Kidney Protection by Hypothermic Total Liquid Ventilation after Cardiac Arrest in Rabbits

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ABSTRACT

Background: Total liquid ventilation (TLV) with perfluorocarbons has been shown to induce rapid protective cooling in animal models of myocardial ischemia and cardiac arrest, with improved neurological and cardiovascular outcomes after resuscitation. In this study, the authors hypothesized that hypothermic TLV can also limit kidney injury after cardiac arrest.

Methods: Anesthetized rabbits were submitted to 15 min of untreated ventricular fibrillation. After resuscitation, three groups of eight rabbits each were studied such as (1) life support plus hypothermia (32°–33°C) induced by cold TLV (TLV group), (2) life support without hypothermia (control group), and (3) Sham group (no cardiac arrest). Life support was continued for 6 h before euthanasia and kidney removal.

Results: Time to target esophageal temperature was less than 5 min in the TLV group. Hypothermia was accompanied by preserved renal function in the TLV group as compared with control group regarding numerous markers including creatinine blood levels (12 ± 1 *vs.* 16 ± 2 mg/l, respectively; mean ± SEM), urinary *N*-acetyl-β-(D)-glucosaminidase (1.70 ± 0.11 *vs.* 3.07 ± 0.10 U/mol of creatinine), γ-glutamyltransferase (8.36 ± 0.29 *vs.* 12.96 ± 0.44 U/mol of creatinine), or β2-microglobulin (0.44 ± 0.01 *vs.* 1.12 ± 0.04 U/mol of creatinine). Kidney lesions evaluated by electron microscopy and conventional histology were also attenuated in TLV *versus* control groups. The renal-protective effect of TLV was not related to differences in delayed inflammatory or immune renal responses because transcriptions of, for example, interferon-γ, tumor necrosis factor-α, interleukin-1β, monocyte chemoattractant protein-1, toll-like receptor-2, toll-like receptor-4, and vascular endothelial growth factor were similarly altered in TLV and control *versus* Sham.

Conclusion: Ultrafast cooling with TLV is renal protective after cardiac arrest and resuscitation, which could increase kidney availability for organ donation. **(Anesthesiology 2014; 120:861-9)**

INSTITUTION of therapeutic hypothermia has been well demonstrated to improve both survival and neurological outcome in patients resuscitated after out-of-hospital cardiac arrest.[1,2] Beyond this neuroprotective effect, it is also important to investigate the effect of hypothermia on the multivisceral dysfunction and the so-called "postcardiac syndrome."[3] As example, acute kidney injury affects approximately 12% of the survivors after cardiac arrest and could worsen the prognosis.[4] In this setting, Susantitaphong *et al.*[5] recently analyzed clinical studies reporting kidney-related outcomes and demonstrated that therapeutic hypothermia prevented neither the development of acute kidney injury nor dialysis requirement. In animal models of cardiac arrest, the benefit afforded by hypothermia however directly depends upon its rapidity of institution after cardiopulmonary resuscitation.[6] This benefit was investigated regarding cardiac and neurological outcomes,[6–10] whereas renal function was not precisely investigated. Therefore, we hypothesized that hypothermia can also exert a renal-protective effect after cardiac arrest if applied very rapidly after the no-flow episode.

What We Already Know about This Topic

- Total liquid ventilation with perfluorocarbons has been shown to induce rapid protective cooling in animal models of myocardial ischemia and cardiac arrest, with improved neurological and cardiovascular outcomes after resuscitation. However, few studies have focused on the effect of hypothermia on renal protection after cardiac arrest.
- This study determined whether ultrafast cooling with total liquid ventilation in a rabbit model can increase kidney resistance to the postcardiac arrest syndrome.

What This Article Tells Us That Is New

- As demonstrated by several renal biomarkers, ultrafast cooling induced by total liquid ventilation protects kidneys in a severe model of cardiac arrest in rabbits.

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An original strategy providing ultrafast cooling is total liquid ventilation (TLV) of the lungs with temperature-controlled perfluorocarbons.[11] TLV can indeed use the lung as a heat exchanger and cool the body while maintaining gas exchanges.[10,12–14] As compared with conventional cooling with combined cold blankets and fluid administration, TLV provided a potent protection on brain and heart in rabbits submitted to equal or less than 10 min of cardiac arrest.[10,15] In this study, we propose to use more severe experimental conditions inducing kidney dysfunction after 15 min of cardiac arrest in rabbits. We hypothesized that ultrafast cooling with TLV can increase kidney resistance to the postcardiac arrest syndrome. Our endpoints were kidney function biomarkers, morphological appearance, and transcriptomic responses.

Materials and Methods

The experiments were conducted in accordance with French official regulations, after approval by the institutional Animal Care Committee (ComEth "Anses/ENVA/UPEC" n°16, Maisons-Alfort; protocol 13/12/11–5). It conformed with the guidelines laid out in the Guide for the Care and Use of Laboratory Animals from the National Academy of Science.

Animal Preparation

New Zealand rabbits (3.0–3.5 kg) were anesthetized with zolazepam, tiletamine, and pentobarbital (all 20–30 mg/kg i.v.). They were intubated and mechanically ventilated (F_{IO_2} = 100%). After administration of pancuronium bromide (200 μg/kg i.v.), two electrodes were implanted upon the chest and inserted into the esophagus for subsequent induction of ventricular fibrillation. Rectal, esophageal, and tympanic temperatures were continuously monitored by using thermal probes (Harvard Apparatus, Paris, France). Throughout the protocol, external electrocardiogram was recorded, as well as arterial blood pressure from a catheter implanted into the ear artery. Data were digitalized and analyzed by using the data acquisition software HEM v3.5 (Notocord, Croissy-sur-Seine, France).

