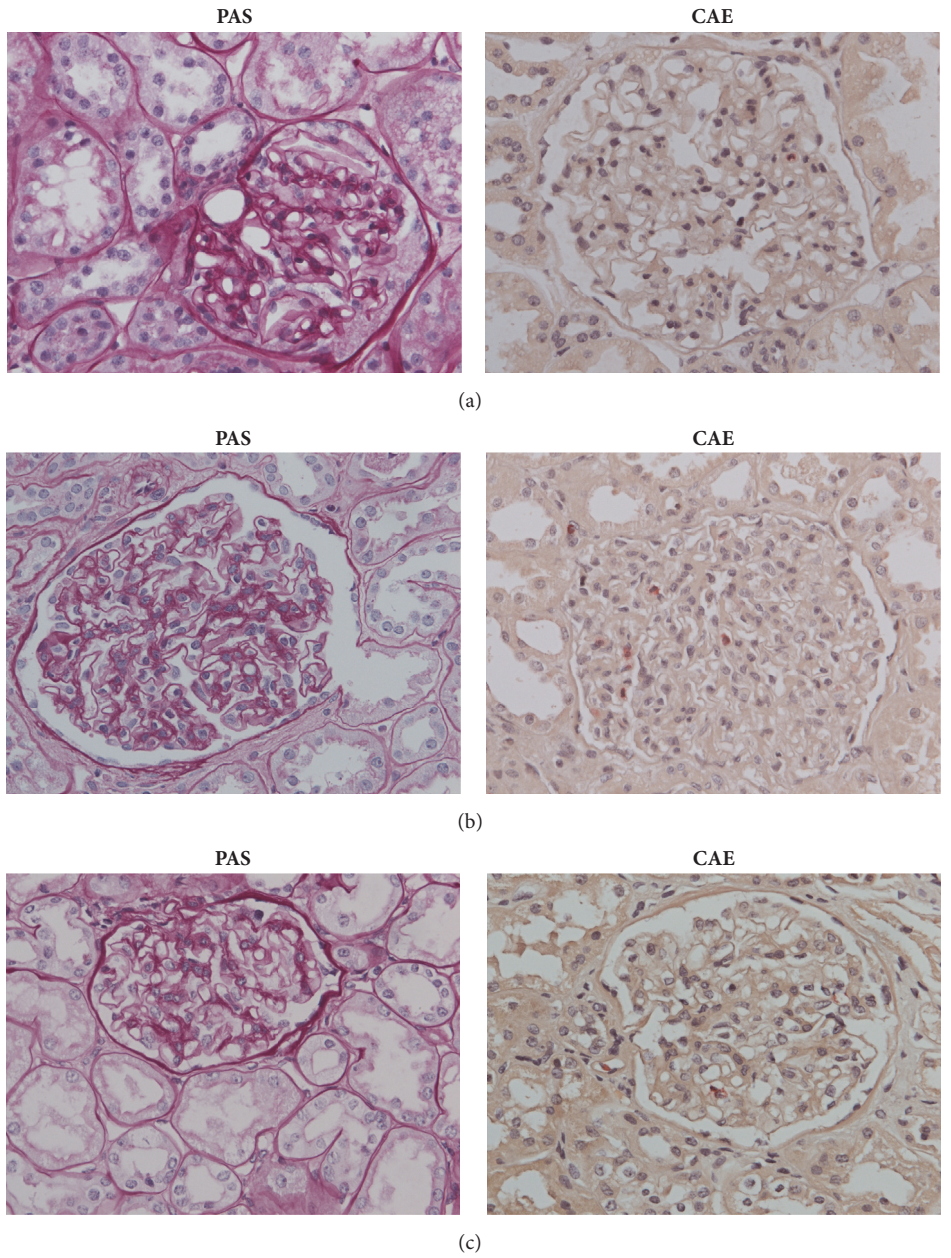

Figure 1: **Light microscopy of kidney graft biopsy:** magnification 400x; periodic acid Schiff (PAS) stain; chloroacetate esterase (CAE) stain. **(a) Biopsy from average deceased donor.** PAS: unremarkable glomerulus and mild acute tubular injury: proximal tubules show epithelial attenuation and focal vacuolization. The lumens appear dilated due to thinning of apical cytoplasm and focal loss of apical brush borders. CAE: in some glomeruli, there is up to one neutrophil (0-1, average 0.4). **(b) Biopsy from deceased donor exposed to ECMO.** PAS: mild acute tubular injury, similar to average preimplantation biopsy. Light microscopy showed unremarkable glomerulus with some neutrophil granulocytes. CAE: special stain revealed intraglomerular neutrophils, up to 6/ glomerulus (1-6, average 3). Sparse neutrophils are also in peritubular capillaries. In small artery above glomerulus, endothelial cells appear shrunken and detached. **(c) Biopsy from deceased donor exposed to Takatsubo cardiomyopathy.** PAS: mild acute tubular injury, similar to average preimplantation biopsy. Light microscopy shows unremarkable glomerulus. CAE: special stain revealed intraglomerular neutrophils, up to 2/ glomerulus (0-2, average 0.8). Sparse neutrophils are also in arterioles and peritubular capillaries.

the vascular anastomosis of the transplanted kidney. Urgent nephrectomy and implantation of the second kidney of the same donor were performed. Two days after implantation the second kidney transplant was also explanted due to large subcapsular and perirenal hematoma leading to hemorrhagic shock. The patient remained dialysis dependent. No signs of antibody-mediated rejection according to Banff criteria (no glomerulitis or peritubular capillaritis, negative C4d along peritubular capillaries) or thrombotic microangiopathy (no fibrinoid necrosis or fibrin/fibrinogen deposits on

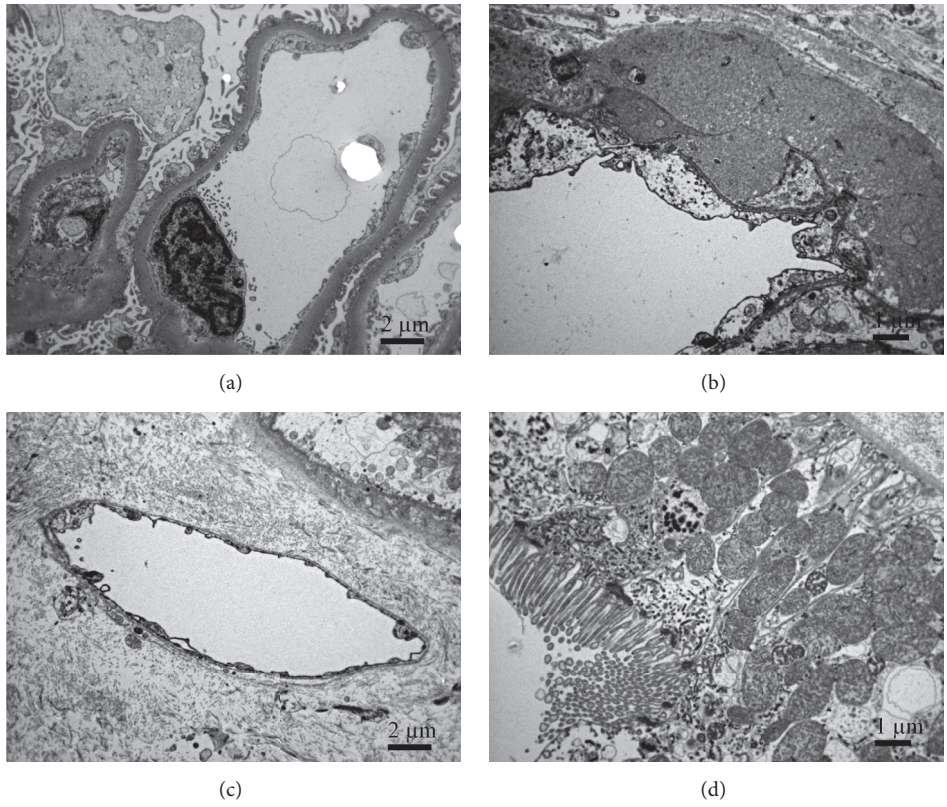

Figure 2: **Ultrastructure of average preimplantation kidney biopsy from deceased donor. (a)** Normal endothelial cell with fenestrated endothelium covers glomerular capillary lumen. Cytoplasmic membrane of the endothelial cell forms scattered endothelial projections (microvilli). **(b)** The normal endothelial cells in arteriole. Beneath endothelial cells, there is transmural hyalinosis indicating arterial hypertensive disease in the donor. **(c)** Normal endothelium in peritubular capillaries. **(d)** Slightly attenuated but preserved tubular epithelial cells with desmosomes. Apical brush border is slightly thinner.

