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1 Article

2 Argon attenuates multi-organ failure in relation with HMGB1 3 inhibition

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15 **Abstract:** Argon inhalation attenuates multiorgan failure (MOF) after experimental ischemic injury.
16 We hypothesized that this protection could involve decreased High Mobility Group Box 1
17 (HMGB1) systemic release. We investigated this issue in an animal model of MOF induced by aor-
18 tic-cross clamping. Anesthetized rabbits were submitted to supra-coeliac aortic cross-clamping for
19 30-minutes, followed by 300-minutes of reperfusion. They were randomly divided into three
20 groups (n=7/group). The Control group inhaled nitrogen [70%] and oxygen [30%]. The Argon
21 group was exposed to a mixture of argon [70%] and oxygen [30%]. The last group received nitro-
22 gen/oxygen [70/30%] with an administration of the HMGB1 inhibitor glycyrrhizin (4 mg/kg i.v.)
23 5-min before aortic unclamping. At the end of follow-up, cardiac output was significantly higher in
24 Argon and Glycyrrhizin vs. Control (60±4 and 49±4 vs. 33±8 ml/kg/min, respectively). Metabolic
25 acidosis was attenuated in Argon and Glycyrrhizin vs. Control, along with reduced amount of
26 norepinephrine to reverse arterial hypotension. This was associated with reduced interleukin-6
27 and HMGB1 plasma concentration in Argon and Glycyrrhizin vs. Control. End-organ damages
28 were also attenuated in liver and kidney in Argon and Glycyrrhizin vs. Control, respectively.
29 Argon inhalation reduced HMGB1 blood level after experimental aortic cross-clamping and pro-
30 vided similar benefits than direct HMGB1 inhibition.

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1. Introduction

Multiorgan failure (MOF) is a syndrome encountered in various clinical settings such as shock and ischemia-reperfusion injuries, generating major medical, ethic and economic issues [1]. Its management is mainly limited to pharmacological or mechanical organ support, along with etiological treatment. Alternative strategies are thus eagerly investigated. Among them, inhalation of noble gases such as xenon or argon could provide organo-protective effects [2,3]. If xenon provided interesting neuroprotective effects, its costs and rarity limited its use and led surge of interest for argon [4]. This more abundant gas extracted from air exerts anti-ischemic effects and provides promising experimental results in animal studies. These benefits have been mainly studied after regional ischemia with “single-organ” dysfunction or after cardiac arrest for neuroprotective purpose [5–10]. Multi-organ protection was also reported by our group in a model of MOF induced by supra-coeliac aorta cross-clamping in rabbits [2].

Mechanistically, argon was shown to increase the resistance to ischemic injuries through direct cytoprotective effects involving the extracellular signal-regulated kinases (ERK ½) pathway [11]. However, other studies reported anti-inflammatory effects with a reduction in interleukins (IL)-6 and IL-1β release, as well as reduced Toll-like receptors 2 and 4 density on cell surface in vitro [12–14]. Yet, the links between the anti-inflammatory and anti-ischemic properties are not established. Determining whether argon could provide specific benefits for MOF prevention with multiple cross-talks between end-organ injuries is thus crucial [3,11]. In the present study, we hypothesized that this link could actually involve High Mobility Group Box 1 (HMGB1), since this well-known activator of innate immunity is released by ischemic cell death and has been shown to be reduced after argon exposure in a rat cardioplegia model [15,16].

Accordingly, the aim of this study was to investigate whether argon-induced attenuation during MOF is associated with HMGB1 release inhibition and provides similar benefits than the direct pharmacological inhibition of HMGB1. For the latter purpose, we used the well-known HMGB1 inhibitor glycyrrhizin [17–19]. We used a model of MOF through aortic cross-clamping in rabbits, that allows to investigate cardiovascular, intestinal, hepatic and renal injuries, as well as blood inflammatory biomarkers.

2. Results

2.1. Hemodynamic parameters

Seven rabbits were included into each experimental group (Figure 1). As illustrated by Figure 2, cardiac output, heart rate and mean arterial pressure were similar among groups before aortic cross-clamping. During aortic cross-clamping, cardiac output was significantly higher in Argon as compared to Control and Glycyrrhizin groups. After aortic cross-clamping, a gradual decrease in cardiac output was further observed in all groups, with higher values in Argon and Glycyrrhizin groups at the end of the follow-up, as compared to Control (60±4, 49±4 and 33±8 ml/min/kg, respectively; Figure 2A; p=0.0002 between Argon and Control and p=0.0213 between Glycyrrhizin and Control). This was not related to any differences in heart rate, which were similar among groups throughout follow-up (Figure 2B). Mean arterial pressure was also not significantly different between groups (Figure 2C) but norepinephrine requirements were reduced in Argon and Glycyrrhizin groups as compared to Control (3.2±1.3, 5.9±2.1 and 12.6±3.0 µg/kg/min at the end of the follow-up, respectively; Figure 2D).

