Hypothermic total liquid ventilation is highly protective through cerebral hemodynamic preservation and sepsis-like mitigation after asphyxial cardiac arrest

Matthias Kohlhauer, DVM, MSc [1,2,3], Fanny Lidouren BSc [1,2,3], Isabelle Remy-Jouet, PhD [4], Nicolas Mongardon, MD, MSc [1,2,3], Clovis Adam, MD [5], Patrick Bruneval, MD [6], Hakim Hocini, PhD [2,7,8], Yves Levy, MD, PhD [2,7,8], Fabiola Blengio, BSc [2,7,8], Pierre Carli, MD, PhD [9], Benoit Vivien, MD, PhD [9], Jean-Damien Ricard, MD, PhD [10], Philippe Micheau, PhD [11], Hervé Walti, MD, PhD [11], Mathieu Nadeau, MSc [11], Raymond Robert, PhD [11], Vincent Richard, PhD [4], Paul Mulder, PharmD, PhD [4], David Maresca, PhD[12], Charlie Demené, PhD [12], Mathieu Pernot, PhD [12], Mickael Tanter, PhD [12], Bijan Ghaleh, MD, PhD [1,2,3], Alain Berdeaux, MD, PhD [1,2,3], Renaud Tissier, DVM, PhD [1,2,3]

[1] Inserm, U955, Equipe 03, F94000, Créteil, France
[2] Université Paris Est, UMR_S955, UPEC, F-94000, Créteil, France
[3] Université Paris Est, Ecole Nationale Vétérinaire d'Alfort, F-94700, Maisons-Alfort, France
[4] Inserm, U1096, plate-forme BOSS, F-76183, Rouen, France
[5] Assistance Publique, Hôpitaux de Paris, Hôpital du Kremlin-Bicêtre, Service d'Anatomie Pathologique, Le Kremlin-Bicêtre, F-94270, France
[6] Inserm, U970, F-75005, Paris, France
[7] Inserm, U955, Equipe 16, F-94000, Créteil, France
[8] Vaccine research Institute, F-94000, Créteil, France
[9] SAMU de Paris, Département d'Anesthésie Réanimation, Hôpital Universitaire Necker-Enfants Malades, Université Paris Descartes – Paris V, F-75015, Paris, France
[10] Inserm, IAME, 1137, Univ Paris Diderot, Sorbonne Paris Cité, F-75018, Paris, and Assistance Publique - Hôpitaux de Paris, Hôpital Louis Mourier, Service de Réanimation Médico-chirurgicale, F-92700, Colombes, France
[11] Sherbrooke Departments of Mechanical Engineering and Physiology, University of Sherbrooke, QC, Canada
[12] Institut Langevin, CNRS UMR 7587, INSERM U979, ESPCI ParisTech, F-75005, Paris, France

Institutions where the work was performed : Affiliations 1-4; 7-8; 10

Corresponding author: Renaud Tissier
Inserm, Unité 955, Equipe 3
Université Paris Est, Ecole Nationale Vétérinaire d'Alfort
7 avenue du Général de Gaulle ; 94704 Maisons-Alfort cedex, France
Tel: +33.1.43.96.73.02; Fax: +33.1.43.96.73.99; E-mail: rtissier@vet-alfort.fr

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Abstract

Objective - Total liquid ventilation (TLV) provides ultra-fast and potently neuro- and cardioprotective cooling after shockable cardiac arrest and myocardial infarction in animals. Our goal was to decipher the effect of hypothermic TLV on the systemic and cerebral response to asphyxial cardiac arrest using an original pressure- and volume-controlled ventilation strategy in rabbits.

Design – Randomized animal study.

Setting - Academic research laboratory.

Subjects – New Zealand Rabbits.

Interventions - Thirty-six rabbits were submitted to 13 min of asphyxia, leading to cardiac arrest. After resumption of spontaneous circulation, they underwent either normothermic life support (Control group, n=12) or hypothermia induced by either 30 min of TLV (TLV group, n=12) or i.v. cold saline (CONV group, n=12).

Measurements and Main Results - Ultra-fast cooling with TLV (32°C within 5 min in the oesophagus) dramatically attenuated the post-cardiac arrest syndrome regarding survival, neurological dysfunction and histological lesions (brain, heart, kidney, liver, lungs). Final survival rate achieved 58% vs 0% and 8% in TLV, Control and CONV groups ($p<0.05$), respectively. This was accompanied by an early preservation of the blood brain barrier integrity and cerebral hemodynamics, as well as reduction in the immediate reactive oxygen species production in the brain, heart and kidney after cardiac arrest. Later on, TLV also mitigated the systemic inflammatory response through alteration of monocyte chemoattractant protein-1, interleukin-1β and interleukin-8 transcripts levels as compared to Control. In the CONV group, cooling was achieved more slowly (32°C within 90-120 min in the oesophagus), providing none of the above-mentioned systemic or organ protection.

Conclusions - Ultra-fast cooling by TLV limits the post-cardiac arrest syndrome after asphyxial cardiac arrest in rabbits. This protection involves an early limitation in reactive oxidative species production, blood-brain barrier disruption anddelayed preservation against the systemic inflammatory response.

Key-words: Cardiopulmonary resuscitation; Cerebral ischemia; Cardiac arrest; Hypothermia; Liquid ventilation; Asphyxia.

Introduction

Beyond neurological and hemodynamic disorders (1), multivisceral dysfunction and respiratory complications (2, 3) participate to the dramatic outcome of resuscitated patients after cardiac arrest. Temperature management has been proposed to improve this outcome (4, 5) but the ideal target temperature is still debated between 32-34°C (mild therapeutic hypothermia) or 36°C (6). This is in apparent contradiction with many animal studies clearly demonstrating potent benefits with therapeutic hypothermia at 32-34°C (7-12). Such discrepancy could be a consequence of the differential ability to achieve rapid cooling in laboratory animals as compared to humans. Indeed, if rodents can be externally cooled within a couple of minutes, humans typically required couple of hours (13). This can in part explain the potent protection observed with intra-arrest experimental hypothermia in laboratory animals (7) as compared to the lack of benefit with pre-hospital cooling using current methods in the clinical arena (13). Thus, a key challenge is to determine whether it is possible to cool a human body as rapidly as in animals and to decipher the mechanisms supporting the benefit of such rapid cooling. Several techniques are currently proposed to achieve this goal including peritoneal lavage, (14) nasal evaporative cooling (15), partial (16) or total liquid ventilation (TLV) (8, 11, 12). The latter technique can use the lungs as a heat exchanger and provides rapid and systemic cooling independently of body weight (8, 17, 18). It can for example cool down the entire body at 32°C within 10-20 min in rabbits, lambs or swine (8, 17-19). After shockable cardiac arrest, hypothermic TLV improved survival (8, 17) as well as neurological (8), cardiac (17) and renal outcomes (20) in rabbits while conventional cooling was not protective (8, 17). Until now, the clinical translation of this concept was limited by the previous demonstration of lung injuries in early trials with partial liquid

ventilation in patients with acute respiratory distress syndrome (21). In contrast, recent experimental studies suggested that TLV could be better tolerated and beneficial in this context of respiratory care (22, 23). Here, we propose to use a new and original specifically dedicated liquid ventilator accurately controlling both tidal volume and pulmonary pressure (24, 25) in order to prevent lung trauma and to provide systemic and rapid hypothermia in the context of non-shockable cardiac arrest.

For these purposes, we investigated TLV in an experimental model of non-shockable and asphyxial cardiac arrest, as the effect of hypothermia is especially debated after non-shockable rhythm (26). Neurological, multivisceral and inflammatory outcomes were therefore assessed after 13 minutes of asphyxia in rabbits. The specific hypothesis was that hypothermic TLV could be highly neuroprotective through mitigation of early reperfusion alterations after cardiac arrest, *i.e.*, cerebral blood flow disturbances, blood brain barrier (BBB) permeability and reactive oxygen species (ROS) production. Importantly, the effects of TLV were compared to conventional cooling with external method and cold saline infusion.

Materials and Methods

Animal instrumentation and the ensuing experiments were conducted in accordance with French official regulations after approval by the local ethical committee (ComEth AnSES/ENVA/UPEC n°16).

Animal preparation and experimental protocol

Male New Zealand rabbits (2.5-3.0 kg) were anaesthetized using zolazepam, tiletamine and pentobarbital (all 20-30 mg/kg i.v.). After intubation and mechanical ventilation (FiO_2 = 30%), rabbits were paralyzed by cisatracurium besylate (0.2 mg/kg, i.v.). Asphyxial cardiac arrest was then induced by disconnecting the endotracheal tube from the ventilator during 13 min. Preliminary experiments demonstrated that cardiac arrest, *i.e.*, null cardiac output, was obtained within 4.5-5.5 min (data not shown). After 13 min of asphyxia, animals were resuscitated using external cardiac massage (200 compressions/min) and epinephrine administration (15 µg/kg i.v.). After resumption of spontaneous circulation (ROSC), animals were randomly assigned to one experimental group (Control, CONV or TLV; Figure 1A). The administration of epinephrine was further permitted during 8 h (target mean blood pressure = 80 mmHg). The Control group did not receive any additional procedure and was maintained under normothermic condition by the use of thermal pads. In the CONV group, hypothermia was induced by conventional cooling through the intravenous administration of 30 ml/kg of cold saline (NaCl, 0.9%, 4°C) during the first 30 min after ROSC. It was combined with the application of cold blankets (0-4°C) upon the skin with no prior shaving. In the TLV group, ultra-fast cooling was induced by TLV started 5 min after ROSC. The lungs were filled by 13 ml/kg of perfluorooctane (F2 Chemicals, Preston, UK), with an initial temperature of 20°C and a progressive increase to 33°C. The liquid ventilator was initially set to a tidal volume of 10 ml/kg, a respiratory frequency of 8 cycle/min and a

positive end-expiratory pressure (PEEP) of 2 cmH_2O, respectively (Figure 1B). We used a previously described algorithm with a volume- and pressure-controlled liquid ventilation mode monitoring PEEP and positive end-inspiratory pressure (PEIP) (Figure 1C). Static lung compliance was calculated by dividing tidal volume by (PEIP-PEEP). After 30 min of TLV, animals were weaned from TLV by prolonged exhalation at -15 cmH_2O. The liquid ventilator was then disconnected and animals were shifted to conventional mechanical ventilation. In the two hypothermic groups (CONV and TLV), the target temperature of 32°C was maintained by cold blankets until the 4^{th} hour after ROSC. Animals were then slowly rewarmed with infrared lights and thermal pads during 4 hours before weaning from conventional ventilation and awakening. Mechanical ventilation was continued until weaning from the mechanical ventilation and awakening of the animals. Rabbits were subsequently returned to cages for survival follow-up. They were housed in a closed cage enriched in O_2 for 24 hours. They received antibiotics (enrofloxacine, 5 mg/kg IM) and analgesics (buprenorphine, 30 µg/kg SC) every days for 3 days.