immunofluorescence, no typical thrombotic microangiopathy signs on electron microscopy) were found in explanted kidneys.

In the second case (Figures 1(c) and 4) the donor was admitted to intensive care unit due to massive subarachnoid and intracerebral hemorrhage, the diagnoses reported as the cause of death. The donor was also reported to have Takotsubo stress cardiomyopathy presenting with diastolic dysfunction and markedly decreased systolic function. At day 3, brain death was established, and one of the kidneys allocated to recipient in our center. The donor was reported to be hemodynamically stable, with normal urine output and unremarkable markers of kidney function and morphology. The number of HLA mismatches was 1 (0, 0, 1). The recipient experienced hemodynamic instability due to subcapsular hematoma, which was recognized few hours after transplantation. Due to primary nonfunction, the recipient needed immediate dialysis. Kidney graft was explanted 3 months after transplantation due to end stage failure.

Transplant kidney biopsy performed 4 days after transplantation revealed glomerular capillary thrombosis and arterial fibrinoid necrosis consistent with acute glomerular and vascular thrombotic microangiopathy, changes not observed in the pretransplant biopsy. Repeated donor specific antibody measurements were negative and there were no other histologic characteristics of antibody-mediated rejection. It was supposed that recipient could have recurrent aHUS, which was confirmed by recipient's complement alternative pathway dysregulation (decreased factor I and C3, elevated urine c5b9).

The time range of cold ischemia storage in 48/50 grafts was 7h21 min-29h25 min, in deceased donor exposed to ECMO the time of cold ischemia was 16h22 min, and in deceased donor exposed to Takotsubo cardiomyopathy it was 9h19 min.

3. Potential Risk Factors of Kidney Graft Endothelial Dysfunction in Both Described Cases

It is known that endothelial dysfunction/activation can be associated with numerous factors, for instance, risk factors originated in the donors (such are hypertension and shear stress, age, inflammation, diabetes-associated factors, and atherosclerosis), transplantation procedures (ischemia-reperfusion), or associated with the recipient (immunologic and/or pathophysiologic (miss)match) [11]. However,

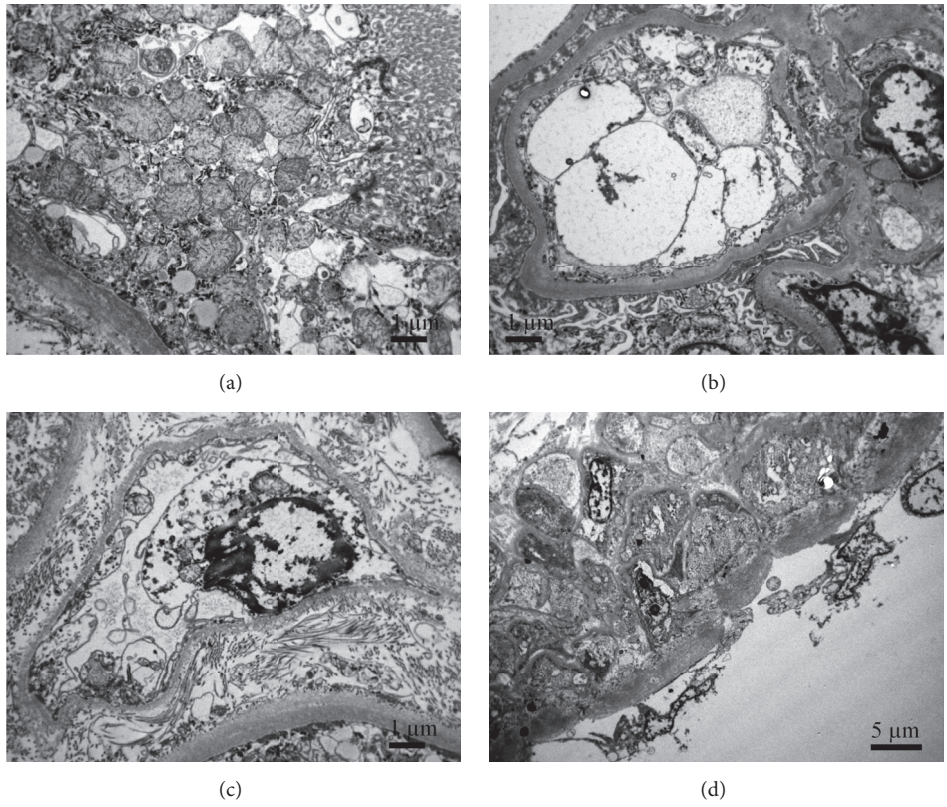

Figure 3: **Ultrastructure of preimplantation kidney biopsy from deceased donor exposed to ECMO. (a)** Tubular epithelial cells are attenuated but preserved showing intact desmosomes. Apical brush border is slightly attenuated, similar to all other deceased kidney biopsies. **(b)** Glomerular endothelial cells are partly detached from glomerular basement membrane, swollen and disintegrated. Swollen endothelial cell, extracellular debris, and numerous microvilli fill the lumen of glomerular capillaries. **(c)** Endothelial cells in peritubular capillary are swollen and the cytoplasmic membrane forms endothelial projections (microvilli). **(d)** Endothelial cells in small artery are completely detached and shrunken.

in deceased donors after brain death (DBD) endothelial dysfunction of kidney transplants due to ischemia and reperfusion, as well as injury associated with time lag from brain death, is almost universal [12].

Therefore, extremely severe and discrepant endothelial injury observed in the two above described kidney graft donors was very unlikely associated with »traditional« risk factors. Discussion on these topics could, however, be extremely broad and exceed the range of this article. Therefore, we would like to stress out and briefly discuss only factors that clearly stood out from other 48 cases and may be importantly associated with the graft outcome. According to the history of the donors two outstanding clinical peculiarities are noticed: in the first case exposure of the donor to ECMO after massive myocardial infarction with successful resuscitation and in the second case donor's experience with Takotsubo cardiomyopathy.

3.1. Extracorporeal Membrane Oxygenation. Extracorporeal membrane oxygenation (ECMO) is used as a standard therapy in critical care for patients with cardiorespiratory failure and nowadays also to manage potential organ donors following cardiopulmonary failure [13, 14]. It is already known that patients on ECMO are at risk of developing acute kidney injury or a systemic inflammatory response syndrome with multiorgan dysfunction [15, 16]. Underlying mechanisms include impaired microcirculatory perfusion [17], gut barrier dysfunction leading to a rise in circulating bacteria products [18], and marked increase in cytokines such as TNFα and IL-8, [19] which altogether affect vascular endothelium. The generation of inflammatory mediators results in the widespread activation of the endothelium. Activated endothelial cells in turn increase their expression of adhesion molecules, leading to the increased transmigration of activated neutrophils [15]. It was proposed that neutrophil infiltration (observed also in our case, Figure 1(b)) may be responsible for the end-organ damage associated with ECMO [19].

