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**Ultra-fast and whole body cooling with total liquid ventilation induces favourable neurological and cardiac outcomes following cardiac arrest in rabbits**

*Short title: Liquid ventilation, hypothermia and cardiac arrest*

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32 **Abstract**

33 **Background:** In animal models of cardiac arrest, the benefit afforded by hypothermia is  
34 closely linked to the rapidity in body temperature decrease after resuscitation. Since total  
35 liquid ventilation (TLV) with temperature controlled perfluorocarbons induces a very rapid  
36 and generalized cooling, we aimed to determine whether this could limit the post-cardiac  
37 arrest syndrome in a rabbit model. We especially focused on neurological, cardiac,  
38 pulmonary, liver and kidney dysfunctions. **Methods and Results:** Anesthetized rabbits were  
39 submitted to either 5 or 10-min of untreated ventricular fibrillation. After cardiopulmonary  
40 resuscitation and resumption of a spontaneous circulation, the animals underwent either  
41 normothermic life support (control) or therapeutic hypothermia induced by TLV. The latter  
42 procedure decreased esophageal and tympanic temperatures to 32-33°C within only 10-min.  
43 After rewarming, the animals submitted to TLV exhibited an attenuated neurological  
44 dysfunction and decreased mortality 7 days later as compared to control. The  
45 neuroprotective effect of TLV was confirmed by a significant reduction in brain histological  
46 damages. We also observed limitation of myocardial necrosis, along with a decrease in  
47 troponin I release and a reduced myocardial caspase 3 activity with TLV. The beneficial  
48 effects of TLV were directly related to the rapidity at inducing hypothermia since neither  
49 conventional cooling (cold saline infusion + external cooling) nor normothermic TLV elicited  
50 a similar protection. **Conclusions:** Ultra-fast cooling instituted by TLV exerts potent  
51 neurological and cardiac protections in an experimental model of cardiac arrest in rabbits.  
52 This could be a relevant approach to afford a global and protective hypothermia against the  
53 post-cardiac arrest syndrome.

54

55 **Key Words:** Cardiopulmonary resuscitation; Fibrillation; Heart arrest; Ischemia; Ventilation.

56 **Introduction**

57 Institution of mild “therapeutic” hypothermia (32-34°C) during 24 to 36 hours after  
58 resuscitation is known to improve survival and neurological recovery in comatose survivors  
59 of cardiac arrest.<sup>1, 2</sup> However, experimental studies in dogs,<sup>3, 4</sup> pigs<sup>5, 6</sup> and rodents<sup>7, 8</sup>  
60 demonstrated that the neuroprotection afforded by hypothermia was related to the rapidity  
61 in body temperature decrease after resuscitation. When achieved rapidly, hypothermia  
62 could also be beneficial for other organs since it can be, for example, also potentially  
63 cardioprotective during myocardial ischemia.<sup>9-12</sup> Accordingly, many strategies were  
64 proposed to afford such a rapid hypothermia, including intravenous infusion of cold fluid,<sup>13</sup>  
65 endovascular<sup>14</sup> or intranasal cooling.<sup>15, 16</sup>

66 Another strategy that can experimentally afford a very rapid and generalized cooling  
67 is liquid ventilation of the lungs with temperature-controlled perfluorocarbons.<sup>11, 17-22</sup> These  
68 liquids can use the lungs as heat exchangers while maintaining normal gas exchanges.<sup>18-20</sup>  
69 In addition, this ventilation procedure also protects the lung integrity.<sup>20</sup> Using a prototype of  
70 total liquid ventilator that alternatively instillates and removes a tidal volume of  
71 perfluorocarbon from the lung, we were able to decrease the left atrial temperature to 32°C  
72 within only 5 min in anesthetized rabbits.<sup>11, 17, 18</sup> This was associated with a very potent  
73 protection against myocardial infarction and subsequent contractile dysfunction in animal  
74 models of coronary artery occlusion.<sup>11, 17, 18</sup> In a swine model of ventricular fibrillation, liquid  
75 ventilation also induced a rapid convective cooling that further improves the chances for  
76 subsequent resumption of spontaneous circulation.<sup>21, 22</sup> However, the effect of hypothermic  
77 total liquid ventilation (TLV) has never been investigated to our knowledge in animal models  
78 of post-cardiac arrest dysfunction when instituted after resumption of spontaneous  
79 circulation.

80 Accordingly, the main purpose of the present study was to investigate the long term  
81 effect of ultrafast cooling induced by TLV in a rabbit model of post-cardiac arrest  
82 dysfunction following ventricular fibrillation and resuscitation. In order to determine whether  
83 hypothermic TLV properly protects through very fast cooling, we investigated two additional

84 groups submitted to a conventional hypothermia (cold saline infusion + external cooling) or  
85 to normothermic TLV. The primary outcome was the survival during 7 days of follow-up.  
86 The secondary outcomes were clinical, biochemical, hemodynamic and histological  
87 parameters describing neurological, cardiac, pulmonary, liver and kidney potential  
88 dysfunctions. We also aimed to investigate whether ultra-fast cooling can protect the heart  
89 through an early inhibition of cardiac cell death. The latter point was also critical to further  
90 support the relevance of very fast cooling to limit the subsequent dysfunction following  
91 cardiac arrest.

92 **Methods**

93 The animal instrumentation and ensuing experiments were conducted in accordance  
94 with French official regulations (agreement A94-046-13) after approval by the local ethical  
95 committee. The investigation conformed to the *Guide for the Care and Use of Laboratory*  
96 *Animals* published by the US National Institutes of Health.

97 **Animal preparation**

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

107 **Cardiac arrest and cardiopulmonary resuscitation**

108 After animal preparation and subsequent stabilisation, ventricular fibrillation was  
109 induced by passing an alternative current (10 V, 4 mA; 2 min) between the implanted  
110 electrodes. Mechanical ventilation was stopped at the onset of fibrillation and throughout  
111 the subsequent period of cardiac arrest. After either 5 or 10 min of untreated fibrillation,  
112 cardiopulmonary resuscitation was started using cardiac massage (~ 200 beats/min),  
113 electric attempts of defibrillation (5-10 J/kg) and intravenous administration of epinephrine  
114 (15 µg/kg i.v.). Resumption of spontaneous circulation (ROSC) was considered as an  
115 organized cardiac rhythm associated with a mean arterial pressure above 40 mmHg during  
116 at least 1 min. After ROSC, administration of epinephrine was further permitted during a  
117 maximum of 7 h at a dosage appropriately adjusted to maintain mean arterial pressure at  
118 ~80 mmHg. Mechanical ventilation was continued until weaning and awakening of the  
119 animals. Rabbits subsequently returned to their cage for a survival follow-up. They received

120 antibiotics (enrofloxacin, 5 mg/kg i.m.) during 7 days and analgesics (buprenorphine, 30  
121 µg/kg s.c.) during 3 days.