Investigated parameters during the survival follow-up

Throughout the protocol, we measured body temperatures, hemodynamics and biochemical parameters (Supplemental Method). In surviving animals, neurological dysfunction was blindly and daily evaluated using a clinical score previously validated in rabbits (0% [normal] - 100% [death]; Supplemental Table 1) (8, 17, 27). Survival was monitored during 3 days before euthanasia and organs sampling. In accordance with our ethical committee, animals eliciting a neurological dysfunction score above 80% at 24 hours or 60% at 48 hours were prematurely euthanized. The heart, kidney, liver, lungs and brain were examined by histopathology and lesions were quantified using a 0-3 score system (0=normal; 3=very severe; ; Supplemental Tables 2 and 3), as previously

described (8, 17, 27). For lungs, two different score systems were used for infectious or cardiogenic congestive lesions, respectively. In the brain, the overall score was obtained by the average of the right and left cortex, hippocampus and cerebellum (8, 17, 27).

Blood was also sampled at different time points for quantification of mRNA expression of inflammation and/or hypoxia markers including interleukins (IL) 1β, 8 and 10, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), hypoxia-inducible factor 1 alpha (HIF1-α) and heme oxygenase-1 (HO-1) as previously described (20) (Supplemental Table 4).

Assessment of reactive oxygen species production, blood brain barrier integrity and cerebral blood volume

Additional rabbits were submitted to the previously described procedure for early organ sampling and fixation, *i.e.*, 30 min after cardiac arrest. Brain cortex and hippocampus, heart, kidneys, lungs and liver were frozen in liquid nitrogen. ROS production was evaluated by electron paramagnetic resonance spectroscopy (detailed protocol in supplemental Method) as previously described (28-30). Other rabbits were submitted to the same procedures of cardiac arrest for BBB integrity evaluation using the Evans Blue Dye leakage method (Supplemental Method). In a last set of experiments, cerebral hemodynamics was assessed during cardiac arrest and after resuscitation using ultrafast ultrasound Doppler imaging, as previously described (31, 32).

Statistical analysis

Data were expressed as mean±SEM. Hemodynamic and biochemical parameters were compared between different groups using a 2-way ANOVA for repeated measures. If necessary, post-hoc analyses were performed at each time point using a Student t-test with Bonferroni correction. Values were not compared between the different time points to avoid multiple comparisons. Neurological dysfunction and histological scores

were compared between groups using a Mann-Whitney nonparametric test. Survival curves were obtained with a Kaplan-Meier analysis and compared between groups with a log-rank test. Significant differences were determined at $p \leq 0.05$.

Results

Total liquid ventilation can be instituted safely after resuscitation

As shown in Table 1, 12 animals were successfully resuscitated in each group with similar times to ROSC. During TLV, tidal volume and PEEP values averaged 8.9±0.3 ml/kg and 2.1±0.2 cmH_2O in the corresponding group. This led to low PEIP values (11.4 ±0.3 cmH_2O) which were well tolerated regarding gas exchanges (Table 2) and static lung compliance (Figure 1D) in the TLV group. The compliance impairment after cardiac massage was even partially reversed after weaning in the TLV group as compared to Control and CONV groups, respectively.

TLV induces rapid cooling without hemodynamic disorders

In the TLV group, body temperatures decreased to 32°C within 5-15 min as illustrated in Figure 2. In comparison, conventional cooling required 90-120 min to achieve the same target (CONV group). As shown in Table 1, heart rate decreased throughout hypothermia in the CONV and TLV groups while mean arterial pressure, blood gases and biochemical parameters were not different between groups. The amount of epinephrine administered to prevent hypotension was however significantly lower in the TLV group as compared to Control.

TLV offers neurological and lung protection and improves survival

As illustrated in Figure 3A, ultra-fast cooling by TLV was associated with a dramatic improvement of the neurological status as compared to Control and CONV groups. For example, neurological dysfunction score was 48% at day 1 in the TLV group as compared to 89 and 90% in the CONV and Control groups (median values), respectively. This reduction was significant when all animals were took into account (including dead animals with 100% score) but also when dead animals were excluded from this analysis (scores of alive animals only). The final survival rate achieved 58% in the TLV group vs

0% and 8% in the Control and CONV groups ($p<0.05$; Figure 3B), respectively. The neuroprotective effect of TLV was associated with a significant limitation of brain ischemic lesions as compared to the Control and CONV groups (Figure 4 and Supplemental Figure 1, Panels A-D).

Beyond cerebral lesions, lung congestive and infectious complications were the most severe consequences of cardiac arrest after pathological examinations (Figure 4). Congestive lesions were attenuated in TLV (Supplemental Figure 1, Panels E-F) vs Control groups (Supplemental Figure 1, Panel G). Conversely, they tended to be aggravated in the CONV group (Supplemental Figure 1, Panel H). A trend toward reduced pulmonary infection was also observed in the TLV vs Control and CONV groups (Figure 4).

TLV limits early blood brain barrier disruption and cerebral hyperemia

We then hypothesized that the multi-organ protection afforded by TLV could be linked to mitigation of early reperfusion injury. For this purpose, we investigated ROS production in additional organ samples withdrawn 30 min after cardiac arrest in rabbits (n=8 in each group). As illustrated in Figure 5, this production was decreased in the brain cortex, heart and kidney in the TLV group as compared to Control ($p<0.05$). A non-significant tendency was also observed in the hippocampus and liver. Interestingly, slow cooling did not reduce ROS production in the CONV group. We then hypothesized that the decrease in ROS production could be a major trigger of neuroprotection through BBB integrity and cerebral hemodynamics protection. Indeed, BBB disruption was observed very early after cardiac arrest through Evans blue dye leakage (Figure 6A-B, n=4 in each group). TLV significantly limited this disruption as compared to Control and CONV groups when assessed 30 min after cardiac arrest (n=4 in each group). In order to

corroborate this vascular effect, cerebral blood volume was further evaluated by ultra-fast ultrasound Doppler in other rabbits (Figure 6C). As shown in Figure 6D, cerebral hyperemia lasted approximately 30 min in the Control group (n=2) and was attenuated in the TLV group (n=2).

TLV mitigates the systemic responses to cardiac arrest

In order to assess the subsequent systemic responses to cardiac arrest, we then investigated the effect of TLV on the sepsis-like syndrome (2). The severity of hypoxic injury was evidenced by a potent up-regulation of HIF1-α and HO-1 (Figure 7A). Interestingly, early cooling with TLV amplified HO-1 up-regulation from the 8th hour after cardiac arrest. As illustrated in Figure 7B, an early pro-inflammatory response was also observed through an increase in IL-1β, IL-8 and MCP-1 transcripts. This response was significantly reduced by TLV vs Control at the 8th hour after cardiac arrest. The anti-inflammatory IL-10 cytokine was simultaneously up-regulated in the Control group and tended to be attenuated by TLV at the 24th hour after cardiac arrest. These data suggest that early cooling with TLV mitigates both the early pro-inflammatory and delayed anti-inflammatory responses induced by cardiac arrest. This was also associated with a down-regulation of IFNγ and TNFα at the 24th hour after cardiac arrest in all groups.

Discussion

In the present study, ultra-fast cooling with TLV potently limited the post-cardiac arrest syndrome after non-shockable and asphyxial cardiac arrest in rabbits while conventional cooling was inefficient. The protection was initiated as early as the first minutes following resuscitation. It was associated with inhibition of ROS production, limitation of BBB disruption and prevention of cerebral hyperemia. An ultimate attenuation of the so-called “sepsis-like” syndrome was also observed, showing that short, rapid and systemic hypothermia could attenuate the entire systemic response to anoxia.

Importantly, our experimental conditions of cardiac arrest were particularly severe as mortality and neurological dysfunctions were maximal in the Control group. Conventional cooling was even unable to provide any benefit as compared to TLV, supporting the concept that the rapidity of cooling plays a decisive protective role in such very severe conditions. Previous studies also demonstrated that immediate hypothermia was dramatically protective in mice after 8 min of asystole but not after a 20 min delay after resuscitation (7). Interestingly, the benefit of conventional hypothermia is also highly challenged in humans after non-shockable cardiac arrest (26).