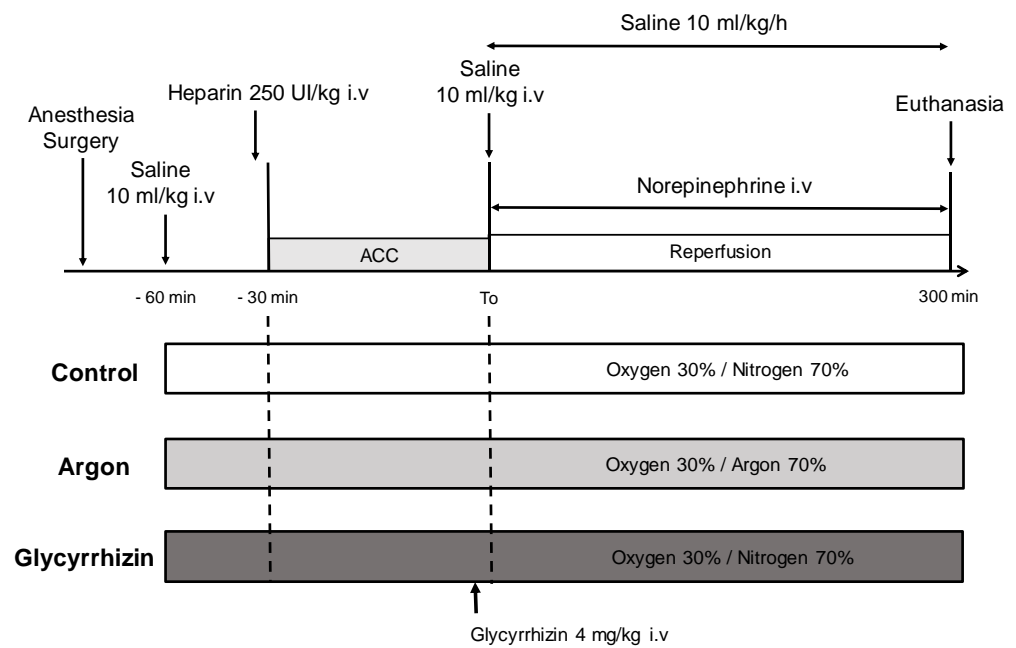


Figure 1. Schematic representation of the experimental protocol. ACC: aortic cross-clamping; Control (n=7), Argon (n=7), Glycyrrhizin (n=7).