### 122 Experimental protocol

123 As shown in Figure 1, rabbits randomly underwent either 5 or 10 min of cardiac arrest  
124 with subsequent cardiopulmonary resuscitation. For each duration of cardiac arrest, rabbits  
125 were randomly allocated to resuscitation under normothermic conditions (Control<sub>5</sub> and  
126 Control<sub>10</sub> groups, respectively) or with hypothermia induced by TLV (H-TLV<sub>5</sub> and H-TLV<sub>10</sub>  
127 groups, respectively). In these last two groups, TLV was started at the 10<sup>th</sup> min following  
128 cardiopulmonary resuscitation (*i.e.*, after ROSC) by filling the lung with 10 ml/kg of  
129 perfluorocarbon (Fluorinert, 3M, Cergy, France) and then connecting the endotracheal tube  
130 to our prototype of liquid ventilator (Supplemental Figure 1).<sup>11, 17, 18</sup> This ventilator was set  
131 to a tidal volume of ~7-10 ml/kg of body weight with a respiratory rate of 6 breaths/min. For  
132 each breath, the ventilator pumped into and out of the lungs the tidal volume of liquids. The  
133 perfluorocarbon mixture was bubbled with 100% O<sub>2</sub>. The temperature of the heat  
134 exchanger was adjusted to maintain esophageal and tympanic temperatures at a target  
135 temperature of ~32°C. After 20 min of TLV and achievement of the hypothermic target  
136 temperature, the perfluorocarbon was evacuated from the lungs by gravity and the  
137 endotracheal tube was again connected to a conventional mechanical ventilator.  
138 Hypothermia was further maintained at 32°C during 3 h, if necessary using cold blankets.  
139 Animals were subsequently rewarmed using infra-red lights and thermal pads until weaning  
140 from conventional ventilation and awakening. Animals were housed in a closed cage  
141 enriched in O<sub>2</sub> during 2-3 days to avoid hypoxic episodes. In order to determine whether  
142 hypothermic TLV properly protects through very fast cooling, we investigated two randomly  
143 allocated additional groups submitted to 10 min of cardiac arrest. The first of these groups  
144 (Saline<sub>10</sub>) was submitted to 3 h of conventional hypothermia through the combination of  
145 cold saline administration (30 ml/kg i.v., NaCl 0.9% at 4°C) and external cooling. The  
146 second additional group was submitted to an episode of TLV with normothermic  
147 perfluorocarbons (N-TLV<sub>10</sub> group) to determine their proper effects.

148 In order to further investigate the effects of hypothermic TLV, additional rabbits were  
149 included in the Control<sub>10'</sub> and H-TLV<sub>10'</sub> groups, respectively. These animals were  
150 euthanized one hour after the cardiac arrest episode for collection of myocardial and blood  
151 samples for caspase activity assays and measurement of circulating troponin I,  
152 respectively.

#### 153 Neurological and cardiac dysfunction assessment

154 Neurological dysfunction was evaluated daily in surviving animals using a clinical  
155 score previously validated in rabbits,<sup>23</sup> as shown in Supplemental Table 1 (0-10% =  
156 normal; 100% = brain death). After 7 days of follow-up, surviving rabbits were  
157 reanesthetized and a pressure catheter (SciSense, London, Ontario, Canada) was  
158 introduced into the left ventricle through the right carotid artery for measurement of end-  
159 diastolic pressures as well as positive and negative left ventricular rate of pressure  
160 development (dP/dt<sub>max</sub> and dP/dt<sub>min</sub>). These parameters were also measured in a group of  
161 Sham rabbits that were neither submitted to cardiac arrest nor hypothermia.

#### 162 Blood chemistry and caspase activity assay

163 Blood pH, carbon dioxide and oxygen partial pressures (pCO<sub>2</sub> and pO<sub>2</sub>, respectively)  
164 were assessed from arterial blood samples with an ABL 77 series analyser (Radio-meter  
165 SA, France). Blood lactate was determined on an Accutrend<sup>®</sup> Plus analyser (Roche  
166 Diagnostics, Mannheim, Germany). Liver and renal functions were evaluated by measuring  
167 the alanine aminotransferase (ALAT) and creatinine concentrations (Prestige 24i, Tokyo-  
168 Boehi, Japan). Troponin I and Creatinine Kinase were measured by an off-site laboratory  
169 (IDEXX Laboratories, Alfortville, France).

170 Caspase 3 activity was assayed from cardiac samples, as previously described.<sup>24</sup>  
171 Briefly, tissues were homogenized in cold buffer (25 mM HEPES pH 7.5, 5 mM MgCl<sub>2</sub>,  
172 2 mM EDTA, 0.1% Triton X-100, 2 mM dithiothreitol, 1 mM PMSF, 5 µl/ml protease cocktail  
173 inhibitor P8340; Sigma-Aldrich, St Louis, MO, USA). Homogenates were centrifuged and  
174 supernatants collected. Proteins (90 µg) were incubated in caspase assay buffer (50 mM  
175 HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, Triton X-100 0.1%,



176 glycerol 10%). Enzymatic reaction was started by addition of 0.2 mM of the fluorogenic  
177 substrates ac-DEVD-AFC (Biomol Research Laboratories, Hambourg, Germany).  
178 Fluorescent arbitrary units were converted into pmol/mg protein/h using a standard curve of  
179 free AFC (Biomol Research Laboratories, Hambourg, Germany).

### 180 Histological analyses

181 After 7 days of follow-up after cardiac arrest, the surviving rabbits were finally  
182 euthanized for pathological analyses of the heart, lung, kidney, liver and brain. These  
183 organs were also removed and analyzed in the animals died before the end of the follow-  
184 up. For lungs, a slice was sampled from each lobe (5 per lung). For the heart, we analyzed  
185 a mid heart transversal biventricular section. For kidneys, two slices were studied for each  
186 organ. We used a 0-3 score system to blindly quantify the severity of each organ alteration,  
187 as shown in Supplemental Tables 2 and 3 (0=normal; 3=very severe lesion). The overall  
188 brain score was the mean value obtained for cortex, hippocampus and cerebellum, as  
189 previously described.<sup>23</sup> For lungs, we assessed two separate scores for cardiogenic lesions  
190 (serous edema and/or congestion) and infectious complication of bronchopneumonia,  
191 respectively. The first panels of Figures 2 and 3 illustrate typical normal and pathological  
192 aspects of the different organs.

### 193 Statistical analyses

194 Data were expressed as mean $\pm$ SEM. Hemodynamic and biochemical parameters  
195 were compared between the different groups and corresponding controls using a two-way  
196 ANOVA for repeated measures. Post-hoc analyses were performed at each time-point as  
197 compared to controls using a Student t-test with Bonferroni correction. Values were not  
198 compared between the different time-points in order to avoid multiple comparisons. In each  
199 experimental group, neurological dysfunction and histological scores were compared to the  
200 corresponding control group using a Mann-Whitney non parametric test. Survival curves  
201 were obtained using a Kaplan-Meier analysis and were compared to the corresponding  
202 control group using a log-rank test. These last analyses took only into account the animals  
203 that achieved ROSC. Significant differences were determined at  $P\leq 0.05$ .

204 **Results**

205            Seventy rabbits were included in the present study and submitted to cardiac arrest  
206 (n=10, 10, 15, 15, 10 and 10 in the Control<sub>5'</sub>, H-TLV<sub>5'</sub>, Control<sub>10'</sub>, H-TLV<sub>10'</sub>, Saline<sub>10'</sub> and N-  
207 TLV<sub>10'</sub> groups, respectively).

208            As shown in Table 1, all rabbits subjected to 5 min of cardiac arrest were successfully  
209 resuscitated (Control<sub>5'</sub> and H-TLV<sub>5'</sub> groups) whereas only 10/15 were successfully  
210 resuscitated in the Control<sub>10'</sub> and H-TLV<sub>10'</sub> groups, respectively. This rate was 7/10 in  
211 Saline<sub>10'</sub> and N-TLV<sub>10'</sub> groups. Regardless the duration of the cardiac arrest, the time to  
212 resumption of spontaneous circulation was not significantly different among groups for each  
213 duration of cardiac arrest.

214            As illustrated in Figure 4, esophageal, tympanic and rectal temperatures were not  
215 significantly different among groups at baseline. A mild and passive decrease in body  
216 temperature was observed in the Control<sub>5'</sub> and Control<sub>10'</sub> groups after cardiac arrest but this  
217 remained within the normothermic range. In H-TLV groups, esophageal and tympanic  
218 temperatures decreased very rapidly after the institution of TLV. As example, tympanic  
219 temperature achieved  $33.3\pm 0.5$  and  $32.5\pm 0.3$  °C in H-TLV<sub>5'</sub> and H-TLV<sub>10'</sub> within 10 min after  
220 the onset of the cooling protocol, respectively. In the Saline<sub>10'</sub> group, such tympanic  
221 temperatures were achieved after ~30 min. Regarding esophageal and rectal temperatures,  
222 the time to achieve 32 to 33°C was less than 5 and 20 min in H-TLV<sub>10'</sub> whereas this was  
223 more than 45 and 60 min in Saline<sub>10'</sub>, respectively. In the N-TLV<sub>10'</sub> group, body  
224 temperatures did not significantly differ from the Control<sub>10'</sub> values throughout the  
225 experimental protocol.