Experimental Protocol

As illustrated in figure 1, the animals were randomly assigned after a period of stabilization to the Sham group or two groups submitted to cardiac arrest (control and TLV groups). In these two groups, cardiac arrest was induced by ventricular fibrillation by passing an alternating current (10 V, 4 mA) between the implanted electrodes. After 15 min of untreated fibrillation, cardiopulmonary resuscitation was started by using cardiac massage (approximately 200 compressions per minute), electric attempts of defibrillation (5–10 J/kg), and intravenous administration of epinephrine (15 μg/kg i.v.). After resumption of spontaneous circulation, administration of epinephrine was still permitted with an infusion pump to maintain mean arterial pressure at approximately 80 mmHg. In the TLV group, the animals were cooled to 32°C by using TLV after resumption of spontaneous circulation. The lungs were filled with 10 ml/kg of perfluorocarbon (Fluorinert, 3M, Cergy, France), and the endotracheal tube was connected to our prototype of liquid ventilator (tidal volume = 7–10 ml/kg; respiratory rate = 6 breaths/min). The temperature of the perfluorocarbon was adjusted to maintain esophageal temperature at a target temperature of approximately 32°C. After 20 min of TLV and achievement of the hypothermic target temperature, the perfluorocarbon was evacuated from the lungs, and the endotracheal tube was again connected to a conventional mechanical ventilator. Hypothermia was maintained externally at 32°C during the subsequent entire follow-up. In all groups, the animals were followed during a total duration

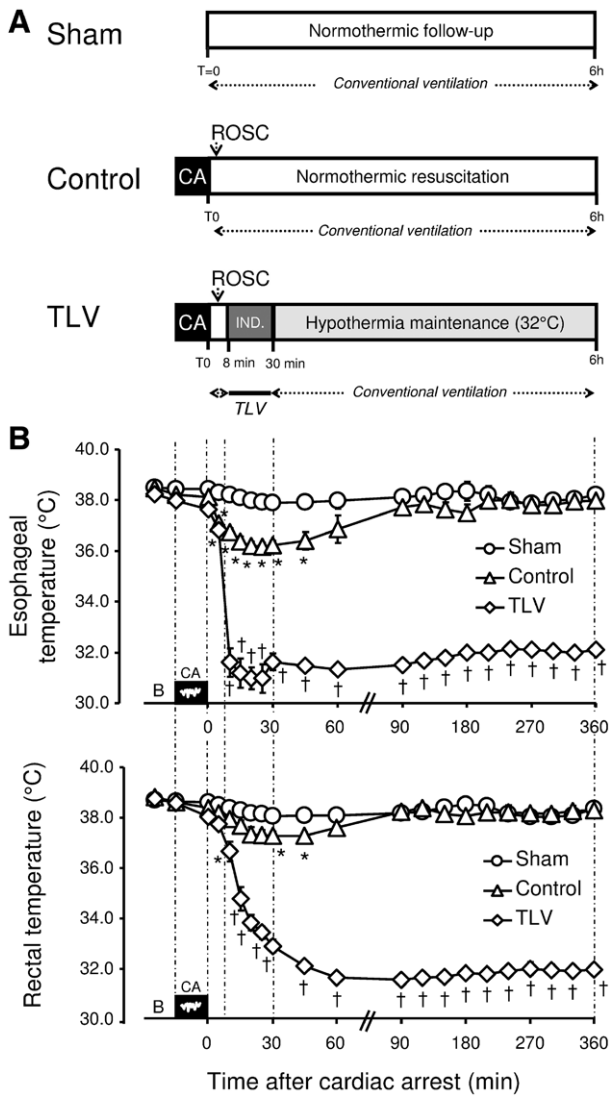

Fig. 1. Schematic representation of the experimental protocol (*A*) and body temperatures throughout follow-up (*B*). *$P < 0.05$ *versus* corresponding control; †$P < 0.05$ *versus* Sham and control. CA = cardiac arrest; IND = induction of hypothermia through TLV; ROSC = resumption of spontaneous circulation; TLV = total liquid ventilation.

of 6 h after cardiac arrest. Blood samples were withdrawn at baseline, 15, 60, 180, and 360 min for the assessment of blood creatinine levels and blood gases partial pressure. Urine production as well as urinary creatinine concentration for calculation of the corresponding clearance was also measured. We also assessed blood and/or urinary osmolarity (freezing point depression osmometer; Roebling Osmometer, Burladingen, Germany) and levels of sodium, glucose, creatine phosphokinase (Hitachi/Roche Cobas; Roche Diagnostic, Meylan, France), *N*-acetyl-β-(D)-glucosaminidase (proximal tubule lysosomal enzyme; Roche Diagnostic, Mannheim, Germany), γ-glutamyltransferase (marker of acute renal injury; Roche Diagnostic), and β2-microglobulin (marker of proximal tubule dysfunction; Roche Diagnostic). Fractional sodium excretion was calculated from blood and urinary sodium levels. At the end of the follow-up, animals were euthanized and kidneys were sampled for electron microscopy, conventional histology, and molecular biology.

Electron Microscopy and Histological Analyses

Kidneys samples were processed by transmission electron microscopy, as previously described.[16] In brief, tissue sections of 1 mm^3 were fixed in glutaraldehyde (3%; 2 h at 4°C), washed and postfixed in osmium tetroxide (1%; 1 h at 4°C). They were dehydrated in graded series of 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). Mitochondria integrity (membrane damage and crest reduction), cellular edema, loss of brush border, cellular vacuolization, and lyses of intracellular organelles were evaluated. The degree of histological lesions was determined in the cortex and medulla by using a semi-quantitative graded scale from 0 to 10 according to lesion extension among the kidney samples (0, no alterations; 1, mild lesions <10% of the kidney; 2, lesions affecting 11–20%; 3, 21–30%; 4, 31–40%; 5, 41–50%; 6, 51–60%; 7, 61–70%; 8, 71–80%; 9, 81–90%; and 10, >91%). Scores were blindly attributed by two independent observers after examination of at least 10 different sections.

Kidneys slices were also fixed in formaldehyde (4%) for conventional histology after hematoxylin–eosin–saffron staining. We used a 0 to 5 score system to blindly quantify the severity of the lesions in the cortex and the medulla (0, normal appearance; 5, extensive necrosis). For each animal, the sum of these two scores (cortex and medulla) led to an overall score from 0 to 10. Detection of macrophages was performed on paraffin tissue sections by using the RAM11 antibody against rabbit macrophages (Dako, Trappes, France), as previously described.[17] The brush-border integrity was also evaluated by immunochemistry staining by using the CD10 antibody (Dako).