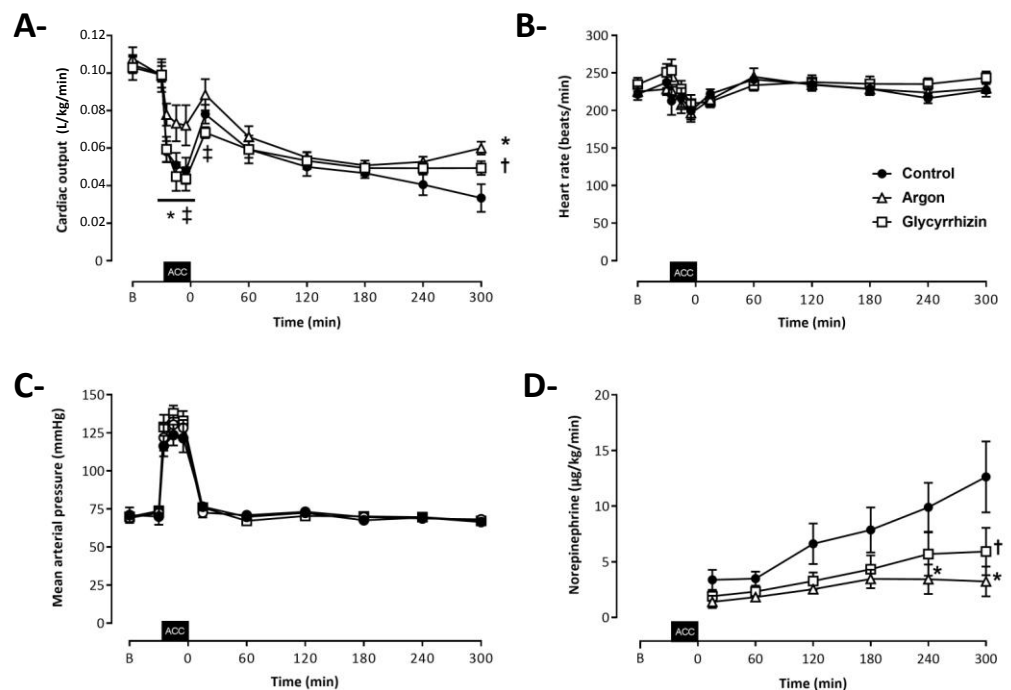


Figure 2. Cardiac output (Panel A), heart rate (Panel B), mean arterial pressure (Panel C) and norepinephrine regimen of administration (Panel D) throughout protocol. ACC: Aortic cross clamping; *, $p < 0.05$ between Control and Argon; †, $p < 0.05$ between Control and Glycyrrhizin; ‡, $p < 0.05$ between Argon and Glycyrrhizin; Control (n=7), Argon (n=7), Glycyrrhizin (n=7).

2.2. Biochemical alterations of shock

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As shown in Figure 3, biochemical hallmarks of shock were evidenced in all animals. In the Control group, aortic cross-clamping resulted in severe metabolic acidosis and renal injury. In comparison, arterial pH was significantly higher at the end of the follow-up in Argon and Glycyrrhizin groups vs Control (7.16 ± 0.06 , 7.21 ± 0.06 and 6.91 ± 0.09 , respectively; Figure 3A; $p=0.0003$ between Argon and Control and $p<0.0001$ between Glycyrrhizin and Control), along with higher plasma bicarbonate concentrations (17.7 ± 2.8 , 15.8 ± 2.3 and 8.3 ± 2.7 mmol/L, respectively; Figure 3B; $p=0.0002$ between Argon and Control and $p=0.0025$ between Glycyrrhizin and Control), lower lactate concentrations (7.8 ± 2.6 , 8.0 ± 2.2 and 14.6 ± 2.4 mmol/L, respectively; Figure 3C; $p=0.0019$ between Argon and Control and $p=0.0025$ between Glycyrrhizin and Control) and reduced creatinine blood levels (132 ± 22 , 123 ± 15 and 181 ± 24 $\mu\text{mol/L}$, respectively; Figure 3D; $p=0.0038$ between Argon and Control and $p=0.0126$ between Glycyrrhizin and Control).

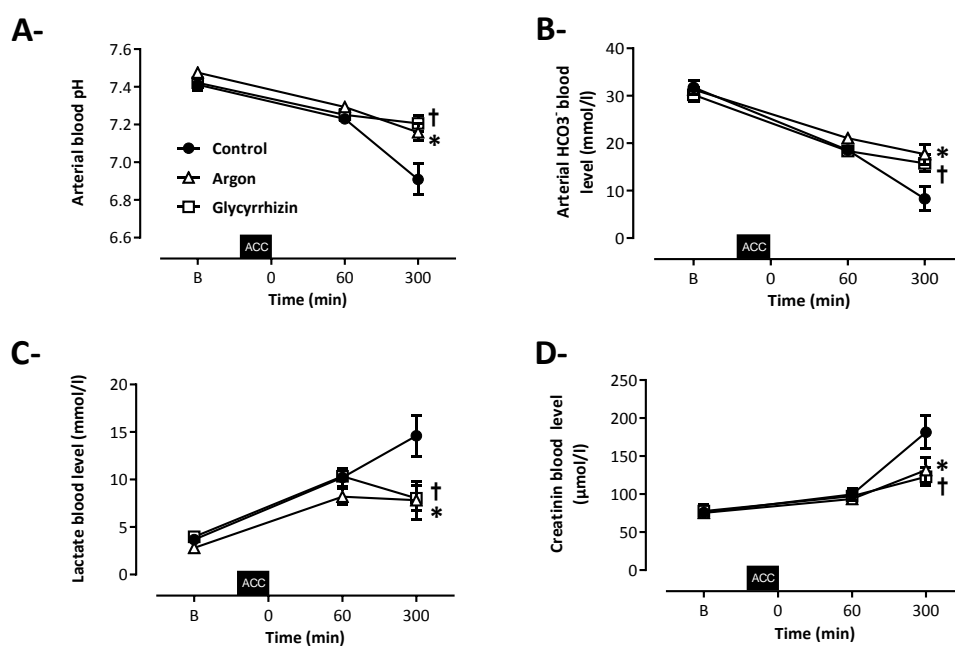


Figure 3. Arterial pH (Panel A) and blood levels of HCO₃⁻ (Panel B), arterial lactate (Panel C) and plasma creatinine (Panel D).

