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Research paper

A new paradigm for lung-conservative total liquid ventilation

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ABSTRACT

Background: Total liquid ventilation (TLV) of the lungs could provide radically new benefits in critically ill patients requiring lung lavage or ultra-fast cooling after cardiac arrest. It consists in an initial filling of the lungs with perfluorocarbons and subsequent tidal ventilation using a dedicated liquid ventilator. Here, we propose a new paradigm for a lung-conservative TLV using pulmonary volumes of perfluorocarbons below functional residual capacity (FRC).

Methods and findings: Using a dedicated technology, we showed that perfluorocarbon end-expiratory volumes could be maintained below expected FRC and lead to better respiratory recovery, preserved lung structure and accelerated evaporation of liquid residues as compared to complete lung filling in piglets. Such TLV below FRC prevented volutrauma through preservation of alveolar recruitment reserve. When used with temperature-controlled perfluorocarbons, this lung-conservative approach provided neuroprotective ultra-fast cooling in a model of hypoxic-ischemic encephalopathy. The scale-up and automating of the technology confirmed that incomplete initial lung filling during TLV was beneficial in human adult-sized pigs, despite larger size and maturity of the lungs. Our results were confirmed in aged non-human primates, confirming the safety of this lung-conservative approach.

Interpretation: This study demonstrated that TLV with an accurate control of perfluorocarbon volume below FRC could provide the full potential of TLV in an innovative and safe manner. This constitutes a new paradigm through the tidal liquid ventilation of incompletely filled lungs, which strongly differs from the previously known TLV approach, opening promising perspectives for a safer clinical translation.

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1. Introduction

The development of protective mechanical ventilation was a major step forward for critically ill patients in intensive care units over the last decades. One of the next medical breakthroughs could be the use of total liquid ventilation (TLV) of the lungs with perfluorocarbons (PFC). This method consists in lungs filling with residual volumes of perfluorocarbons, above which a tidal volume of liquid is added and removed at each respiratory cycle. Importantly, TLV should not be confounded with partial liquid ventilation (PLV), which

Abbreviations: ANOVA, analysis of variance; CT, computerized tomography; EV, expiratory volume of liquid; FiO2, inhaled fraction of oxygen; FRC, functional residual capacity; HIE, hypoxic-ischemic encephalopathy; MRI, magnetic resonance imaging; PEEP, positive end-expiratory pressure; PFOB, perfluorooctylbromide; PCO2, arterial blood carbon dioxide partial pressure; PO2, arterial blood oxygen partial pressure; PLV, partial liquid ventilation; PFC, perfluorocarbon; ROI, region of interest; TLV, total liquid ventilation; TV, tidal volume

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Research in context

Evidence before this study

Total liquid ventilation (TLV) consists in lungs filling with residual volumes of perfluorocarbons, above which a tidal volume of liquid is added and removed at each respiratory cycle using a dedicated liquid ventilator. This should not be confounded with the so-called "partial liquid ventilation", which consists in lung filling with perfluorocarbons and further gaseous ventilation with a conventional ventilator. Until now, it was usually admitted that TLV should abolish the air-liquid interface through a complete filling of the lungs with liquids. When afforded with temperature-controlled perfluorocarbons, it can also use the lungs as heat exchangers and induce ultra-fast cooling and potent experimental neuroprotection after resuscitated cardiac arrest. The clinical translation of TLV was limited by the lack of dedicated ventilators and the need for long-term studies in clinically relevant conditions. Moreover, precise recommendations regarding ideal residual and tidal volumes during TLV were still needed until now.

Added value of this study

Here, we propose a radically new way of pulmonary ventilation for critical care situations. It consists in an automatized TLV of the lungs with liquid volumes below functional residual capacity (FRC) throughout the procedure. Using sophisticated and up-to-date engineering and imaging techniques, we evaluated both immediate and delayed effects of this procedure in several animal models, including non-human primates. We also demonstrated that TLV provides ultrafast cooling even in large animals and prevent neurological sequels following hypoxic brain injury.

Implications of all the available evidence

This study demonstrates that TLV could be induced in a totally safe and efficient manner using an accurate control of perfluorocarbon volume below FRC. This constitutes a new paradigm as our method consists in tidal liquid ventilation of incompletely filled lungs, which strongly differs from the previously known TLV approach, opening promising perspectives for a safer clinical translation. This also differs from partial liquid ventilation, that was shown to increase the rate of lung trauma due to "high-volume" ventilation in patients presenting with acute respiratory distress. This new paradigm of TLV below FRC could relaunch the clinical investigations with liquid ventilation in a safe manner. For instance, the clinical translation of TLV for ultra-fast cooling and neuroprotection could be considered in the near future after cardiac arrest.

consists in lung filling with PFC and further gaseous ventilation with conventional ventilator [1–3]. This failed to provide benefits in patients presenting with acute-respiratory distress syndrome [2]. More generally, PFC are also proposed for other medical applications such as aerosols for respiratory diseases [4,5], blood substitutes [6] or contrast product for imaging [7,8].

Due to the high solubility of the PFC for gases, TLV can ensure normal gas exchanges and provides pulmonary benefits [9], as shown in animal models of respiratory diseases [10–12]. When afforded with temperature-controlled PFC, it can also use the lungs as heat exchangers [13–16] and afford ultra-fast cooling and potent experimental neuroprotection after resuscitated cardiac arrest [13,14]. However, its clinical translation was limited by the lack of liquid ventilators able to adequately control PFC pulmonary flows during TLV and the absence of consensus regarding adequate respiratory parameters [17]. Recently, our group has developed a prototype that can continuously regulate expiratory flow as well as PFC volumes and pressures, which was a great cornerstone for TLV translation [18,19]. At this step, precise recommendations are still needed to provide an efficient procedure, regarding targeted PFC volumes, filling pressures and PFC target temperatures. This is particularly required after the negative clinical experiences with PLV, which produced volu- or barotrauma in patients presenting with acute-respiratory distress syndrome [2]. Even if TLV should not produce the same side effects through the primary of control liquid volumes throughout the respiratory cycle, this invites to be very cautious prior to any other trial with liquid ventilation. Here, our goal was therefore to properly assess the short and long-term effect of TLV at different levels of lung filling in highly clinically relevant conditions.

Therefore, we propose a new, fully safe and protective approach for TLV that allows tidal ventilation of the lungs after incomplete lung filling with PFC. We tested different strategies with various filling and tidal volumes and determined the procedure with the best tolerance through liquid redistribution into initially partly filled lungs rather than inflation of fully filled lungs. This showed that TLV could be much better tolerated when the lungs are filled below the expected volume of the functional residual capacity (FRC), despite incomplete initial degassing. We tested this approach in healthy piglets and further confirmed our results in pathophysiological conditions in a piglet model of hypoxic-ischemic encephalopathy. This confirmed that beyond being safe, this procedure could still be beneficial through its ultra-fast cooling properties. Then, we attempted to integrate the concept of TLV using liquid volumes below FRC using a new liquid ventilator. Beyond the automating of the whole process, we scaled up the technology and confirmed that TLV at residual volumes below FRC can provide a safe procedure while enabling the full potential of TLV in human adult-sized animals, as well as non-human primates. Such tidal liquid ventilation strongly differs from the previously known TLV approach, opening promising perspectives for a safer clinical translation.

2. Material and methods

The animal instrumentation and the ensuing experiments were conducted in accordance with French official regulations after approval by the ethical committee for animal use. Experimental protocols, designs of liquid ventilator and imaging methods are described as Supplementary Material and Methods.

2.1. Piglets preparation and follow-up

Piglets were anesthetized by zolazepam (25 mg/kg i.m.), tiletamine (25 mg/kg i.m.) and propofol (5–10 mg/kg i.v.). After endotracheal intubation, animals were submitted to conventional mechanical gas ventilation with tidal volume of 8 ml/kg and respiratory rate of 25 per min (Minerve, Esternay, France). Inhaled fraction of oxygen (FiO_2) was set at 30%. Animals received analgesics (buprenorphine, 30 μg/kg i.v.) and were paralyzed by the administration of vecuronium bromide (0.1 mg/kg i.v.). Catheters were inserted into the cephalic vein and the femoral artery for the continuous evaluation of mean arterial pressure and heart rate, respectively. Rectal temperature was also recorded throughout the procedures. Hemodynamic data were digitized (Hem, Notocord v4.2, Noisy-sur-Seine, France).

In a first set of experiments (safety experiment), newborn piglets were submitted to normothermic TLV without any initial injury, in order to evaluate proper pulmonary effects of the procedure. As described in detail as supplemental material, different conditions of expiratory volume of liquid (EV = 15 or 30 ml/kg) or tidal volume of liquid (TV = 8 or 16 ml/kg) were tested. In some experiments, a CT-scan was conducted and animals were euthanized at the end of the procedure. In other experiments, animals resumed to conventional mechanical ventilation after 30 min of TLV. They returned to spontaneous breathing after 5 h. Clinical recovery

was then evaluated during 3 days before euthanasia and lung removal for post-mortem analysis. Animals were also submitted to a similar surgical procedure with 5 h of conventional mechanical ventilation without TLV (Sham group).

