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# Strategies to Restore Adenosine Triphosphate (ATP) Level After More than 20 Hours of Cold Ischemia Time in Human Marginal Kidney Grafts

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

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**Background:** The persisting organ shortage in the field of transplantation recommends the use of marginal kidneys which poorly tolerate ischemic damage. Adenosine triphosphate (ATP) depletion during cold ischemia time (CIT) is considered crucial for graft function. We tested different strategies of kidney perfusion before transplantation in the attempt to improve the technique.


**Material/Methods:** Twenty human discarded kidneys from donors after brain death and with at least 20 hours of CIT were randomized to the following experimental groups (treatment time three-hours at 4°C): a) static cold storage (CS); b) static cold hyperbaric oxygenation (Hyp); c) hypothermic perfusion (PE); d) hypothermic perfusion in hyperbaric oxygenation (PE-Hyp); and e) hypothermic oxygenated perfusion (PE-O<sub>2</sub>).

**Results:** Histological results showed that perfusion with or without oxygen did not produce any endothelial damage. A depletion of ATP content following the preservation procedure was observed in CS, PE, and Hyp, while PE-Hyp and PE-O<sub>2</sub> were associated with a net increase of ATP content with respect to baseline level. In addition, PE-Hyp was associated with a significant downregulation of endothelial isoform of nitric oxide synthase (eNOS) gene expression and of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ).

**Conclusions:** Hyperbaric or normobaric oxygenation with perfusion improves organ metabolic preservation compared to other methods. This approach may prevent the onset of delayed graft function, but clinical trials are needed to confirm this.

**MeSH Keywords:** Adenosine Triphosphate • Delayed Graft Function • Organ Preservation • Organs at Risk • Pulsatile Flow

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## Background

One of the major challenges in the field of transplantation is the shortage of donor organs. Many patients waiting for transplant die during the waiting time or wait many years for kidney transplantation, with detrimental effects on their quality of life and increasing morbidity and related costs [1–4]. Effective strategies, which safely extend the donor pool, are therefore advocated.

During the last 20 years, the two main policies attempting to achieve this purpose were living donation and expanded criteria donors [5–8]. These donors are presumed to provide a worse outcome than conventional donors and many research protocols have been developed to reduce preservation injury (PI) and PI-related complications [9]. Static cold storage (SCS) has been the standard technique in clinical practice for liver and kidney preservation using specific solutions (Belzer UW, Custodiol, Celsior) able to prevent cellular swelling [10,11].

Hypothermic machine perfusion (HMP) of the kidney and the liver in the preimplantation phase has been widely explored and shown that high tissue adenosine triphosphate (ATP) levels at reperfusion time and the continuous perfusion of the microcirculation improve graft dysfunction and survival [9,12–18]. Oxygen during perfusion seems to add further improvements to marginal grafts, including donation after circulatory death [19–22].

Furthermore, organ preservation by normothermic machine perfusion (NMP) has proven to be superior to SCS in animal and clinical studies [23–27]. NMP mimics the physiological environment by maintaining a normal temperature and providing substrates for cellular metabolism, oxygen and nutrition [23,26–27].

Another possible strategy for reducing PI could be treatment with hyperbaric oxygen that has been shown to improve lipid peroxidation and mitochondrial oxidative phosphorylation capacity in rat livers [28,29] and protects against the ischemia-reperfusion injury in rat kidneys [30].

The present study was designed in order to assess the impact of normobaric and hyperbaric oxygenation combined with hypothermia and pulsatile perfusion on the structure and metabolic activity of discarded human kidneys.

## Material and Methods

Our research group developed an innovative machine able to perfuse organs at hypothermic temperature (4°C), also providing oxygen to the graft in normobaric or hyperbaric condition.

We report the experimental results of 20 kidneys discarded for kidney transplantation due to clinical reasons and with at least 20 hours of static cold ischemia time (CIT) which underwent different organ preservation strategies.

### Study design

From May 2014 to June 2015, kidneys from donors after brain death not suitable for transplantation and offered for research after informed consent from the relatives were randomized in the following experimental groups: a) Static cold storage (CS); b) Static cold hyperbaric oxygenation (Hyp); c) Hypothermic perfusion (PE); d) Hypothermic perfusion in hyperbaric oxygenation (PE-Hyp); and e) Hypothermic oxygenated perfusion (PE-O<sub>2</sub>).

Ethical approval was granted by the Ethics Committee of University of Bologna Sant'Orsola- Malpighi Hospital and the experimental phases reported were conducted in accordance with institutional guidelines. Organ retrieval was performed by our surgical team according to the current protocols of the National Transplant Center. All kidneys initially were stored in Celsior (Waters Medical Systems of Institute Georges Lopez) at 4°C until organ preparation following standard back-table procedures. For kidneys of group c, d, and e, the renal artery was dissected and cannulated by means of an 8 Fr, 10 Fr, or 12 Fr vascular cannula (Medtronic-UK), based on renal artery size. Each treatment was accomplished under hypothermic conditions (4°C) for three hours and using 1 L of Celsior.