The outcomes of DBD kidney transplants procured from donors on ECMO have been shown to be comparable to those from non-ECMO donors [20]. However, there are number of possible ECMO-related complications that may have detrimental effect on organs. They are likely dependent on the type of ECMO (i.e., venovenous (pulsatile circulation) or venoarterial (mostly nonpulsatile circulation)) and very likely also on its duration [14]. Importantly, when ECMO is used as a bridge to transplantation, the donor is usually supported by ECMO only for a short time (for instance, in

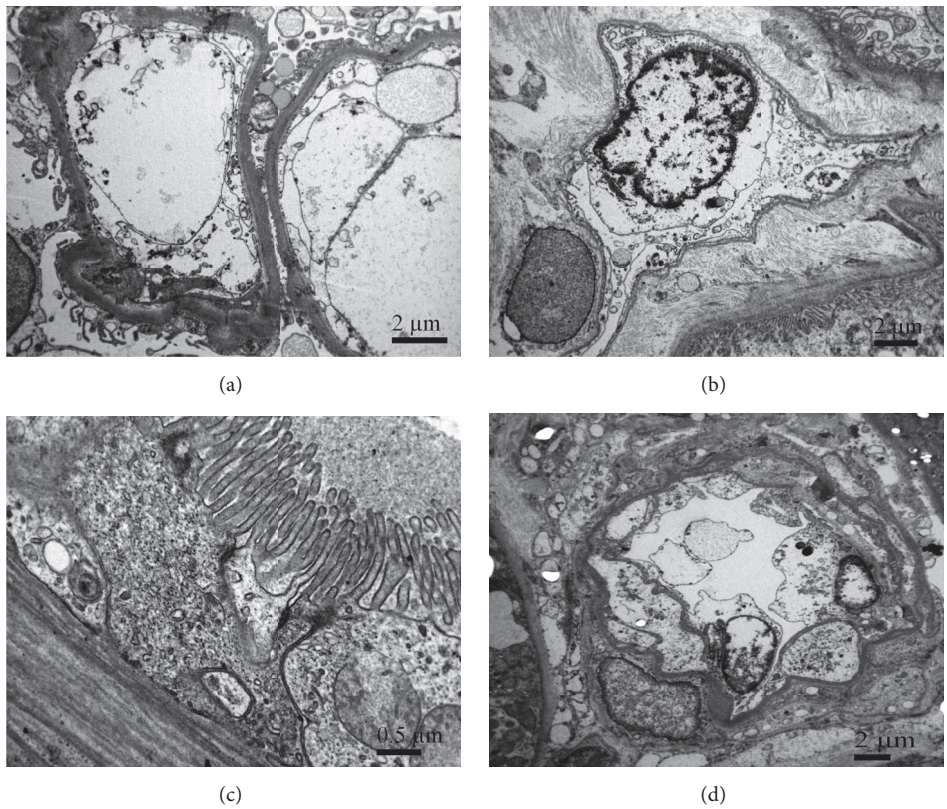

Figure 4: **Ultrastructure of preimplantation kidney biopsy from deceased donor exposed to Takotsubo cardiomyopathy (TCM). (a)** Glomerular endothelial cells are swollen, disintegrated, and partly detached from glomerular basement membrane. Swollen endothelial cell and extracellular debris fill the lumen of glomerular capillary. **(b)** Swollen endothelial cell in peritubular capillary. Their **c**ytoplasmic membrane forms endothelial projections (microvilli). **(c)** Tubular epithelial cells are preserved but attenuated. Desmosomes are clearly seen. Apical brush border is thinner. **(d)** Arteriole with swollen and partly detached endothelial cells.

the study of Carter et al. [20] the average ECMO duration was 87 minutes). In contrast, when ECMO is used as a life bridge, patients are exposed to ECMO for days or weeks. According to all above-mentioned risk factors of ECMO, the time of exposure to ECMO is very likely associated with the ECMO-related complications, including endothelial dysfunction. However, to the best of our knowledge, the impact of long-term effect of ECMO on ultrastructure of endothelial cells of donated organs has not been explored yet. Nevertheless, our results suggest that 4 days of ECMO exposure in patient with cardiac arrest may lead to severe endothelial dysfunction.

3.2. Takotsubo Stress Cardiomyopathy and Myocardial Stunning. Takotsubo stress cardiomyopathy occurs in the setting of a severe mental or physical stressor, predominantly affecting postmenopausal females, and in the absence of obstructive coronary artery disease. Typically, there is transient reduction in left ventricular systolic function with specific regional wall motion abnormalities [21]. Although the underlying mechanisms are still not known, recent findings indicate that Takotsubo cardiomyopathy can cause chronically impaired peripheral vascular reactivity, including impaired peripheral endothelium-dependent vasodilation, excessive vasoconstriction, and augmented sympathetic activation [22]. The only mechanism known by cardiologists to cause similar transient left ventricular dysfunction as Takotsubo is myocardial stunning [23, 24] which is usually related to transient coronary occlusion and postresuscitation period after the restoration of spontaneous circulation [25].

The results show that while myocardial function has already recovered after acute insult, endothelial cells are more severely impaired than smooth muscle cells, and that this injury persists beyond myocardial stunning. Thus, endothelial-dependent dysfunction can still impair vasodilatation, while ventricular dysfunction is successfully mechanically supported or has already resolved [26]. This foundation refers to coronary endothelium. It would be certainly interesting to explore whether the kidney endothelium responds the same way in such pathophysiologic instances.

4. Conclusions

Our observations show that severe endothelial injury of kidney graft microvasculature seen only on ultrastructural level may occur before any significant injury is observed on pathohistological level in preimplantation kidney graft

biopsies. These extreme injuries to microvasculature, which are associated with early graft failure, could be promoted by some specific conditions in donor and/or recipient such as long-term ECMO employment, postresuscitation myocardial stunning, or Takotsubo cardiomyopathy. Further studies are needed to determine prognostic significance of severe ultrastructural microvasculature lesions and to evaluate other disease states and conditions that could be associated with severe endothelial dysfunction of kidney allograft.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that no financial interest or conflicts of interest exist.

Authors' Contributions

N. Kojc and Ž. Večerić-Haler contributed equally to this work.

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Research Article

Impact of Hypothermia and Oxygen Deprivation on the Cytoskeleton in Organ Preservation Models

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Ischemia reperfusion (IR) lesions are an unavoidable consequence of organ transplantation. Researching new therapeutics against these lesions requires the definition of early mechanisms. The cytoskeleton is composed of 3 types of filaments: microfilaments, intermediate filaments, and microtubules. We aimed to characterize the influence of preservation on their phenotype. In an *in vitro* model using primary human endothelial cells reproducing the conditions of organ preservation, two aspects were explored: (a) the impact of IR and cold ischemia time on each filament type, evaluating the roles of temperature, solution, and oxygen; and (b) the potential of cytoskeleton-mediated therapy to alleviate cell death. Results showed that intermediary filaments were unaffected, while microfilaments showed radical changes with disappearance of the structure replaced by a disorganized array of nodules; moreover, microtubules almost completely disappeared with time. Furthermore, temperature, and not oxygen deprivation or the solution, was the determining factor of the cytoskeleton's loss of integrity during preservation. Finally, pharmaceutical intervention could indeed preserve fiber structure but did not alter survival. Our work shows that improvement of preservation must include a more adapted temperature before considering oxygen, as it could profoundly improve cytoskeleton organization and thus cell fate. This highlights the importance of this structure for the development of new therapeutics and the definition of graft quality biomarkers.