From a biochemical point of view, the severity of the ischemic injury is associated with a potent up-regulation of HIF-1α in all groups in the present study. This transcription factor is well known to be enhanced during hypoxia (33) and could lead to various consequences such as vascular endothelium growth factor secretion, apoptosis or oxidative stress (34). Its upregulation could be linked to the activation of HO-1 protective pathway (35) which occurred earlier in the TLV group as compared to Control or CONV groups. Such an earlier activation could promote ubiquitous benefits

regarding ROS production and inflammation in the TLV group (36). In addition, ultra-fast cooling with TLV potently limited multi-organ ROS production as soon as 30 min after CPR initiation. As this was observed not only in the brain but also in the heart and kidney, it emphasizes the importance of systemic hypothermia by TLV.

In order to further investigate the neuroprotective effect of TLV, we also investigated BBB permeability and showed very rapid impairment in Control conditions, *i.e.*, 30 min after cardiac arrest. This impairment was totally prevented by TLV, in agreement with the previously shown limitation in ROS production and reduced hyperemia observed with ultrafast ultrasound imaging. The lack of benefit of conventional cooling in these conditions also suggests that hypothermia should be achieved at least before 90 min after cardiac arrest to efficiently inhibit the triggering events of neurological injury.

Since one major interest of TLV is to provide rapid and systemic cooling, we also investigated the systemic responses to cardiac arrest and the "sepsis-like" biochemical disorders (2). Importantly, the balance between the early pro-inflammatory and the later anti-inflammatory responses was modified by TLV but not through conventional cooling. This is consistent with previous studies showing a lack of benefit with delayed hypothermia in rats submitted to asphyxial cardiac arrest (37). The benefit of TLV persisted far into the rewarming phase (*i.e.*, 8 h after cardiac arrest), demonstrating that mitigation of early resuscitation disorders (*e.g.*, ROS generation) resulted in delayed benefits during the entire post-cardiac arrest syndrome. The alterations in MCP-1 levels are especially of interest in this regard, as it is associated with brain injury during stroke (38). Conversely, IFNγ and TNFα were not really modified in our conditions, which is likely related to a selective regional activation and/or different time-courses of activation (39).

In agreement with our previous studies (8, 17, 20), we confirmed here that TLV was well tolerated regarding lung function and histology. For this purpose, we used an original and advanced liquid ventilator that accurately controls both liquid filling pressures and volumes within the lungs (25). Beyond its capacity to induce an ultrafast and systemic hypothermia, such liquid ventilator was also able to preserve lung function and ultrastructure at difference with conventional hypothermia with cold fluid which promoted lung congestion (13). Alteration in lung static compliance after cardiac massage was even reversed by TLV, several hours after cardiac arrest as compared to others groups. This impairment in Control conditions could be consequence of pulmonary ischemia-perfusion and/or direct effect of the cardiac massage. TLV and perfluorocarbons may directly and independently reduce the congestive lesions and pulmonary infection. This shows that TLV could be well tolerated with such sophisticated and dedicated liquid ventilator, as compared to previous studies using partial liquid ventilation which were associated with lung injury (21). This new ventilator could therefore open promising perspectives for the applications of TLV and for the future management of cardiac arrest.

Our study presents however several limitations. As previously discussed, we only investigated some time-points for biochemical, ROS and imaging studies. Other experiments with additional time-points could be highly relevant to better understand the kinetic of alterations. It could also be relevant to investigate normothermic TLV or hypothermic TLV with a different schedule of institution (e.g., delayed initiation and/or duration). However, normothermic TLV did not provide any benefit after shockable arrest (8), supporting that TLV is only protective through its cooling properties. Longer episode of hypothermia could also be tested, as currently done in the clinical setting. However, if hypothermia needs to be prolonged to provide benefits after experimental

stroke (>12h) (40), it is well known that shorter episodes can be maximally protective after cardiac arrest (7, 41).

In conclusion, ultra-fast cooling with TLV limits the post-cardiac arrest syndrome after asphyxial cardiac arrest in rabbits with potent neurological and survival benefits. This protection involves an early and global limitation of ROS production, a rapid BBB preservation and an ultimate mitigation of the systemic inflammatory response. This was achieved using a "latest-generation" of liquid ventilator providing TLV safely and using the lungs as a unique medium for therapeutic hypothermia

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Legend of Figures

Figure 1: Experimental protocol and design of the liquid ventilator

Panel A: Experimental protocol.

Panel B: Schematic representation and picture of the liquid ventilator.

Panel C: Typical waveforms of airway pressures and liquid flows during total liquid ventilation.

Panel D: Lung compliance at baseline and after cardiac arrest in the different groups.

*CONV group; CPR, Cardio pulmonary resuscitation; PTUh, programmable thermic unit of hypothermia; TLV, Total Liquid Ventilation; *, p<0.05 vs Control; †, p<0.05 vs CONV.*

Figure 2: Tympanic, oesophageal and rectal temperatures in the different groups.

*CONV, Conventional cooling; TLV, Total Liquid Ventilation; *, p<0.05 vs Control; †, p<0.05 vs CONV.*

Figure 3: Neurological outcome and survival

Panel A: Neurological dysfunction scores at days 1 and 3 following resuscitation. Open circles represent individual scores and thick lines represent the median value of the corresponding group.

Panel B: Kaplan-Meyer curves of survival in the different groups.

*CONV, Conventional cooling; TLV, Total Liquid Ventilation; *, p<0.05 vs Control; †, p<0.05 vs CONV.*

Figure 4: Lesional scores of the different organs in the different groups

*Open circles represent individual scores and thick lines represent the median value of the corresponding group. CONV, Conventional cooling group; TLV, Total liquid ventilation; *, p<0.05 vs Control; †, p<0.05 vs CONV.*

Figure 5: Reactive oxygen species production

The reactive oxygen species production was assessed in the brain cortex, hippocampus, heart, kidney, lungs and liver at t=30 min after cardiac arrest (n=8 in each group) by electron paramagnetic resonance spectroscopy (EPR). Data are expressed in arbitrary unit per gram of protein per minute.

*CONV, conventional cooling; TLV, total liquid ventilation; ROS, reactive oxygen species; *, p<0.05 vs control; †, p<0.05 vs CONV.*

Figure 6: Assessment of blood brain barrier integrity and cerebral blood volume after cardiac arrest

Panel A: Blood brain barrier permeability assessed by cerebral Evans blue dye concentration. Evans blue was distributed in the cerebral tissue through vascular leakage after *in vivo* administration at t=30 min after cardiac arrest (n=4 in each group).

Panel B: Typical pictures of brain slices in the different groups. Blue areas represent Evans blue dye vascular leakage.

Panel C: Ultra-fast ultrasound Doppler imaging after cardiac arrest. The echographic frontal views show the visual repartition of cerebral blood volume at the maximum intensity in representative rabbits of the Control and TLV groups. The corresponding hippocampus and cortical blood volumes were calculated in one control and one TLV rabbit.

Panel D: Overall cortical blood volume from Ultra-fast ultrasound Doppler imaging in one control and one TLV rabbits.

*CONV, conventional cooling; CPR, Cardio pulmonary resuscitation; TLV, total liquid ventilation; *, p<0.05 vs control; †, p<0.05 vs CONV; A.U., arbitrary unit.*

<u>Figure 7:</u> Blood transcriptomic profiles determined by quantitative real-time PCR

Panel A: Transcript levels of hypoxia pathway.

Panel B: Transcripts levels of blood immunity and inflammation markers.

*Data are expressed as relative fold change as compared to baseline, i.e., prior to cardiac arrest. CONV, conventional cooling; HIF1-α, hypoxia-inducible factor 1 alpha; HO-1, heme oxygenase-1; IFNγ, interferon-γ; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TLR, toll-like receptor; TLV, total liquid ventilation; TNF α, tumor necrosis factor-α; *, p<0.05 vs Control.*

Table 1: Resuscitation parameters

	Control	CONV	TLV
Body weight (kg)			
	2.8±0.1	2.7±0.1	2.7±0.1
Time to systolic blood pressure < 40 mmHg during asphyxia (min)			
	4.7±0.3	5.2±0.2	5.2±0.1
Time to ROSC during cardiopulmonary resuscitation (min)			
	1.5±0.3	1.5±0.3	1.5±0.2
Total amount of epinephrine (µg/kg)			
	164±22	133±23	101±11[a]

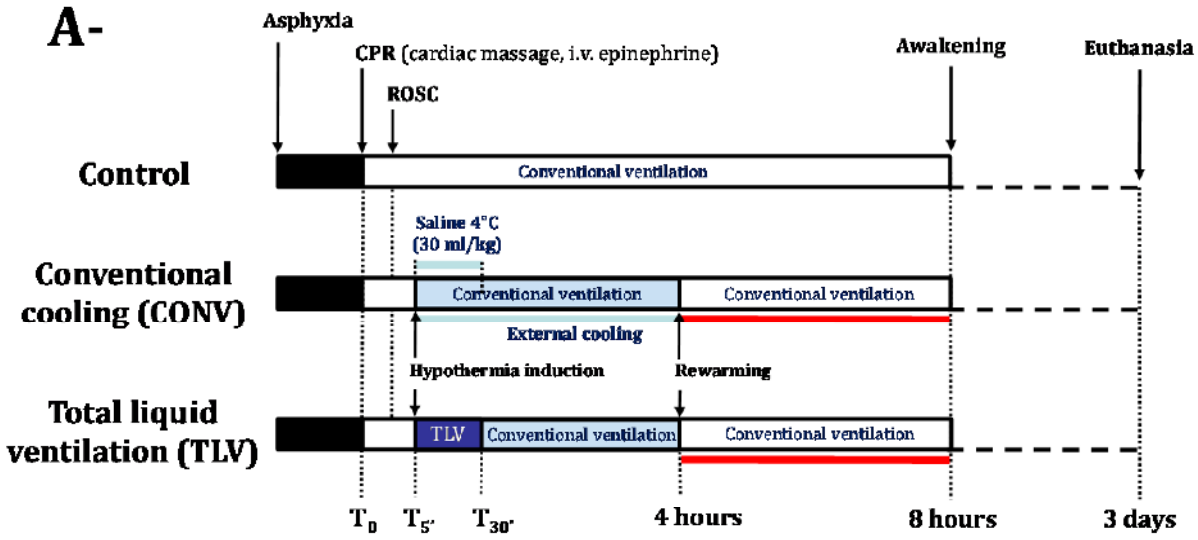
CONV, Conventional cooling; TLV, Total liquid ventilation; ROSC, resumption of spontaneous circulation;