### Machine perfusion

The perfusion device was developed by Medica S.P.A and Centro Iperbarico S.R.L. under the scientific management of the author MR. It provides pulsatile perfusion through three peristaltic pumps, each driven by an individual motor with the dual purpose of perfusing the organ with the preservation solution and, at the same time, maintaining constant liquid circulation around the organ within the hyperbaric chamber. The first pump, used only in hyperbaric treatments, breaks the gas-liquid interface facilitating the diffusion of the compressed gaseous mixture into the preservation solution. The second and third peristaltic pumps are used to perfuse the organ through suction and discharge pipes connected to the organ's blood vessels.

The organ is connected through the cannulated vascular access with sterile tubing (PVC and silicon) to the machine. Flow and pressure values during perfusion are assessed by means of specific sensors, auto-regulated and displayed on the device's screen in real time. To achieve oxygenation of the organs during perfusion, it is possible to use a membrane oxygenator integrated in the perfusion circuit that provides oxygen to the preservation fluid (normobaric oxygenation) or a pressurizable

chamber where the graft is placed during the treatment (hyperbaric oxygenation).

### **Normobaric oxygenation**

The oxygenator is a component of the tubing system that provides oxygen to the organ preservation fluid. It is a microporous hollow fiber membrane oxygenator consisting of a gas exchange module with an integrated heat exchanger. The device, supplied as single-use and ethylene oxide sterile, is intended to be used in an extracorporeal perfusion circuit to oxygenate and remove carbon dioxide from the fluid during routine perfusion procedures for up to six hours in duration.

### **Hyperbaric oxygenation**

The device consists of a pressurizable (“hyperbaric”) chamber designed to accommodate the organ fully immersed in the preservation solution. The chamber is compressed with a mixture of medical oxygen (95%) and carbon dioxide (5%) until a higher pressure than the atmospheric one is obtained. The minimum partial pressure of oxygen ( $pO_2$ ) was inversely proportional to the normalized flow (mL/min/g of the organ); the device allows pressurization up to 200 kilopascals by a certified medical gas cylinder. An appropriate reduction gear, certified for use with medical oxygen, reduces the gas pressure. The mixture is dissolved in the preservation solution in a concentration directly proportional to the pressure exerted by the gas on the gas-liquid interface (Henry’s law). The hyperbaric chamber is placed inside a thermal conditioning apparatus, which can be powered from 12 to up to 220 volts. The device has the ability to maintain the hypothermic (or, where appropriate, hyper-thermic) condition within the range of  $-5^{\circ}\text{C}/40^{\circ}\text{C}$  ( $23^{\circ}\text{F}/104^{\circ}\text{F}$ ), for all the time necessary. To guarantee effective organ preservation during transport, there is a retro-actuated control of the refrigerator temperature and of the storage chamber for the organ.

### **Kidney perfusion and oxygenation parameters**

The renal perfusion rate was set in order to reach an arterial pressure of 25–30 mmHg. To provide an adequate oxygen supply with  $pO_2$  level of the preservation solution close to 750 mmHg, the oxygenation setting was between 4 L and 2 L of  $O_2$  in normobaric condition and at 1.5 atm in hyperbaric condition. All the parameters were chosen based on preliminary experiments performed during machine tuning.

### **Histological analysis**

Kidney biopsies were obtained prior to (T0) and after treatment (T1). All core-needle biopsies were fixed in formalin and embedded in a paraffin block by standard procedures (see

supplemental file). Sections underwent histochemical staining (Hematoxylin-Eosin, Trichrome stain, Periodic-acid Schiff) and examination to reveal any tissue change possibly related to preservation damage.

In T0 and T1 biopsies, all the components of kidney parenchyma were histologically evaluated for the purposes of the study: glomeruli: signs of glomerular ischemia, occurrence and diffusion of glomerular sclerosis; interstitium: occurrence of fibrosis and/or inflammatory infiltrate; tubules: occurrence of tubulocyte atrophy, tubulocyte vacuolization, luminal cylinders, acute tubular necrosis; and arteries: occurrence of myointimal thickening, detachment of endothelial cells and other signs of arterial/arteriolar damage.