ACC: Aortic cross clamping; *, $p<0.05$ between Control and Argon; †, $p<0.05$ between Control and Glycyrrhizin; Control ($n=7$), Argon ($n=7$), Glycyrrhizin ($n=7$).

2.3. Cytokine blood levels

As illustrated in Figure 4A, a progressive and large increase in blood IL-6 concentrations was observed after unclamping in the Control group, with a significant reduction in Argon and Glycyrrhizin groups vs Control (7504 ± 2481 , 3251 ± 1229 and 13420 ± 383 pg/ml, respectively; $p=0.0296$ between Argon and Control and $p<0.0001$ between Glycyrrhizin and Control). Conversely, blood IL-1 β concentrations were not modified during follow-up and among groups (47.4 ± 12.8 , 39.4 ± 14.4 and 54.4 ± 50.5 pg/ml at the end of follow-up; Figure 4B; $p=0.4273$ between Argon and Control and $p=0.6536$ between Glycyrrhizin and Control).

At baseline, blood HMGB1 concentration did not differ between groups. After 60 minutes of reperfusion, an early and significant decrease in blood HMGB1 concentration was observed in Argon and Glycyrrhizin groups vs Control (14.2 ± 4.2 , 4.5 ± 0.1 and 37.5 ± 9.7 pg/ml, respectively; $p=0.0021$ between Argon and Control and $p<0.0001$ between Glycyrrhizin and Control). At 300 minutes of reperfusion, blood HMGB1 concentration was still significantly lower in Argon and Glycyrrhizin groups, while a secondary increase was observed in Control (3.4 ± 1.9 and 4.7 ± 0.1 vs. 35.8 ± 14.1 pg/ml, respectively;

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Figure 4C; $p < 0.0001$ between Argon and Control and $p < 0.0001$ between Glycyrrhizin and Control).

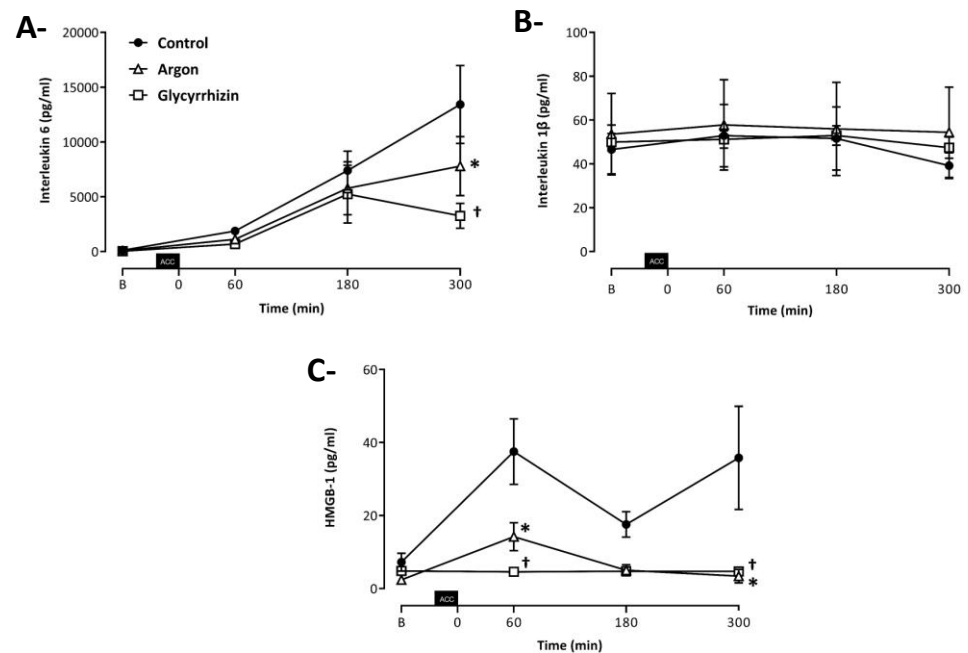
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Figure 4. Blood levels of interleukin 6 (Panel A), interleukin 1β (Panel B) and High Mobility Group Box 1 (Panel C).

ACC: Aortic cross clamping; *, $p < 0.05$ between Control and Argon; †, $p < 0.05$ between Control and Glycyrrhizin; Control ($n=7$), Argon ($n=7$), Glycyrrhizin ($n=7$).