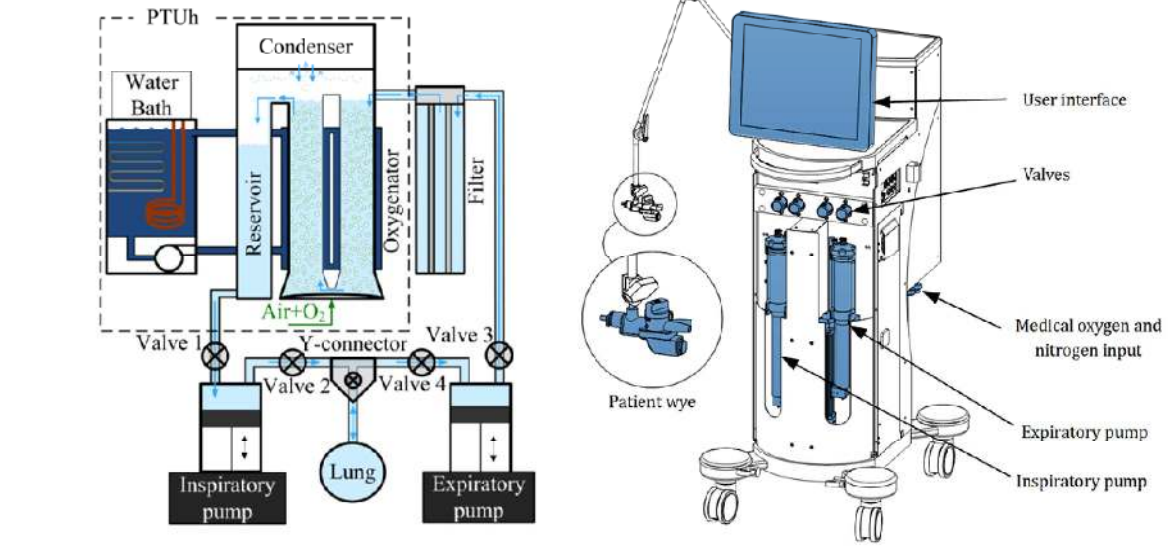
[a]*, p<0.05 vs Control.*

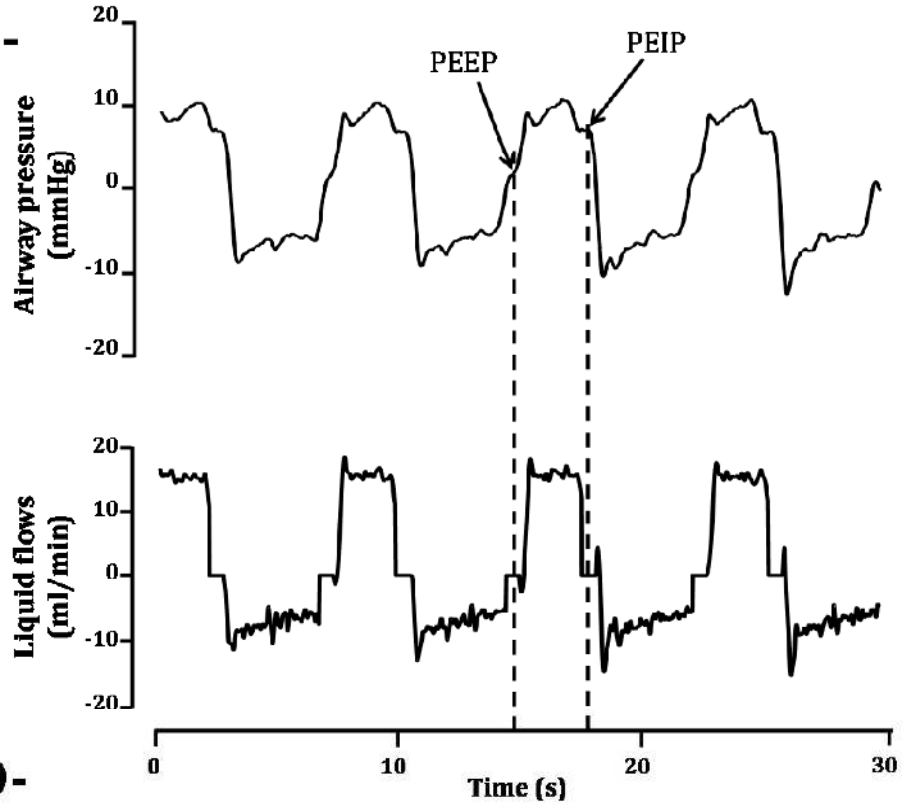
Table 2: Hemodynamic and biochemical parameters

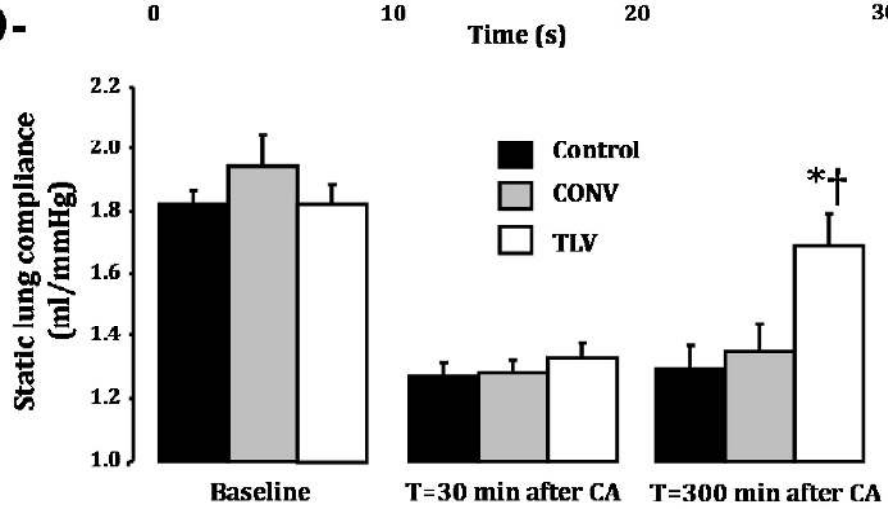
		After cardiac arrest			
	Baseline	**30 min**	**180 min**	**360 min**	**Day 1**
Number of rabbits					
Control	12	12	12	12	8
CONV	12	12	12	12	8
TLV	12	12	12	12	12
Mean arterial blood pressure (mmHg)					
Control	69 ± 3	83 ± 2	82 ± 3	77 ± 3	96 ± 5
CONV	78 ± 2	79 ± 2	83 ± 3	83 ± 3	82 ± 11
TLV	72 ± 4	81 ± 4	83 ± 3	79 ± 2	86 ± 8
Heart rate (beats/min)					
Control	241 ± 9	198 ± 3.0	202 ± 10.5	228 ± 9.1	265 ± 23.3
CONV	260 ± 12	209 ± 11	149 ± 5[a]	201 ± 8.4	224 ± 28.2
TLV	238 ± 13	124 ± 6[ab]	148 ± 6[a]	195 ± 6.8	237 ± 27.8
Arterial pCO_2 (Torr)					
Control	47 ± 3	58 ± 3	-	46 ± 2	30 ± 3
CONV	51 ± 3	65 ± 4	-	43 ± 1	44 ± 11
TLV	46 ± 3	65 ± 3	-	39 ± 2	55 ± 4
Arterial pO2 (Torr)					
Control	172 ± 7	109 ± 13	-	146 ± 8	82 ± 9
CONV	148 ± 10	84 ± 4	-	154 ± 15	67 ± 12
TLV	168 ± 8	123 ± 15	-	170 ± 7	71 ± 6
Arterial pH					
Control	7.42 ± 0.04	7.14 ± 0.02	-	7,34 ± 0.03	7.39 ± 0.03
CONV	7.36 ± 0.02	7.07 ± 0.03	-	7,28 ± 0.02	7.36 ± 0.09
TLV	7.42 ± 0.02	7.08 ± 0.02	-	7.28 ± 0.03	7.31 ± 0.02
HCO^-_3 blood levels (mmol/L)					
Control	28 ± 1	19 ± 2	-	23 ± 1	18 ± 3
CONV	29 ± 1	20 ± 1	-	21 ± 1	22 ± 1
TLV	28 ± 1	21 ± 1	-	19 ± 1	26 ± 11
Glucose blood levels (mg/dL)					
Control	1.8 ± 0.1	4.4 ± 0.2	-	1.8 ± 0.3	1.5 ± 0.3
CONV	2.2 ± 0.2	4.2 ± 0.3	-	2.5 ± 0.4	1.6 ± 0.2
TLV	1.9 ± 0.1	3.7 ± 0.4	-	2.7 ± 0.2	1.6 ± 0.1
Creatinine blood levels (mg/dL)					
Control	77 ± 7	75 ± 7	-	81 ± 5	145 ± 35
CONV	67 ± 5	58 ± 4	-	66 ± 6	136 ± 15
TLV	73 ± 5	86 ± 5	-	94 ± 6	107 ± 8
Alanine transaminase (UI/L)					
Control	46 ± 3	-	128 ± 21	129 ± 18	142 ± 23
CONV	55 ± 9	-	129 ± 16	111 ± 14	157 ± 40
TLV	45 ± 7	-	135 ± 19	144 ± 23	124 ± 20
Lactate dehydrogenase (UI/L)					
Control	306 ± 37	-	885 ± 217	460 ± 72	498 ± 128
CONV	341 ± 31	-	923 ± 180	602 ± 97	542 ± 210
TLV	365 ± 38	-	808 ± 136	585 ± 134	516 ± 64