In the second set of experiments (efficiency experiment), animals were submitted to hypoxic-ischemic injury in order to evaluate the benefits of hypothermic TLV. Hypoxia-ischemia encephalopathy (HIE) was induced by 30 min of hypoxia at FiO_2 = 10%, followed by an interruption of the mechanical ventilation and clamping of the endotracheal tube. After 7 min of respiratory arrest, and subsequent cardiac arrest, a cardiopulmonary resuscitation was started with manual chest compression, resumption of mechanical ventilation, increase of the inhaled fraction of oxygen (FiO_2) to 100% and bolus administration of epinephrine (4 μg/kg, i.v.). The experimental design was set after preliminary experiments determining the balance between the severity of hypoxic-ischemic insult and the ability to obtain successful resuscitation after apnea. After resumption of spontaneous circulation, piglets were randomly allocated to the Control or TLV group. In the control group, piglets were maintained under conventional mechanical ventilation during 6 h after HIE induction. In the TLV group, piglets were rapidly cooled to 32 °C by TLV according to the safest setting previously determined (EV = 15 ml/kg and TV = 8 ml/kg). An additional group of piglets was submitted to the surgical procedure with no hypoxia-ischemia (Sham group).

In all set of experiments, arterial blood samples were taken for the evaluation of blood pH, pCO_2, pO_2 and lactate levels (Epoc, Kitvia, Labarthe-Inard, France). After awakening, animals were followed during 3 or 7 days in the safety or efficiency experiments, respectively. They received analgesics (buprenorphine, 30 μg/kg i.m. b.i.d.) and appropriate cares throughout follow-up. Survival and clinical recovery was assessed. We evaluated the daily neurological function with a notation grid developed for piglets (Table S1). In accordance with our ethical committee, animals with a severe dyspnea or neurological dysfunction score higher than 70% at day 1 or 40% at day 2, respectively, were euthanized with an overdose of pentobarbital. At the end of the follow-up, all animals were euthanized and the lungs were withdrawn. Lung morphology was evaluated by histological evaluation. For the evaluation of the neurological protection afforded by hypothermic TLV (efficiency experiment), two catheters were inserted into the internal carotid arteries after euthanasia and brain was perfused with 4% paraformaldehyde. Brain was then collected and stained with Fluorojade-C. The number of degenerating neurons was evaluated in the cortex, caudate and putamen nuclei and hippocampus.

2.2. Adult pigs preparation and follow-up

Adult pigs weighing 60–70 kg were anesthetized by acepromazine (1 mg/kg, i.m.), ketamine (10 mg/kg, i.m.), propofol (5–10 mg/kg, i.v.) and morphine (0.1 mg/kg, i.v.). Anesthesia was maintained by inhaled isoflurane (2.5%). After endotracheal intubation, pigs were paralyzed (rocuronium, 6 mg/kg, i.v.) and submitted to conventional mechanical ventilation (TV of 8 ml/kg and respiratory rate of 10 per min; FiO_2 = 30%). An arterial catheter was inserted into the caudal artery for mean arterial pressure monitoring and arterial blood sampling. Rectal, tympanic, oesophageal and vesical temperature probes were inserted for the evaluation of body cooling in different compartments during TLV. The exact procedure is described as supplemental material.

After 30 min of TLV, conventional mechanical ventilation was resumed. They were progressively rewarmed and awakened after 6 h. They returned to animal facility with no oxygen supplementation. All pigs were followed for 10 days after the TLV procedure for clinical respiratory function assessment. They received analgesics and appropriate care. At the end of the follow-up, animals were euthanized by an overdose of pentobarbital and heart-lungs were withdrawn for immediate evaluation of PFC residues in lungs by ex vivo CT-scan imaging. After imaging, we sampled representative tissue of each lobe of the lungs and stored them in formaldehyde 4%. Conventional histology was then performed on these samples.

2.3. Macaques preparation and follow-up

Two 13-years aged adults monkeys (*Macaqua Fascicularis*) weighting 10.6 and 11.7 kg were enrolled in the study. They underwent an initial anesthesia using a mixture of dexmedetomidine (15 μg/kg i.m.) and ketamine (3 mg/kg i.m.) for an initial CT-scan examination, as described in supplemental material. Two-weeks later, they were reanesthetized and intubated for mechanical ventilation (respiratory rate = 20 cycles/min; VT = 8 ml/kg). Anesthesia was maintained by alphaxan (6 mg/kg/h). After the administration of rocuronium (6 mg/kg, i.v.), animals underwent 20 min of hypothermic TLV using the automated and upscaled ventilator previously used in large pigs. EV and TV were maintained at 15 and 8 ml/kg throughout TLV. Respiratory rate was set at 6 cycles/min. The temperature of the PFC was controlled as previously described to cool body core to 32 °C. After TLV, animals resumed to conventional mechanical ventilation during 6 h for slow rewarming using external pads. After awakening, animals underwent a close clinical follow-up. Three weeks later, they were sedated for a very short period using dexmedetomidine for blood samples withdrawing and respiratory gas exchanges evaluation. Animals were awakened rapidly using the anesthetics antagonist atipamezole. A last anesthesia was conducted four weeks later for a final CT-scan examination and evaluation of lung parenchyma integrity. Since no any abnormality was observed, animals were thereafter hosted in a rehabilitation center.

2.4. Statistical analysis

Data were expressed as mean ± SEM or, when indicated, as individual values and medians. Statistical analysis were conducted using a dedicated software (Sigmastat, Systat software, San Jose, CA, USA). Continuous parameters were compared between groups using either one-way ANOVA or 2-way ANOVA for repeated measures, depending upon the parameters. These analyses were followed by a Hold-sidak analysis if necessary. Values were not compared between the different time points to avoid multiple comparisons. Neurological scores were compared using a Kruskall-Wallis analysis followed by a Mann-Whitney test. Survival was compared between groups using a log-rank test. Significant differences were determined at $p \leq 0.05$.

3. Results

3.1. Acute effects of total liquid ventilation with different conditions of lung filling

In preliminary experiments, we assessed lung volume by chest computerized tomography (CT-scan) in four anesthetized piglets. As illustrated by Fig. 1, lung end-expiratory volume achieved 13.8 ± 1.8 ml/kg and 37.7 ± 8.8 ml/kg at PEEP = 0 and 5 cmH_2O, respectively. It is consistent with previous findings showing physiological FRC in the middle of this range, around 25–30 ml/kg in babies [20]. Accordingly, we decided to evaluate the effect of TLV with end-expiratory volumes of PFC (EV) close to these “extreme” physiological volumes, i.e., below or close to estimated FRC at either 15 or 30 ml/kg, respectively. We used a dedicated device for small animals [18,19], as illustrated by Fig. 2A and Suppl. Fig. S1. TLV was induced with perfluoctylbromide (PFOB) TLV [21,22]. As illustrated in Fig. 2B, we crossed the evaluation of the two selected EV levels with two different levels of tidal volume (TV) set at either 8 or 16 ml/kg (TV_8-EV_{15}, TV_{16}-EV_{15}, TV_8-EV_{30}, TV_{16}-EV_{30} groups, respectively). In all groups, animals were submitted to 30 min of TLV (n = 5 in each group), with respiratory rate fixed to maintain similar respiratory minute volume in all groups (i.e., 9 vs 4.5 cycles in the groups with TV = 8 or 16 ml/kg, respectively). An additional group of Sham animals were submitted to conventional gas ventilation without TLV (n = 5).

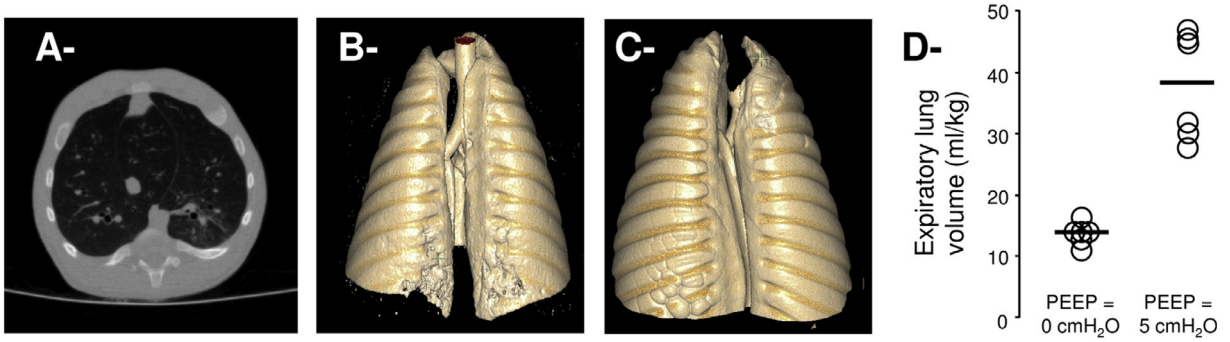


Fig. 1. Evaluation of lung volumes in normal conditions in anesthetized piglets submitted to gas ventilation. A- Picture of a thoracic computerized tomography (CT)-scan in one anesthetized piglet during mechanical ventilation. Images were obtained during prolonged expiratory pause. B- 3D reconstruction of the lung with an acquisition during a prolonged expiratory pause with positive end-expiratory pressure (PEEP) set at 0 cmH_2O. C- 3D reconstruction of the lung with an acquisition during a prolonged expiratory pause with positive end-expiratory pressure (PEEP) set at 5 cmH_2O. D- Measured lung volumes in 6 piglets during a prolonged expiratory pause at PEEP = 0 or 5 cmH_2O. Circles represent individual values and bold line mean values in each condition, respectively.