Real-time Quantitative Polymerase Chain Reaction

In all animals, kidney samples were fixed immediately after organ removal by using liquid nitrogen. For cortical tissue RNA extraction, we used a commercial kit (Macherey Nagel, Hoerdt, France). Genomic DNA was removed by using DNA-free kit (Applied Biosystems, Saint Aubin, France), and first-strand reverse transcription (Applied Biosystems) was performed. Real-time polymerase chain reaction assays were performed on a RotorGene Q (Qiagen, Courtaboeuf, France) following the manufacturer's recommendations. Rabbit DNA primers were designed using OligoPerfect™ (Invitrogen, Carlsbad, NM), QuantPrim (Universität Potsdam, Max-Planck-Gesellschaft), and OligoAnalyzer (Integrated DNA Technologies, Coralville, IA), with the sequences detailed in Supplemental Digital Content 1, http://links.lww.com/ALN/B5. Finally, expression level of messenger RNA, relative to expression in healthy kidneys, was quantified with the Pfaffl method (expressed as relative fold change), using ribosomal L19, β-actine, and ribosomal protein large P0 as gene references.

Statistics

Data were expressed as mean ± SEM. Statistical analyses were performed with the use of a statistical software (SigmaStat 3.5; Systat Software Inc., Chicago, IL). Hemodynamic and biochemical parameters were compared between groups by using a two-way ANOVA for repeated measures. *Post hoc* analyses were performed between groups at each time point using a Student *t* test with Bonferroni correction (two-tailed). Values were not compared between the different time points to avoid multiple comparisons. Histological scores and molecular biology markers were compared between groups by using a Kruskall–Wallis nonparametric test. Significant differences were determined at a *P* value of 0.05 or less.

Results

Twenty-four animals were included in the different groups (n = 8 per group), with no missing data. In control and TLV groups, resumption of spontaneous circulation was obtained in 4.1 ± 0.7 and 3.7 ± 0.5 min after cardiac arrest, respectively.

TLV Affords a Very Rapid Cooling and Preserves Hemodynamic

As illustrated in figure 1B, a mild and reversible decrease in esophageal and rectal temperatures was observed in the control group after cardiac arrest. In comparison, temperatures decreased very rapidly in the TLV group and achieved 32°C within 5 min after the onset of TLV in the esophagus. As shown in table 1, this was associated with a strong decrease in heart rate in the TLV group as compared with control and Sham groups (*e.g.*, −32% in TLV *vs.* control groups at t = 360 min after cardiac arrest). Only minor changes were observed regarding mean blood pressure as our goal was to support values of approximately 80 mmHg using epinephrine infusion. The total doses administered to achieve this goal were significantly higher in control *versus* TLV groups (990 ± 179 and 361 ± 23 μg/kg, respectively),

Table 1. Heart Rate, Mean Arterial Pressure, and Blood Biochemical Parameters

		After Cardiac Arrest (min)			
	Baseline	15	60	180	360
Heart rate, beats/min					
Sham	25 ± 12	234 ± 8	239 ± 12	244 ± 11	235 ± 8
Control	254 ± 8	204 ± 15*	184 ± 9*	187 ± 5*	203 ± 11*
TLV	250 ± 7	151 ± 6†	131 ± 3†	137 ± 2†	139 ± 5†
Mean blood pressure, mmHg					
Sham	75 ± 3	72 ± 3	80 ± 3	87 ± 3	83 ± 5
Control	81 ± 4	84 ± 4*	80 ± 5	84 ± 3	63 ± 5*
TLV	78 ± 3	98 ± 3†	84 ± 4	74 ± 2†	74 ± 5†
Lactates blood levels (mM)					
Sham	3.0 ± 0.3	2.8 ± 0.5	2.6 ± 0.3	2.2 ± 0.3	2.2 ± 0.5
Control	2.3 ± 0.4	13.0 ± 1.7*	12.9 ± 1.3*	13.1 ± 0.8*	11.6 ± 1.3*
TLV	2.7 ± 0.2	9.6 ± 0.9†	12.1 ± 1.0*	10.6 ± 0.8*	9.8 ± 1.0*
Glucose blood levels, mg/dl					
Sham	183 ± 23	180 ± 19	130 ± 15	133 ± 6	126 ± 4
Control	184 ± 31	480 ± 27*	536 ± 35*	700 ± 28*	550 ± 47*
TLV	153 ± 58	375 ± 64*	460 ± 76*	477 ± 88†	474 ± 82*
Creatine phosphokinase blood levels, mg/dl					
Sham	42 ± 1	—	—	—	56 ± 1
Control	43 ± 1	—	—	—	69 ± 2*
TLV	44 ± 1	—	—	—	67 ± 1*
Blood pH					
Sham	7.41 ± 0.02	7.42 ± 0.03	—	7.44 ± 0.02	7.45 ± 0.02
Control	7.42 ± 0.02	7.05 ± 0.05*	—	7.06 ± 0.08*	7.05 ± 0.07*
TLV	7.42 ± 0.05	6.92 ± 0.06†	—	7.11 ± 0.05*	7.04 ± 0.05*
Blood pCO_2, mmHg					
Sham	46 ± 3	37 ± 4	—	37 ± 3	38 ± 2
Control	46 ± 5	44 ± 3	—	43 ± 3	43 ± 6
TLV	42 ± 3	77 ± 8†	—	34 ± 7	32 ± 4
Blood pO_2, mmHg					
Sham	532 ± 31	558 ± 34	—	550 ± 29	559 ± 38
Control	504 ± 34	185 ± 41*	—	269 ± 72*	250 ± 65*
TLV	520 ± 57	245 ± 61*	—	310 ± 58*	201 ± 71*

* $P < 0.05$ *vs.* corresponding Sham group; † $P < 0.05$ *vs.* corresponding Sham and control groups.
TLV = total liquid ventilation.

showing favorable hemodynamic effects of hypothermia. Despite epinephrine administration, blood pressure was moreover significantly decreased in the control group at the end of the follow-up (t = 360 min after cardiac arrest) as compared with the TLV group. In these two groups, we also observed a dramatic increase in glucose and lactate blood levels, as well as acidosis and decrease in blood oxygen partial pressure (table 1). During the TLV episode (15 min after cardiac arrest), blood carbon dioxide partial pressure was also significantly higher in the TLV group as compared with control and Sham. Creatine phosphokinase blood levels were similarly increased in TLV and control *versus* Sham.