### **Immunohistochemistry**

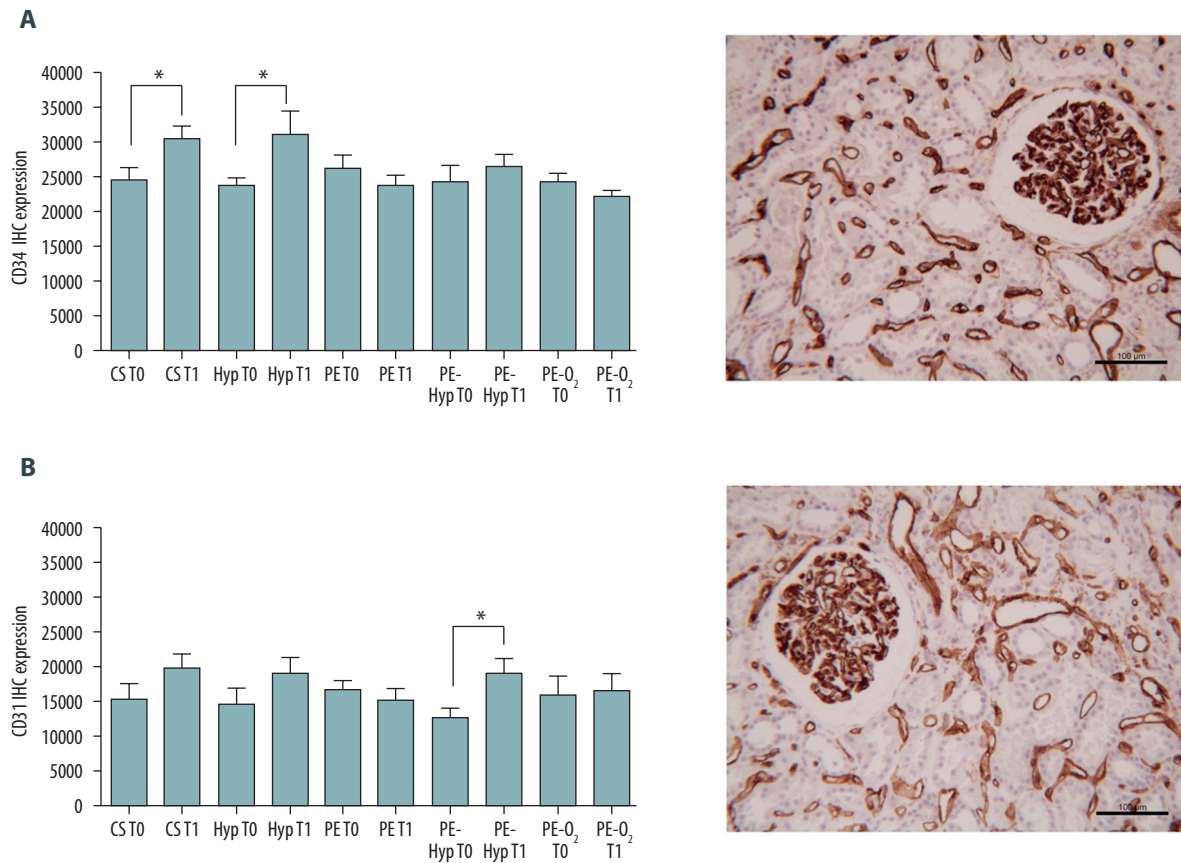
Immunohistochemistry (IHC) for CD34 (mouse monoclonal antibody directed against human CD34, clone QBEnd/10, Roche Diagnostics) and CD31 (mouse monoclonal antibody directed against human CD31, clone JC70, Roche Diagnostics) was automatically performed with BenchMark XT<sup>®</sup> Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) following the manufacturer’s instructions. IHC was carried out on formalin-fixed paraffin embedded 2- $\mu\text{m}$ -thick sections. Slides were first dewaxed in xylol (30 minutes) and rehydrated through grade washes of ethanol: 100% (five minutes), 95% (three minutes) and 70% (one minute). Nuclei were counterstained with Gills hematoxylin (Sigma Chemicals). Negative controls were obtained by omitting the primary antibody.

### **Semi-quantitative immunohistochemistry**

Immunohistochemical images were acquired using a Leitz Diaplan light microscope (Wetzlar, Germany) connected to a JVC 3CCD video camera (KY-F55B, Yokohama, Japan). Semi-quantitative analysis was performed with Image-Pro Plus<sup>®</sup> 6 software (Media Cybernetics, Silver Spring, MD, USA) using digital images taken at 10 $\times$  of magnification. For each experimental condition, the positive area intensely stained with endothelial cell markers (CD31, CD34) was quantified; at least three randomly selected tissue areas corresponding to an average area of 303622.56  $\mu\text{m}^2$  were recorded; to make the selection comparable we decided to select images that exclusively contained a single glomerulus (as seen in Figure 1, right column); the values were scored separately.

### **Transmission electron microscopy**

Small tissue fragments were fixed in Karnovsky fixative (2% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer) and processed for transmission electron microscopy (TEM) analysis.



**Figure 1.** Representative immunohistochemical images (right column) and semi-quantitative analysis (left column) of kidney tissue expressing CD34 (A) and CD31 (B) endothelial cell markers. TO and T1 values are reported separately. (A, B) Scale bars=100  $\mu$ m. (A) \*  $p$ -value=0.0187 for CS group, \*  $p$ -value=0.0466 for Hyp. (B) \*  $p$ -value=0.0109 for Hyp-PE group; unpaired Student's  $t$ -test.

Each specimen was rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for one hour at room temperature, dehydrated with graded ethanol (from 30% to 100%) and embedded in Araldite resin. The ultrathin sections were counterstained with uranyl acetate and lead citrate before examination in a Philips CM10 (FEI Company, Milan, Italy) transmission electron microscope equipped with a Gatan camera; digital images were captured using the FEI proprietary software Olympus SIS Megaview SSD digital camera.