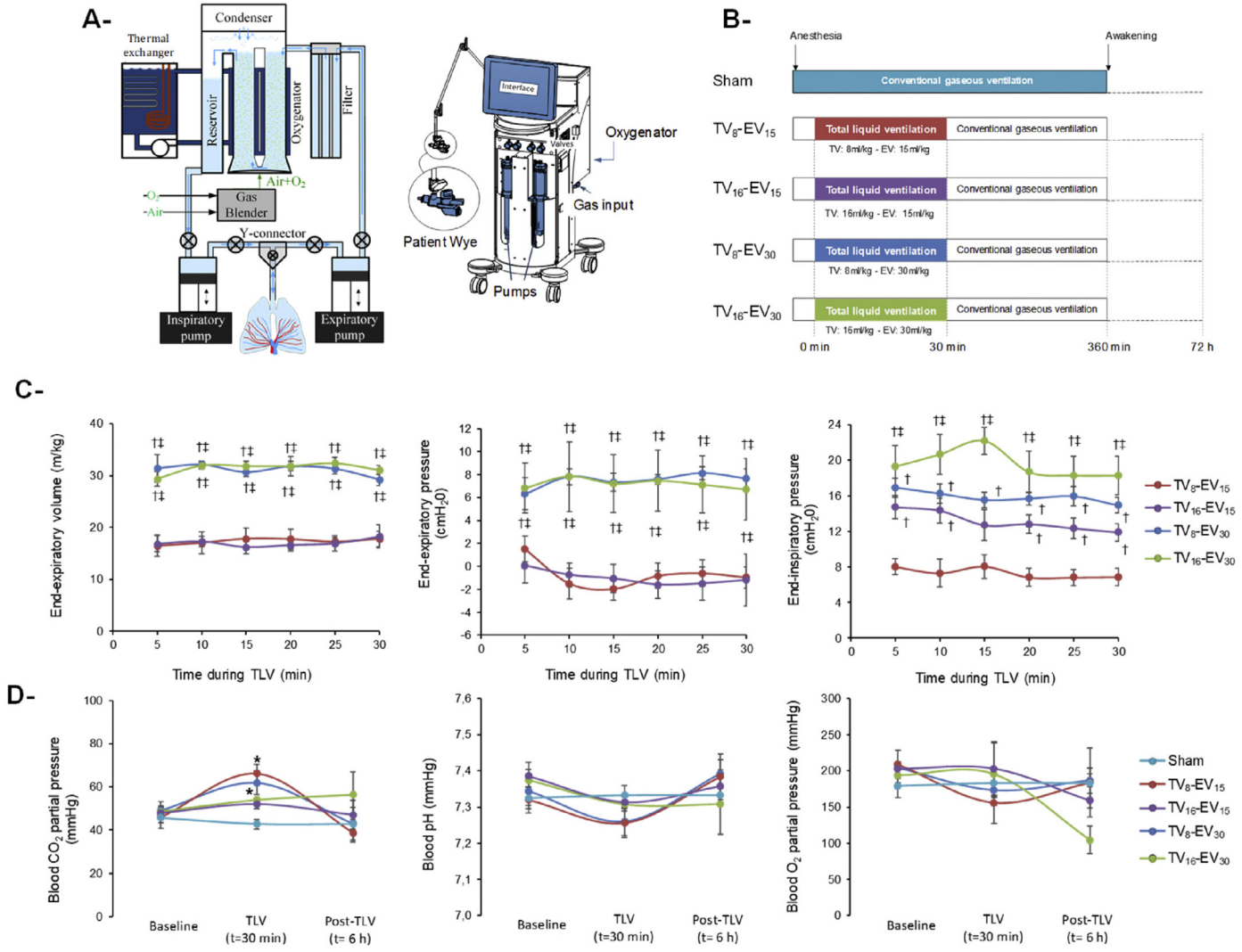


Fig. 2. Evaluation of different ventilation strategy for total liquid ventilation in piglets. A- Schematic representation of the liquid ventilators for TLV, including piston pumps and valves to drive the liquid into and from the lung, thermal exchanger, condenser for perfluorocarbon condensing and oxygenator. The liquid ventilator also includes sensors, graphic user interface and electronic algorithm to control the entire process [19]. B- Experimental protocol including five groups of piglets submitted to 30 min of TLV with different tidal volumes (TV of 8 or 16 ml/kg) and end-expiratory volumes (EV of 15 or 30 ml/kg), as compared to Sham animals with conventional mechanical ventilation only. The four corresponding groups are so-called TV_8-EV_{15}, TV_{16}-EV_{15}, TV_8-EV_{30} and TV_{16}-EV_{30}, respectively. C- Volumes of perfluorocarbons within the lungs at the end of expiration during TLV and static pulmonary pressures measured during end-expiratory and end-inspiratory pauses, respectively. D- Blood pH and carbon dioxide and oxygen partial pressure (pCO_2 and pO_2, respectively). *, $p < 0.05$ vs Sham; †, $p < 0.05$ vs TV_8-EV_{15}; ‡, $p < 0.05$ vs TV_{16}-EV_{15}.

As illustrated by Fig. 2C, the target EV of 15 and 30 ml/kg were roughly maintained throughout TLV in the corresponding groups. Surprisingly, end-expiratory static pressure was negative in the two groups with EV set at 15 ml/kg, as compared to +6−8 cmH_2O in the groups filled with 30 ml/kg of EV (Fig. 2C). This peculiar finding can be explained by the active exhalation driven by the piston pump during TLV. In the TV_8-EV_{15} and TV_8-EV_{30} groups, this led to slight depression and could suggest that the actual EV was yet below FRC. End-inspiratory alveolar pause pressure also increased along with TV and EV in the different groups, achieving a maximal value ≈20 cmH_20 in TV_8-EV_{30}. As shown in Fig. 2D, blood oxygenation and pH were not significantly modified during TLV in the different groups vs Sham animals. However, a significant increase in arterial blood CO_2 pressure (pCO_2) was observed in TV_8-EV_{15} and TV_{16}-EV_{15} groups as compared to Sham. This could be expected as TLV parameters were fixed by the study design and no change in respiratory rate was allowed during TLV. Heart rate and mean arterial pressure were not modified by TLV as compared to Sham (Suppl. Fig. S2).

3.2. Animal recovery after total liquid ventilation

After the episode of 30 min of TLV, the piglets were submitted to 5 h of conventional mechanical ventilation, after which they were weaned from ventilation and awakened. Oxygen enrichment was permitted during 24 h using semi-hermetic cages. After return to spontaneous breathing, gas exchange and hemodynamic parameters were not significantly modified in animals previously submitted to TLV vs Sham (Fig. 2D and Suppl. Fig. S2). A non-significant decrease in pO_2 was observed in TV_{16}-EV_{30} as compared to other groups (Fig. 2D). Yet, two animals of this very group rapidly presented severe acute respiratory failure after awakening. They were euthanized and gross post-mortem analysis showed macroscopic lung congestion and hemorrhage.

The days after TLV, animals from the TV_8-EV_{15}, TV_{16}-EV_{15} and TV_8-EV_{30}, groups did not show any sign of respiratory dysfunction as compared to Sham. These animals were followed during 3 days with no sign of acute respiratory discomfort. Conversely, respiratory discomfort and dyspnea were observed in the three surviving animals from the TV_{16}-EV_{30} group. Respiratory rate achieved 145 ± 9 breaths/min after 24 h, as compared to 41 ± 8 breaths/min in Sham animals ($p < 0.05$). Two animals were euthanized for persistent polypnea after 24 h in the TV_{16}-EV_{30} group and the last one after 48 h following TLV, respectively.