TLV Limits Kidney Injury after Cardiac Arrest

As shown in table 2, blood creatinine levels were significantly increased at the end of the follow-up (360 min) in both groups submitted to cardiac arrest as compared with the Sham group. They were significantly higher in the control group as compared with TLV. Creatinine clearance was also significantly reduced in both control and TLV groups when compared with Sham. This reduction tended to be more important in the control *versus* TLV group (+50%), but this did not achieve statistical significance. Total urine output also nonsignificantly decreased in control as compared with Sham and TLV groups. As shown in table 3, the beneficial effect of TLV on tubule function was evidenced by preserved fractional sodium excretion and urine concentration capacity. We also observed a limited glucose urinary excretion in TLV as compared with control animals, despite similar blood glucose levels (table 1). The urinary concentrations of selective markers of tubular damages, that is, *N*-acetyl-β-(D)-glucosaminidase, β2-microglobulin, and γ-glutamyl transferase, were also significantly decreased in TLV *versus* control despite not completely normalized as compared with Sham animals.

Table 2. Blood Creatinine Levels, Urine Production, and Creatinine Clearance

	Baseline	After Cardiac Arrest T = 360 min
Blood creatinine levels, mg/l		
Sham	7.5 ± 0.4	7.8 ± 0.8
Control	7.4 ± 0.6	16.1 ± 1.8*
TLV	6.9 ± 0.7	12.3 ± 1.2†
Total urine output, ml/h		
Sham	—	6.3 ± 1.6
Control	—	3.5 ± 0.6
TLV	—	7.1 ± 1.1
Creatinine clearance, ml min^{-1} kg^{-1}		
Sham	—	2.8 ± 0.5
Control	—	0.6 ± 0.2*
TLV	—	0.9 ± 0.2*

* $P < 0.05$ *vs.* corresponding Sham group; † $P < 0.05$ *vs.* corresponding Sham and control groups.
TLV = total liquid ventilation.

These functional alterations were supported by kidney lesions in animals submitted to cardiac arrest as compared with Sham animals (figs. 2 and 3). As shown in figure 2, electron microscopy revealed altered microvilli (brush border) and loss in cytosolic and mitochondrial crest density in the cortex and medulla in the control group (fig. 2, C and G). In comparison, appearance of cortex and medulla was better preserved in the TLV group (fig. 2, D and H). In Sham animals, the appearance was normal at electron microscopy (fig. 2, B and F). This led to a significant decrease in lesion score in TLV *versus* control group with a significant increase for both groups when compared with Sham (fig. 2A). The glomerular apparatus was preserved in all groups (fig. 2E). These differences were confirmed using conventional histology (fig. 3A). The injuries were indeed particularly marked in the cortex of the control group as illustrated by a tubular necrosis in figure 3C. In the TLV group, lesions were attenuated with "only" dilation of the proximal tubes (fig. 3D). Virtually no macrophage infiltrations were detected by using the RAM11 antibody (fig. 3, E–H), even in territories with extensive necrosis in the control group (fig. 3F). Figure 3G illustrates one of the rare macrophages in a territory with a normal appearance in this same group. Immunochemistry marking of the brush-border membrane showed extensive degradation in control animals (fig. 3J) as compared with Sham animals (3I). These alterations were not prevented in all animals in the TLV group. Some kidneys indeed showed normal appearance (fig. 3K) whereas others had clear brush-border membrane alteration (fig. 3L).

Table 3. Urinary Markers of Renal Function

	Baseline	After Cardiac Arrest T = 360 min
Fractional sodium excretion, %		
Sham	1.04 ± 0.03	0.84 ± 0.03
Control	1.01 ± 0.03	4.58 ± 0.67*
TLV	1.04 ± 0.02	2.09 ± 0.11†
Ratio between plasmatic and urinary osmolarity		
Sham	0.93 ± 0.01	0.91 ± 0.01
Control	0.94 ± 0.01	1.31 ± 0.02*
TLV	0.96 ± 0.01	1.01 ± 0.00†
Glucose urinary levels, g/l		
Sham	0.06 ± 0.01	0.10 ± 0.02
Control	0.06 ± 0.01	1.27 ± 0.02*
TLV	0.06 ± 0.01	0.40 ± 0.03†
N-acetyl-β-(D)-glucosaminidase urinary levels, U/mol of creatinine		
Sham	0.84 ± 0.05	0.87 ± 0.07
Control	0.94 ± 0.04	3.07 ± 0.10*
TLV	1.0 ± 0.03	1.70 ± 0.11†
β2-microglobulin urinary levels, U/mol of creatinine		
Sham	0.18 ± 0.01	0.20 ± 0.01
Control	0.17 ± 0.01	1.12 ± 0.04*
TLV	0.17 ± 0.01	0.44 ± 0.01†
γ-glutamyl transferase urinary levels, U/mol of creatinine		
Sham	5.31 ± 0.59	5.18 ± 0.39
Control	4.69 ± 0.34	12.96 ± 0.44*
TLV	4.82 ± 0.49	8.36 ± 0.29†

* $P < 0.05$ *vs.* corresponding Sham group; † $P < 0.05$ *vs.* corresponding Sham and control groups.
TLV = total liquid ventilation.

Cardiac Arrest Strongly Up-regulates Hypoxia and Inflammation Markers in Both Control and TLV Groups

As shown in figure 4A, we observed a dramatic increase in the expression of hypoxia markers in both control and TLV groups as compared with Sham (heme oxygenase-1, erythropoïetin, hypoxia-inducible factor-1α, and vascular endothelial growth factor). The expression of the apoptotic marker Fas and the mobility marker RhoA was also similarly expressed in TLV and control groups (fig. 4B). As illustrated in figure 4, C and D, the expression of endothelial activation and innate immunity markers was also not different between these two groups, including E-selectin, vascular cell adhesion molecule-1, interleukin-10, interleukin-1β, interferon-γ, tumor necrosis factor-α, interleukin-18, monocyte chemoattractant protein-1, and toll-like receptors 2 and 4.