226            As shown in Table 2, heart rate significantly decreased during the hypothermic phase  
227 in hypothermic groups as compared to corresponding controls (e.g., -21%, -28% and -31%  
228 at 60 min after cardiac arrest in H-TLV<sub>5'</sub>, H-TLV<sub>10'</sub> and Saline<sub>10'</sub> vs corresponding controls,  
229 respectively). Mean arterial pressure was not significantly different between groups  
230 throughout the experimental protocol since epinephrine administration was permitted to  
231 maintain a ~80 mmHg value during 7 h after cardiac arrest. As shown in Table 1, the total

232 amount of epinephrine administered throughout cardiopulmonary resuscitation was  
233 however significantly lower in H-TLV<sub>10'</sub> vs Control<sub>10'</sub> (128±128 vs 684±118 µg/kg,  
234 respectively), suggesting a favorable hemodynamic effect of hypothermic TLV. We did not  
235 observe such a significant difference in H-TLV<sub>5'</sub> vs Control<sub>5'</sub> but epinephrine dosages were  
236 much lower (174±81 vs 207±58 µg/kg, respectively). In Saline<sub>10'</sub> and N-TLV<sub>10'</sub> groups,  
237 epinephrine dosages were also not different from Control<sub>10'</sub>. After discontinuation of any  
238 pharmacological support (e.g., at 8 h after cardiac arrest), the lactate levels were  
239 significantly lower in H-TLV<sub>5'</sub> vs Control<sub>5'</sub> (1.2±0.2 vs 4.8±1.7 mmol/l) and in H-TLV<sub>10'</sub> vs  
240 Control<sub>10'</sub> (3.6±0.7 vs 7.0±1.7 mmol/l). Those levels were not significantly different among  
241 Saline<sub>10'</sub> and N-TLV<sub>10'</sub> groups as compared to Control<sub>10'</sub> (5.9±0.7 and 7.6±0.6 vs 7.0±1.7  
242 mmol/l).

243 As shown in Figure 5, we observed a severe acidosis with an increase in pCO<sub>2</sub> and a  
244 decrease in pO<sub>2</sub> in all groups following cardiac arrest. In H-TLV<sub>5'</sub>, pO<sub>2</sub> was lower 15 min  
245 after cardiac arrest as compared to Control<sub>5'</sub>. This could be expected as control animals  
246 were ventilated with oxygen whereas TLV rabbits underwent liquid ventilation by that time.  
247 At 180 min, gas exchanges were conversely improved in H-TLV groups as compared to  
248 controls. As example, blood pH and pO<sub>2</sub> increased whereas pCO<sub>2</sub> decreased in H-TLV<sub>10'</sub> vs  
249 Control<sub>10'</sub>, respectively. Importantly, all the animals were submitted to conventional  
250 ventilation at that time point, with standardized ventilation parameters. As illustrated in  
251 Figure 2, the safety of TLV for lungs was also documented by histology demonstrating  
252 cardiogenic lesions (serous edema and/or congestion) or infectious complications of  
253 bronchopneumonia to a similar extent in TLV groups vs corresponding controls.

254 As shown in Table 2, renal function was not affected after cardiac arrest in all groups  
255 since plasma creatinine levels remained within usual values. Conversely, we observed an  
256 increase in the liver enzyme ALAT with no difference among TLV and corresponding  
257 controls. Kidney and liver lesions were mild with no difference among groups (Figure 2,  
258 panel B).

259 As illustrated in Figure 6, neurological dysfunction was significantly attenuated in H-  
260 TLV groups as compared to controls. This difference was significant as early as the 2<sup>nd</sup> day  
261 following cardiac arrest in H-TLV<sub>5'</sub> vs Control<sub>5'</sub> (panel A) whereas this was observed within  
262 24h of follow-up in H-TLV<sub>10'</sub> vs Control<sub>10'</sub> (panel B). In Saline<sub>10'</sub>, a transient improvement in  
263 neurological recovery was observed at day 1 but this was no longer significant at day 2  
264 after cardiac arrest. As illustrated in Figure 3 (panel B), the neuroprotective effect of  
265 hypothermic TLV was further demonstrated by a significant decrease in the severity of the  
266 ischemic disorders in the brain in H-TLV<sub>5'</sub> and H-TLV<sub>10'</sub> as compared to Control<sub>5'</sub> and  
267 Control<sub>10'</sub> groups, respectively. No any protection was conversely seen in Saline<sub>10'</sub> and N-  
268 TLV<sub>10'</sub> as compared to Control<sub>10'</sub>.

269 A significant difference in survivals was also evidenced between H-TLV groups and  
270 corresponding controls, as illustrated in Figure 6 (panels C and D). At the end of the follow-  
271 up, 9/10 and 7/10 rabbits survived in the H-TLV<sub>5'</sub> and H-TLV<sub>10'</sub> groups as compared to 5/10  
272 and 0/10 in Control<sub>5'</sub> and Control<sub>10'</sub>, respectively. Conversely, survival was not significantly  
273 improved in Saline<sub>10'</sub> and N-TLV<sub>10'</sub> as compared to Control<sub>10'</sub>.

274 As illustrated in Figure 3 (panel B), myocardial foci of necrosis were less frequent in  
275 H-TLV<sub>10'</sub> vs Control<sub>10'</sub>, demonstrating a cardioprotective effect of hypothermic TLV. No  
276 difference was conversely seen between Saline<sub>10'</sub> and N-TLV<sub>10'</sub> as compared to Control<sub>10'</sub>.  
277 In surviving animals, the functional myocardial sequels of cardiac arrest were also  
278 evaluated after 7 days of follow-up. As shown in Supplemental Table 4, mean blood  
279 pressure and heart rate in the conscious state were not different among groups as  
280 compared to a Sham group. After anesthesia, end diastolic left ventricular pressure,  
281 dP/dt<sub>max</sub> and dP/dt<sub>min</sub> were also not different between groups, suggesting that there was  
282 no major functional myocardial alterations in those surviving animals.

283 To further explore the cardioprotective effect of hypothermic TLV, eight additional  
284 rabbits were included in the Control<sub>10'</sub> and H-TLV<sub>10'</sub> groups for a surrogate study dedicated  
285 to the caspase activity assays and measurement of troponin I levels. As shown in  
286 supplemental Figure 2, troponin I measured 60 min after cardiac arrest significantly

287 decreased in H-TLV<sub>10'</sub> as compared to Control<sub>10'</sub> (1.3±0.3 vs 70.7±30.4 ng/ml, respectively).  
288 The cardioprotective effect of hypothermic TLV was also supported by a decrease in  
289 caspase 3 activity as compared to control (6.2±1.2 vs 10.0±1.2 pmol/mg prot/h,  
290 respectively).

291 **Discussion**

292 The present study provides the proof of concept that ultra-fast whole body cooling  
293 with hypothermic TLV limits the post-cardiac arrest syndrome when instituted after ROSC in  
294 a rabbit model of ventricular fibrillation. Interestingly, we observed potent neuro- and  
295 cardioprotections with hypothermic TLV which remains a safe procedure for the lungs. As  
296 we used only 3 h of hypothermia, this also suggested that very early hypothermia after  
297 ROSC does not need to be prolonged to produce a strong clinical benefit. Importantly, this  
298 benefit was directly related to cooling rapidity with TLV since a conventional cooling with  
299 cold saline and external blankets was not significantly protective in similar conditions.  
300 Proper effects of the perfluorocarbon are unlikely as related to the lack of protection with  
301 normothermic TLV.