3.3. Morphological effects of total liquid ventilation on lung structure and evaluation of perfluorocarbons residues

All animals were euthanized at the end of the clinical follow-up for post-mortem analysis. A dual-nuclei magnetic resonance imaging (MRI)

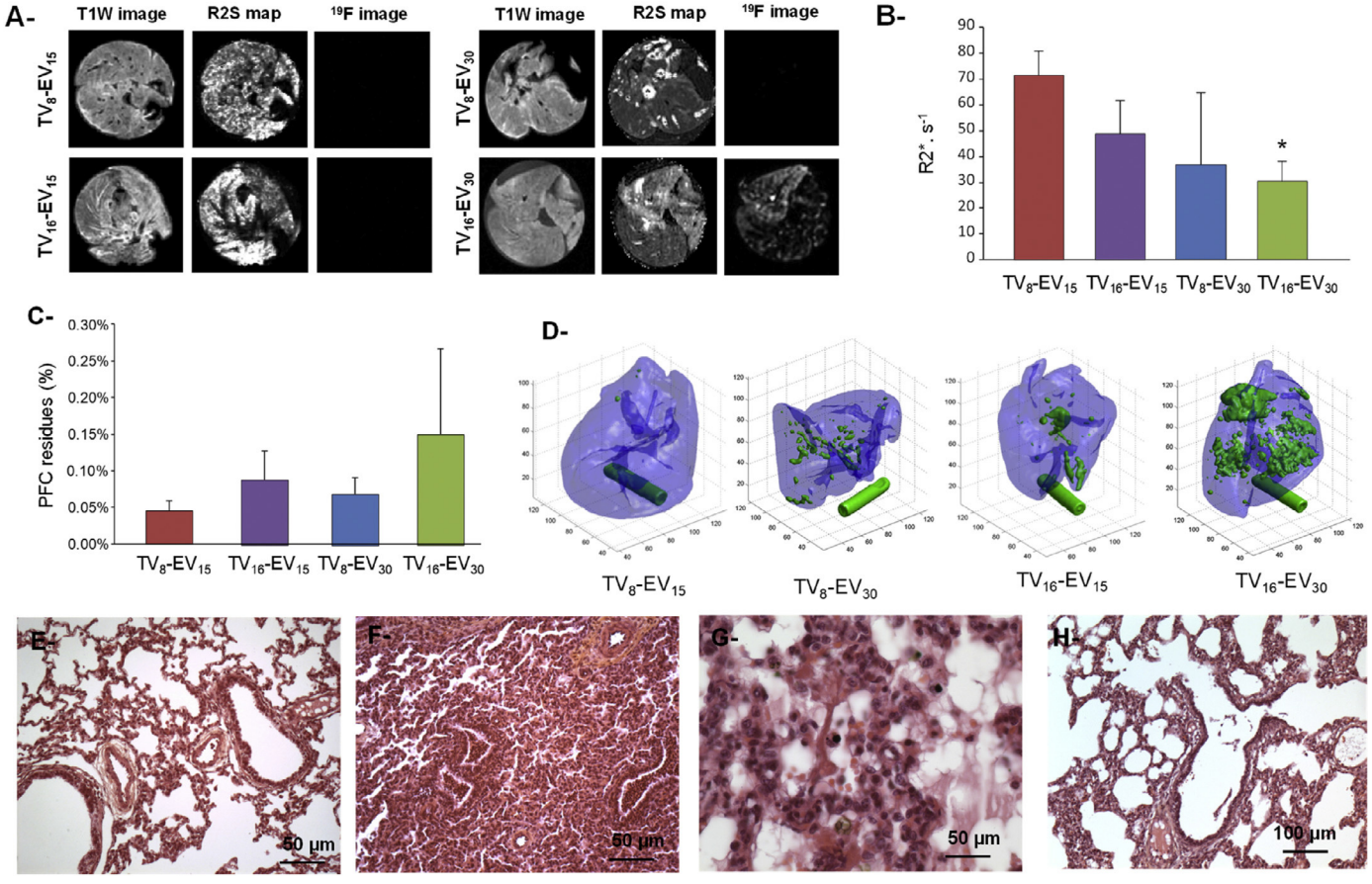


Fig. 3. Morphological alterations and perfluorocarbon (PFC) residues in the different groups of piglets submitted to total liquid ventilation (TLV). A- Typical appearance of lung parenchyma on magnetic resonance imaging using T1W sequence, apparent transverse relaxation rate (R2*) map and ^{19}F dual-nuclei imaging in piglets from the different groups. The T1W allows visualizing lung parenchyma and anatomy. Reduced R2* suggests enhanced hemorrhage or edema in the TV16-EV30 group [32]. ^{19}F signal shows PFC residues. B- Average value of apparent transverse relaxation rate R2* in the entire lungs in the different groups. Low values indicate morphological alterations including hemorrhage and pulmonary edema. C- Average volume of PFC residues in the different groups (as percentage of entire lung volume). D- 3-D reconstruction of entire lungs after ^{19}F dual-nuclei imaging. Blue and green areas represent lung parenchyma and PFC-filled spots, respectively. A tube filled with 100% PFC is located under the sample for absolute quantification of ^{19}F images. E- Normal pulmonary histological appearance in a Sham piglet. F- Abnormal pulmonary histological appearance in a Sham piglet demonstrating interstitial inflammation and foci of bronchiolitis. G- Abnormal pulmonary histological appearance in a piglet from the TV_{16}-EV_{30} group including hyaline membranes, alveolitis, hemorrhage and serous edema. H- Abnormal pulmonary histological appearance in a piglet from the TV_{16}-EV_{30} group demonstrating alveolar and bronchiolar distension. *, vs TV_8-EV_{15}; See legend in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protocol was developed for the concomitant analysis of lung lesions and PFC residues in the entire lungs (Fig. 3A and Suppl. Fig. S3). As illustrated in Fig. 3A and B, average relaxation rate R2* was significantly decreased in TV_{16}-EV_{30}, but not TV_8-EV_{30} and TV_{16}-EV_{15}, as compared to TV_8-EV_{15}, evidencing hemorrhage or pulmonary edema in the first group. Along with these abnormalities on R2* mapping, ^{19}F dual imaging also showed a non-significant increase in the amount of PFC residues in the TV_{16}-EV_{30} group as compared to the three other groups (Fig. 3C). As illustrated by Fig. 3D, the 3D reconstruction of lung parenchyma (in blue) indeed showed that PFC residues (in green) were more frequent and localized in the TV_{16}-EV_{30} group, as compared to others. It is reasonable to hypothesize that PFC persistence was linked to parenchymal alterations that slow or inhibit PFC elimination.

The histological examinations of the lungs confirmed severe pulmonary alterations in the TV_{16}-EV_{30} as compared to all other groups. Indeed, we observed normal appearance in the Sham, TV_8-EV_{15}, TV_{16}-EV_{15} and TV_8-EV_{30} groups (Fig. 3E, respectively). We only observed non specific foci of infection in some areas (Fig. 3F). In the TV_{16}-EV_{30}, we observed typical alterations of diffuse alveolar damage including severe alveolitis, alveolar hemorrhage and hyaline membranes (Fig. 3G). Some areas showed alveolar or bronchiolar dilation with a typical "balloon-like" pattern compatible with overdistension in the latter group (Fig. 3H).

3.4. *Repartition of perfluorocarbons during total liquid ventilation and consequences on lung mechanics*

In order to understand PFC distribution during TLV, we submitted two additional anesthetized piglets to chest CT-scan imaging during TLV with 15 min at TV_8-EV_{15} and then 10 min at each other conditions (TV_{16}-EV_{15}, TV_8-EV_{30} and TV_{16}-EV_{30}). PFOB could be identified easily as it is highly hyperattenuating as compared to lung parenchyma. As illustrated by Fig. 4A, CT-scan confirmed incomplete lung filling in the TV_8-EV_{15}, TV_{16}-EV_{15} and TV_8-EV_{30} conditions, as compared to TV_{16}-EV_{30}. Under the first two conditions (EV of 15 ml/kg), upper lobes appeared completely free of PFC. Under the two other conditions (EV of 30 ml/kg), upper lobes contains PFC but the density was attenuated as compared to the lower lobes, suggesting still incomplete filling in those upper lobes. The relative abundance of PFOB was then further assessed within different pulmonary regions of interest (Fig. 4B). The calculated percentage of PFOB within each region of interest was plotted against the total volume of liquid into the lung (Fig. 4C). It confirmed that lower and intermediate regions of interest are maximally filled by the PFC, even at low total volumes. Conversely, the upper regions of interest are empty at low volumes and received the additional PFC when the volume increases (Fig. 4D). This area could act as a protective alveolar recruitment reserve at low EV, while overdistension could occur at higher EV, e.g., in the TV_{16}-EV_{30} group. In the latter group, the PFC content was dramatically increased in the upper (sternal) ROI at inspiration (46 ml/kg of PFOB in the entire lungs), confirming that TLV exceeding FRC in those conditions.