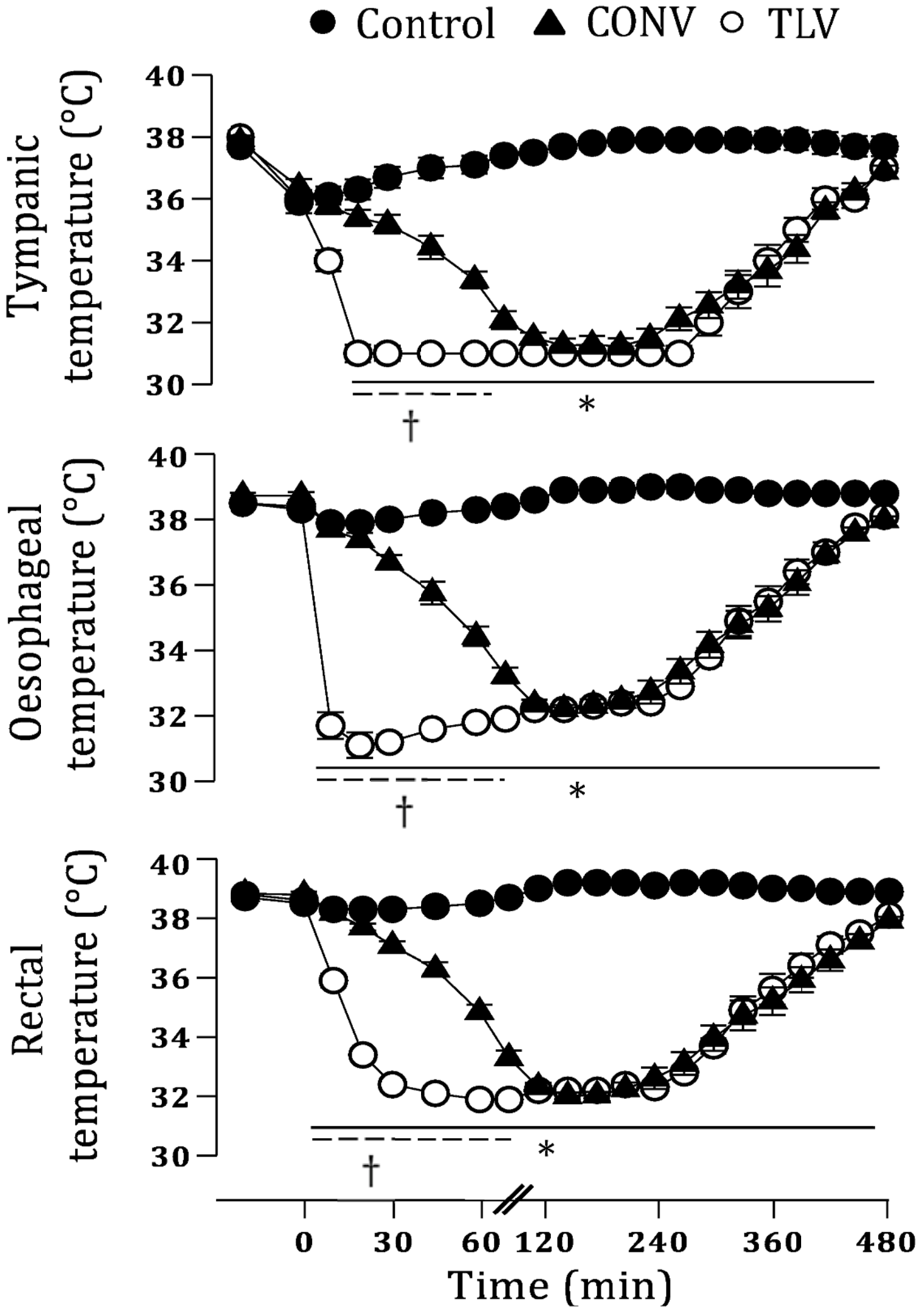
CONV, Conventional cooling; TLV, Total liquid ventilation ; [a], $p<0.05$ vs Control; [b], $p<0.05$ vs CONV.

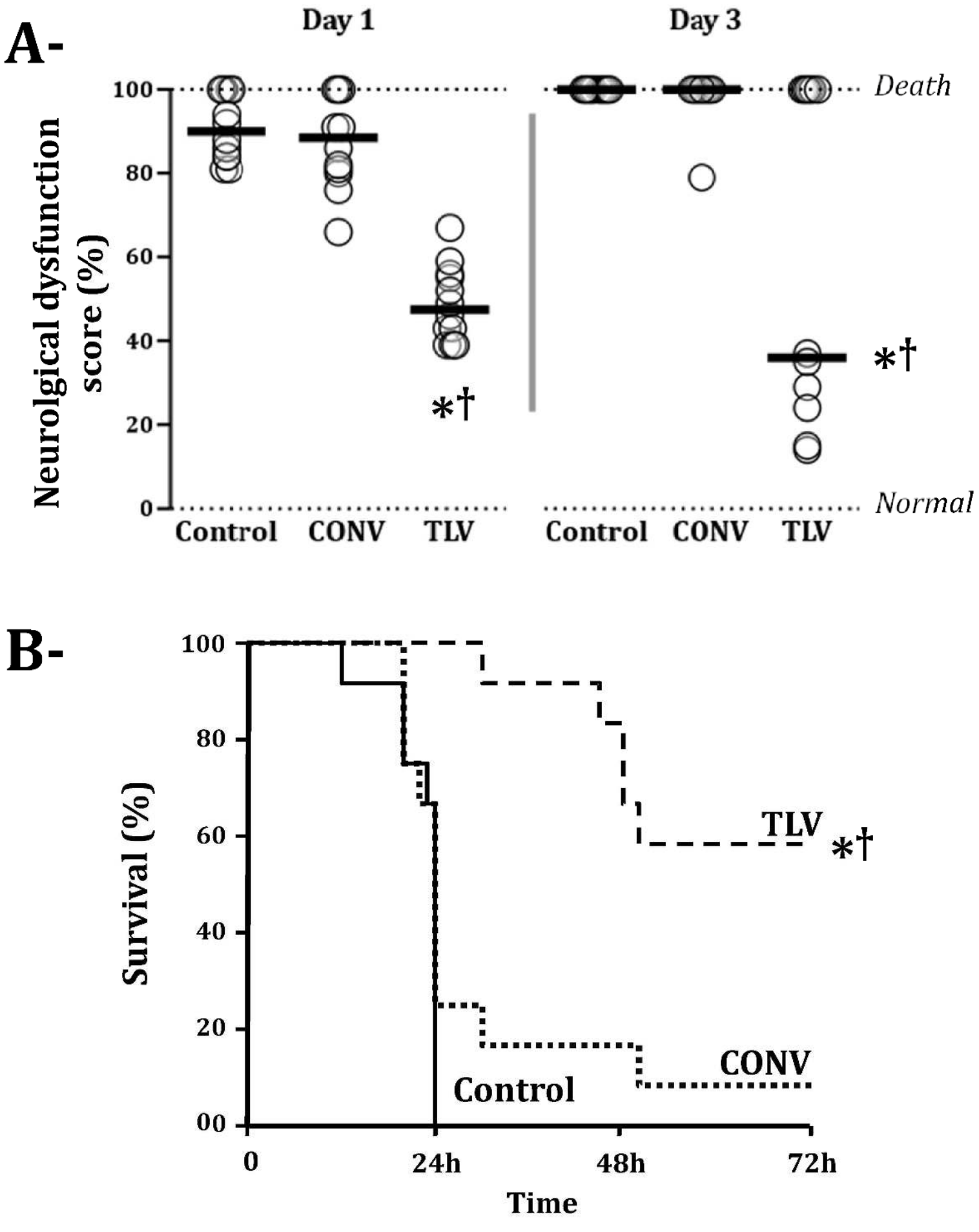

A-
Asphyxia
CPR (cardiac massage, i.v. epinephrine)
ROSC
Awakening
Euthanasia
Control
Conventional ventilation
Saline 4°C
(30 ml/kg)
Conventional cooling (CONV)
Conventional ventilation
Conventional ventilation
External cooling
Hypothermia induction
Rewarming
Total liquid ventilation (TLV)
TLV
Conventional ventilation
Conventional ventilation
T0
T5'
T30'
4 hours
8 hours
3 days