302 Our first finding is the rapidity of TLV-induced cooling since esophageal and brain  
303 temperatures achieved ~32-33°C within only 10 min. In comparison, a conventional  
304 hypothermic protocol (cold saline infusion + external cooling) requires ~30 and 45 min to  
305 similarly reduce these temperatures. The rapid cooling elicited by TLV was directly related  
306 to the tidal exchange of the liquid since simple repetitive pulmonary lavages with a 4°C  
307 perfluorocarbon requires more than 60 min to decrease the tympanic temperature to 32°C  
308 in the same species.<sup>20</sup> In large animals, hypothermic TLV was also reported to afford a very  
309 fast cooling and to reduce the pulmonary artery temperature to 32°C within 9-10 min when  
310 instituted intra-arrest in a ventricular fibrillation model in swine.<sup>22</sup>

311 Importantly, the rapid hypothermia elicited by TLV was associated with a potent  
312 neurological protection and an increase in survival rate as compared to control conditions.  
313 In animal studies, it is admitted that the neuroprotective effect of hypothermia is time  
314 dependent and that a large part of the protection is lost when cooling is delayed.<sup>25</sup> For  
315 example, in a canine model of cardiac arrest, the neurological protection was lost after only  
316 15 min of delay before the onset of hypothermia after ROSC.<sup>25</sup> In the present study, we  
317 observed a very potent benefit of hypothermia when achieved rapidly after ROSC with TLV  
318 whereas a conventional hypothermia was not significantly protective. Recent experiments

319 have also shown that hypothermia started before ROSC (e.g., intra-arrest hypothermia) can  
320 afford an additional benefit<sup>7,8</sup> but this might be difficult to translate this concept into human  
321 clinical practice. All these findings demonstrate that most of the possible benefits of  
322 hypothermia can be lost within minutes after ROSC, further supporting the need of devices  
323 eliciting ultra-fast cooling such as TLV in the present study.

324           Importantly, the benefit of hypothermic TLV observed in our conditions was  
325 produced by a short hypothermic episode (3 h), whereas the current recommendations in  
326 humans are maintenance of hypothermia during 24-36 h.<sup>1,2</sup> We choose this short duration  
327 since previous experiments have shown that when achieved very early, the duration of the  
328 hypothermia does not need to be prolonged to afford an effective neuroprotection, e.g., in a  
329 gerbil model of global ischemia.<sup>26</sup> Mice studies also noted that 1 h of cooling after ROSC  
330 was sufficient to generate a significant clinical benefit.<sup>7,8</sup> When the severity of the ischemic  
331 insult increases or when the onset of cooling is delayed, it is conversely well established  
332 that prolonging hypothermia is critical for achieving a maximal neurological protection.<sup>27,28</sup>  
333 As example, a prolonged cooling allowed to provide enduring behavioral and histological  
334 protection in rats submitted to permanent middle cerebral artery occlusion, even when  
335 delayed after onset of ischemia.<sup>27</sup>

336           Another important beneficial effect of hypothermic TLV is the cardioprotection  
337 observed here like that previously shown in animal models of coronary artery occlusion.<sup>11,</sup>  
338 <sup>17,18</sup> This was especially observed after 10 min of cardiac arrest since myocardial lesions  
339 were minor in the groups submitted to only 5 min of cardiac arrest. This was evidenced by a  
340 limitation in myocardial necrosis and a preservation of myocardial functional performance in  
341 surviving rabbits. Cardioprotection was also observed very early after cardiac arrest since  
342 troponin I release and caspase 3 activity were significantly decreased within 60 min after  
343 resuscitation in H-TLV<sub>10'</sub> vs Control<sub>10'</sub>. In animal models of focal myocardial ischemia, the  
344 window of protection with hypothermia is virtually limited to the ischemic phase, whereas  
345 cooling at reperfusion is ineffective at reducing infarct-size in most experimental studies.<sup>12</sup>  
346 In the present study, hypothermia was instituted after global reperfusion (ROSC) but it is

347 reasonable to speculate that the myocardium remains momentarily and partially ischemic  
348 even after ROSC. This can explain that a very rapid cooling with TLV can still afford a  
349 beneficial effect even if instituted after ROSC and systemic reperfusion. Improved post-  
350 resuscitation myocardial function have interestingly also be observed with intra-arrest rapid  
351 head cooling.<sup>29</sup> Generalized hypothermia could even potentially afford a protection of the  
352 liver and/or the kidney.<sup>30</sup> As these organs were mildly altered in control conditions in the  
353 present study, we were not able to show any difference with hypothermic TLV.

354 Importantly, TLV was a safe procedure for the lungs. Even, we observed improved  
355 gas exchanges using standardized ventilatory parameters in TLV vs control groups at 3 h  
356 after cardiac arrest. After weaning from ventilation, animals were however maintained in a  
357 cage enriched in oxygen to avoid hypoxic episodes.<sup>11</sup> In pigs, intra-arrest liquid ventilation  
358 was indeed demonstrated to alter lung function since activation of pulmonary macrophages  
359 might alter gas exchanges after resumption to conventional ventilation.<sup>21, 22</sup> In our study, the  
360 tolerance of TLV was shown by histological examinations and this is supported by several  
361 reports from the literature demonstrating that liquid ventilation can even protect the lungs.<sup>19,</sup>  
362 <sup>20</sup> Several prototypes of liquid ventilator have been developed and the clinical translation of  
363 this concept might accordingly be feasible when those devices will be available for a clinical  
364 use.<sup>31</sup> To date, the current prototypes are mostly developed for a paediatric use<sup>31</sup> and the  
365 translation of TLV-induced hypothermia would be accordingly first possible in newborns  
366 presenting global ischemia. Further developments might also ultimately permit a translation  
367 in adult patients.

368 Our study has several limitations. First, neurological dysfunctions were assessed on  
369 the basis of clinical and histological parameters. Other more functional tests or imaging  
370 would also be important. Second, histological analyses were performed in all animals,  
371 irrespectively of their survival time. This would have lead to an underestimation of the  
372 histological scores in some animals who died very early after the cardiac arrest. However,  
373 since the lower scores were observed in the group that lived for the longer time (H-TLV<sub>10</sub>),  
374 the latter limitation should not actually impact our conclusions.



375           In conclusion, ultra-fast cooling instituted by hypothermic TLV limits the post-cardiac  
376 arrest dysfunction with associated neuro- and cardioprotective effects. Importantly, TLV  
377 was a safe procedure for the lungs in our experimental conditions. The beneficial effects of  
378 hypothermic TLV were probably directly related to the rapidity in temperature decrease  
379 since myocardial cell death inhibition was evidenced even very early following resuscitation.

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396 **Disclosure**

397 None.

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496 **Legends of Figures**

497

498 **Figure 1:** Experimental protocol

499 *CA, cardiac arrest; TLV, total liquid ventilation initiated; H-TLV, hypothermic TLV; N-TLV,*  
500 *normothermic TLV; Saline, hypothermia induced by intravenous administration of cold*  
501 *saline combined to external cooling; ROSC, resumption of spontaneous circulation.*

502

503 **Figure 2:**

504 ***Panel A:*** Examples of normal or pathological histological appearances of the kidney, liver  
505 and lungs in the TLV and control groups, respectively. In kidney, lesions consisted in dilated  
506 regenerative proximal tubules (arrows, bar=120  $\mu\text{m}$ ). In liver, we observed systematized  
507 clarification of hepatocytes (arrows, bar=120  $\mu\text{m}$ ). In lungs, lesions were congestion and  
508 serous edema (arrows in the left lung panel, bar=120  $\mu\text{m}$ ) or foci of bronchopneumonia  
509 (arrows in the right lung panel, bar=120  $\mu\text{m}$ ).