We further evaluated the pressure–volume relationship in two piglets during lung filling with increasing volumes of PFC. As illustrated in Fig. 4D, two slope breaks were evidenced after the administration of approximately 20 and 40 ml/kg of PFC, respectively. At 20 ml/kg, the first change likely corresponds to the beginning of

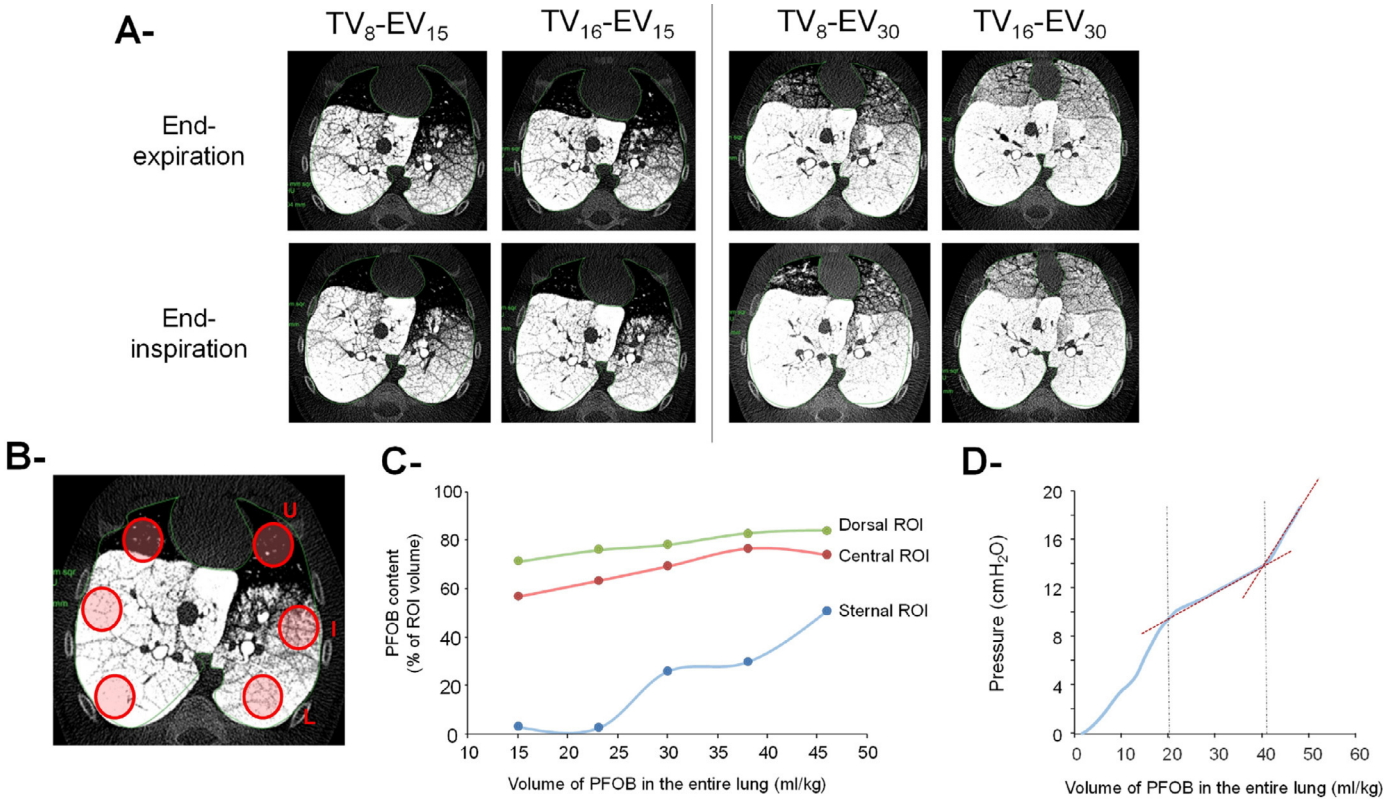


Fig. 4. Evaluation of perfluorocarbons (PFC) pulmonary repartition during total liquid ventilation (TLV) in piglets. A- Transverse images of thoracic computerized tomography (CT-scan) during prolonged pauses at end-inspiration or end-expiration in one anesthetized piglet positioned in dorsal recumbency and submitted to TLV with various end-expiratory or tidal volumes (EV and TV set at 8/16 and 15/30 ml/kg, respectively). B- Lung regions of interest (ROI) on a typical thoracic CT-scan picture. The amount of perfluoctylbromide was calculated in each ROI, taking into account its own attenuation as compared to lung normal attenuation (+2300 and −600 Houndsfield Units, respectively). The so-called zones U, I and L corresponds to upper (sternal), intermediate and lower (dorsal) ROI. C- Amount of PFOB in each ROI, along with the total volume of PFC in the lungs. A pooled analysis was done among inspiration and expiration measurement. Five points were analyzed with the following expected volumes: 1) 15 ml/kg (expiration in TV_8-EV_{15} and TV_{16}-EV_{15} conditions), 2) 24 ml/kg (inspiration in TV_8-EV_{15} conditions), 3) 30–31 ml/kg (inspiration in TV_{16}-EV_{15} and expiration in TV_8-EV_{30} and TV_{16}-EV_{30} conditions), 4) 38 ml/kg (inspiration in TV_8-EV_{30} conditions) and 5) 46 ml/kg (inspiration in TV_{16}-EV_{30}). Mean values were calculated in case of replication, at 15 and 30–31 ml/kg. D- Relationship between pulmonary pressure and PFC volume during a slow instillation into the trachea in a piglet. Dashed red lines emphasizes inflexion points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alveolar recruitment of the upper region of the lung. At 40 ml/kg, the second slope break likely corresponds to an overdistension of already completely filled lungs. Interestingly, this happens at rather low alveolar pressures, averaging 15 cmH_20.

3.5. Protective approach for total liquid ventilation after experimental hypoxic-ischemic encephalopathy in newborns

Beyond determining safe parameters, we then assessed whether this conservative approach of TLV could still provide the full potential of TLV benefits in piglets. Accordingly, we aimed at investigating the neuroprotective effect of hypothermic TLV in a model of pediatric cardiac arrest and neonatal hypoxic-ischemic encephalopathy (HIE). HIE was induced by 30 min of hypoxia (FiO_2 lowered at 10%) and 7 min of apnea in anesthetized and paralyzed piglets. Animals were resuscitated using external chest compression and epinephrine administration. As illustrated by Fig. 5A, piglets were either submitted to a normothermic follow-up (38 °C, Control group; n = 6) or to hypothermic TLV started 5 min after resuscitation (TLV group; n = 6). In the latter group, TLV was maintained during 30 min with a target temperature of 33 °C and lung-conservative liquid ventilation (EV and TV = 15 and 8 mL/kg, respectively). Animals subsequently resumed to conventional gas ventilation for hypothermia maintenance with external blankets during 3 h under conventional mechanical ventilation. In both Control and TLV groups, animals were weaned from mechanical ventilation after 6 h following HIE injury. A third group was submitted to conventional mechanical ventilation without hypoxia-ischemia (Sham group; n = 4).

As illustrated by Suppl. Fig. S4, the lung-conservative approach of TLV did not alter its capacity to maintain correct gas exchanges as compared to conventional ventilation and its ability to induce ultrafast cooling in pathophysiological conditions. For instance, temperature achieved the 33 °C threshold within 10 to 20 min after TLV in oesophageal and rectal compartments, respectively (Suppl. Fig. S4A).

Animals were followed during 7 days after awakening. In the Control group, one animal died before the neurological evaluation at day 1 and all others were euthanized prematurely for severe neurological dysfunctions such as cerebral palsy or seizures. In the TLV group, neurological recovery was improved and only three animals presented a neurological dysfunction requiring premature euthanasia. Neurological dysfunction score was significantly attenuated in TLV vs Control groups at day 2 after HIE (Fig. 5B). Blood concentrations of brain injury biomarker S100B also evidenced a high increase at the time of euthanasia in Control but not TLV groups as compared to Sham (Fig. 5C). The benefits of TLV are also illustrated by survival analysis over the 7 days of follow-up in Fig. 5D. Histological analyses of the brain further confirmed a significant reduction in degenerating neurons count after euthanasia in the TLV vs Control groups (Fluoro-jade C staining, Fig. 5E and F). This shows the potent neuroprotective potential of hypothermic TLV.