Discussion

In the current study, we demonstrate that ultrafast cooling induced by TLV protects kidneys in a severe model of cardiac arrest in rabbits. This was strongly supported by improved renal function and preserved morphology by using electron microscopy and conventional histology. Transcriptomic profiles were not much affected by TLV regarding numerous genes involved in innate immunity and hypoxic responses.

We previously showed that ultrafast hypothermic TLV can strongly prevent both cardiovascular and neurological dysfunctions after cardiac arrest.[10] These investigations were conducted in rabbits after shorter duration of cardiac arrest comprised between 5 and 10 min.[10] In these conditions, we

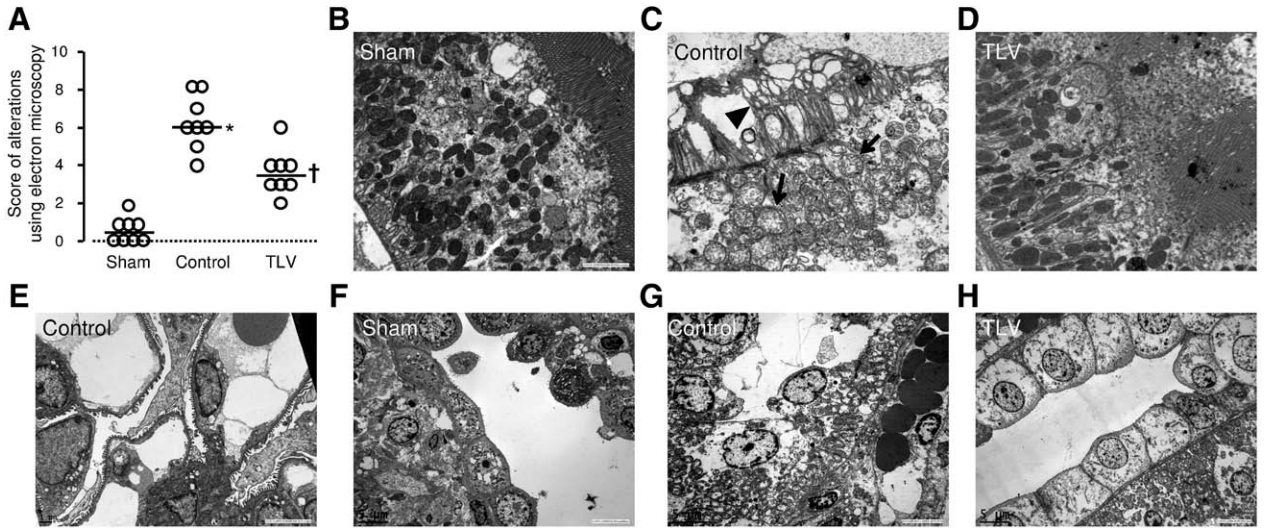

Fig. 2. Kidney lesions assessed by electron microscopy. (*A*) Individual scores and medians of the different groups of renal alterations by using electron microscopy. (*B–D*) Typical histological appearance of epithelial cells (proximal tubes) in the renal cortex from Sham, control, and total liquid ventilation (TLV) groups, respectively. In Sham and TLV groups, brush-border microvilli and mitochondria are well preserved. In control group, microvilli are altered (*arrowhead*) and mitochondria elicited a dramatic loss in density (*arrows*). (*E*) Typical histological appearance of the glomerular apparatus in a kidney from control group. This apparatus was well preserved in all groups. (*F–H*) Typical histological appearances of epithelial cells (connecting tubes) in the renal medulla from Sham, control, and TLV groups, respectively. Major loss in cytosolic density can be seen in the control group but not in Sham and TLV groups. **P* < 0.05 *versus* corresponding control; †*P* < 0.05 *versus* Sham and control.

observed dramatic cardiac and neurological dysfunctions, whereas renal dysfunction was very mild. In this study, we used a prolonged episode of 15 min of no-flow to obtain acute renal dysfunction and likely very poor neurological prognosis. TLV preserved urine output and significantly limited the increase in blood creatinine levels. The latter benefit was importantly not related to a decreased release of creatinine with hypothermia because creatine phosphokinase blood levels were similarly enhanced in TLV and control groups. Surprisingly, we did not observe any significant improvement in creatinine urinary clearance in TLV *versus* control. It is rather difficult to analyze this result according to the complex effect of hypothermia on urine production. After out-of-hospital cardiac arrest, Zeiner *et al.*[18] also showed a delayed recovery in creatinine clearance with therapeutic hypothermia (32°–34°C). One could also argue that hypothermia directly decreased urine production or conversely increased the production of diluted urine as previously showed in hypothermic patients treated for acute ischemic stroke[19] or with profound hypothermia.[20,21] This was not the case in our experimental conditions as hypothermia preserved urine production and osmolarity in the TLV group as compared with control. Importantly, the functional benefit afforded by TLV was also supported by a significant improvement of all markers of tubular damage, including *N*-acetyl-β-(D)-glucosaminidase, β2-microglobulin, and γ-glutamyl transferase. It would also be relevant to investigate original and specific biomarkers such as neutrophil gelatinase–associated lipocalin and kidney injury molecule-1.[22,23]

Morphological data definitely confirm the renal-protective effect of TLV in our study by using either conventional histology or electron microscopy. Such a beneficial effect of moderate hypothermia has also been shown in animal models of regional renal ischemia–reperfusion in rats.[24] In the latter study, body temperature during ischemia dramatically affected the severity of injury, again showing the importance of per-ischemic and early cooling. Intraischemic hypothermia is however difficult to achieve during cardiac arrest, especially regarding abdominal and renal temperatures. In a recent meta-analysis including 19 studies and 2,218 patients, Susantitaphong *et al.*[5] showed that therapeutic hypothermia prevented neither the development of acute kidney injury nor dialysis requirement but was associated with lower mortality. The current study suggests that the benefit of hypothermia could be still evidenced in very severe experimental conditions when it is induced very rapidly.