510 ***Panel B:*** Histological scores of alteration in kidney, liver and lungs of rabbits from the  
511 different groups. For lungs, we assessed two separate scores for cardiogenic lesions and  
512 infection complications, respectively. Open circles represents individual scores and the  
513 thick line represents the median value of corresponding group.

514 *\*  $p < 0.05$  vs corresponding control; TLV, total liquid ventilation; H-TLV, hypothermic TLV;*  
515 *N-TLV, normothermic TLV; Saline, hypothermia induced by intravenous administration of*  
516 *cold saline combined to external cooling.*

517

518 **Figure 3:**

519 ***Panel A:*** Examples of normal or pathological histological appearances of the brain and the  
520 heart in the TLV and control groups, respectively. In brain, ischemic disorders consisted in  
521 ischemic pyramidal cells with pycnotic nucleus in the hippocampus (arrows, bar=30  $\mu\text{m}$ ), in  
522 laminar necrosis of Purkinje cells in the cerebellum (arrows, bar=30  $\mu\text{m}$ ) or in numerous

523 ischemic neurons in the cortex (arrows, bar=30  $\mu$ m), respectively. In the myocardium, we  
524 observed foci of cardiomyocytes necrosis (arrows, bar=120  $\mu$ m).

525 **Panel B:** Histological scores of alteration in the brain and heart of rabbits from the different  
526 groups. Open circles represents individual scores and the thick line represents the median  
527 value of the corresponding group.

528 \*  $p < 0.05$  vs corresponding control; TLV, total liquid ventilation; H-TLV, hypothermic TLV;  
529 N-TLV, normothermic TLV; Saline, hypothermia induced by intravenous administration of  
530 cold saline combined to external cooling.

531

532 **Figure 4:** Esophageal, tympanic and rectal temperatures in the different experimental  
533 groups.

534 \*  $p < 0.05$  vs corresponding control;  $n = 10$  in each experimental group; TLV, total liquid  
535 ventilation; H-TLV, hypothermic TLV; N-TLV, normothermic TLV; Saline, hypothermia  
536 induced by intravenous administration of cold saline combined to external cooling.

537

538 **Figure 5:** Blood pH, pCO<sub>2</sub> and pO<sub>2</sub> in the different experimental groups.

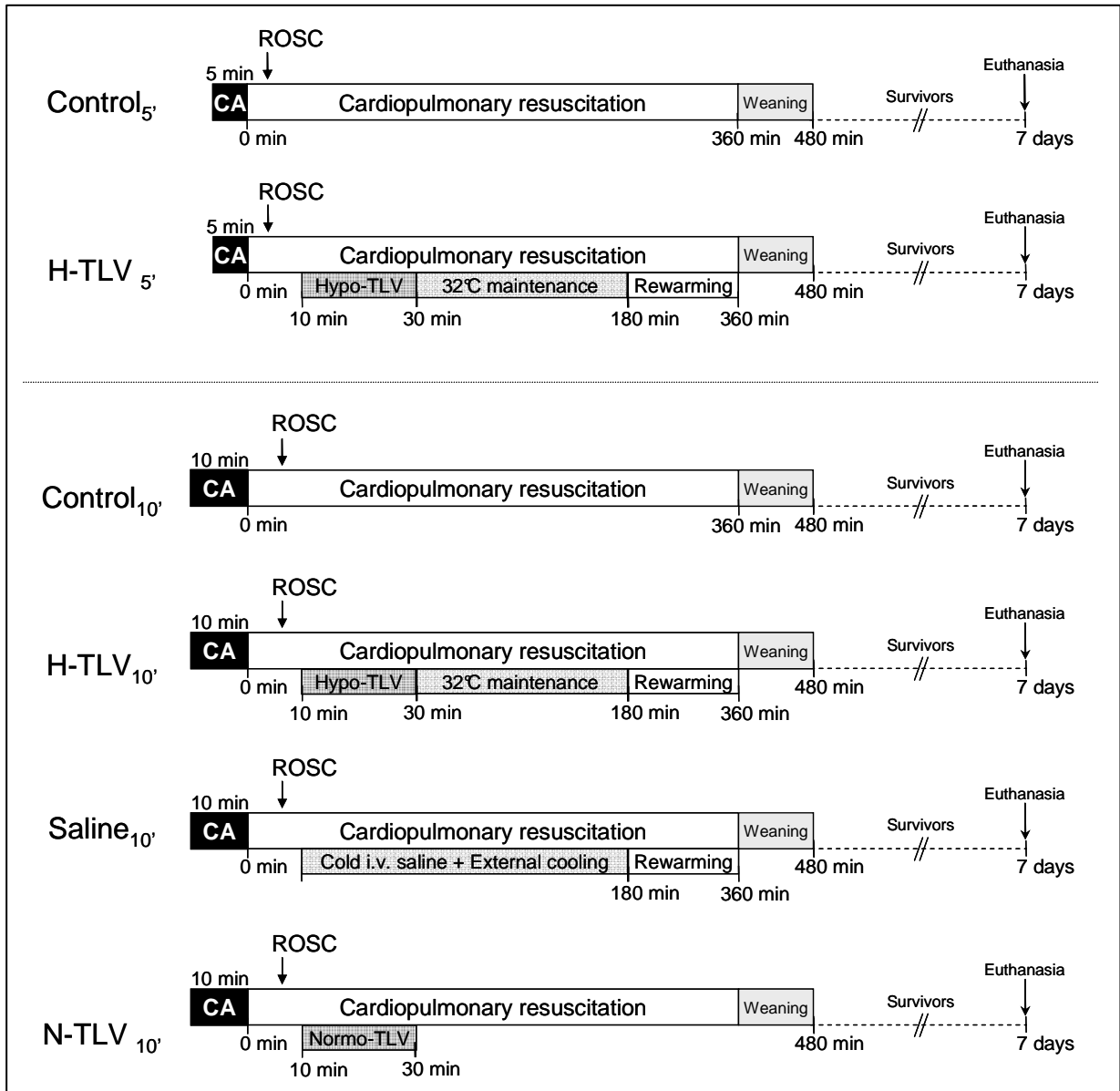
539 \*  $p < 0.05$  vs corresponding control;  $n = 10$  in each experimental group; TLV, total liquid  
540 ventilation; H-TLV, hypothermic TLV; N-TLV, normothermic TLV; Saline, hypothermia  
541 induced by intravenous administration of cold saline combined to external cooling.