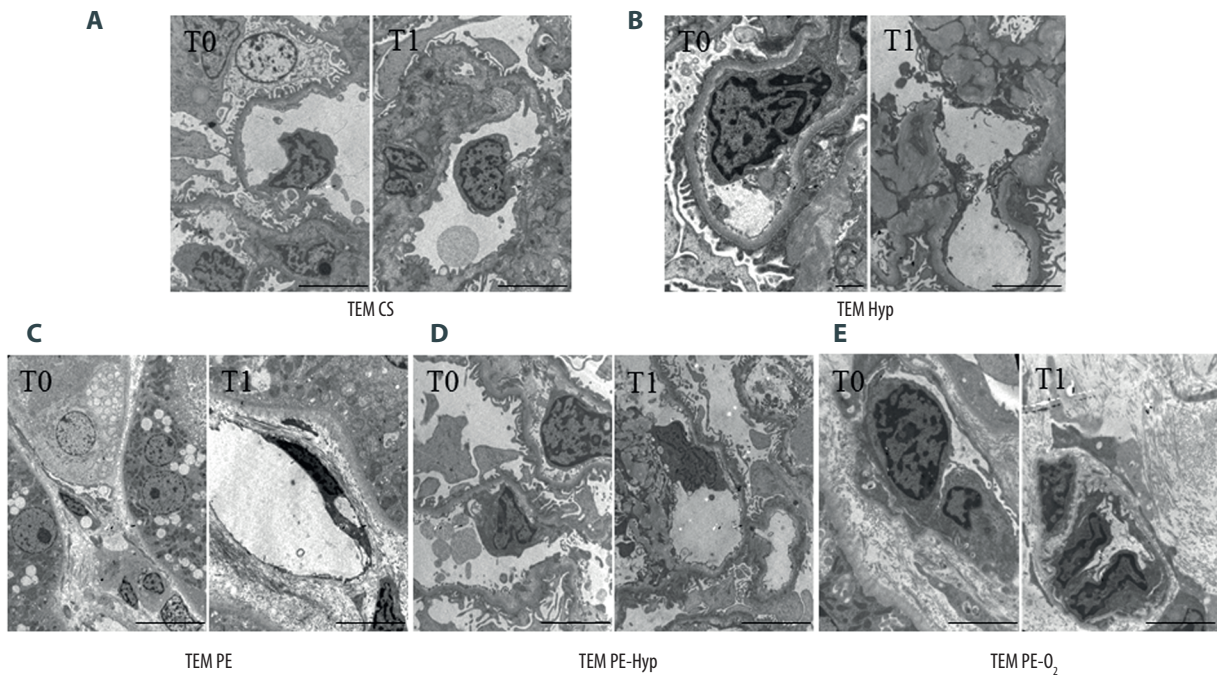
#### Assessment of metabolic parameters

The pH, lactate concentration, oxygen ( $pO_2$ ), and carbon dioxide ( $pCO_2$ ) partial pressure were assessed in the perfusate collected from the organ reservoir at T0 and T1 by means of hemo gas analyzer (Gem Premier 3500, Instrumentation Laboratory-Werfen S.P.A., Barcelona, Spain).

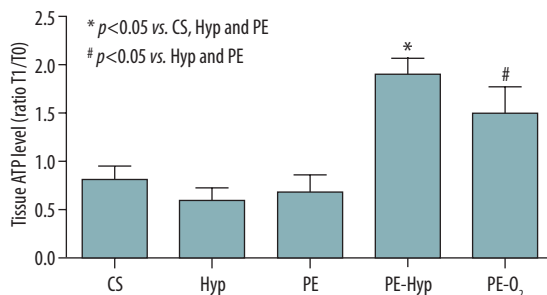
#### Tissue ATP level determination

Tissue samples were collected and immediately frozen in liquid nitrogen at T0 and T1. After protein extraction, ATP content was assessed through an ATP determination kit (Cat. N A22066, Thermo Fisher, Waltham, MA, USA).

Total proteins were extracted from each tissue sample using a buffer containing 150 mM NaCl, 20 mM  $Na_2HPO_4/NaH_2PO_4$ , 10% glycerol, 1% Triton X-100, 100 mM PMSF, 100 mM DTT, and a mix of protease inhibitors (Roche; Basel, Swiss). Total protein concentration was determined by the Lowry method; then each sample was diluted to a final concentration of 1  $\mu$ g/ $\mu$ L. Then 5  $\mu$ g of total protein were used for the determination of the tissue ATP content on a Glomax 20/20 single tube luminometer (Promega, Fitchburg, WI, USA) following the manufacturer's protocol. For each sample, the absolute ATP concentration was obtained by interpolating the luminescence value on a standard curve, then the ATP level was expressed as the ratio of T1 over T0 (Figure 3).



**Figure 2.** Representative ultrastructural images of endothelial cells seen in glomerular loop and interstitium. (A) Scale bars=5 μm; (B) T0 scale bar=2 μm, T1 scale bar=5 μm; (C) T0 scale bar=5 μm, T1 scale bar=5 μm; (D, E) scale bars=5 μm.