In the current study, the severity of the ischemic insult was also supported by kidney transcriptomic alterations. As an example, hypoxic stress led to a 30- to 50-fold increase in heme oxygenase-1 expression, as well as a 10- and 6-fold increases for erythropoïetin and hypoxia-inducible factor-1α, respectively. These expressions were not different between TLV and control groups, suggesting that the renal-protective effect of TLV was not directly mediated through these pathways. However, one could speculate that the potent up-regulation of erythropoïetin and heme oxygenase-1 could be associated with a facilitation of tissue repair, as previously suggested after tubulointerstitial injury and progressive nephritis.[25] We also did not observe

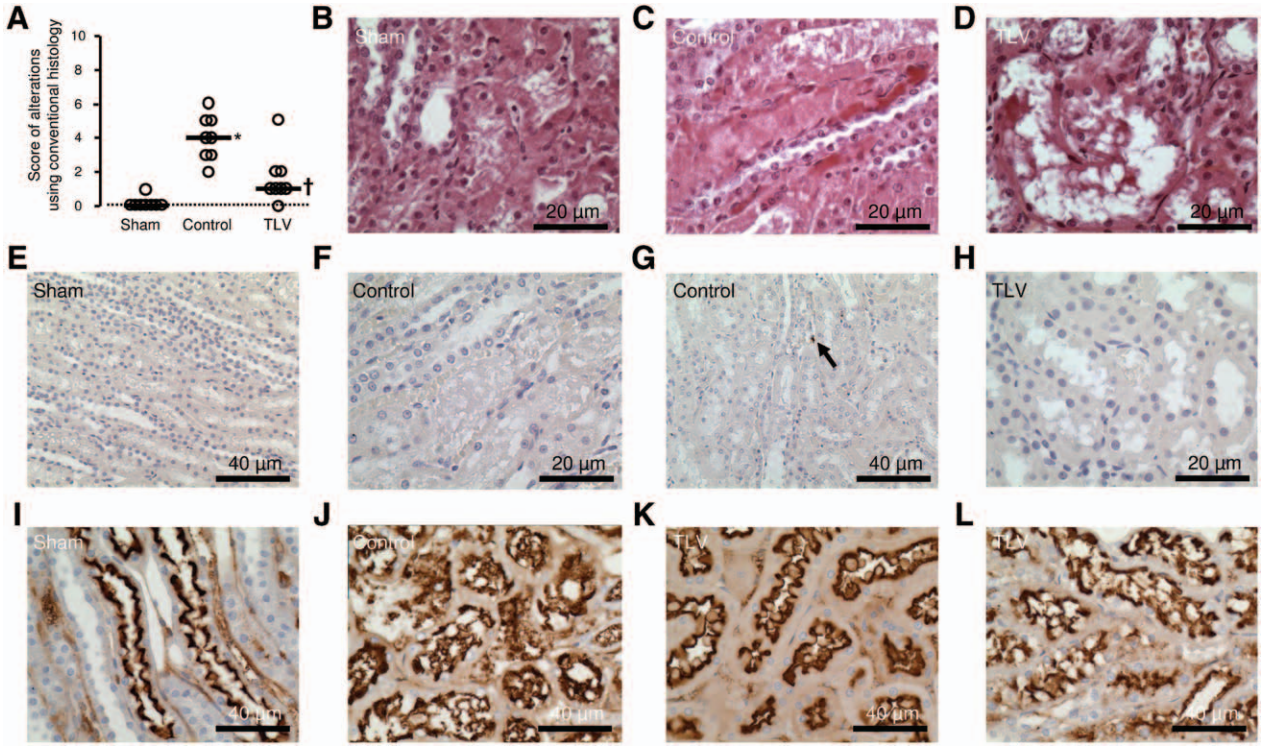

Fig. 3. Kidney lesions assessed by conventional histology. (*A*) Individual scores and medians of the different groups of renal alterations by using conventional histology. (*B–D*) Typical histological appearance of the renal cortex from Sham, control, and total liquid ventilation (TLV) groups, respectively. In kidney from the Sham group (*B*), no particular lesion can be observed. In kidney from the control group (*C*), a coagulation necrosis of epithelial cells can be observed in the proximal tubes. In kidney from the TLV group (*D*), a dilation of the proximal tube can be observed. (*E–H*) Typical appearance of the kidney from the different groups after immunohistochemistry with RAM11 antibody (marker of macrophage). No positive cells can be observed in most kidneys (*E*, *F*, and *H*), including in a foci of tube necrosis in the control groups (*F*). As shown in (*G*), very rare macrophages (*arrows*) were identified and usually remote from the foci of necrosis and/or tube dilation. (*I–L*) Typical appearance of the kidney from the different groups after immunohistochemistry with CD10 antibody (marker of the brush-border membrane). A preserved membrane was observed in all kidneys from the Sham group (*I*). In kidney from the control group, a loss in density was observed (*J*). Two kidneys from the TLV groups are shown with either preserved (*K*) or altered (*L*) patterns.*$P < 0.05$ *versus* corresponding control; †$P < 0.05$ *versus* Sham and control.

any significant difference in transcriptomic profiles of cellular mobility, endothelial activation, and innate immunity markers between control and TLV groups. A nonsignificant decrease in toll-like receptor-2 and proinflammatory tumor necrosis factor-α and interferon-γ markers was however observed in the TLV group, as well as a mild and nonsignificant up-regulation of the regulatory interleukin-10 cytokine. Interestingly, the latter cytokine was shown to mediate delayed protection afforded by remote ischemic preconditioning against myocardial ischemia–reperfusion injury.[26] Conversely, expressions of toll-like receptor-2 and toll-like receptor-4 are well known to mediate kidney ischemia–reperfusion damages.[27,28] The lack of differences between groups could be a consequence of the early time point of organ removal (6 h after cardiac arrest). This also suggests that the protective effect of TLV is at least in part related to other mechanisms, likely an early antinecrotic effect through ultrafast hypothermia. Improvement in the hemodynamic status could also participate in the renal-protective effect.