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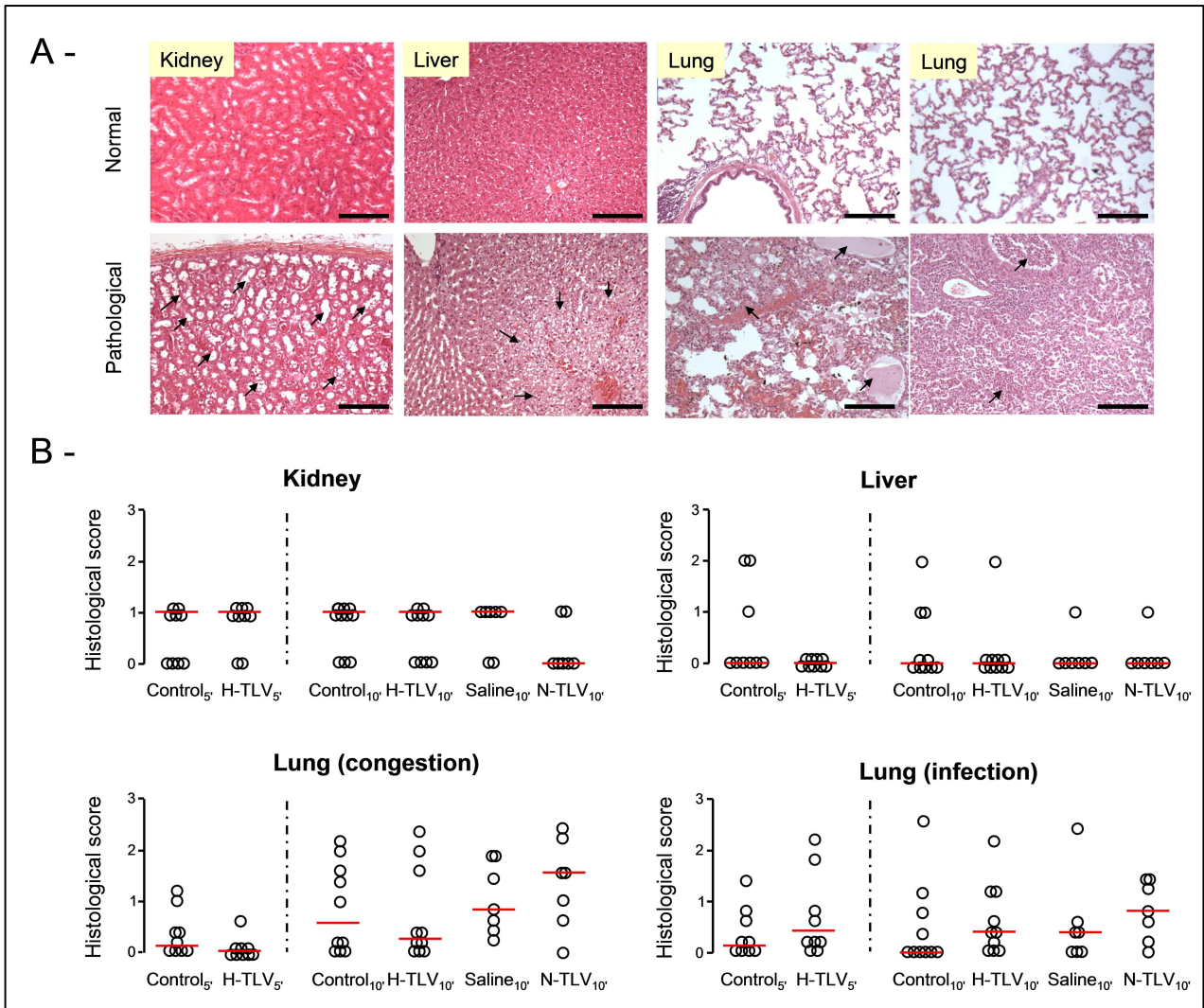
543 **Figure 6:**

544 **Panels A and B:** Neurological dysfunction scores at days 1, 2 and 7 following resuscitation  
545 in the different experimental groups submitted to 5 min or 10 min of cardiac arrest,  
546 respectively. Open circles represent individual scores and the thick line represents the  
547 median value of the corresponding group. Only animals achieving resumption of  
548 spontaneous circulation were included.

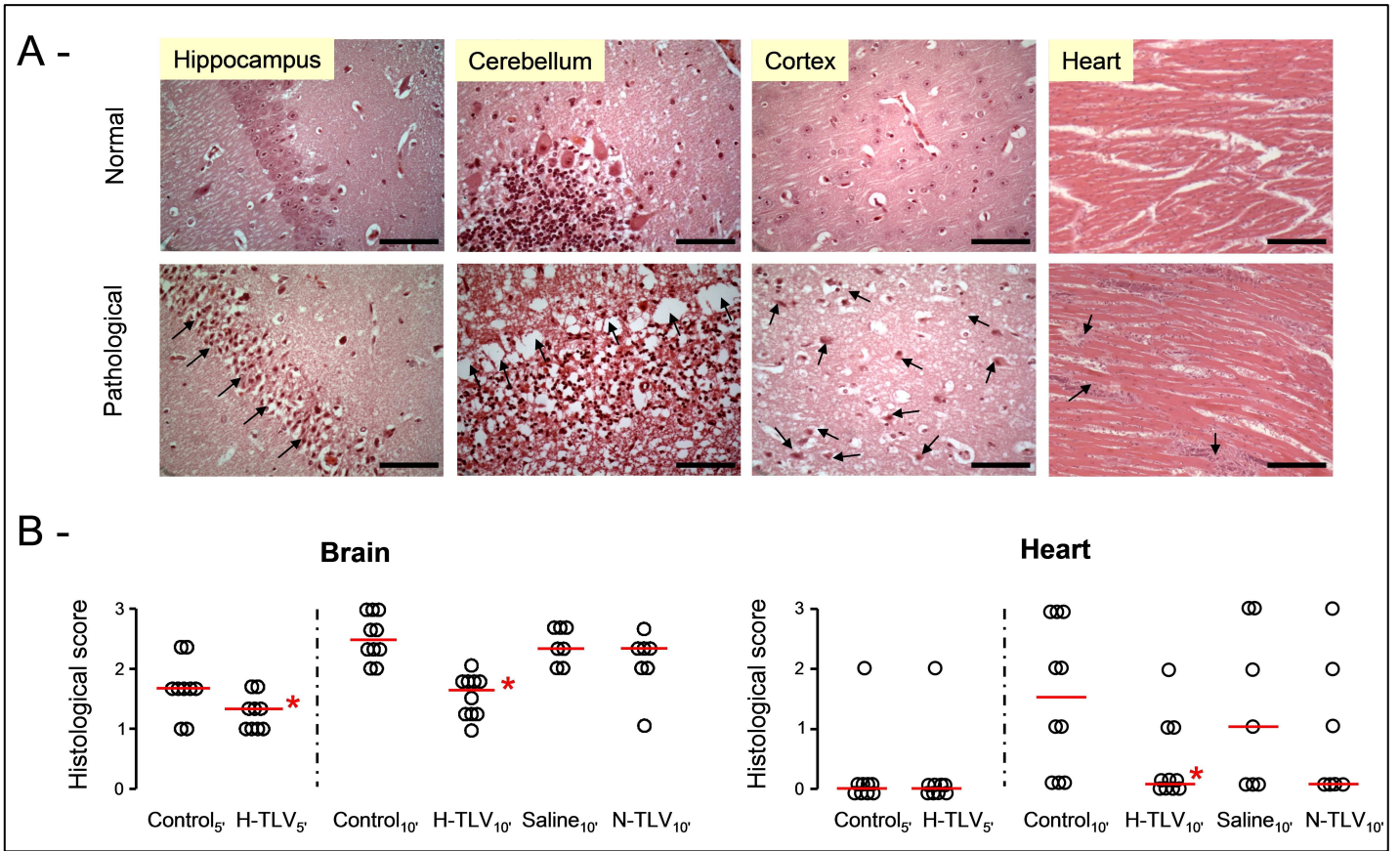
549 **Panels C and D:** Kaplan-Meyer survival curves in the different experimental groups  
550 submitted to 5 min or 10 min of cardiac arrest, respectively. Only animals achieving  
551 resumption of spontaneous circulation were included.  
552 \*  $p < 0.05$  vs corresponding control; TLV, total liquid ventilation; H-TLV, hypothermic TLV; N-  
553 TLV, normothermic TLV; Saline, hypothermia induced by intravenous administration of cold  
554 saline combined to external cooling.  
555