**Figure 3.** Tissue adenosine triphosphate (ATP) level. Results are expressed as the ratio between ATP content at T1 and T0. The results of post-hoc analysis are indicated by asterisk.

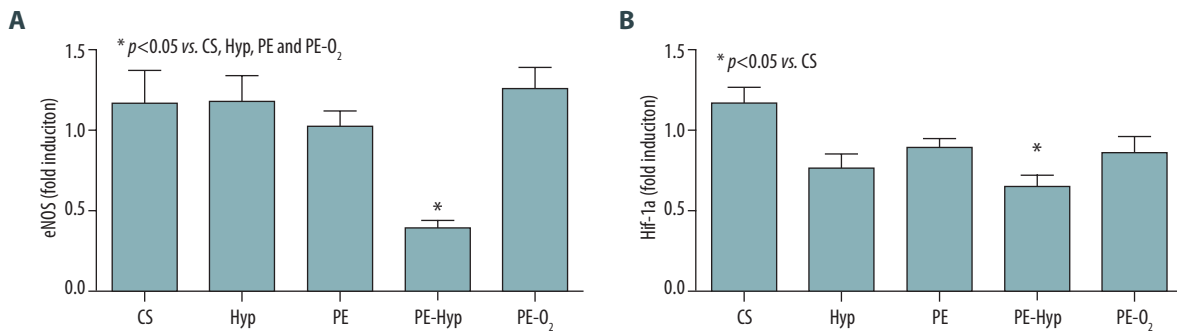
### Gene expression analysis

Total RNA was extracted from each tissue sample, collected as aforementioned, following the Trizol RNA isolation protocol and reverse transcribed by using a SuperScript Vilo Master Mix (Invitrogen; Waltham, MA, USA). The cDNA was amplified through a semi-quantitative real-time qPCR on an iCycler (Biorad, Hercules, CA, USA) using SYBR GreenER qPCR SuperMix (Invitrogen, Waltham, MA, USA). The primers used were: hypoxia inducible factor 1 alpha (HIF-1 $\alpha$ ) (FW AACATAAAGTCTGCAACATGGAAG; RV TTTGATGGGTGAGGAATGGG), endothelial nitric

oxide synthase (eNOS) (FW GTGGCTGGTACATGAGCACT; RV GTCTTTCCACAGGGACGAGG), beta actin (FW CCTGGACTT CGAGCAAGAGATG; RV GGAAGGAAGCTGGAAGAGTG) and beta-2microglobulin (FW CTTTCTGGCCTGGAGGCTATC; RV CTTTCTGGCCTGGAGGCTATC). Data were analyzed according to the  $2^{-\Delta\Delta Ct}$  method in which the expression of each gene at T0 was used as the reference value. Gene expression level was therefore reported as fold induction of T1 over T0 (Figure 4A, 4B).

### Data analysis

Data are expressed as the ratio between values measured at T0 and T1 or absolute value. For continuous variables, data are presented as mean and SD. For all variables, the Kolmogorov-Smirnov test was used to test the normality of the distribution while the homogeneity of variance was evaluated through the Levene test, then differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test for intergroup comparisons. The correlation between ATP level and pCO<sub>2</sub> was performed following Pearson's method. Differences between sample categories following IHC semi-quantification were determined using an unpaired Student's *t*-test. All tests used were two-sided and *p* values less than 0.05 were considered statistically significant. SPSS version 20.0 and GraphPad Prism 5.0 software were used for statistical analysis.



**Figure 4.** Gene expression of endothelial nitric oxide synthase (eNOS) (A) and hypoxia inducible factor 1α (HIF-1α) (B). Data presented are fold induction of sample at T1 over T0. The results of post-hoc analysis are indicated by asterisk.

**Table 1.** Features of the discharged kidney enrolled in the 5 study groups.

	CS (4)	Hyp (4)	PE (4)	PE-Hyp (4)	PE-O <sub>2</sub> (4)	p
Mean donor age	74±6	74±6	69±5	69±5	70±4	n.s.
Mean cold ischemia time	22.3±2.2	22.3±2.2	26.8±3.3	22.8±1.6	26.5±1.4	n.s.
Mean Remuzzi score	5	5	6	5.5	4.5	n.s.
Mean pO <sub>2</sub> at T0	67±4	65±7	75±5	72±3	68±7	n.s.
Mean pO <sub>2</sub> at T1	176±48	282±29*	106±37	366±83*	710±39*	<0.001*
Mean pCO <sub>2</sub> at T0	7±2.5	6.3±2.5	6.5±4.4	5.3±1.3	4.3±0.5	n.s.
Mean pCO <sub>2</sub> at T1	8.3±3.3	12±3.5*	7±2.5	40±19.6*	4.8±1	<0.001*
Mean lactic acid at T0	0.3±0.2	0.2±0	4.3±5.9	0.7±0.8	2.2±0.6	n.s.
Mean lactic acid at T1	0.5±0.4	0.3±0.2	12.7±9.9*	2.1±2.6	9.5±5.5*	<0.001*
Mean pH at T0	6.99±0.05	7.03±0.04	6.96±0.08	6.98±0.01	7.04±0.03	n.s.
Mean pH at T1	6.96±0.07	6.85±0.02	6.85±0.08	6.80±0.01	7±0.21	n.s.