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2.4. Histopathological evaluation

Histopathological lesions revealed ischemic lesions in most organs (score > 0). In myocardium and pulmonary samples (Figure 5A and 5B), myocardial necrosis and lung congestion lesions were similar in all groups. In contrast, tubular necrosis lesions were less pronounced in Argon group (without significant difference) but significantly lower in Glycyrrhizin group, as compared to Control (Figure 5C). Conversely, liver lesions of centrilobular clarification were significantly attenuated with Argon but not Glycyrrhizin, as compared to Control (Figure 5D). No significant difference was observed on intestinal necrosis lesions (Figure 5E).

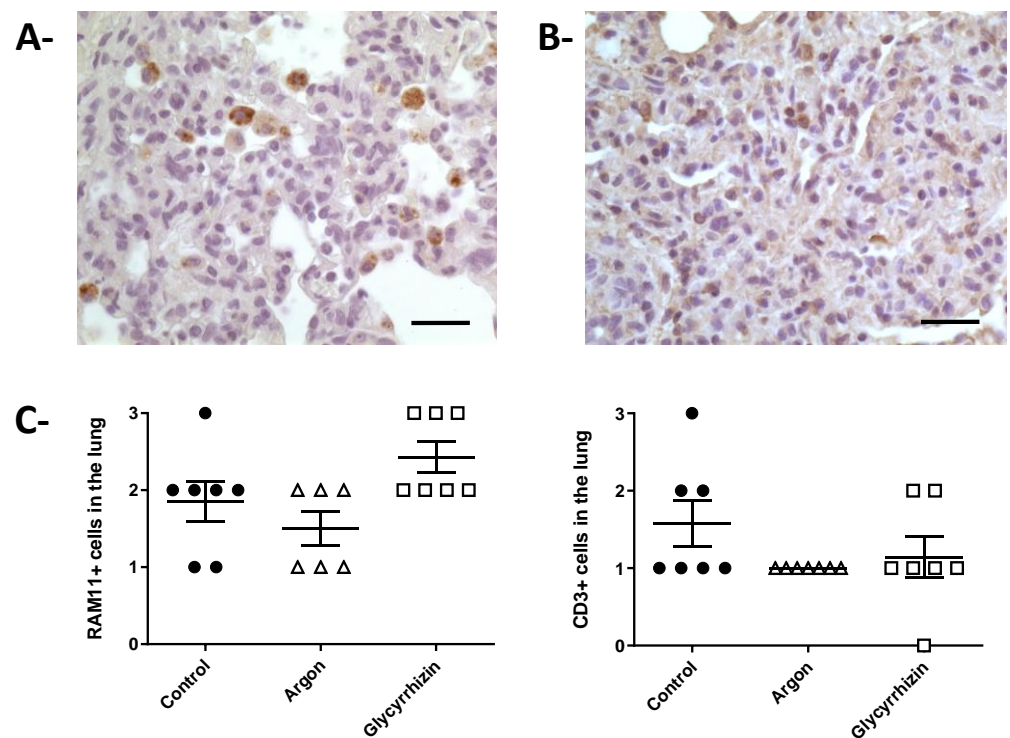


Figure 6. Immunohistochemistry in lung parenchyma.

A- Morphological appearance of the immunohistochemical staining for macrophage identification using the RAM11 polyclonal antibody.

B- Morphological appearance of the immunohistochemical staining for T cells identification using a CD3 antibody.

C- Semiquantification of RAM11 and CD3 positive cells in the pulmonary samples from the different groups.

3. Discussion

In the present study, argon inhalation and HMGB1 inhibition by glycyrrhizin attenuated MOF induced by aortic cross-clamping. This situation mimics various medical or postoperative scenes encountered in critically ill patients. The benefits were observed on cardiac output, norepinephrine requirements, metabolic acidosis, and renal or hepatic damages. In the Argon group, cardiovascular benefits followed a multi-phasic pattern of improvements, with an initial improvement in cardiac output during aortic cross-clamping, and then a deterioration with an ultimate re-increase at the end of the follow-up. In the Glycyrrhizin group, cardiac output was only improved at the end of the follow-up vs. Control. In both groups, blood levels of HMGB1 and IL-6 were significantly reduced, as compared to Control.