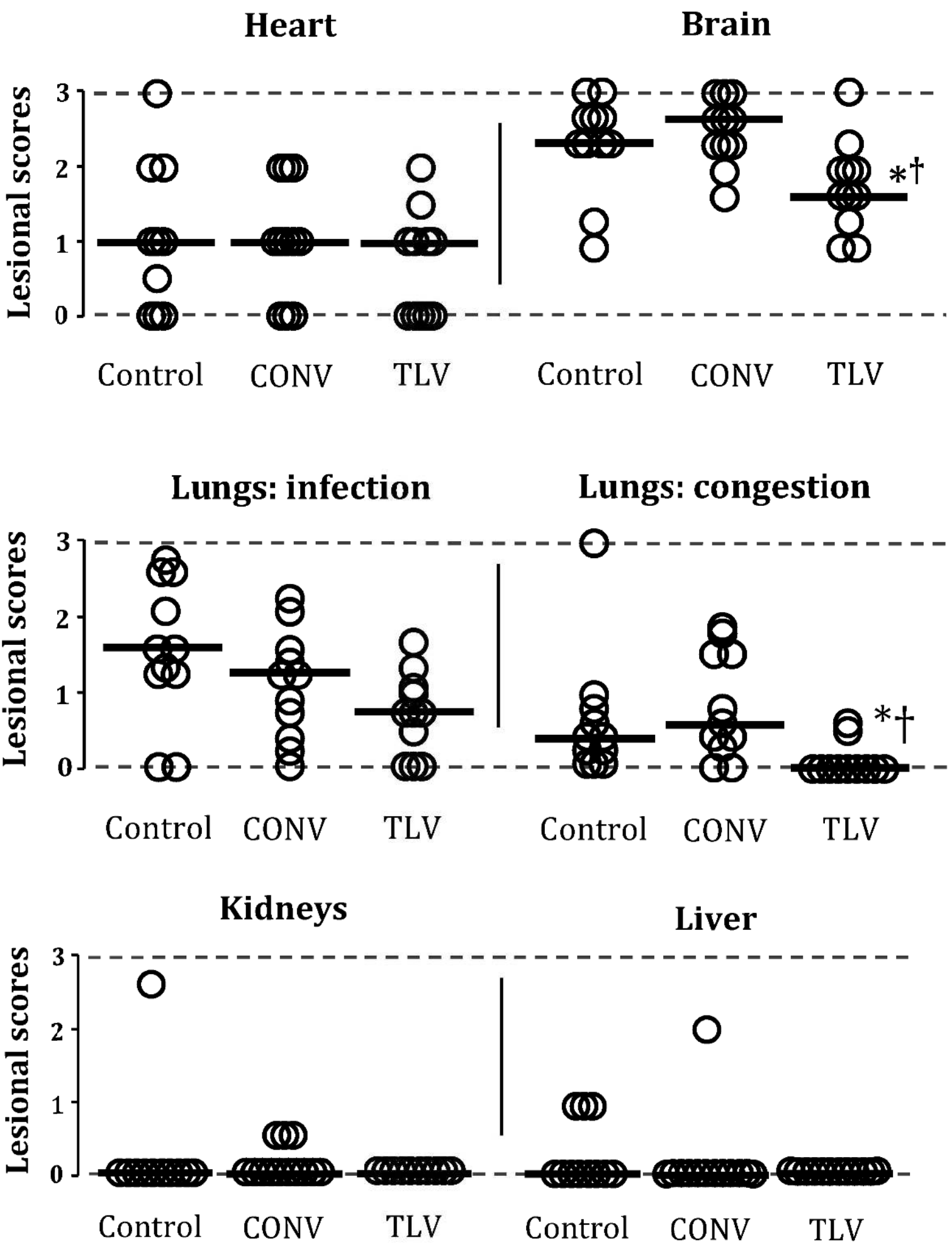

B-
PTUh
Condenser
Water Bath
Reservoir
Oxygenator
Filter
Air+O2
Valve 1
Y-connector
Valve 3
Valve 2
Valve 4
Lung
Inspiratory pump
Expiratory pump
User interface
Valves
Medical oxygen and nitrogen input
Patient wye
Expiratory pump
Inspiratory pump

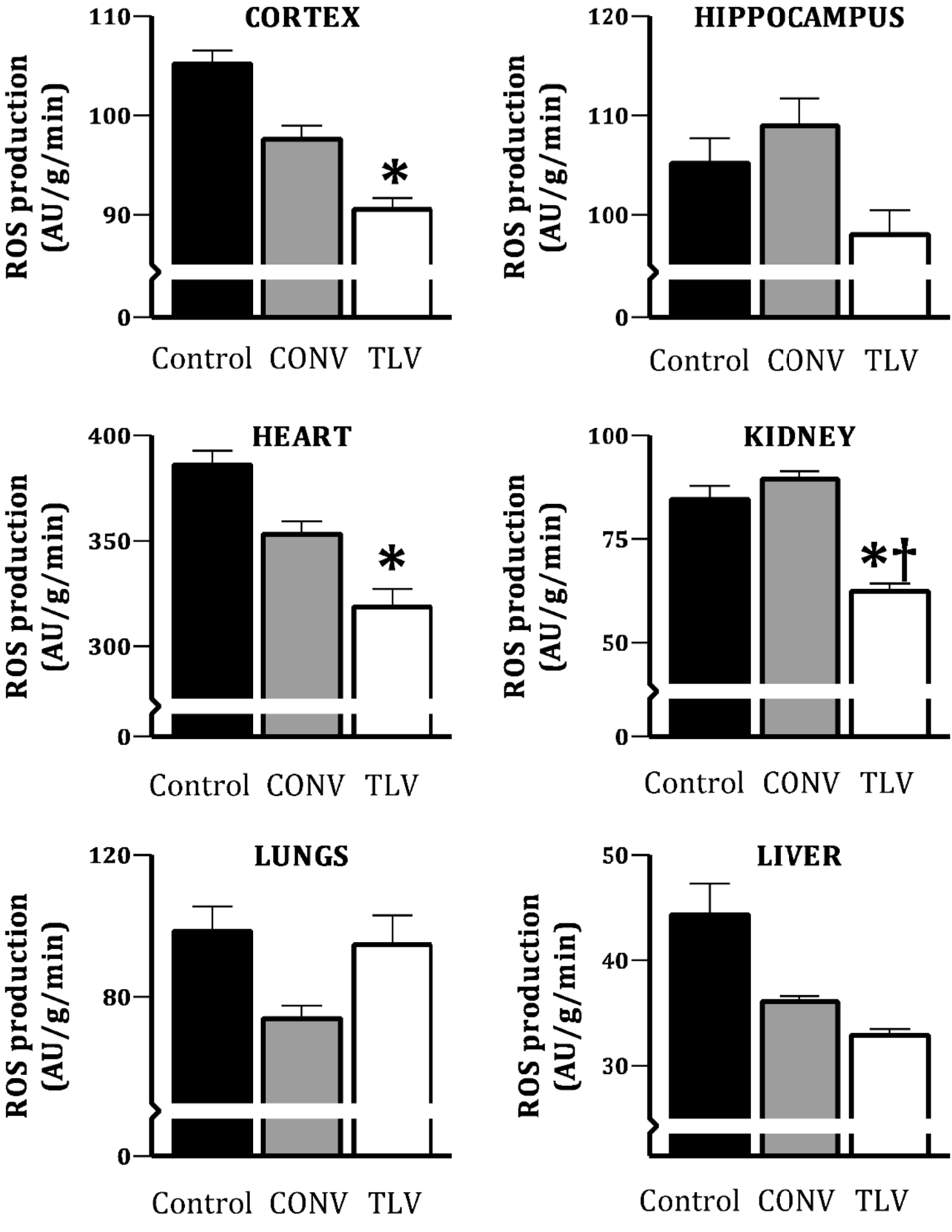

C-
Airway pressure (mmHg)
PEEP
PEIP
Liquid flows (ml/min)
Time (s)

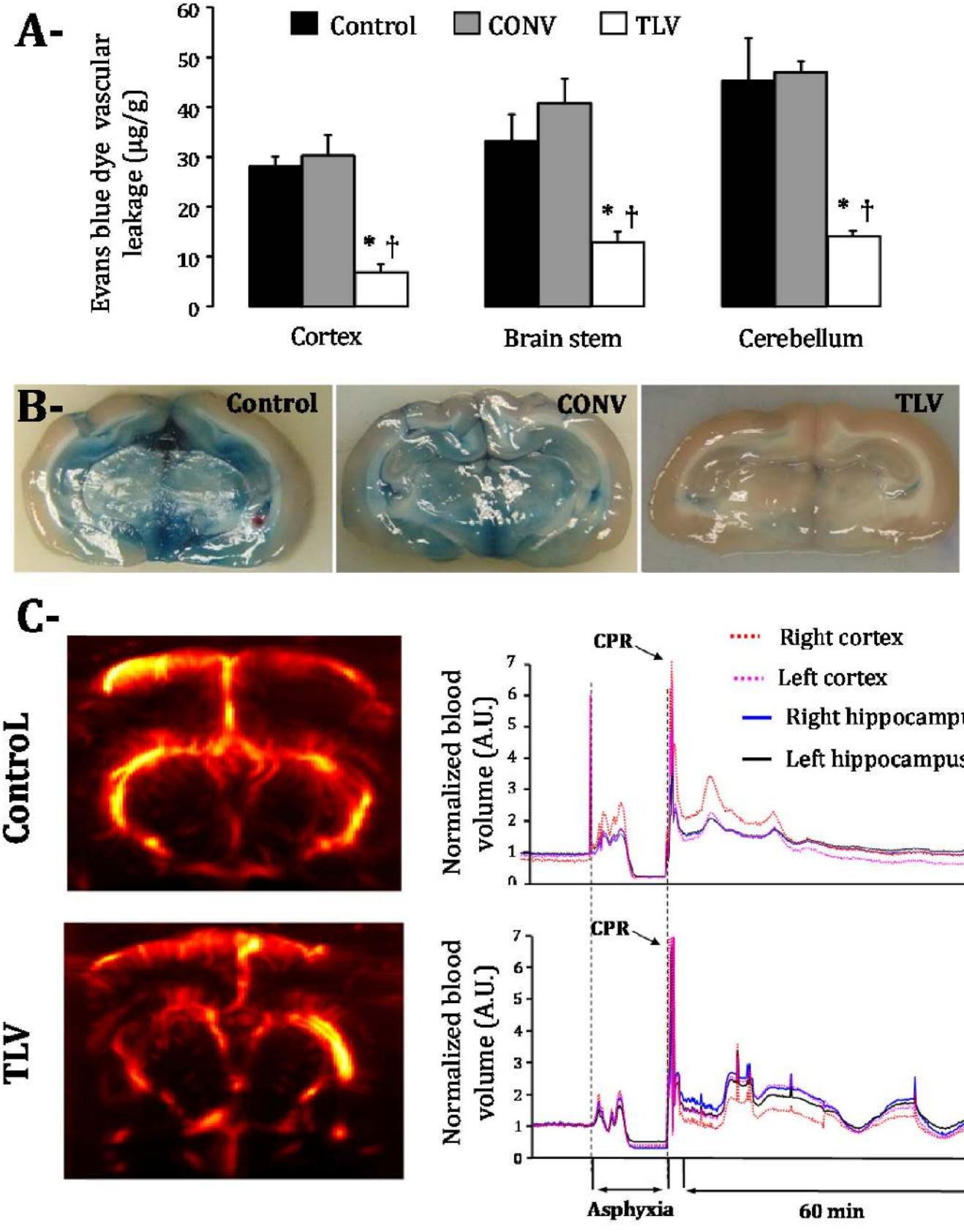

D-
Static lung compliance (ml/mmHg)
Control
CONV
TLV
*†
Baseline
T=30 min after CA
T=300 min after CA