CS – static cold storage; Hyp – static cold hyperbaric oxygenation with 1.5 atm; PE – hypothermic perfusion with arterial pressure of 25–30 mmHg; PE-Hyp – hypothermic perfusion in hyperbaric oxygenation with arterial pressure of 25–30 mmHg and with 1.5 atm; PE-O<sub>2</sub> – hypothermic oxygenated perfusion with arterial pressure of 25–30 mmHg and with 2–4 L of O<sub>2</sub> level maintaining pO<sub>2</sub> close to 700 mmHg; T0 – before starting the treatment; T1 – at the end of three hours of treatment. All preservation treatments were performed for three hours at 4°C with 1 L Celsior solution.

## Results

### Features of the discarded kidneys

The kidneys were discarded for various reasons: high Remuzzi score [31], donor age, or too long CIT. The mean donor age was 70±4 years while the minimum CIT before the start of the planned treatment was 20 hours. Data were comparable among all experimental groups (Table 1).

### Histological analysis and immunohistochemistry

Each experimental group included organs (minimum one and maximum three) at T0 with pathological evidences of glomerular

sclerosis, tubular injury, mild interstitial fibrosis, interstitial chronic fibrosis, or arterial myointimal thickening. After T1, endothelial damage appeared in one or two cases for all groups without any correlation to perfusion or oxygen. An increased expression of CD31 and CD34, indicating a well-preserved tissue, was observed in the CS, Hyp, and PE-Hyp groups; instead, expression was not significant for PE and PE-O<sub>2</sub> (Figure 1, left column). TEM showed endothelial injury only for the PE-group (Figure 2C) respect to the CS, Hyp, PE-Hyp and PE-O<sub>2</sub> groups (Figure 2A, 2B, 2D, 2E, respectively).

### Metabolic evaluation of the preservation solution

As expected,  $pO_2$  was significantly higher in the Hyp, PE-Hyp, and PE- $O_2$  groups compared to the CS and PE groups. At T1, the lactate concentration was significantly higher in all perfusion groups except for PE-Hyp. The  $pCO_2$  was significantly higher in the Hyp and PE-Hyp groups with respect to all the other groups. Of note,  $pCO_2$  levels were not evaluated in the PE- $O_2$  group since the oxygenator was equipped with a  $CO_2$  scavenger. Data expressed as mean and SD are reported in Table 1.

### Tissue ATP content

The amount of ATP content for each preservation group is shown in Figure 3 and presented as the ratio of T1 over T0 samples. In the CS, Hyp, and PE groups a net depletion of ATP content following the preservation procedure was observed. On the contrary PE-Hyp as well as PE- $O_2$  were associated with a net increase of ATP content with respect to baseline levels. As a result, at the end of the preservation the amount of ATP was significantly higher in the PE-Hyp group with respect to the CS, Hyp, and PE groups, while ATP level in PE- $O_2$  was significantly increased only with respect to the PE and Hyp groups. Additionally, grouping all samples with the exception of PE- $O_2$  for the  $CO_2$  of the oxygenator device scavenger showed a significant correlation between ATP content and  $pCO_2$  (Pearson correlation 0.759,  $p=0.001$ ).

### Gene expression of eNOS and HIF1 $\alpha$

As shown in Figure 4A, the mRNA level of eNOS was reduced in the PE-Hyp group, while in the other groups, including PE- $O_2$ , the mRNA level was not decreased. As a result, PE-Hyp was associated with a significant downregulation of eNOS gene expression with respect to CS, PE, Hyp, and PE- $O_2$  groups. The gene expression of HIF-1 $\alpha$  was also evaluated. All preservation modalities were associated with a slight reduction in the expression of HIF1 $\alpha$  with respect to CS, reaching statistical significance only in the PE-Hyp group (Figure 4B).

Expression of genes involved in the inflammatory response (interleukin-6) and cellular apoptosis (caspase-3) were also evaluated; however, no significant differences between groups were seen when the gene expression levels in T1 samples were compared to that of T0 samples (data not shown).

## Discussion

The present experimental study indicated that three hours of hypothermic perfusion with hyperbaric or normobaric oxygenation restored ATP levels, a signal of organ functional activity. These improvements were possible even with extended criteria

grafts declared not suitable for transplantation and after a prolonged CIT (more than 20 hours). An increased ATP content during organ preservation may lead to better outcomes and reduced risks of organ dysfunction after transplantation [32]. Furthermore, the reduced gene expression of both eNOS and HIF1 $\alpha$  in the PE-Hyp group may suggest optimal delivery of both oxygen and nutritional support to the graft.