**Figure 1**

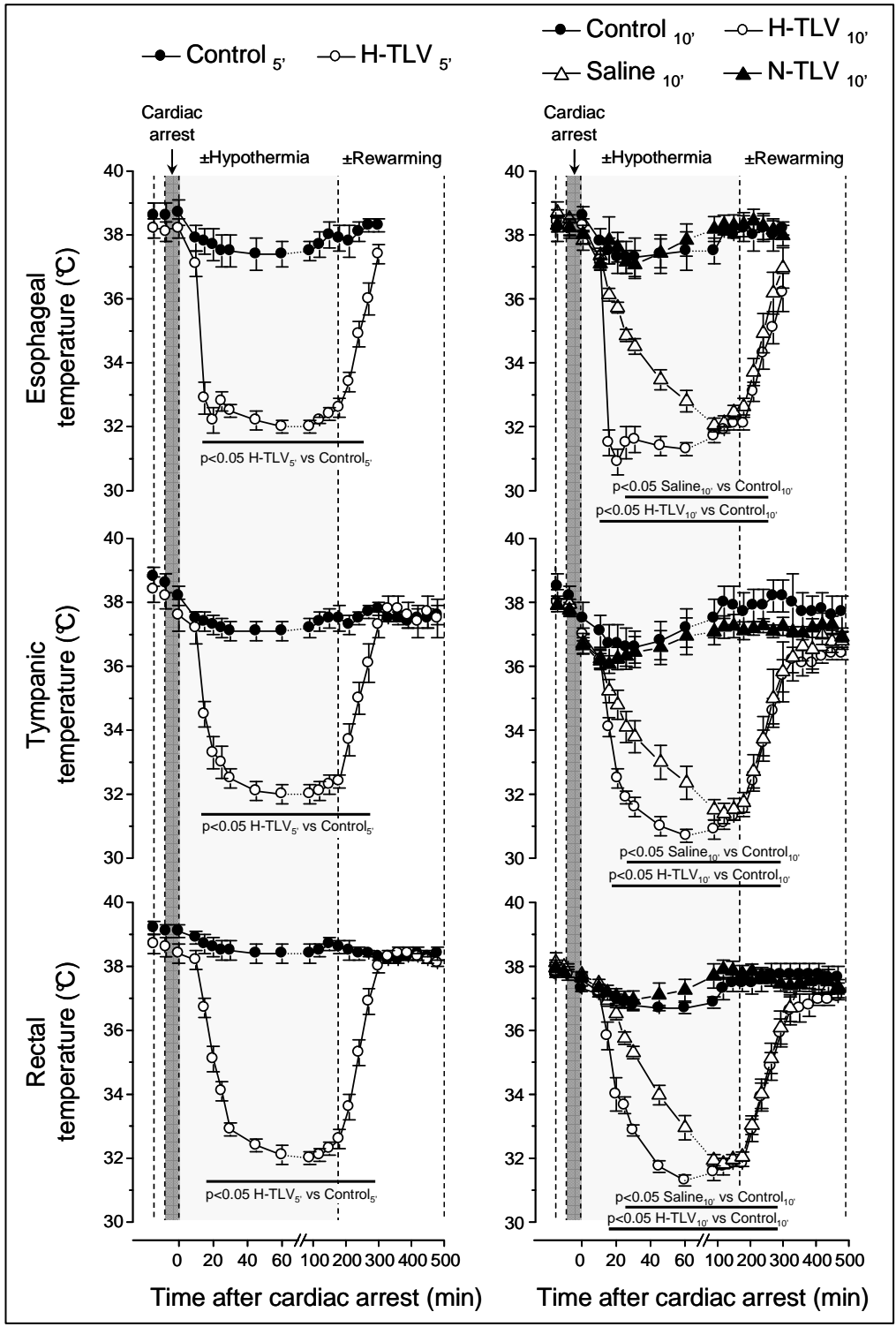
**Figure 2**



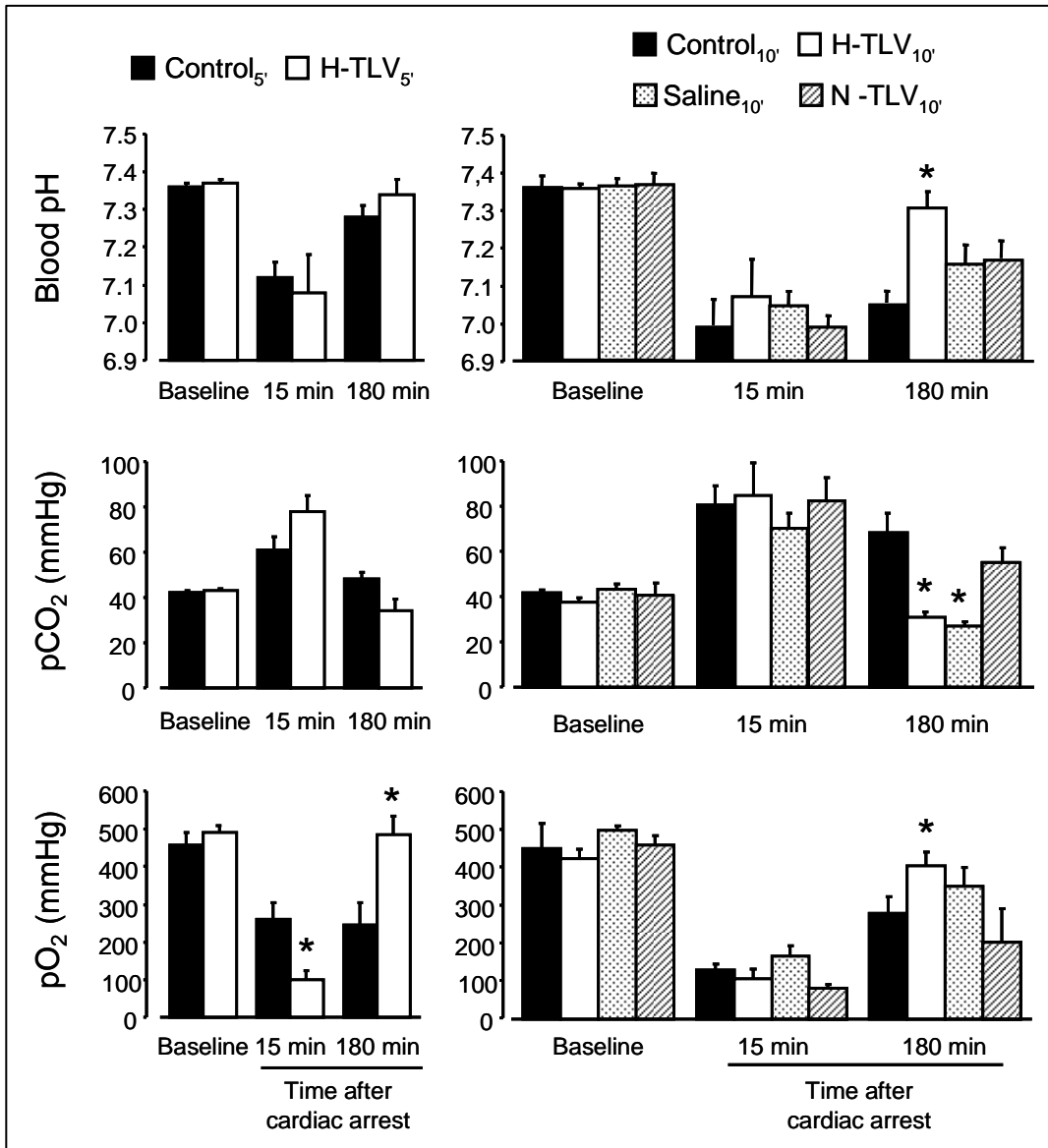
**Figure 3**



**Figure 4**

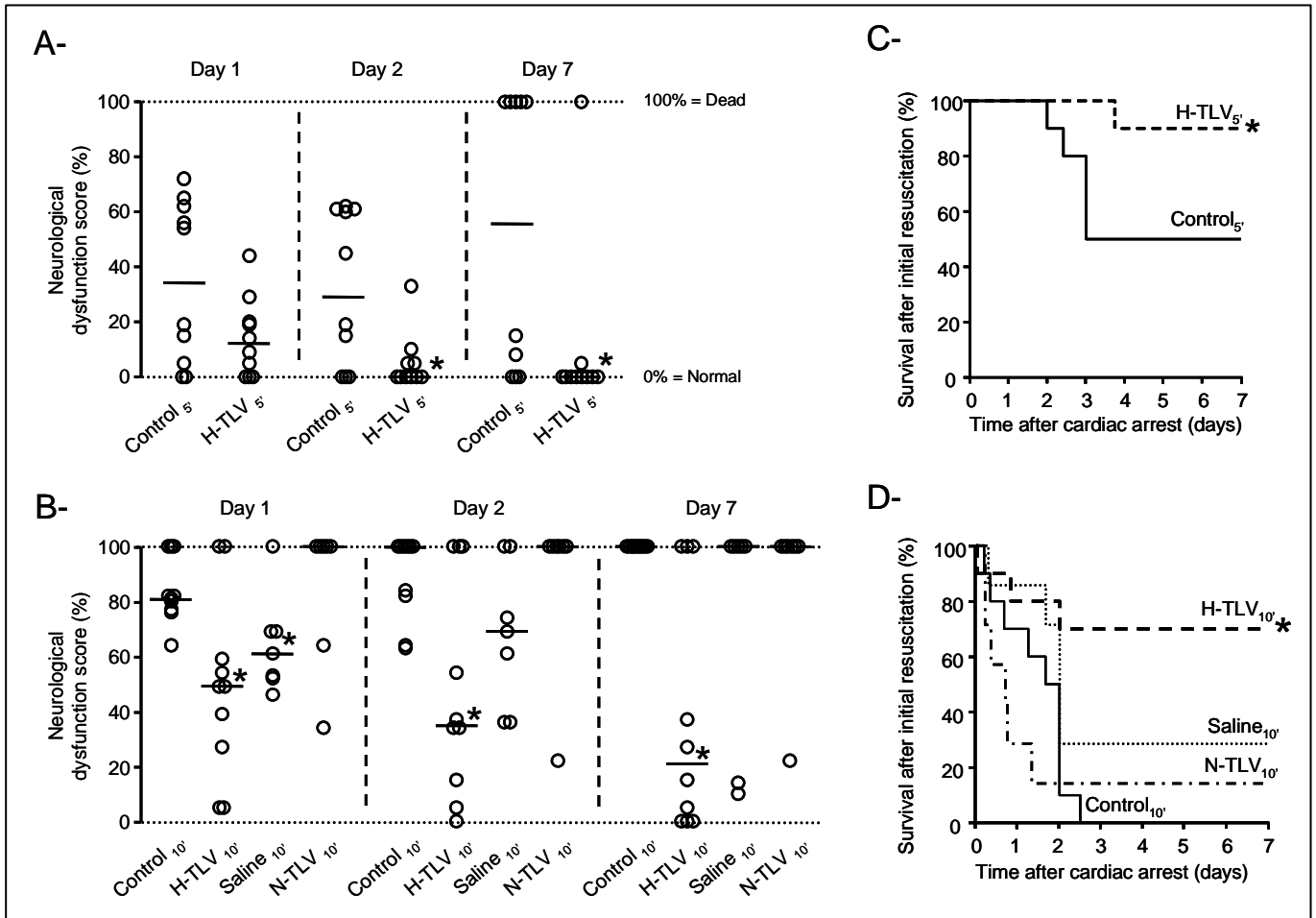


**Figure 5**





**Figure 6**



562 **Table 1 :** Groups characteristics during cardiopulmonary resuscitation, including the rate of  
 563 successful resuscitation, the time to resumption of spontaneous circulation (ROSC) and the  
 564 total amount of epinephrine administered throughout the protocol.

	n	Rate of successfull resuscitation	ROSC (min)	Epinephrine dose ( $\mu\text{g}/\text{kg}$ )
Control <sub>5'</sub>	10	10/10	2.4 $\pm$ 0.3	207 $\pm$ 58
H-TLV <sub>5'</sub>	10	10/10	2.3 $\pm$ 0.3	174 $\pm$ 81
Control <sub>10'</sub>	15	10/15	4.8 $\pm$ 0.4	684 $\pm$ 118
H-TLV <sub>10'</sub>	15	10/15	4.2 $\pm$ 0.8	128 $\pm$ 128 *
Saline <sub>10'</sub>	10	7/10	3.7 $\pm$ 0.7	430 $\pm$ 126
N-TLV <sub>10'</sub>	10	7/10	3.7 $\pm$ 0.4	509 $\pm$ 64

565 \*  $p < 0.05$  vs corresponding control value; TLV, total liquid ventilation; H-TLV, hypothermic  
 566 TLV; N-TLV, normothermic TLV; Saline, hypothermia induced by intravenous administration  
 567 of cold saline combined to external cooling.

568 **Table 2 :** Mean arterial pressure, heart rate, plasma creatinine and alanine aminotransferase  
 569 concentrations (ALAT) throughout the experimental protocol in the different groups.

	n	Baseline	Cardiopulmonary resuscitation					Day 1 (n)
			15min	60min	180min	360min	480min	
<u>Epinephrine perfusion</u>		No	Yes	Yes	Yes	Yes	No	
<u>Heart rate (beat/min)</u>								
Control 5'	10	257±11	222±8	221±7	243±11	216±7	220±9	234±8 (10)