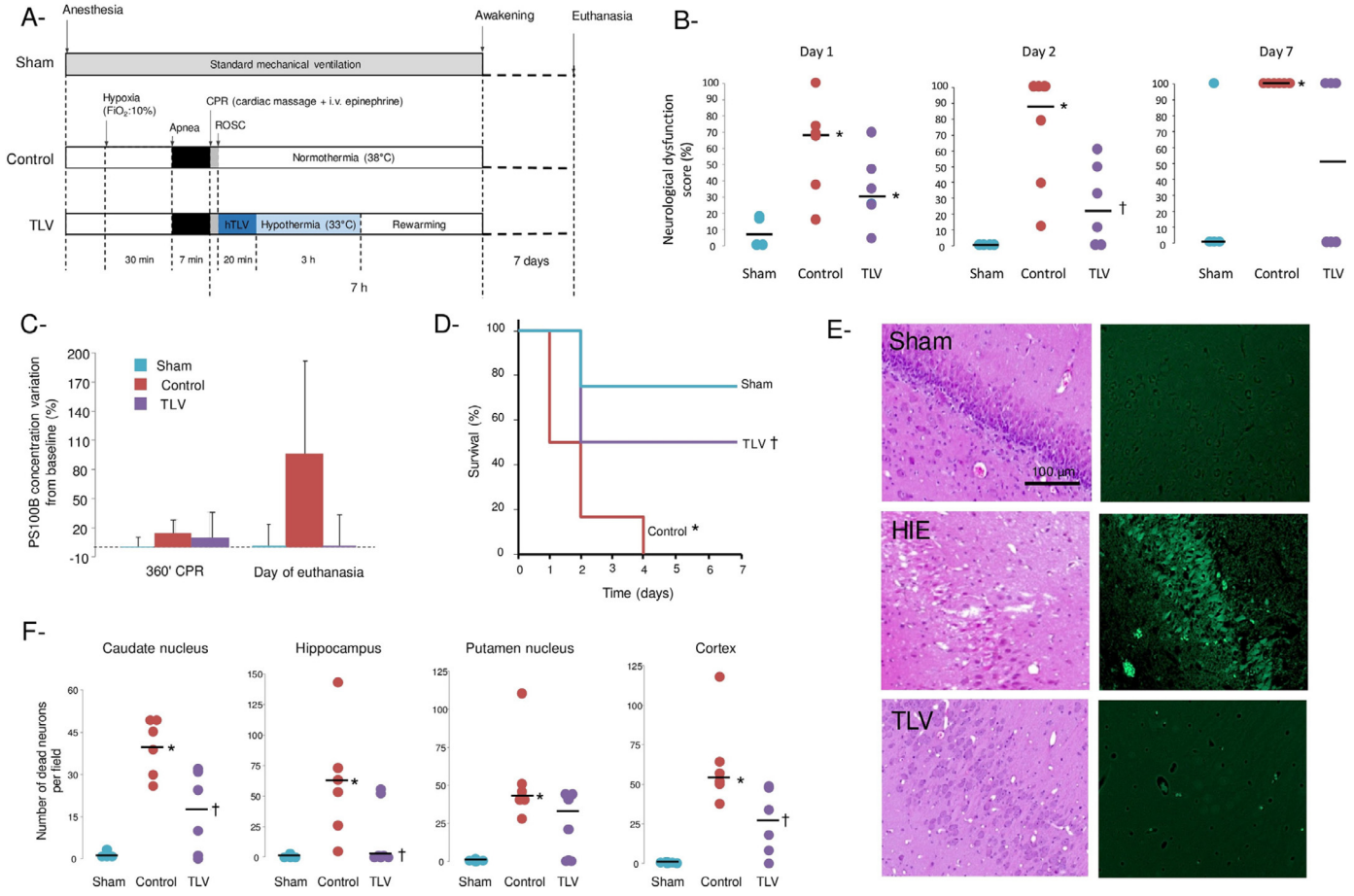
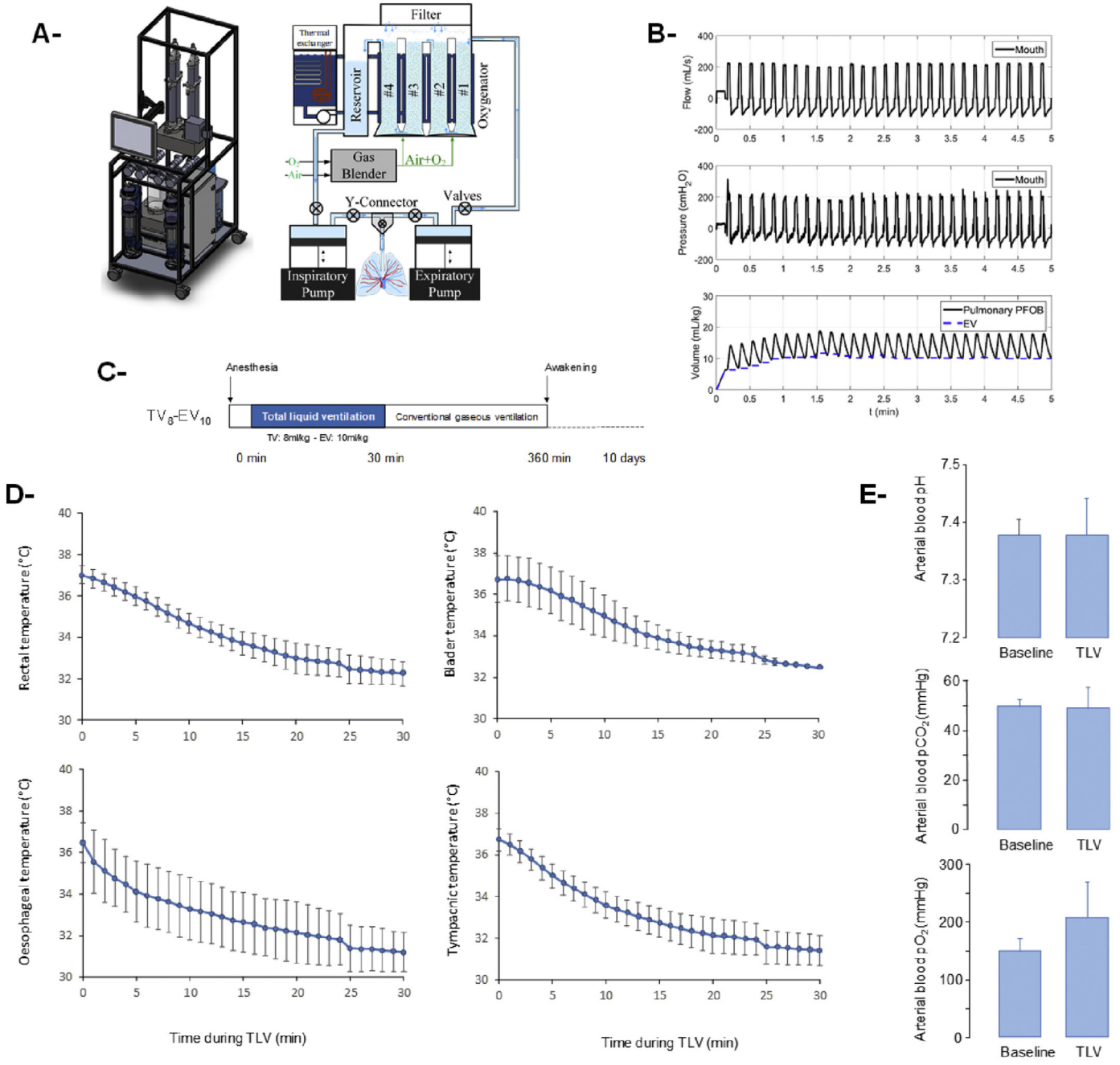


Fig. 5. Evaluation of lung-conservative total liquid ventilation (TLV) in pathophysiological condition of hypoxic-ischemic encephalopathy (HIE) in piglets. A- Experimental protocol describing the hypoxic-ischemic injury. After resuscitation, animals were either treated by conventional mechanical ventilation under normothermia (Control group) or hypothermic TLV. A third group was submitted to a Sham procedure with no HIE induction. B- Neurological dysfunction scores after HIE induction or Sham procedure (0% = lack of dysfunction; 100% = death). Open circles and bold lines represent individual and median values, respectively. C- Blood concentration of the S100B protein as a marker of brain injury. D- Kaplan-Meyer survival curves in all experimental groups. E- Typical histological appearance of the hippocampus after hemalun-eosin and fluorojade-C staining (left and right column rows, respectively). The latter staining show no or very few degenerating neurons in Sham and TLV groups, as compared frequent degenerating neurons in Control group. F- Number of positive fluoro-Jade C cells, expressed ad mean number per analyzed field, in each areas of interest of the central nervous system. *, $p < 0.05$ vs Sham; †, $p < 0.05$ vs Control.


A-
Filter
Thermal exchanger
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Oxygenator
Gas Blender
Air+O2
Y-Connector
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Expiratory Pump
B-
Flow (mL/s)
Mouth
Pressure (cmH2O)
Mouth
Volume (mL/kg)
Pulmonary PFOB
EV
t (min)
C-
Anesthesia
Awakening
TV8-EV10
Total liquid ventilation
Conventional gaseous ventilation
TV: 8ml/kg - EV: 10ml/kg
0 min
30 min
360 min
10 days
D-
Rectal temperature (°C)
Bladder temperature (°C)
Oesophageal temperature (°C)
Tympanic temperature (°C)
Time during TLV (min)
Time during TLV (min)
E-
Arterial blood pH
Arterial blood pCO2 (mmHg)
Arterial blood pO2 (mmHg)
Baseline
TLV
F-
10 cm
G-
100 µm
100 µm

3.6. Technology up-scale for automatized TLV in large animals

The previous experiments showed that lung-conservative approach of TLV could provide safe TLV with full benefits in both physiological and pathophysiological conditions in piglets. One would argue that those findings could not be extrapolated in large animals due to higher body mass, chest size and lung maturity. Accordingly, we up-scaled our liquid ventilator and designed a technology for large animals up to 100 kg. All the components were adapted with specific materials for medical applications (Fig. 6A). In addition, we aimed at automatizing the TLV process. A specific algorithm was developed to estimate the volume of liquid into the lungs and maintain EV at a given target, as we demonstrated that it was a critical parameter. Thereby, expiratory TV was continuously and automatically modified by the ventilator to exactly maintain the EV set by the investigator (Fig. 6B). Similarly, PFC initial temperature and rewarming rate was calculated [16], as our main goal was here to use TLV for the induction of rapid hypothermia.