1. Introduction

Chronic kidney disease prevalence in the US population now reaches 14% [1]. With a large amount of these patients evolving towards end stage renal disease, the need for organs to be transplanted, the most adapted treatment is constantly increasing. Organ demand is now fourfold higher than the donation rate, leading procurement organizations to extend donor criteria [2], to the detriment of organ quality. These suboptimal organs are more sensitive to ischemia reperfusion injury (IRI) [3], partly explaining the increased rate of short-term [4–6] and long-term [7, 8] complications. In this ongoing donor demographic change, it is now pivotal to better understand IRI mechanisms in order to design better preservation protocols and organ quality biomarkers.

Cell function is intimately related to its structure and thus to the integrity of this network composed of three types of fibers: (a) microfilaments of actin, responsible for cell shape and movement (contraction, crawling), as well as the intracellular movement of organelles; (b) intermediate filaments, responsible for cell shape and anchorage of organelles; and (c) microtubules, composed of α- and β-tubulin dimers, in charge of cellular movement (flagella, cilia), chromosome movement during division, and organelle movement and particularly of vesicular trafficking. Studies on the influence of preservation on the cytoskeleton have been conducted.

In pulmonary endothelial cells, hypothermia induced actin and tubulin filaments disorganization [9]. In cultured hepatocytes, hypothermia induced microfilament precipitation and microtubule shortening [10]. In kidney epithelial cells, similar conditions caused disorganized microtubules while leaving intact the microfilaments, with a particular sensitivity of the proximal tubule cells [11]. Finally, in isolated kidney tubules hypothermia coupled to hypoxia induced a dysfunction of the microfilament-membrane interface and depolymerized microtubules [12]. However, the conditions in some of these studies were very different to those of organ preservation, regarding oxygenation, cold ischemia time (reaching days for some studies), and preservation solution. Hence, these conclusions are sometimes contradictory and difficult to apply to organ transplantation-related IRI.

Herein, we focused on the endothelium, due to its ubiquitous presence in all transplanted organs and the major role played as interface between the organ and the organism, particularly its immune system. Endothelial cells are indeed the principal regulators of the vasculature, in constant interaction with the blood and component of the vessel wall [13]. Moreover, they play a central role in the regulation of the inflammatory response, itself at the heart of several pathologies, including IRI [14]. The mechanisms underlying the hypothermia-induced injury on endothelial cells have only been partly resolved [15]; they include nuclear deformation, loss of adhesion, and cell death. Recently, mild hypothermia was shown to increase apoptosis resistance and reduce inflammatory cytokines production, except IL6 which was upregulated [16]. However, our own results show that organ preservation level hypothermia induces endothelial cell activation and a proinflammatory phenotype [8, 17].

In the present study, we determined the influence of organ preservation conditions on endothelial cells cytoskeleton, following each of the three subtypes of filaments during storage, as well as attempting to identify the individual influence of each of the three parameters altered during preservation: temperature, oxygenation, and solution.

2. Results

2.1. Evolution of Cytoskeletal Filament Organization during Cold Ischemia Time

2.1.1. Microfilaments (Figure 1). Actin staining was performed at different times during cold storage of endothelial cells. Results showed that control cells displayed regular and well organized stress fibers. However, as early as 6 hours after the start of hypothermic/hypoxic storage in UW, signs of disorganization appeared, with amorphous nodules of actin in the cytoplasm and shortened stress fibers. These features appeared more frequently with the elongation of cold ischemia time, leading to a complete loss of fibers within the cytoplasm as well as a peculiar organization at the cellular perimeter, in which short fibers appear to strike out of the cell.

2.1.2. Microtubules (Figure 2). α and β tubulin staining on control cells revealed a concentration of signal near the nuclei (centrosome region) and well-defined filaments reaching towards the extremity of the cell. Six hours after the start of storage, the signal was more diffuse with less defined filament. At 12h, the signal intensity was decreased, with diffuse staining in the perinuclear region and appearance of dot-like staining at the extremities of the cell instead of fibers. Further time points revealed a similar staining pattern, albeit with decreased intensity.

2.1.3. Intermediate Filaments (Supplementary Figure 1). Vimentin was the main protein for these filaments in our cells. Staining showed that, in control cells, the filaments were organized in a clearly defined network within the cell, with concentrated staining near the nuclei. Unlike the other filament subtypes however, cold storage did not appear to affect the staining pattern, at any time point during the 24h period.

2.2. Impact of Cold Ischemia on Cytoskeleton Proteins. To uncover the mechanism underlying the observed rearrangement in tubulin and actin structure, we performed a separation of cell proteins permitting us to differentiate between soluble proteins (i.e., not attached to the cytoskeleton) and insoluble proteins (Figure 3).

Tubulin probing (Figure 3 (top)) showed that the majority of the protein was found in the insoluble fraction in the control cells, indicating that the majority of α and β tubulin were polymerized. After 24h cold ischemia, we observed a reduction of the amount of protein present in the insoluble fraction, while there was no change in the soluble fraction. This indicated that the phenotype observed by immunocytochemistry was likely due to a decrease of the total tubulin present, either through degradation or arrest of production.

We then detected β actin on the membranes (Figure 3 (bottom)), showing that in control cells there was protein in both fraction, with a higher amount present in the insoluble fraction. This was concordant with the constant renewal of actin microfilament, which requires a constantly rotating pool of nonpolymerized proteins. However, in the cells subjected to 24h cold ischemia, all proteins were present in the insoluble fraction, with no significant change in the amount of total actin.

2.3. Individual Role of Solution, Temperature, and Oxygen in the Cytoskeleton. To further understand the impact of cold ischemia on the cytoskeleton, we attempted to study the influence of each variable individually. In these figures (Figures 4 and 5 as well as Supplementary Figures 2–5) culture at 37°C in M200 with oxygen was the control condition: the condition in which these cells were cultured in the incubator, in accordance with the supplier's recommendations. Although intermediate filaments did not appear altered by the protocol, we investigated the effect of each parameter on vimentin staining (Supplementary Figures 4 and 5). In accordance with previous findings, no alterations were observed in this staining, with the exception of UW preserved cells at 37°C; however, these are likely due to the use of UW at 37°C.