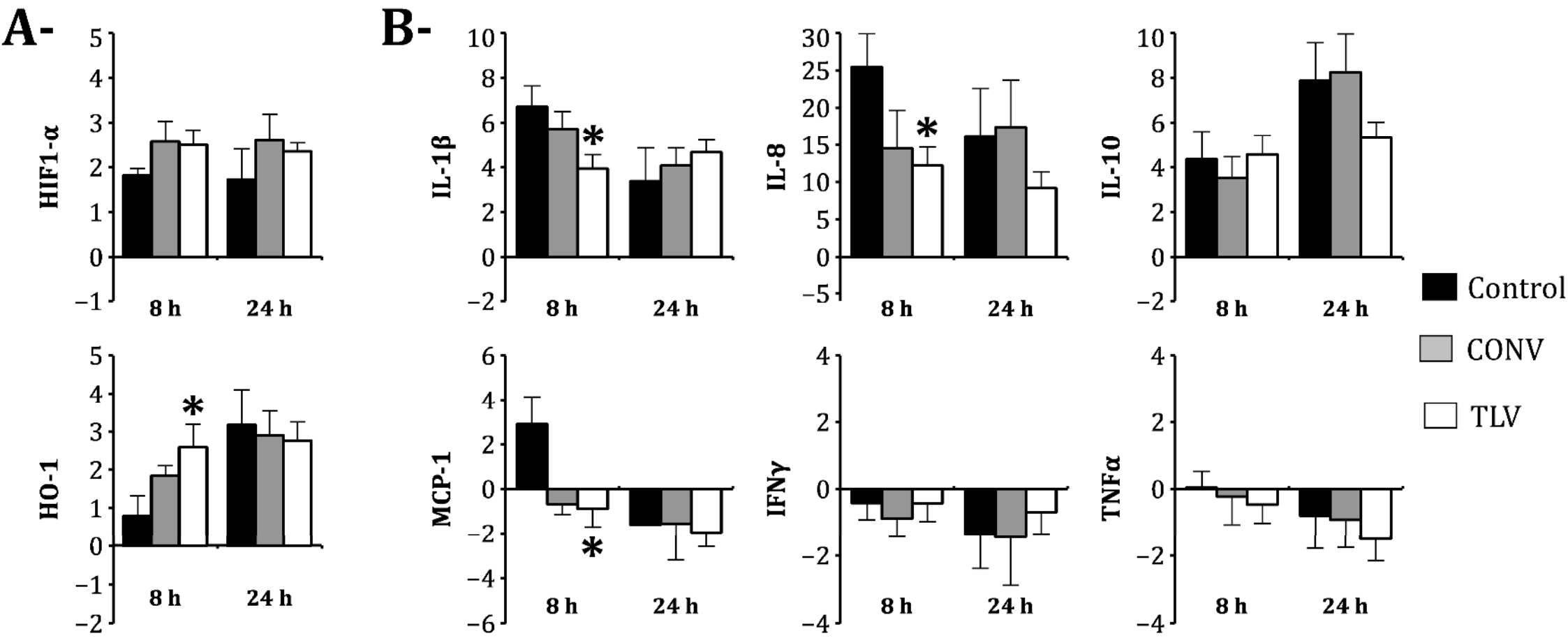

Control
CONV
TLV
Tympanic temperature (°C)
Oesophageal temperature (°C)
Rectal temperature (°C)
40
38
36
34
32
30
†
*
0
30
60
120
240
360
480
Time (min)


A-
Day 1
Day 3
Neurolgical dysfunction score (%)
100
80
60
40
20
0
Death
Normal
*†
*†
Control
CONV
TLV
Control
CONV
TLV
B-
Survival (%)
100
80
60
40
20
00
TLV
*†
CONV
Control
0
24h
48h
72h
Time


Heart
Brain
Lesional scores
3
2
1
0
*†
Control
CONV
TLV
Control
CONV
TLV
Lungs: infection
Lungs: congestion
Lesional scores
3
2
1
0
*†
Control
CONV
TLV
Control
CONV
TLV
Kidneys
Liver
Lesional scores
3
2
1
0
Control
CONV
TLV
Control
CONV
TLV


CORTEX
ROS production (AU/g/min)
110
100
90
0
*
Control CONV TLV
HIPPOCAMPUS
ROS production (AU/g/min)
120
110
100
0
Control CONV TLV
HEART
ROS production (AU/g/min)
400
350
300
0
*
Control CONV TLV
KIDNEY
ROS production (AU/g/min)
100
75
50
0
*†
Control CONV TLV
LUNGS
ROS production (AU/g/min)
120
80
0
Control CONV TLV
LIVER
ROS production (AU/g/min)
50
40
30
Control CONV TLV


A-
Control
CONV
TLV
Evans blue dye vascular leakage (µg/g)
60
50
40
30
20
10
0
* †
* †
* †
Cortex
Brain stem
Cerebellum
B-
Control
CONV
TLV
C-
ControL
TLV
CPR
Right cortex
Left cortex
Right hippocampus
Left hippocampus
Normalized blood volume (A.U.)
7
6
5
4
3
2
1
0
Asphyxia
60 min
D-
Overall cortical blood volume (norm A.U.)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
TLV
Asphyxia
60 min


A-
B-
HIF1-α
HO-1
IL-1β
MCP-1
IL-8
IFNγ
IL-10
TNFα
8 h
24 h
Control
CONV
TLV

Supplemental Method

Investigated parameters during the survival follow-up

Rectal, oesophageal and tympanic temperatures were monitored using thermal probes (Harvard Apparatus, Paris, France). External electrocardiogram and invasive arterial blood pressure were continuously recorded (Notocord, Croissy-sur-Seine, France). Blood samples were withdrawn at baseline and at 30 min, 180 min and 360 min after cardiac arrest for blood gases and biochemical analyses.

Quantitative real-time PCR procedure

Total RNA was extracted from blood using Tempus™ Spin RNA Isolation Kit (Applied Biosystems). The cDNA synthesis was performed on 1 µg of tot RNA using AffinityScript QPCR cDNA Synthesis kit (Stratagene). Quantitative PCR was performed using Brilliant II SYBR Green Master Mix (Stratagene) on a Biosystems ABI PRISM 7900HT Real-time PCR System. The amplification conditions were 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 59°C and 15 seconds at 72°C. For IL18 and RPL5 the qPCR conditions were 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60,5°C and 15 seconds at 72°C. Expression levels were normalized to housekeeping gene RPL5. Transcripts levels were expressed as fold change from baseline levels.

Reactive oxygen species production measurement with electron paramagnetic resonance (EPR) spectroscopy

Tissue were incubated in Krebs-HEPES buffer containing the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CMH; Noxygen, Elzach, Germany). Spectra of the oxidized product of CMH (CM°) were recorded with an X-band spectrometer (MS-400; Magnettech, Berlin, Germany) and expressed arbitrary unit normalized per milligram of protein and per minute.

Blood brain barrier assessment using Evans Blue Dye administration

In another set of experiments, rabbits were submitted to the same procedures of cardiac arrest and resuscitation for blood brain barrier integrity evaluation. Thirty minutes after cardiac arrest, Evans Blue Dye (EBD 4%, 3 ml/kg) was administered intravenously. Life support was further maintained during 2 h after which the rabbits were euthanized and the brain washed by 250 ml of saline (0.9%) through each carotid arteries. The brain was then sampled and slices of 1 mm were prepared and photographed. Cerebral cortex, cerebral trunk and cerebellum were further isolated and homogenized using formamide (1 ml / 100g) during 48 h at 37°C. After centrifugation, the absorbance was measured at 620 nm. The corresponding concentration of EBD was calculated using a calibration curve. It was expressed in µg/g of brain tissue.

Cerebral blood volume measurement

A last set of experiments consisted in cerebral hemodynamics investigation during cardiac arrest and resuscitation using ultrafast ultrasound Doppler imaging, as previously described (1, 2). The rabbits underwent craniotomy and a linear ultrasound probe (192 elements, 7 MHz, 200 µm pitch; SuperSonic Imaging®, Aix-en-Provence, France) was positioned on the brain. Animals were subsequently submitted to the protocol described previously (Control and TLV groups only). Life support and brain imaging were pursued during one hour after cardiac arrest.