H-TLV 5'	10	259±10	202±12	174±6 *	177±9 *	245±9	234±8	244±10 (10)
Control 10'	10	263±10	219±6	220±10	198±8	221±11	231±13	256±17 (7)
H-TLV 10'	10	267±8	167±10 *	158±8 *	167±11	208±12	240±11	252±7 (8)
Saline 10'	7	266±7	200±10	153±7 *	155±13 *	219±10	218±10	226±16 (6)
N-TLV 10'	7	256±13	216±19	207±9	213±12	207±9	221±15	240±28 (2)
<u>Mean arterial pressure (mmHg)</u>								
Control 5'	10	81±3	83±4	82±3	83±1	83±4	80±4	83±4 (10)
H-TLV 5'	10	80±7	81±3	82±5	82±3	83±3	79±3	82±4 (10)
Control 10'	10	80±5	82±3	83±3	81±4	83±2	80±3	79±4 (7)
H-TLV 10'	10	83±4	81±4	82±3	81±3	81±4	80±4	79±6 (8)
Saline 10'	7	80±8	86±6	89±2	78±5	82±5	76±6	83±9 (6)
N-TLV 10'	7	78±7	78±5	78±1	78±4	78±5	75±7	88±4 (2)
<u>Plasma creatinine concentrations (mg/l)</u>								
Control 5'	10	10±1	11±1	-	10±1	-	-	10±1 (10)
H-TLV 5'	10	10±0	12±1	-	11±1	-	-	10±1 (10)
Control 10'	10	9±1	13±1	-	14±2	-	-	11±1 (7)
H-TLV 10'	10	10±0	13±1	-	12±1	-	-	11±1 (8)
Saline 10'	7	9±1	10±1	-	10±1	-	-	10±1 (6)
N-TLV 10'	7	9±1	11±1	-	12±1	-	-	13±6 (2)
<u>Plasma ALAT concentrations (U/l)</u>								
Control 5'	10	29±5	31±4	-	33±4	-	-	35±9 (10)
H-TLV 5'	10	25±3	26±2	-	43±5	-	-	30±6 (10)
Control 10'	10	44±13	79±25	-	115±32	-	-	60±17 (7)
H-TLV 10'	10	48±3	65±5	-	111±27	-	-	83±14 (8)
Saline 10'	7	32±2	48±4	-	101±30	-	-	62±27 (6)
N-TLV 10'	7	31±5	66±10	-	96±13	-	-	94±37 (2)

570 \*  $p < 0.05$  vs corresponding control value; TLV, total liquid ventilation; H-TLV, hypothermic  
571 TLV; N-TLV, normothermic TLV; Saline, hypothermia induced by intravenous administration  
572 of cold saline combined to external cooling.