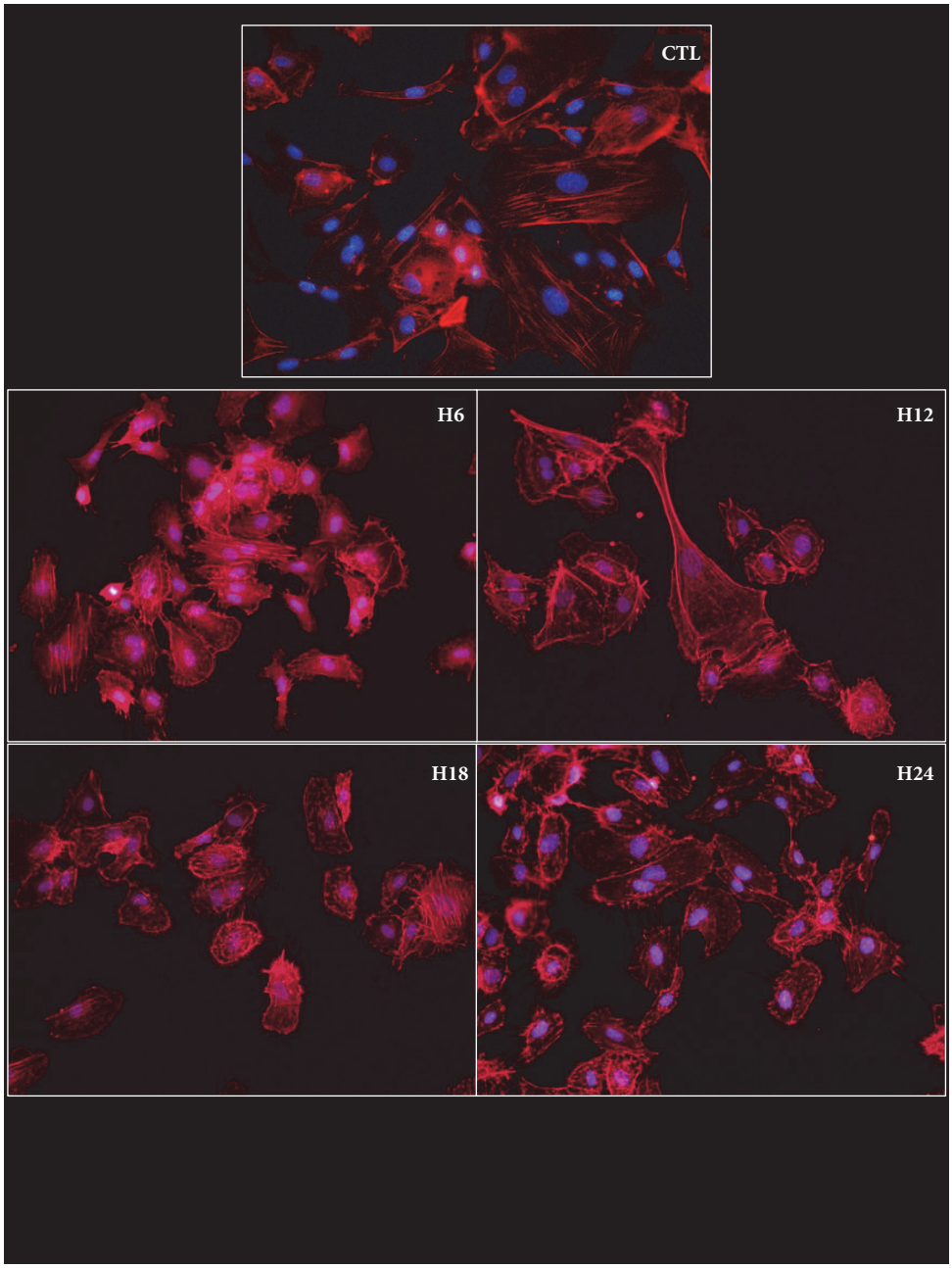

Figure 1: **Actin microfilament phenotype during cold ischemia**. HAEC were cultured in hypoxia/hypothermia using UW solution for different lengths of time then stained with phalloidin as per the Materials and Methods. Representative staining is shown for each duration.

2.3.1. Solution Effect. Indeed, the inadequacy of UW to maintain cell integrity at 37°C was evidenced as early as 6 hours after the start of the experiment (Pictures (c) and (d) of Figures 4 and 5 as well as Supplementary Figures 2, 3, 4, and 5). Hence, we were only able to compare the solution effect on the cells at 4°C.

Regarding microfilaments, at 6h the level of alterations in M200 appeared higher than in UW preserved cells (Figure 4, (e) and (f) versus (g) and (h)), as the later displayed more prominent stress fibers and appeared more spread out on the culture surface. However, these differences were not found after 24h of preservation (Supplementary Figure 2, E and F versus G and H).

Concerning microtubules, we observed different phenotypes in cells preserved with the two solutions after 6h (Figure 5, (e) and (f) versus (g) and (h)): UW preserved cells displayed decreased tubulin staining around the cells nuclei and dot-like signal towards the extremities; however, M200 cells showed an even reduced signal, with only faint tubulin staining in the perinuclear region. At 24h, the staining patterns were similar between conditions.

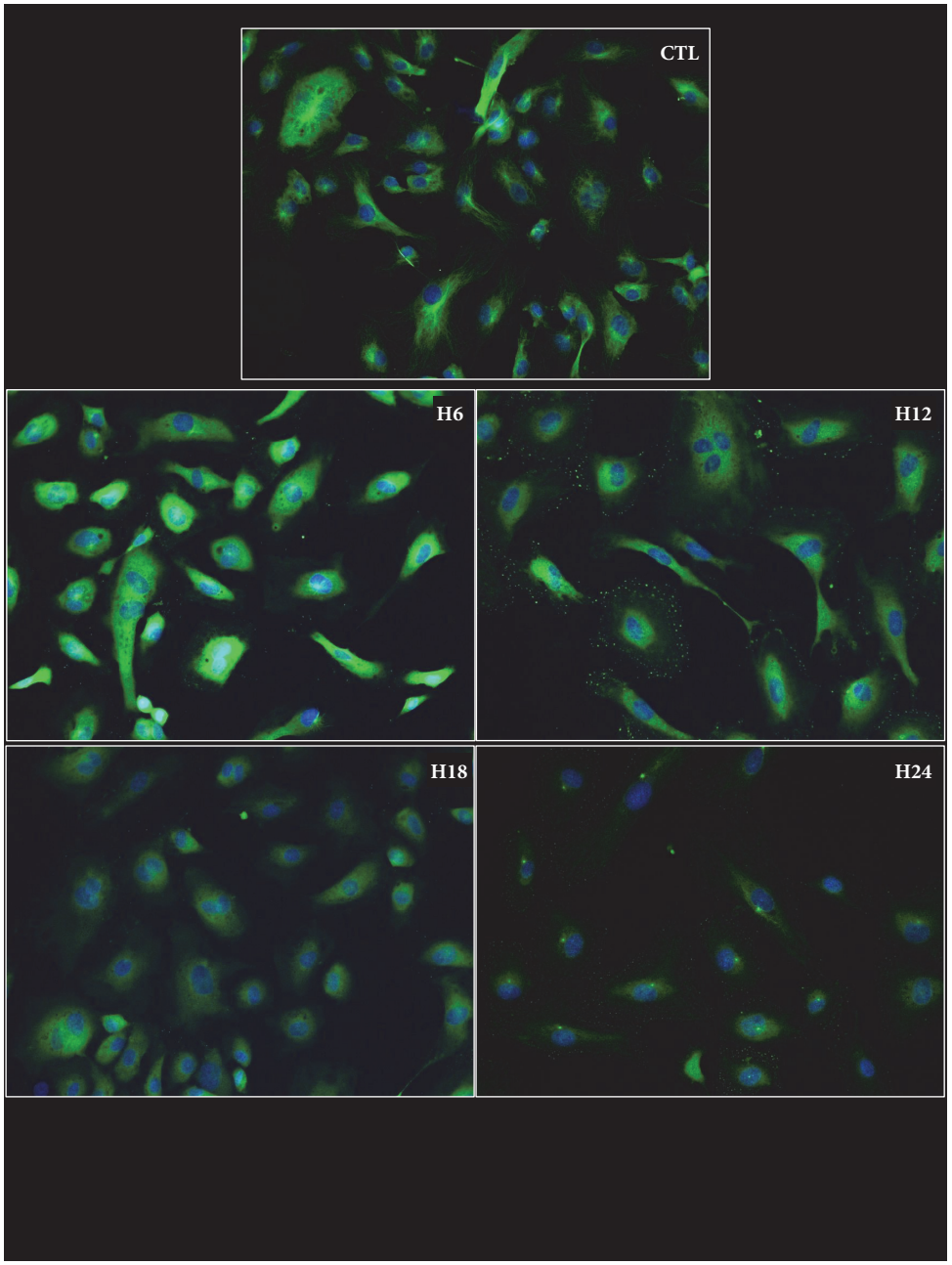

Figure 2: **Microtubules phenotype during cold ischemia**. HAEC were cultured in hypoxia/hypothermia using UW solution for different lengths of time and then stained with an anti α and β tubulin antibody as per the Materials and Methods. Representative staining is shown for each duration.