The present results reinforce our previous observations with argon alone in similar experimental conditions in rabbits [2]. In this previous study, argon inhalation reduced hemodynamic alterations with maximal benefits when inhaled throughout aortic cross-clamping and unclamping as compared to post-clamping only. As opposite to the present study, we did not observe any increase in cardiac output with argon during aortic cross-clamping as compared to Control, but only after unclamping. During the previous study, the mechanical ventilation led to a positive end-expiratory pressure (~3-5 cmH₂O), which was not the case in the current experiment with zero end-expiratory pressure. This difference could have hampered the vasodilatory effect of argon on pulmonary vessels, which might contribute to the cardiac output increase in the present study [20]. Obviously, this is still only a hypothesis that deserves further investigation

181 but that could open perspectives for argon use during cardiogenic shock. In the same
182 line, the recent study of Fumagalli et al. in a porcine model of cardiac arrest also reported
183 hemodynamic benefits with argon after resuscitation [9].

184 More importantly, the present study shows that argon reduced HMGB1 blood levels
185 while the direct inhibition of this protein with glycyrrhizin also provided benefits. Then,
186 argon could exert an initial direct anti-ischemic effect that reduces the passive release of
187 the nuclear HMGB1. Several studies have indeed shown that argon could exert, for in-
188 stance, direct anti-apoptotic effects through caspase-3 inhibition or modulation of the
189 Bcl2 pathway [13,21]. Argon was also shown to increase Extracellular signal-Regulated
190 Kinases (ERK $\frac{1}{2}$) phosphorylation, which increases cells resistance to ischemic damages
191 [6,14]. These effects could reduce the initial drop in HMGB1 blood levels, which could
192 explain the later anti-inflammatory effects of argon on IL-6 blood levels [16].

193 Indeed, the use of the direct HMGB-1 inhibitor glycyrrhizin just before aortic
194 cross-unclamping reduced HMGB1 and IL-6 blood levels with a similar magnitude than
195 argon. This liquorice-derived glycoside is known to act through a direct binding to
196 HMGB1, which reduced subsequent cytokine production [22]. For instance, it was shown
197 to provide neuroprotection after spinal cord ischemia–reperfusion injury or
198 cardioprotection after myocardial infarction model [18,23]. Since glycyrrhizin completely
199 blunted HMGB1 blood concentration increase after unclamping, this protein is probably
200 promoting a proper vicious circle after aortic cross-clamping with an initial minor release
201 due to ischemia and then a continuous active secretion promoted by the protein itself on
202 immune cells. Similar auto-promotion of HMGB1 release was indeed well demonstrated
203 in other conditions and could perpetuate the inflammatory reactions during MOF or after
204 ischemic injuries [24]. Then, argon might exert its benefit through an initial reduction of
205 HMGB1 by pure anti-ischemic effect and then lead to secondary benefits with the sub-
206 sequent blunting of HMGB1 inflammatory vicious circle. It is supported by our previous
207 study showing that argon administration before and just after aortic-cross clamping
208 prevented MOF with a magnitude close to that afforded by argon inhalation throughout
209 the ischemic and reperfusion period [2]. It could be important since it suggests that argon
210 may not directly alter the inflammatory reaction, but rather exerts pure anti-ischemic ef-
211 fect that might just attenuate the subsequent innate immunity activation. As shown by
212 the lung immunohistochemical analyses, this attenuation did not blunt lung immune
213 cells infiltration, even if a trend toward less T cells infiltration was importantly observed.

214 This study has several limitations. First, it was not possible to address directly the
215 link between argon and HMGB1, since it would require a direct activation of HMGB1
216 pathway on top of argon, expecting a loss of protection. We would need analogs of
217 HMGB1 which are activating its receptors, such as the receptor for advanced glycation
218 end-products (RAGE), while HMGB1 is known to exert more complex effects, depending
219 upon its redox status and multiple receptors [24]. The direct activation of RAGE would
220 then lead to much stronger inflammatory reaction with multiple deleterious effects that
221 could limit the relevance of this experiment. We have then chosen to compare argon and
222 glycyrrhizin, with a concomitant evaluation of HMGB1 blood level. Second, glycyrrhizin
223 is acting through HMGB1 inhibition but might also exert other pharmacological effects
224 on mineralocorticoids pathways [25]. However, if the latter effect is known to participate
225 to the hypertensive effect of glycyrrhizin after chronic exposure, it is believed to be ir-
226 relevant after a single exposure, explaining why this molecule is typically used for
227 HMGB1 mitigation [26]. In addition, we conducted preliminary experiments that con-
228 firmed that glycyrrhizin did not exert any proper hemodynamic effects in sham animals
229 (data no shown). Finally, one would be surprised by the large increase in IL-6 blood lev-
230 els in our conditions, while IL-1 β blood levels were not modified. In previous report after
231 generalized ischemia, we indeed also observed that IL-6 was activated very early,