3.7. Total liquid ventilation using the same approach could provide ultrafast cooling and safety in large pigs

Relevant settings for TLV in large animals were determined by preliminary experiments (See Supplemental Material and Methods). As illustrated in Fig. 6C, four pigs weighing 67 ± 3 kg were submitted to 30 min of TLV with TV and EV set at 8 and 10 ml/kg, respectively (Suppl. Fig. S5). It allowed to maintain EV below FRC. The target temperature range of 31−33 °C was achieved within <20 min in the entire body (Fig. 6D). Gas exchanges were normal after 30 min of TLV as compared to baseline ventilation (Suppl. Fig. S5). After 30 min of TLV, animals resumed to conventional gas ventilation and were slowly rewarmed. They were weaned from ventilation within 4 to 6 h, after which they returned to the animal room without any oxygen supplementation. All animals presented an excellent recovery with no sign of respiratory discomfort. Blood oxygen saturation remained above 97−98% in all animals from the first day after TLV to the end of the follow-up (Fig. 6E). After 10 days, they were euthanized for lung harvesting. As illustrated in Fig. 6F, CT-scan imaging of explanted lungs did not show any visible macroscopic foci of PFC residues since the entire lung parenchyma was diffusely hypoattenuating. Histological analysis also revealed normal appearance in both inferior and superior lung regions. In some areas, mild alveolar or bronchiolar dilation were only observed with minor thickening of the alveolar wall, as typically observed after mechanical ventilation (Fig. 6G). No any lesion of alveolitis or alveolar damage was observed. This confirms that the incomplete lung filling with low volumes of PFC was perfectly tolerated in the different regions. The lung-conservative approach of TLV with lung filling below FRC then provided a safe procedure with full potential regarding gas exchanges and ultrafast cooling properties in large animals.

3.8. Validation of the concept of lung-protective liquid ventilation in non-human primates

In order to demonstrate the ultimate safety of the lung-conservative approach of TLV with lung filled below FRC, we confirmed our results in mature and aged non-human primates. Indeed, previous results were only obtained in new born or young adults pigs. One would also argue that the poor tolerance of partial liquid ventilation in a previous clinical trial in humans could suggest a particular sensitivity of primates to liquid ventilation [2]. Therefore, we conducted an ultimate experiment in aged non-human primates in order to rule out any species or age specificity. We enrolled two 13-years monkeys (*Macaca fascicularis*) weighting 10.6 and 11.7 kg, respectively. This allowed evaluating lung-conservative TLV in old animals for which ageing could alter the response to TLV, which was never tested previously to our knowledge. Animals underwent a first anesthesia for CT-scan examination of the lung parenchyma (Fig. 7A) and evaluation of baseline FRC after 3D-reconstruction of the lung (Fig. 7B). We obtained FRC values averaging 26 and 32 ml/kg for the two animals, respectively. This is consistent with the expected FRC in primates. Then, we decided to test lung-conservative TLV targeting EV and TV of 15 and 8 ml/kg, respectively, which allowed remaining below FRC throughout TLV. Two weeks after the initial CT-scan, monkeys were indeed reanesthetized and submitted to 20 min of TLV with temperature-controlled PFC. As illustrated by Fig. 7C, rectal temperatures decreased very rapidly and achieved a target temperature of 32 °C in the entire body within 15 min. Lung compliance was not modified by TLV, demonstrating a lack of immediate lung trauma (Fig. 7D). The acute tolerance of TLV was also excellent regarding hemodynamics (Suppl. Table 2) or gas exchange (Fig. 7E). After TLV, monkeys were rewarmed under conventional mechanical ventilation during 6 h. Then, they were weaned from ventilation and awakened without any sign of dyspnea or hypoxemia. During the further follow-up, respiratory function was completely normal and no behavioral change was observed. Three weeks after the TLV procedure, gas exchanges were again evaluated and showed normal values for arterial pH, CO_2 partial pressure or oxygen saturation (Fig. 7E). Another CT-scan analysis was also conducted 4 weeks after TLV, which demonstrated no any sign of lung parenchyma abnormalities (Fig. 7F and G). Thanks to the excellent clinical status of the animals and the lack of any sequels following the TLV episode, we decided to transfer both monkeys to a rehabilitation center.

4. Discussion

Here, we propose a new approach for TLV through incomplete lung filling with PFC below FRC and subsequent tidal liquid ventilation. This represents a radical paradigm shift as compared to previous beliefs [10,11], that considered that lungs should be primarily completely filled with PFC and fully degassed since the filling phase. This lung-conservative approach of TLV was further automatized with an up-scaled device for large animals continuously controlling EV below FRC ranges. PLV was tested in humans but the largest trial raised skepticism regarding the actual safety of this procedure [2]. Those negative results were poorly deciphered a posteriori and it was often overstated that any way of liquid ventilation enhanced trauma risks by itself, regardless its exact way of induction. Therefore, it was critical to evaluate lung mechanics precisely during TLV and its delayed consequences after resumption to spontaneous breathing. Here, we show that TLV could be induced safely when controlling EV below expected FRC. This procedure was still able to provide ultrafast cooling in piglets and large pigs, as well as in non-human primates, reinforcing previous results in small animals. We confirmed

Fig. 6. Evaluation of total liquid ventilation with a new dedicated technology for large pigs. A- Schematic representation of the new specifically designed liquid ventilator. B- Typical perfluocarbon flow (upper raw), pressure at mouth and pulmonary volume of perfluocarbon during the first 5 min of total liquid ventilation (TLV) in a 63 kg pig. C- Schematic representation of experimental protocol in large pigs submitted to 30 min of hypothermic TLV followed by conventional gaseous ventilation and rewarming, before awakening. Animals were followed during 10 days before euthanasia for post-mortem analyses. D- Body temperatures in the different compartments during the TLV episode, showing a rapid decrease of target temperature (32−33 °C) within 20 min in all compartments. E- Blood pH and carbon dioxide and oxygen partial pressure (pCO_2 and pO_2, respectively). F- Thoracic computerized tomography (CT-scan) of an explanted lung in a pig at the end of the follow-up. No macroscopic foci of perfluorocarbons can be observed, suggesting complete elimination. G- Morphological appearance of the lung upon histological examinations. The left panel shows normal appearance. The right panel show an area with dilation of bronchioles (arrow) and alveolae, as typically observed after mechanical ventilation.