The main limitation of the current study was therefore probably the short duration of follow-up before organ sampling and analyses after cardiac arrest. Animals were followed during only 6 h as it was difficult to maintain animals alive for much longer duration (*e.g.*, 24 h) according to the severity of the cardiac arrest insult (15 min of no-flow). In future studies, it could be relevant to analyze kidney function at later time points, for example, after transplantation in recipient animals. Hypothermic TLV could indeed offers quite new and promising therapeutic perspectives for uncontrolled organ donation after cardiac arrest or for controlled organ donation after brain death in initially resuscitated patients.[29] It was not possible to conduct transplantation experiments in rabbits in the current experimental conditions, but we currently are working on a new technology of liquid ventilation that could be used in a relevant porcine model for a definitive proof-of-concept using organ transplantation.[30] There is indeed a high degree of proximity between human and pig kidneys with multilobular, multipapillary architecture, whereas mice, rats, dogs, and rabbits have unilobular, unipapillary kidneys.[31] In

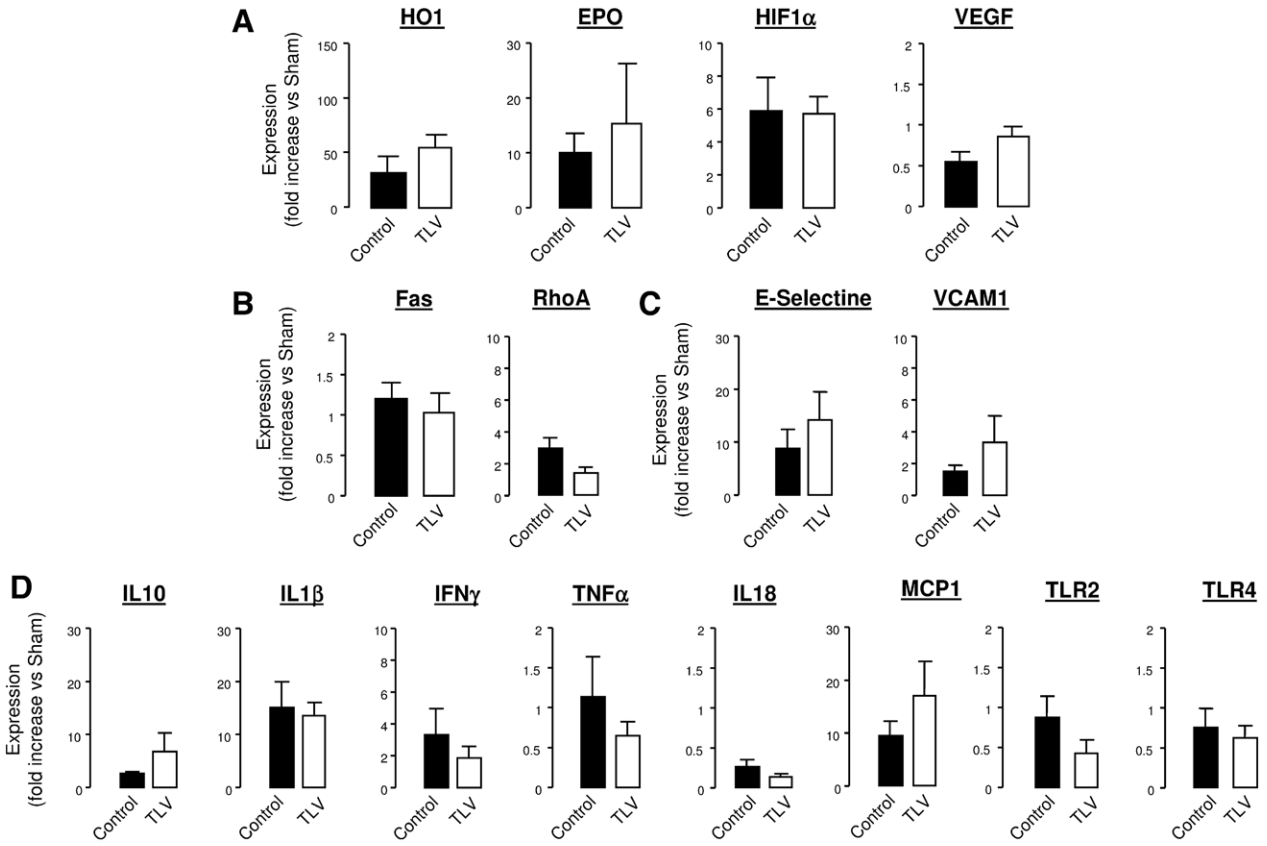

Fig. 4. Transcriptomic evaluation by real-time quantitative polymerase chain reaction of (*A*) hypoxic preconditioning markers, (*B*) apoptosis and mobility cellular markers, (*C*) endothelial activation markers, and (*D*) innate immunity markers. Results are expressed as mean ± SEM in relative folds change to healthy kidney. EPO = erythropoïetin; HIF1α = hypoxia-inducible factor-1α; HO1 = heme oxygenase-1; IFNγ = interferon-γ; IL = interleukin; MCP-1 = monocyte chemoattractant protein-1; TLR = toll-like receptor; TLV = total liquid ventilation; TNFα = tumor necrosis factor-α; VCAM1 = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor.

dogs and rodents, segmental arteries are bypassed due to the lack of multiple medullary pyramids, whereas in humans and pigs an elaborated system of interlobar and segmental arteries is present to supply the numerous kidney lobes.[30]

In conclusion, ultrafast cooling with TLV is renal protective after cardiac arrest and resuscitation. Further experiments with organ transplantation might be relevant to afford a proof-of-concept in this setting.

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Competing Interests

The authors declare no competing interests.