Metabolic evaluations showed that the high  $pO_2$  level detected at T1 in the Hyp, PE- $O_2$ , and PE-Hyp groups was related to a higher level of  $pCO_2$  for Hyp and, mostly, to the PE-Hyp group showing an increased metabolic activity of the graft. Therefore, the different  $pCO_2$  level between the Hyp and PE-Hyp groups suggests that only hyperbaric oxygenation without perfusion may lead renal cells to not efficiently exploit the  $O_2$  dissolved in the storage solution to maintain their basal metabolism. A high  $pCO_2$  level was not detected in the PE- $O_2$  group, due to the  $CO_2$  scavenger of the oxygenator device. In the PE group  $pCO_2$  did not change at T1 with respect to T0; only perfusion was less effective in maintaining the renal basal metabolism. Like the perfusion groups, PE and PE- $O_2$  had higher levels of lactic acid in the preservation solution because the organs were flushed, but PE-Hyp did not show this increased level. During perfusion, the hyperbaric oxygenation probably stimulated a better aerobic metabolism, yielding higher ATP and lower lactate concentrations, as previously reported by other authors in different experimental settings [33–35]. The reduced lactate production may contribute to the beneficial effect of the hyperbaric oxygenation in ischemia/reperfusion injury. Furthermore, hyperbaric perfusion showed a significantly different gene expression of HIF-1 $\alpha$  and eNOS. These results again suggested a reduction of the preservation injury and a better delivery of oxygen to the tissue. HIF1 $\alpha$  expression normally occurs during SCS as an adaptive response to sub-optimal oxygen tension, therefore its reduced expression in the PE-Hyp group indicates optimal oxygen delivery to the graft [36]. The expression of eNOS in the same group also supports this hypothesis. Indeed, it was reported that eNOS gene expression was upregulated during hypoxia, thus favoring vasodilation in order to ensure an adequate blood supply [37].

The principal mechanisms of action of hyperbaric oxygenation are based on antioxidant enzymes induction, inflammatory cytokine suppression, angiogenesis, vasculogenesis, and enhancement of stem cell mobilization [38–45]. An early event associated with post-ischemic tissue reperfusion is the adherence of circulating neutrophils to vascular endothelium by  $\beta_2$  integrin. Some authors have shown that in many tissues, including liver and kidney, hyperbaric oxygen, unlike normobaric oxygen, temporarily arrests the adherence/sequestration of neutrophils by inhibiting the  $\beta_2$  integrin function and induces the release of antioxidant enzymes and anti-inflammatory proteins [40].

Our study explored only some aspects of these beneficial effects, but the absence of damage at histological examination, the improved metabolic activity, and a different gene expression seem to establish a good basis for the novel application of PE-Hyp of grafts in clinical settings.

All experimental procedures in each group were conducted setting arterial pressure and the time of perfusion after preliminary validation. We selected 30 mmHg as arterial pressure for the perfusion because, as reported by other authors [46] and in our initial experimental phase (data not reported), higher arterial pressure values (50–60 mmHg) were associated with endothelial damage and altered parenchymal architecture. Confirming our hypothesis, IHC and ultrastructural analysis showed that the glomerular and interstitial endothelial cells were well preserved.

We set the timing of treatment at three hours in order to keep the CIT as short as possible, due to its relation to organ dysfunction and survival [47,48]. Additionally, in our preliminary experiments, longer treatment times were not associated with better features of kidneys in terms of metabolic activity and tissue damage (data not reported). Other groups have reported one hour as an effective time of treatment with oxygen for liver transplantation and in the setting of donation after circulatory death [20].

Many case series have reported CIT values between 10 and 18 hours [8,9] and >30 hours [7]. Often, CIT is secondary to recipient selection and dialysis before transplantation. In many circumstances, therefore, the kidneys are available for pre-transplant treatments during recipient preparation and three hours seemed a reasonable compromise between a short CIT and the opportunity of improving the quality of the preserved graft.

Other clinical strategies for improving graft function are currently under debate, such as reduced donor temperature, sub-normothermic perfusion, or use of a cell-free oxygen carrier [49–52]. Up until now, there has been no evident clinical data in favor of one strategy compared to the others, but in agreement with some authors' suggestions [53], our group believes that oxygenation during hypothermia is effective and simple. In addition, we used hyperbaric oxygenation, which has been reported as kidney perfusion procedure since 1966 [54,55], and has been used by many researchers in the past on animal bloodless perfusion kidneys. Hyperbaric oxygen therapy (HBO) continues to be investigated in the field

of renal disease. Heiachi et al. confirmed that the HBO after renal ischemia reperfusion injury of rats decreased apoptosis and increased cellular proliferation [56]. We have applied hyperbaric oxygen in kidney perfusion *ex-situ*, cited among oxygenation techniques [22], and have not yet applied it for clinical human kidney preservation. This represents a novel aspect of our research work on organ perfusion.

Some limitations of our study have to be acknowledged. We did not have a healthy control kidney to use as a reference; ethical issues do not allow us to use organs suitable for transplantation. Furthermore, study cases were not transplanted and so we have no data about graft function and survival. However, our results may be the basis for advanced clinical investigations into the beneficial effect of hypothermic oxygenated perfusion in normobaric or hyperbaric condition for marginal kidneys before transplantation.