2.3.2. Temperature Effect. Since UW did not permit cell survival at 37°C, the only acceptable solution to be used for proper comparison of temperature was M200.

Regarding microfilaments, we confirmed the high degree of disturbance found in filament organization caused by hypothermia regardless of oxygen levels at both 6 (Figure 4(b) versus (f)) and 24 hours (Supplementary Figure 2).

Microtubule organization was again severely disturbed at both 6 (Figure 5 (b) versus (f)) and 24 hours (Supplementary Figure 3) by hypothermia.

2.3.3. Oxygen Effect. Microfilaments staining revealed little to no difference between cells preserved in the absence or in the presence of oxygen after 6 hours, both at 37°C in M200 (Figure 4, (a) versus (b)) and at 4°C in both solutions (Figure 4, (e), (g) versus (f), (h)). The absence of oxygen effect was also evident after 24h incubation (Supplementary Figure 2).

Study of microtubule alterations revealed a similar conclusion regarding the absence of influence for oxygen supply. As shown in Figure 5, restauration of oxygen to the cell does not alter the phenotype.

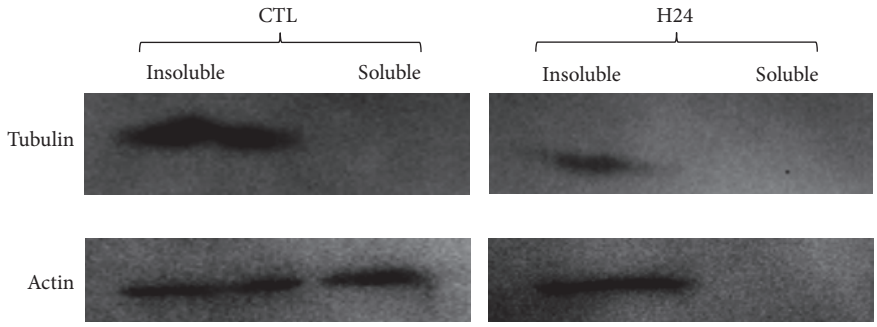

Figure 3: **Western blot analysis of cytoskeleton proteins during cold ischemia**. HAEC were cultured in hypoxia/hypothermia using UW solution for 24h; then the soluble and insoluble protein fractions were separated as per the Materials and Methods.

The same observations were performed after 24h incubation, at which time a similar lack of involvement of oxygen could be observed (Supplementary Figures 2 and 3).

2.4. Relationship between Cytoskeleton Stability and Cell Death. To evaluate if the observed alteration in cytoskeletal played a role in cell fate, we used pharmacological modulators of microfilaments and microtubules and tested their ability to improve primary endothelial cell survival after 24 hours cold hypoxic storage followed by 6 hours of regular culture conditions (mimicking reperfusion).

We first confirmed that these compounds were able to maintain cytoskeletal organization in a dose response manner (Supplementary Figure 6). Then, we observed that maintaining either microfilaments or microtubule organization throughout the 24 hours of simulated cold ischemia did not significantly improve cell survival (Figure 6) above the level of standard preservation conditions (UW, 65% survival rate).

3. Discussion

With the necessary evolution in donor demographics, the need for a better understanding of ischemia reperfusion becomes paramount for the establishment of more consistent and adapted protocols. In addition, characterizations of better biomarkers to quantify the quality of the organ are needed, as well as new therapeutic targets to improve viability and insure a better outcome. In this study, we investigated the impact of cold storage conditions on the cytoskeleton, a core structure of cells exposed to CS.

We used an in vitro model of hypoxia/hypothermia to mimic cold storage, using two different medium: on the one hand, the culture medium used for these cells, on the other the University of Wisconsin solution, currently one of the most used preservation solutions in transplantation [18]. Of the three types of filaments composing the cytoskeleton, only two were affected by these conditions: actin microfilaments and tubulin microtubules. Intermediate filaments were not affected.

While fluorescent images revealed a general disorganization of both filament types, the resulting effect was specific to each:

(1) Microfilaments were rapidly reduced to amorphous nodules and shortened stress fibers, leading to a loss of fibers and reorganization of the actin proteins to the cell periphery. This suggests that the cell responded to the changes in conditions with a prioritization of resources to the cell-extracellular space interface, possibly anchoring the cell.

(2) Microtubules rapidly lost their fine definition after the start of storage and reorganized near the nuclei, with loss of signal following the extension of cold ischemia time. Due to the role of microtubule in intracellular trafficking, this may imply that the cell reorganizes its transit around the nucleus to favor *de novo* protein production in order to resist organ preservation conditions.

Further probing by western blot showed that these changes appeared to stem from two different phenomena: while actin protein level remains constant overall, it appears that the ratio of organized/free actin favors the free actin portion; on the other hand, total tubulin level decreases over time, suggesting that the loss of organization and signal is due to degradation and/or production arrest.

These observations are concurrent with the bioenergetics aspect of cytoskeletal organization. Indeed, sensitivity of the cytoskeleton to hypothermia is intimately linked to the dependence of the fibers to intracellular energy: actin relies on ATP hydrolysis for its polymerization (16 ATP per 37nm) and its contractile function [19, 20], while microtubule formation requires important input of GTP (16 GTP per 8nm) [21] and vesicular transport demands 1 ATP for each 8nm traveled [22]; on the other hand, intermediate filaments have a slow turnover and their activity does not need energy; hence, the absence of influence from IR highlighted herein is logical.

Since cold storage translates into the change of 3 important parameters for the cell: temperature, environment, and oxygen tension; we endeavored to test the individual contribution of each of these factors to the observed phenotype.

UW was used herein as it remains the leading solution used worldwide and has shown to remain on par with the other solutions in terms of organ protection [23]. Unfortunately, UW is not adapted for cell preservation at 37°C; this condition was thus tested for the sake of thoroughness but could not be used to test the effect of temperature. UW