Supplemental Table 1: Rabbit neurological deficit grading scale (3-5)

		Maximum score
Level of conciousness		
Normal	0	
Clouded	5	
Stuporous	10	
Comatose	25	25
Respiration		
Normal	0	
Abnormal	5	5
Cranial nervs		
Vision	1	
Light reflex	1	
Oculocephalic	1	
Corneal	1	
Facial sensation	1	
Auditory	1	
Gag reflex	1	7
Motor and sensory function		
Flexor response to pain (Front)	2	
Flexor response to pain (Rear)	2	
Righting reflex	10	14
Gait		
Normal	0	
Minimal ataxia	5	
Moderate ataxia	10	
Able to stand	15	
Unable to stend	20	
No purposeful movement	25	25
Behavior		
Grooming	4	
Eating/drinking	10	
Exploring	10	24
Total		**100**

Supplemental Table 2: Histological cerebral lesion severity grading scale (3-5)

	Score
Neocortex	
Normal	0
Rare ischemic neurons (<10%)	1
Frequent ischemic neurons (10-50%)	2
Majority of neurons ischemic (>50%)	3
Hippocampus	
Normal	0
Rare ischemic pyramidal or granule cells	1
Focal ischemic damage	2
Severe, diffuse ischemic damage	3
Cerebellum	
Normal	0
Rare ischemic Purkinje cells	1
10-50% ischemic Purkinje cells	2
>50% ischemic Purkinje cells	3

Supplemental Table 3: Histological lesion severity grading scale for the kidney, liver, heart and lung (3-5). For this last organ, we assessed two separate scores for the cardiogenic lesions and the infectious complication, respectively.

	Score
Kidney	
Normal	0
Dilated regenerative proximal tubule	1
Focal scar fibrosis	2
Extensive scar fibrosis	3
Liver	
Normal	0
Limited clarification of hepatocytes	1
Moderate clarification of hepatocytes	2
Extensive clarification of hepatocytes	3
Heart	
Normal	0
Very rare foci of cardiomyocyte necrosis	1
Rare foci of cardiomyocyte necrosis	2
Frequent foci of cardiomyocyte necrosis	3
Lung (cardiogenic lesion)	
Normal	0
Limited congestion and/or serous edema	1
Moderate congestion and/or serous edema	2
Extended congestion and/or serous edema	3
Lung (infection)	
Normal	0
Limited foci of bronchopneumia	1
Moderate foci of bronchopneumia	2
Extended foci of bronchopneumia	3

Supplemental Table 4: Primers used for the quantification of mRNA expression of immunity, inflammation and/or hypoxia pathway markers with Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-QPCR),

Name	Sequence (5'-> 3')
IFN gamma FB - F	TGCCAGGACACACTAACCAGAG
IFN gamma FB - R	TGTCACTCTCCTCTTTCCAATTCC
TNF alpha FB - F	AGATTGAGCCCGGAACATC
TNF alpha FB - R	GCCTAGGTCTGGGTGACAAC
RPL5 - F	TCCCTCACAGTACCAAACGA
RPL5 -R	TTCTGCCCCATAATGTGCTT
RPLP0 - F	CGACGTGCAGCTGATAAAGA
RPLP0 - R	GGGTTGTAGATGCTGCCATT
HO - F	CCTTCGCAGGCACCAG
HO - R	CTGGGAGAGGCTGCTGAG
HIF - F	GGCTTCTGTTATGAGGCTTACC
HIF -R	AAACAGTTCATCTGTGCCTTCA
MCP1 - F	CTCAGTGAAGAGGCTAATGAGCTA
MCP1 - F	TCTGCTTGGGGTCAGCA
IL 1 beta - F	CCACAGTGGCAATGAAAATG
IL 1 beta - R	GCTGGATGCCCTCGTCT
IL 8 - F	CCCAAATTTATCAAAGAATTGAGAG
IL 8 - R	TTGGGGTCCAGGCAGA
IL 10 - F	ATGCCAAGCCTTGTCGGAGATG
IL 10 - R	TGATGGCTGGACTGTGGTTCTCAG

HIF1, hypoxia-inducible factor; HO, heme oxygenase ; IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; RP, ribosomal protein, TLR, toll-like receptor; TNF α, tumor necrosis factor-α.

Legend of Supplemental Figure 1

Supplemental Figure 1: Histological appearance of brain and lungs in the different groups

Panel A: Normal histological appearance of the cerebral cortex in a rabbit from the TLV group.

Panel B: Pathological appearance with multifocal necrosis (arrows) of the cerebral cortex in a rabbit from the Control group.

Panel C: Normal histological appearance of the hippocampus in a rabbit from the TLV group.

Panel D: Pathological appearance with multifocal necrosis (arrows) of the hippocampus in a rabbit from the Control group.

Panel E: Normal histological appearance of the lungs in the TLV group.

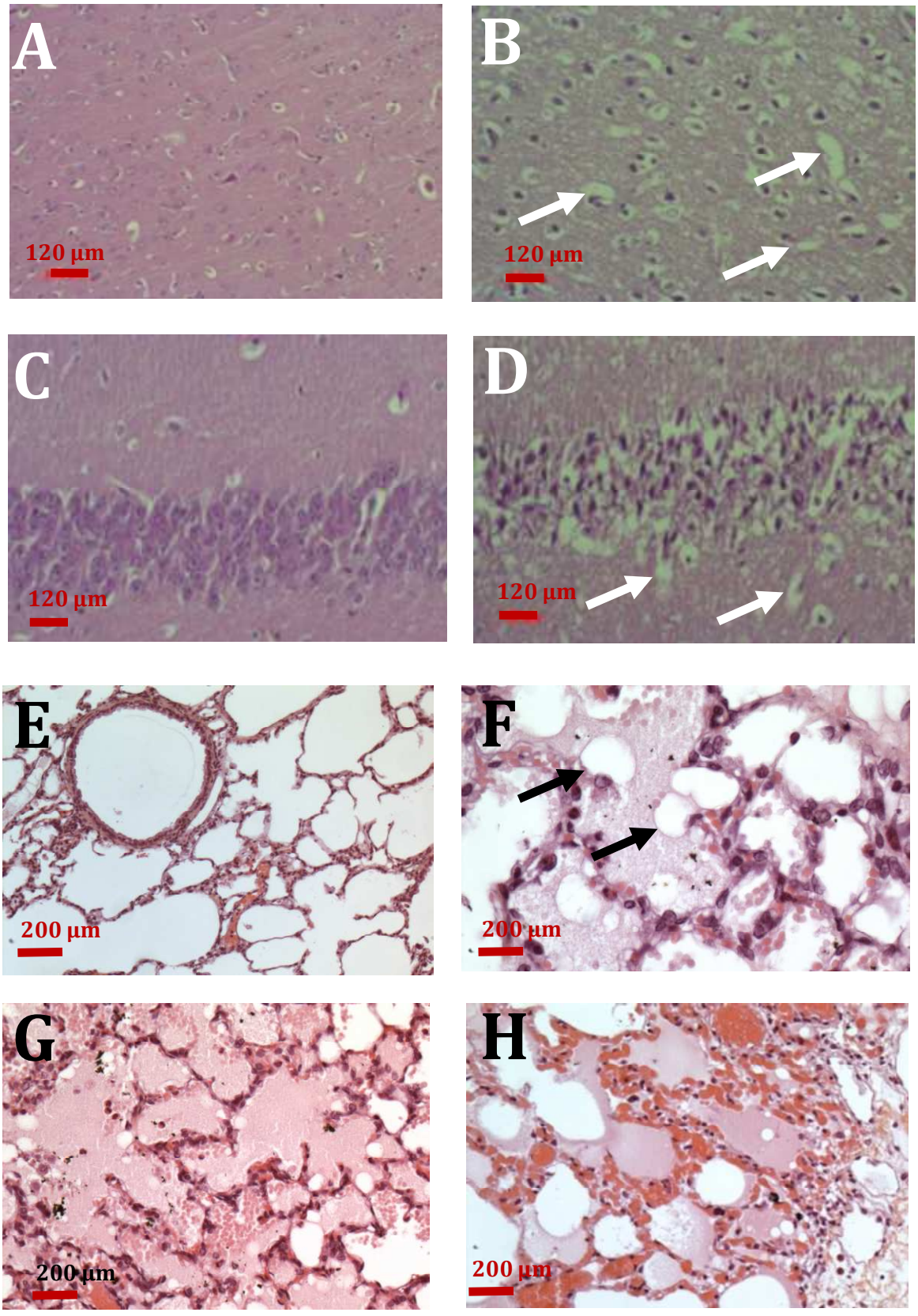
Panel F: Congestive lesions of the lungs in a rabbit from the TLV group (black arrows).

Panel G: Extensive alveolar edema in rabbits from the Control.

Panel H: Extensive alveolar edema in rabbits from the CONV group.

CONV, Conventional cooling group; TLV, Total liquid ventilation.

Supplemental Figure 1



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2. Tanter M, Fink M. Ultrafast imaging in biomedical ultrasound. IEEE Trans Ultrason Ferroelectr Freq Control 2013;61:102-119.
3. Baker AJ, Zornow MH, Grafe MR, et al. Hypothermia prevents ischemia-induced increases in hippocampal glycine concentrations in rabbits. Stroke 1991;22:666-673.
4. Chenoune M, Lidouren F, Adam C, et al. Ultrafast and whole-body cooling with total liquid ventilation induces favorable neurological and cardiac outcomes after cardiac arrest in rabbits. Circulation 2011;124:901-911, 901-907.
5. Darbera L, Chenoune M, Lidouren F, et al. Hypothermic Liquid Ventilation Prevents Early Hemodynamic Dysfunction and Cardiovascular Mortality After Coronary Artery Occlusion Complicated by Cardiac Arrest in Rabbits. Crit Care Med 2013;41:e457-465.