## Conclusions

Hyperbaric or normobaric oxygenation and dynamic hypothermic perfusion improve organ metabolic preservation compared to other treatments that were tested in our study. Thus, these advanced organ preservation techniques may avoid the risk of delayed graft function; however, clinical trials are needed to confirm our study findings (ClinicalTrials.gov ID: NCT03031067).

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## Conflict of interest

None.



## Supplementary file

### Results of histological analysis and immunohistochemistry in detail

#### a) CS

The 4 grafts of the control group had mainly mild interstitial fibrosis, tubular atrophy and glomerular sclerosis at T0.

After CS (T1), 2/4 grafts showed a histologically visible tubular injury, characterized by simplification and focal vacuolization of tubulocytes, and appearance of ATN was observed in 2 cases. One graft showed endothelial damage. At T1, IHC showed an increased expression of the investigated proteins (Figure 1, left column); in particular, CD34 increased significantly. These results, indicating well preserved tissue, were confirmed by ultrastructural examination; indeed, in T1 samples, endothelial cells of glomeruli and interstitium showed excellent morphology including nuclei with dispersed chromatin and small nucleoli as well as a normal complement of cytoplasm organelles (Figure 2A).

#### b) Hyp

Among the 4 grafts of this group, 1 had some degree of tubular injury with ATN at T0.

At T1, 3 grafts showed the same morphological picture as to T0 and endothelial damage appeared in 1 graft. The expression of CD31 and CD34 increased (Figure 1, left column); a significant CD34 increase was seen. At ultrastructural examination, endothelial cells were normal without any feature of subtle cell damage (Figure 2B).

#### c) PE

Among the 4 grafts of this group, 2 had some degree of tubular injury with ATN at T0, and diffuse glomerular sclerosis was seen in one. After PE, 2/4 grafts showed the same morphology as T0, while 2 grafts had some degree of endothelial injury. Unlike the previous group, a decreased and non-significant expression of CD31 and CD34 was seen at IHC (Figure 1, left column). TEM showed endothelial cell injury; markedly electron dense shrunken cells with cytoplasm vacuolization (Figure 2C).

#### d) PE-Hyp

In this group only 1 case was characterized by diffuse glomerular sclerosis, tubular atrophy with interstitial chronic phlogosis and arterial myointimal thickening. At T1, 2 grafts showed the same morphology as at T0, while the other 2 showed a global

worsening of histologic appearance, with glomerular simplification, tubular atrophy and endothelial loss.

The CD31 and CD34 expression increased in this group too, and CD31 was significantly expressed after the treatment (Figure 1, left column). Accordingly, TEM revealed unremarkable endothelial cells (Figure 2D).

#### e) PE-O<sub>2</sub>

Among the 4 grafts of this group, 3 showed some degree of tubular injury with ATN at T0.

After PE-O<sub>2</sub>, 3/4 grafts showed the same morphology as at T0, while 1 graft had some degree of endothelial injury. The CD31 and CD34 expression did not vary after the treatment; in particular, CD31 showed a slightly increased expression whereas CD34 decreased a little (Figure 1, left column). TEM of endothelial cells did not show any difference after the treatment (Figure 2E).

### Formal-fixed paraffin-embedded tissues processing protocol

#### 1. Preparation and cassetting

- a. Fixed tissue is sent to the Histology facility.
- b. If not done yet, grossing, slicing and cassetting are achieved by our technical staff.

#### 2. Processing: wax infiltration step inside tissue sample

- a. Dehydration: 3 alcohol baths with growing concentrations, 70–85–90%, preventing tissue damage (distortion, hardening). Water is finally removed by 3 final absolute alcohol baths.
- b. Clearing: 3 toluene baths will enable to replace alcohol trapped inside tissues and to be a miscible solvent with wax.
- c. Wax infiltration: hot wax baths (44–60°C) will solidify the tissue.

#### 3. Tissue embedding

- a. Tissue is orientated inside a mold filled with hot paraffin.

#### 4. Tissue sectioning using a microtome.

- a. Most of the time, histology sections are 4 µm thick. Wax ribbons are transferred onto a warm water bath (43–45°C) and spread on a microscope glass slide before drying at least for one hour at 45°C.

#### 5. Routine stain

- a. HE (Hematoxylin-Eosin) or HPS (Hematoxylin-Phloxin-Safran)
  - Deparaffinize
  - 2 baths of toluene (95–100%) 3 minutes each
  - 3 baths of alcohol (80–95–100%) 3 minutes each

- Rinse in tap water
- Gill Hematoxylin for 3 to 10 minutes
- Sodium Bicarbonate for 5 to 10 seconds
- Rinse in tap water
- Eosin/Phloxin 1 minute
- Rinse in tap water

- 3 baths of absolute alcohol 40 seconds each. (Safran. for 3 to 10 minutes)
  - 3 baths of absolute alcohol 40 seconds each
  - 3 baths of toluene
- b. Special stains

## 6. Pathologist analysis.

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