232 whereas IL-1 β or tumor necrosis factor-alpha were not [27]. An increase in IL-6 has been
233 also reported in swine undergoing infra-renal clamping [28]. Conversely, argon did not
234 significantly modify mRNA blood levels of several cytokines in a previous study in sim-
235 ilar conditions, suggesting that argon only reduced reactivity of inflammatory cells
236 through reduced HMGB1 level but not transcription levels. However, we only investi-
237 gated a few markers and did not decipher the immune response after aortic
238 cross-clamping, which was not the goal of the study. More importantly, argon could also
239 likely modify other pro-inflammatory markers of cell injury even if we here focused on
240 HMGB1.

241 4. Materials and Methods

242 All experiments were conducted in compliance with the French legislation govern-
243 ing animal research. The experimental procedure was approved by the institutional re-
244 view board for animal research (APAFIS#17800-2020012017214953).

245 4.1. Animal preparation

246
247 As previously described [2,29], male New Zealand rabbits (2.0-3.0 kg) were anes-
248 thetized using a mixture of zolazepam and tiletamine (12.5 mg/kg i.v), thiopental (10
249 mg/kg i.v) and methadone chlorhydrate (0.6 mg/kg i.v). After tracheal intubation, me-
250 chanical ventilation was started with inspired fraction of oxygen set at 30%, tidal volume
251 at 10 ml/kg and respiratory rate at 28-30 cycles/minute. Electrocardiogram was continu-
252 ously monitored, as well as blood pressure through a pressure catheter inserted into the
253 right carotid artery. A rectal temperature probe was inserted for body temperature con-
254 trol at 38.0 \pm 0.5 $^{\circ}$ C with heating pads. After left thoracotomy, a 4-mm diameter flow probe
255 was placed around the ascending aorta to continuously measure cardiac output
256 (PS-Series Probes, Transonic, NY, USA). Then, a left laparotomy incision was performed
257 to isolate the abdominal supra-coeliac aorta. After instrumentation, saline (NaCl 0.9%, 10
258 mL/kg i.v) and heparin (250 IU/kg i.v) were administered. Bladder was emptied before
259 aortic cross-clamping to measure urinary output.

260 4.2. Protocol of aortic cross-clamping

261
262 After a period of stabilization, supra-coeliac aorta was occluded with a vascular
263 clamp. The occlusion was released after 30 min of aortic cross-clamping, as previously
264 described [2,28]. To optimize hemodynamics, additional administration of saline (10
265 mL/kg i.v) was performed just after unclamping, followed by a continuous infusion (10
266 mL/kg/h i.v) until the end of experiment. Norepinephrine administration was adjusted to
267 target a mean arterial pressure of 70 mmHg. Animals were then monitored for 300
268 minutes of reperfusion after aortic unclamping. Then, they were euthanized using a le-
269 thal dose of pentobarbital (60 mL.kg-1 i.v). Lungs, heart, liver, gut and kidneys were
270 sampled and fixed in formaldehyde (4%) for blinded histological analysis.

271 4.3. Experimental protocol

272
273 Prior to aortic cross-clamping, the animals were assigned to one of the 3 experi-
274 mental groups using a block randomization (Figure 1). In the Control group, animals
275 underwent conventional mechanical ventilation with a mixture of nitrogen (inspired
276 fraction 70%) and oxygen (30%). In the Argon group, animals inhaled mixture of argon
277 (70%) and oxygen (30%). Argon inhalation started 30 minutes before aortic
278 cross-clamping and was maintained until the end of the protocol, i.e. 300 minutes after
279 reperfusion. In the Glycyrrhizin group, mechanical ventilation was performed with ni-
280 trogen/oxygen mixture (70%/30%) with an administration of glycyrrhizin (4 mg/kg i.v)
281 five minutes before reperfusion [30]. The investigator taking care of the animals was not
282 blinded of the treatment group.
283

4.4. Immuno-assay and histology

Plasma concentrations of IL-6, IL1- β and HMGB1 were evaluated by enzyme-linked immunosorbent assay (IL-6 and IL1- β : R&DSystems®, Minneapolis, USA; HMGB1: Abxexa®, Cambridge, UK). Heart, lungs, kidneys, liver and gut were submitted to blinded histopathological analyses after haematoxylin-eosine-safran staining. For the heart, kidney and liver, ischemic injury was blindly graded using a score from 0 (normal tissue) to 3 (extensive cell necrosis). For the lung, a score of lung congestion and alveolar edema was attributed from 0 (no congestion and edema) to 3 (severe congestion and serous alveolar edema). Finally, intestinal damages were graded using a modified Chiu score from 0 (normal) to 5 (severely damaged), as previously described [2].