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References

1. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K: Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 2002; 346:557–63
2. The Hypothermia After Cardiac Arrest Study Group: Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 2002; 346:549–56
3. Adrie C, Laurent I, Monchi M, Cariou A, Dhainaou JF, Spaulding C: Postresuscitation disease after cardiac arrest: A sepsis-like syndrome? Curr Opin Crit Care 2004; 10:208–12
4. Domanovits H, Schillinger M, Müllner M, Thoennissen J, Sterz F, Zeiner A, Druml W: Acute renal failure after successful

cardiopulmonary resuscitation. Intensive Care Med 2001; 27:1194–9

5. Susantitaphong P, Alfayez M, Cohen-Bucay A, Balk EM, Jaber BL: Therapeutic hypothermia and prevention of acute kidney injury: A meta-analysis of randomized controlled trials. Resuscitation 2012; 83:159–67
6. Kuboyama K, Safar P, Radovsky A, Tisherman SA, Stezoski SW, Alexander H: Delay in cooling negates the beneficial effect of mild resuscitative cerebral hypothermia after cardiac arrest in dogs: A prospective, randomized study. Crit Care Med 1993; 21:1348–58
7. Yu T, Barbut D, Ristagno G, Cho JH, Sun S, Li Y, Weil MH, Tang W: Survival and neurological outcomes after nasopharyngeal cooling or peripheral vein cold saline infusion initiated during cardiopulmonary resuscitation in a porcine model of prolonged cardiac arrest. Crit Care Med 2010; 38:916–21
8. Yannopoulos D, Zviman M, Castro V, Kolandaivelu A, Ranjan R, Wilson RF, Halperin HR: Intra-cardiopulmonary resuscitation hypothermia with and without volume loading in an ischemic model of cardiac arrest. Circulation 2009; 120:1426–35
9. Abella BS, Zhao D, Alvarado J, Hamann K, Vanden Hoek TL, Becker LB: Intra-arrest cooling improves outcomes in a murine cardiac arrest model. Circulation 2004; 109:2786–91
10. Chenoune M, Lidouren F, Adam C, Pons S, Darbera L, Bruneval P, Ghaleb B, Zini R, Dubois-Randé JL, Carli P, Vivien B, Ricard JD, Berdeaux A, Tissier R: Ultrafast and whole-body cooling with total liquid ventilation induces favorable neurological and cardiac outcomes after cardiac arrest in rabbits. Circulation 2011; 124:901–11, 1–7
11. Shaffer TH, Forman DL, Wolfson MR: Physiological effects of ventilation with liquid fluorocarbon at controlled temperatures. Undersea Biomed Res 1984; 11:287–98
12. Chenoune M, Lidouren F, Ghaleb B, Couvreur N, Dubois-Rande JL, Berdeaux A, Tissier R: Rapid cooling of the heart with total liquid ventilation prevents transmural myocardial infarction following prolonged ischemia in rabbits. Resuscitation 2010; 81:359–62
13. Tissier R, Couvreur N, Ghaleb B, Bruneval P, Lidouren F, Morin D, Zini R, Bize A, Chenoune M, Belair MF, Mandet C, Douheret M, Dubois-Rande JL, Parker JC, Cohen MV, Downey JM, Berdeaux A: Rapid cooling preserves the ischaemic myocardium against mitochondrial damage and left ventricular dysfunction. Cardiovasc Res 2009; 83:345–53
14. Tissier R, Hamanaka K, Kuno A, Parker JC, Cohen MV, Downey JM: Total liquid ventilation provides ultra-fast cardioprotective cooling. J Am Coll Cardiol 2007; 49:601–5
15. Darbera L, Chenoune M, Lidouren F, Kohlhauer M, Adam C, Bruneval P, Ghaleb B, Dubois-Rande JL, Carli P, Vivien B, Ricard JD, Berdeaux A, Tissier R: Hypothermic liquid ventilation prevents early hemodynamic dysfunction and cardiovascular mortality after coronary artery occlusion complicated by cardiac arrest in rabbits. Crit Care Med 2013 Oct 11. [Epub ahead of print]
16. Goujon JM, Hauet T, Menet E, Levillain P, Babin P, Carretier M: Histological evaluation of proximal tubule cell injury in isolated perfused pig kidneys exposed to cold ischemia. J Surg Res 1999; 82:228–33
17. Aouam K, Tissier R, Bruneval P, Mandet C, Berdeaux A, Ghaleb B: Preconditioning of salvaged myocardium in conscious rabbits with postinfarction dysfunction. Am J Physiol Heart Circ Physiol 2005; 288:H2763–9
18. Zeiner A, Sunder-Plassmann G, Sterz F, Holzer M, Losert H, Laggner AN, Müllner M: The effect of mild therapeutic hypothermia on renal function after cardiopulmonary resuscitation in men. Resuscitation 2004; 60:253–61
19. Guluma KZ, Liu L, Hemmen TM, Acharya AB, Rapp KS, Raman R, Lyden PD: Therapeutic hypothermia is associated with a decrease in urine output in acute stroke patients. Resuscitation 2010; 81:1642–7
20. Gil-Rodriguez JA, O'Gorman P: Renal function during profound hypothermia. Br J Anaesth 1970; 42:557
21. Knight DR, Horvath SM: Urinary responses to cold temperature during water immersion. Am J Physiol 1985; 248(5 Pt 2):R560–6
22. Arthur JM, Hill EG, Alge JL, Lewis EC, Neely BA, Janech MG, Tumlin JA, Chawla LS, Shaw AD: Evaluation of 32 urine biomarkers to predict the progression of acute kidney injury after cardiac surgery. Kidney Int 2013 Sept 4. [Epub head of print]
23. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, Barasch J, Devarajan P: Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol 2003; 14:2534–43
24. Delbridge MS, Shrestha BM, Raftery AT, El Nahas AM, Haylor JL: The effect of body temperature in a rat model of renal ischemia-reperfusion injury. Transplant Proc 2007; 39:2983–5
25. Tanaka T, Matsumoto M, Inagi R, Miyata T, Kojima I, Ohse T, Fujita T, Nangaku M: Induction of protective genes by cobalt ameliorates tubulointerstitial injury in the progressive Thy1 nephritis. Kidney Int 2005; 68:2714–25
26. Cai ZP, Parajuli N, Zheng X, Becker L: Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10. Basic Res Cardiol 2012; 107:277
27. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 2005; 115:2894–903
28. Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U, Wyburn KR, Chadban SJ: HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010; 21:1878–90
29. Rodríguez-Arias D, Deballon IO: Protocols for uncontrolled donation after circulatory death. Lancet 2012; 379:1275–6
30. Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, Hauet T: Contribution of large pig for renal ischemia-reperfusion and transplantation studies: The preclinical model. J Biomed Biotechnol 2011; 2011:532127
31. Simmons MN, Schreiber MJ, Gill IS: Surgical renal ischemia: A contemporary overview. J Urol 2008; 180:19–30