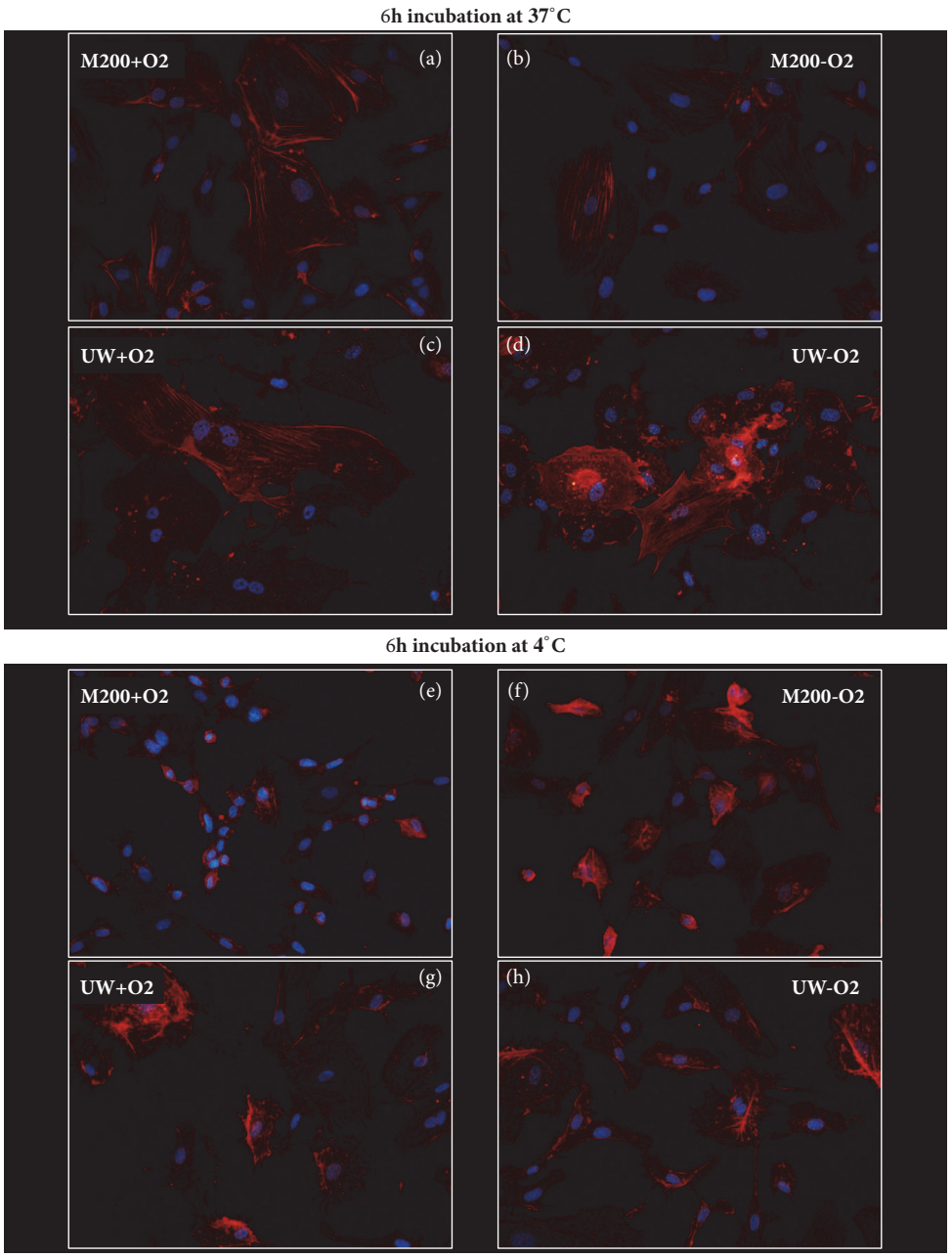

FIGURE 4: **Microfilament phenotype alteration after 24h: influence of solution, temperature, and oxygenation level**. HAEC were cultured in different conditions for 24h and then stained with phalloidin as per the Materials and Methods. Representative staining is shown for each condition.

was however more effective than M200 in preserving cellular integrity at 4°C, which is concordant with the rationale behind the formulation of each solution. Nevertheless, the deleterious effect of UW at 37°C did not permit us to compare the effect of different temperatures on UW stored cells.

However, use of M200 did reveal the important dichotomy between the roles played by temperature and the involvement of oxygen. Indeed, while temperature had a profound impact on both actin and tubulin organization, oxygen tension did not show any significant impact. This strongly suggests that the structures of the cytoskeleton are highly dependent on the temperature and little affected by the lack of oxygen.

One limit of our study is the limited number of temperatures tested. However, since the role of oxygen was not elucidated, the number of conditions would have been too important to permit clear conclusions to be drawn. Further studies will be necessary to elucidate the effect of varying temperature in an anoxic environment.

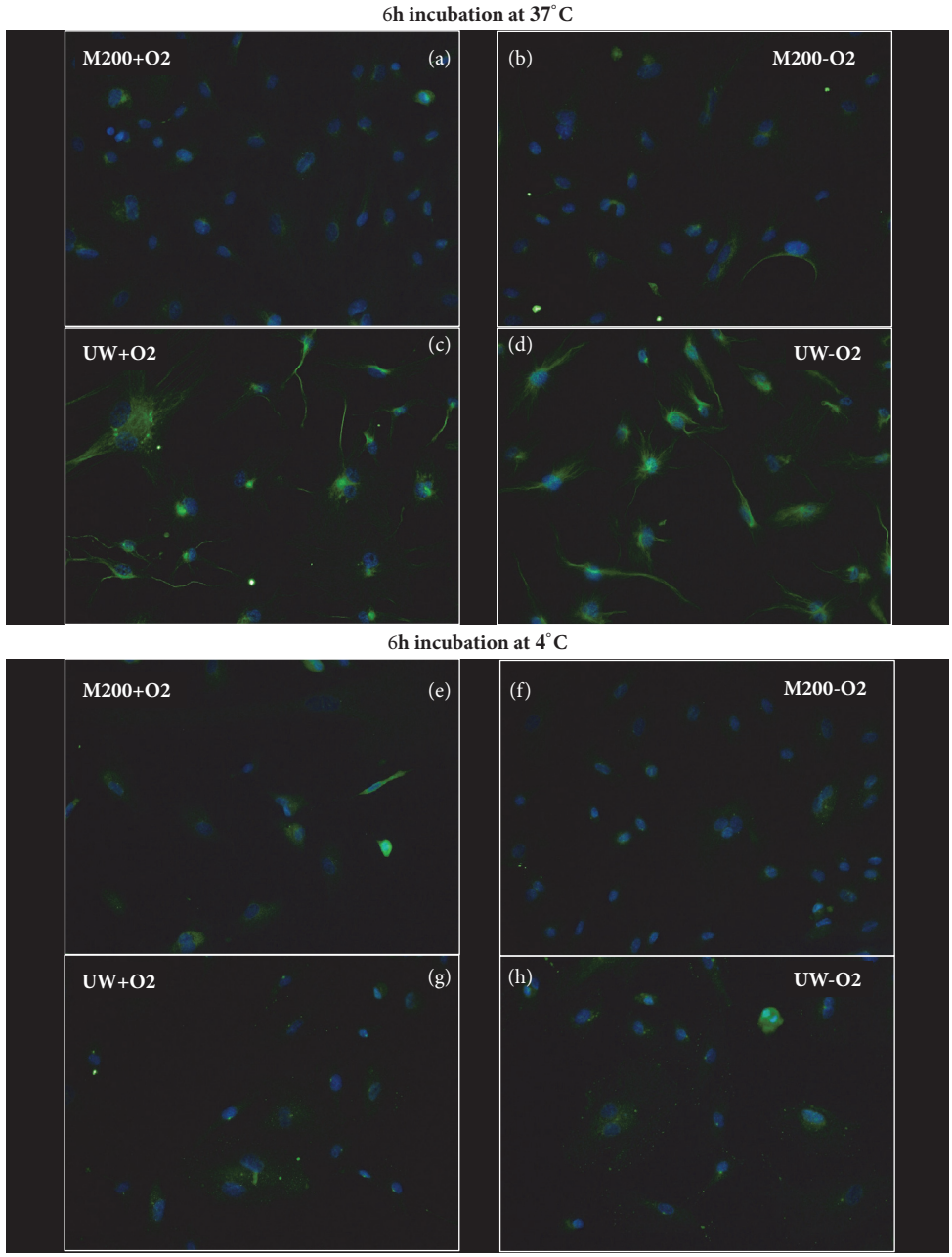

FIGURE 5: **Microtubules phenotype alteration after 24h: influence of solution, temperature, and oxygenation level**. HAEC were cultured in different conditions for 24h and then stained with an anti α and β tubulin antibody as per the Materials and Methods. Representative staining is shown for each condition.