Since we observed lung inflammation during histopathological evaluation, immunohistochemical analyses were further performed to quantify the invasion of the pulmonary parenchyma by immune cells. T-cells were detected with a monoclonal antibody against a CD3 antigen (Orb323391, BIORBYT, Cliniscience, Nanterre, France). Macrophages were detected with a monoclonal antibody (RAM11, VWR, Fontenay-sous-Bois, France). Cell infiltration was blindly graded from 0 to 3.

4.5. Statistical analysis

Data are expressed as mean \pm SEM for continuous parameters and individual values and medians for histological scores, respectively. The primary outcome was the cardiac output at the end of the follow-up. Secondary outcomes were other hemodynamic, biochemical and histopathological parameters. Hemodynamic parameters were compared between groups by a two-way analysis of variance (ANOVA) for repeated measurements at baseline, during aortic cross-clamping (at 5, 15 and 25 minutes) and during reperfusion (at 60, 120, 180, 240 and 300 minutes). If necessary, a post-hoc Holm-Sidak test was performed for comparison to Control value at each time (no between-time comparison). Biochemical parameters (except cytokines) were compared between groups by the same statistical analysis, except for the baseline value, where a non-parametric Student's test was performed (one time only). Inflammatory markers were compared with a Kruskal-Wallis analysis followed if necessary by a Mann-Whitney test. Significant differences were determined at $p \leq 0.05$.

5. Conclusions

Both argon and glycyrrhizin attenuated MOF in a rabbit model of aortic-cross clamping, leading to improved cardiac output and attenuated shock and renal and hepatic ischemic lesions. The action of argon could involve multiple mechanisms including direct hemodynamic benefits during aortic-cross clamping, reduced cell death at reperfusion and attenuation of damages perpetuation through HMGB1 release initial inhibition.

6. Patents

Quentin de Roux, Matthieu Chalopin, Géraldine Farjot, Catherine Billoet, Nicolas Mongardon and Renaud Tissier are named as co-inventors on a patent application related to the use of inhaled argon in patients cardiovascular diseases.

Author Contributions: Conceptualization, Quentin de Roux, Matthieu Chalopin, Géraldine Farjot, Catherine Billoet, Nicolas Mongardon and Renaud Tissier; Data curation, Quentin de Roux, Fanny Lidouren and Emilie Boissady; Formal analysis, Quentin de Roux, Matthias Kohlhauer, Matthieu Chalopin, Géraldine Farjot, Catherine Billoet, Bijan Ghaleh, Nicolas Mongardon and Renaud Tissier; Funding acquisition, Nicolas Mongardon and Renaud Tissier; Investigation, Quentin de Roux, Fanny Lidouren, Agathe Kudela, Lina Slassi, Emilie Boissady, Patrick Bruneval and Renaud Tissier; Methodology, Quentin de Roux, Géraldine Farjot, Catherine Billoet, Patrick Bruneval and Renaud Tissier; Writing – original draft, Quentin de Roux, Nicolas Mongardon and Renaud Tissier;

333 Writing – review & editing, Matthias Kohlhauer, Matthieu Chalopin, Géraldine Farjot, Catherine
334 Billoet, Patrick Bruneval, Bijan Ghaleh, Nicolas Mongardon and Renaud Tissier.

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337 **Institutional Review Board Statement:** All experiments were conducted in compliance with the
338 French legislation governing animal research. The experimental procedure was approved by the
339 review board for animal research (APAFIS#17800-2020012017214953).

340 **Informed Consent Statement:** Any research article describing a study involving humans should
341 contain this statement. Please add “Informed consent was obtained from all subjects involved in
342 the study.” OR “Patient consent was waived due to REASON (please provide a detailed justifica-
343 tion).” OR “Not applicable.” for studies not involving humans. You might also choose to exclude
344 this statement if the study did not involve humans.

345 **Data Availability Statement:** In this section, please provide details regarding where data sup-
346 porting reported results can be found, including links to publicly archived datasets analyzed or
347 generated during the study. Please refer to suggested Data Availability Statements in section
348 “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this
349 statement if the study did not report any data.

350 **Conflicts of Interest:** M.C., G.F. and C.B. are employees of Air Liquide Santé International.

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