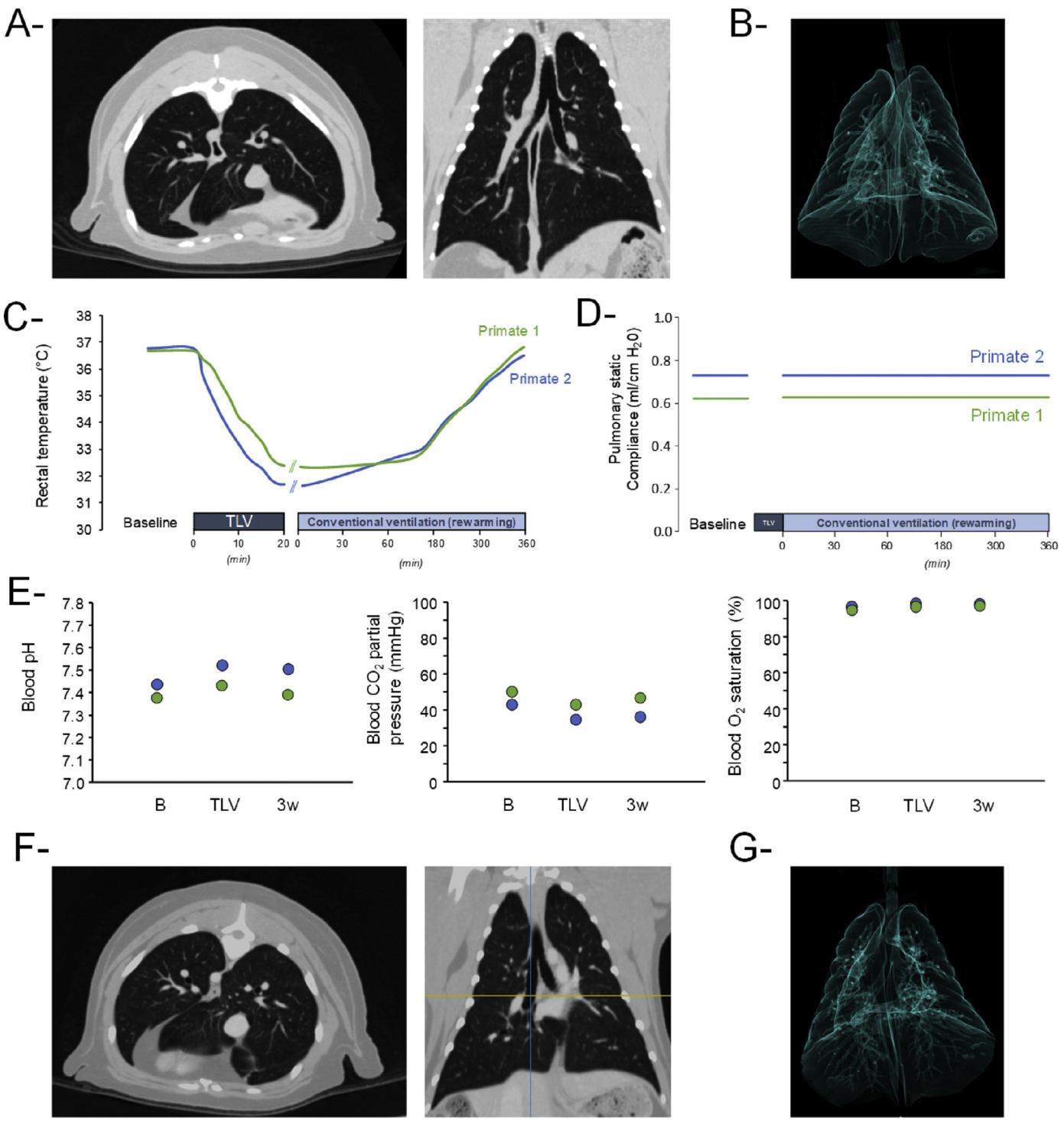

Fig. 7. Evaluation of the long-term tolerance of lung conservative total liquid ventilation (TLV) in two aged non-human primates (*Macaca fascicularis*). A- Transverse images of thoracic computerized tomography (CT-scan) at the initial stage (i.e., two weeks before the TLV episode). B- 3D reconstruction of the lung in the same animal at the initial stage. C- Rectal temperature of the two primates submitted to an episode of hypothermic TLV, followed by rewarming during conventional mechanical ventilation before awakening. D- Pulmonary compliance of the two primates before and after the episode of hypothermic TLV. E- Arterial blood pH, partial pressure of CO_2 and hemoglobin oxygen saturation in the two primates at baseline, during the episode of TLV and 3 weeks later. F- CT-scan four weeks after the TLV episode. G- 3D reconstruction of the lung in the same animal four weeks after the TLV episode.

that such fast cooling with lung-conservative TLV could still be protective in pathophysiological conditions using a piglet model of HIE. This opens promising perspectives for target temperature management in cardiac arrest patients, beyond the other applications of liquid ventilation for lung lavage, drug delivery or lung imaging.

Until now, most reports with TLV were done in animal models of pediatric respiratory diseases with EV and TV averaging 20–30 ml/kg and 15–30 ml/kg, respectively [10,11,23]. For instance, Tredici et al. induced TLV in rabbits using an initial filling with 20 ml/kg of PFC and TV of 17.5–20 ml/kg [23]. The main rationale was that TLV should completely abolish the air-liquid interface and optimize pulmonary recruitment. However, the long-term pulmonary recovery was rarely evaluated [24] after resumption to spontaneous breathing, which obviously often limited the translation of the results. Here, we

show that such approach could actually be deleterious and that incomplete filling with low EV should be preferred, even if the air-liquid interface is not fully abolished in the initial phase. As illustrated by the pressure-volume relationship, an inflexion point occurs around 40 ml/kg of liquid volume and 15 cmH_20 of alveolar pressure, suggesting that beyond this point, hyperinflation and alveolar overdistension might happen [25]. This could also bring possible explanations for the failure of PLV in patients with acute respiratory distress syndrome [2]. Actually, the previously mentioned pivot trial tested the static intra-tracheal administration of 10 or 20 ml/kg of perflubron during conventional gas ventilation at PEEP = 13 cmH_2O and TV = 8−10 ml/kg [2]. This led to high end-inspiratory alveolar pressure averaging 30 cmH_2O, which is far above the alveolar pressures observed in the present study. This could have led to very high lung volume that completely compromised the putative benefits of PLV. Overall, our finding suggests that the best-tolerated conditions of TLV are associated with a lung filling below FRC, which could be responsible for a certain level of derecruited alveoli in upper pulmonary regions. This alveolar reserve could allow subsequent and safe addition of tidal volume of liquid during liquid ventilation. A certain level of heterogeneity in liquid distribution at expiration could therefore be paradoxically more conservative.

An important finding is also that lung-conservative TLV exerts very fast cooling in both piglets and adult pigs. This is the first study to confirm this finding in animals weighing up to 80 kg, further emphasizing the body-weight independent cooling rate of TLV [19,26]. Such cooling was shown to provide potent neurological benefits after cardiac arrest in adult rabbits. However, we here further show that benefits can also be observed in a neonatal model of cardiac arrest after hypoxic-ischemic encephalopathy. This supports the hypothesis of a very narrow therapeutic window of hypothermia after ischemic injury. In humans treated by therapeutic hypothermia, target temperature is usually achieved after at least 3−4 h of cooling while TLV affords whole-body cooling in <30 min [27,28]. Some techniques were shown to provide rapid regional cooling but TLV is able to cool the entire body rapidly, and not a single body compartment such as the brain with helmets or intra-sinusal cooling [29].

Here, we demonstrate that the rapid achievement of mild hypothermia after resuscitation could be also neuroprotective after neonatal cardiac arrest and hypoxic brain injury. It reinforces previous findings showing the superiority of hypothermic TLV in animal models of adult cardiac arrest as compared to normothermic TLV [14,30]. It also opens promising perspectives for the application of liquid ventilation after delivery in newborns combining severe respiratory distress, meconial aspiration and secondary cardiac arrest. The combined benefit of ultra-fast cooling [14] and lung lavage [10] could lead to potent benefits, as compared to the current management with slow hypothermia and bronchoalveolar lavage with saline. Importantly, the effect of hypothermia may also be mitigated by its duration. Here, TLV was only used for cooling induction and hypothermia was maintained for only 3 h, as compared to 72 h in patients. We are indeed suggesting that early institution after ischemia is sufficient to stop further damages, and thus, longer maintenance of hypothermia might not be necessary. Therefore, these important findings reinforces our previous conclusion in animal models of adult cardiac arrest in rabbits [13,14]. For the latter conditions, we also further show that large animals can be cooled as fast as small animals, allowing to expect similar benefits after resuscitation. Together, these findings support the fact that the clinical translation of TLV could have a maximal benefit-to-risk ratio after pediatric and adult cardiac arrest.

Finally, we also overcame a technological challenge in the present study. For the first time, we developed and used an automatized liquid ventilator able to perform TLV in large animals up to 80 kg. To our knowledge, this is also the first demonstration of the pulmonary consequences of TLV in large animals after resumption to spontaneous breathing. Previous results were observed in dogs with cyclic lung lavage with PFC [31] but this was not allowing a liquid ventilation with the corresponding myriad of benefits. Here, we also showed that lung-conservative TLV was extremely well tolerated in old non-human primates, ruling out a peculiar sensitivity of the mature primate lungs to liquid filling or to PFC. The latter hypothesis could also have explained the poor tolerance of liquid ventilation in humans in previous clinical trials with PLV [2]. Our results were obtained in 13-years old monkeys, further showing that the good tolerance persisted in mature or aged lungs. Until now, the delayed effect of liquid ventilation after resumption to spontaneous breathing was indeed very poorly evaluated, especially in adult or aged animals as compared to newborn animals. Our findings then makes again TLV a realistic strategy for further applications in humans. At this step, the technology simply needs to be assessed for regulatory purposes before considering a clinical translation.

In conclusion, this study demonstrated that TLV with an accurate and reliable control of lung volumes of perfluorocarbons below FRC could provide the full potential of TLV in a novel and safe manner, despite incomplete initial degassing. This constitutes a paradigm shift through the “tidal” liquid ventilation of partly filled lungs, which strongly differs from the previously known TLV approach, opening promising perspectives for a safe clinical translation.

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Author contributions

Conception and design of the experiments (MK, EB, LDR, AC, JDR, HW, AB, BG, PM, RT); In vivo experiments (MK, EB, FL, AH, JR, NM, RT); Lung pathology (PB); Imaging (LDR, RMD, GG, VP, PP, EB, FL, MK, RT); Liquid ventilator (EFP, MS, MN, PM); Statistical analyses (MK, RT); Data analyses (All authors); Drafting and revision the manuscript (All authors); Approving the final version (All authors).

Declaration of Competing Interest

R Tissier and A Berdeaux are named as inventor on a patent on cooling with liquid ventilation (US20120226337 A1). P Micheau, M Nadeau and H Walti declares owning patents on liquid ventilation (US Patents # 7,726,311; Preliminary US patent 61/838,896). A Berdeaux, M Kohlhauer, H Walti, M Nadeau, P Micheau and R Tissier are shareholders of a start-up company dedicated to the clinical research on total liquid ventilation (Orixha).

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Appendix A. Supplementary materials

Supplementary material associated with this article can be found in the online version at doi: https://doi.org/10.1016/j.ebiom.2019.08.026.

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