Finally, we tested the hypothesis that cytoskeleton protection could improve cell survival. Previous studies have shown that Taxol, preventing tubulin depolymerization, could improve viability in a canine kidney isolated tubule model [12]. In our hands, however, a similar observation could not be produced as addition of a dose range of Taxol failed to rescue cell death at the end of our cold ischemia/warm reperfusion protocol. This may be due to the difference in models (isolated tubules preserved for days versus plated endothelial cells preserved for hours) and in cell types used. Conversely, we attempted to measure the influence of actin microfilament stabilization on cell death. Here also, previous studies have shown that the use of Jasplakinolide could protect rat kidneys against cold ischemia induced apoptosis [24]. However, in our hands endothelial cells could not be protected against cell death by this compound. This may also be due to the difference in model and targeted cell type. In our endothelial cell model, cytoskeletal adaptation may be downstream from death-inducing mechanisms, such as mitochondrial dysfunction which could both influence

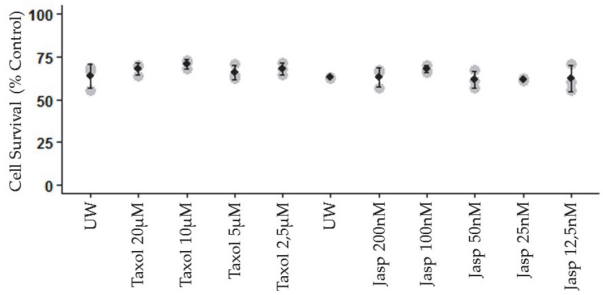

FIGURE 6: **Impact of cytoskeleton pharmaceutical stabilization on cell survival**. HAEC were cultured in hypoxia/hypothermia for 24h using either Taxol or Jasplakinolide (Jasp) and then processed for reperfusion as per the Materials and Methods. Cell survival was assessed using XTT. Statistics: * $p<0.05$ between groups.

survival and cytoskeletal organization: in this light, stabilizing the cytoskeleton would not prevent the induction of cell death. This pathway would thus not be the route of choice to establish a therapeutic strategy towards preserving the endothelium, a cell type at the center of IR and playing a central role in chronic lesion development [25].

In conclusion, we demonstrate in an *in vitro* model that, during cold storage, the endothelial cytoskeleton is deeply disorganized for both microtubules and microfilaments. These changes occur rapidly and are particularly influenced by temperature, rather than oxygenation.

4. Materials and Methods

4.1. In Vitro Model. Primary human aortic endothelial cells (HAEC) were cultured on 1% gelatin (Sigma, France) in medium 200 supplemented with 2% Low Serum Growth Factor (LSGS) and 10% fetal bovine serum (FBS) (Invitrogen, France) in a humidified atmosphere at 5% CO2 and 37°C. Cells were used at passage 5. At confluence and after synchronization, cells were subjected to several conditions: cold ischemia like conditions *via* incubation in a hermetic chamber at 4°C containing an hypoxic atmosphere: 0% O2, 5% CO2, and 95% N2 (Bactal 2 gaz, Air Liquide, France) during 24h, in University of Wisconsin solution (UW, Bridge to Life, France); several parameters were then changed to study the influence of temperature (cells kept at 37°C instead of 4°C), oxygenation (maintained in normal atmosphere rather than hypoxic), and solution (using medium 200 with 2% FBS, thereafter referred to as M200, instead of UW). At different times of the conservation period, cells were collected for analysis. Reperfusion, when testing for cell survival, was mimicked by restoring normal culture conditions.

4.2. Pharmacological Modulation. To modulate cytoskeleton fibers, we used Jasplakinolide (Sigma-Aldrich, St. Quentin Fallavier, France) and Taxol (Paclitaxel, Sigma), at the concentrations indicated in the figures.

4.3. Immunofluorescent Staining. Cells were grown on microscope glass slides, subjected to indicated conditions and fixed with paraformaldehyde then processed for immunofluochemistry staining. Actin staining was performed with phalloidin (Sigma); α and β tubulin antibody and vimentin antibody were purchased from Cell Signaling Technologies (Ozyme, Montigny-le-Bretonneux, France).

4.4. Cell Proliferation Assay. Cell proliferation was assessed after 6 hours of reperfusion by measurement of the mitochondrial succinate dehydrogenase activity with the XTT kit (Sigma) following the manufacturer's guidelines. Reactions were quantified by spectrophotometer (Victor3, Perkin-Elmer, France).

4.5. Western Blotting. Cells were grown in culture flasks. The soluble fraction of the cytoskeleton was separated from the insoluble fraction using a specific buffer (12) (1 mM MgCl2, 2 mM ethylene glycol-bis ([beta]-aminoethyl ether)-N,N,N′,N′-tetraacetic acid [EGTA], 0.5% Nonidet (P-40, Sigma, St. Louis, MO), 0.25 mg/mL aprotinin, 200 μg/mL soybean trypsin inhibitor, 5 mM [epsilon]-aminocaproic acid, 1 mM benzamide, 20 mM Tris-HCl, pH 6.8 with NaOH). Each fraction was separated on a 4-15% TGX gel (Biorad, Marnes-la-Coquette, France) and transferred on a PVDF membrane. Actin staining was performed with anti actin antibody (Sigma); α and β tubulin antibody was purchased from Cell Signaling Technologies (Ozyme, Montigny-le-Bretonneux, France).

4.6. Statistics. Data were analyzed using NCSS software [26] and are expressed as the mean ± SD. Data normality was assessed by performing a Shapiro-Wilk W Test. Normally distributed data were analyzed with one-way ANOVA followed by pairwise t-test with pooled SD with Bonferroni correction. Nonnormally distributed data were analyzed with Kruskal-Wallis test followed by a Dunn's post hoc test. For comparisons between two groups, a Wilcoxon-Mann–Whitney test was performed. A p value < 0.05 was considered statistically significant.

Abbreviations

MDPI: Multidisciplinary Digital Publishing Institute
DOAJ: Directory of open access journals
IRI: Ischemia reperfusion injury
HAEC: Primary human aortic endothelial cells
LSGS: Low serum growth factor
UW: University of Wisconsin solution
FBS: Fetal bovine serum
EGTA: Ethylene glycol-bis ([beta]-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Raphael Thuillier and Thierry Hauet conceived and designed the experiments; Raphael Thuillier performed the experiments; Raphael Thuillier analyzed the data; Raphael Thuillier and Thierry Hauet wrote the paper.

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Supplementary Materials

Supplementary 1. Supplementary Figure 1: **intermediate filament phenotype during cold ischemia**. HAEC were cultured in hypoxia/hypothermia using UW solution for different lengths of time and then stained with an anti-vimentin antibody as per the Materials and Methods. Representative staining is shown for each duration.

Supplementary 2. Supplementary Figure 2: **microfilament phenotype alteration after 6h: influence of solution, temperature, and oxygenation level**. HAEC were cultured in different conditions for 24h and then stained with phalloidin as per the Materials and Methods. Representative staining is shown for each condition.

Supplementary 3. Supplementary Figure 3: **microtubules phenotype alteration after 6h: influence of solution, temperature, and oxygenation level**. HAEC were cultured in different conditions for 24h and then stained with an anti α and β tubulin antibody as per the Materials and Methods section. Representative staining is shown for each condition.

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Supplementary 6. Supplementary Figure 6: **confirmation of effectiveness for Taxol or Jasplakinolide**. The effect of each compound was visually verified on HAEC cultured in different conditions for 24h. **Top**: staining with falloidin showed dose effect on actin cytoskeleton stabilization, using as low dose as 50nM Jasplakinolide. **Bottom**: staining with an anti α and β tubulin showed Taxol's ability to stabilize microtubules in a dose-effect manner.

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