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ORIGINAL RESEARCH

High-Mobility Group Box 1–Signaling Inhibition With Glycyrrhizin Prevents Cerebral T-Cell Infiltration After Cardiac Arrest

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BACKGROUND: High-mobility group box 1 (HMGB1) is a major promotor of ischemic injuries and aseptic inflammatory responses. We tested its inhibition on neurological outcome and systemic immune response after cardiac arrest (CA) in rabbits.

METHODS AND RESULTS: After 10 minutes of ventricular fibrillation, rabbits were resuscitated and received saline (control) or the HMGB1 inhibitor glycyrrhizin. A sham group underwent a similar procedure without CA. After resuscitation, glycyrrhizin blunted the successive rises in HMGB1, interleukin-6, and interleukin-10 blood levels as compared with control. Blood counts of the different immune cell populations were not different in glycyrrhizin versus control. After animal awakening, neurological outcome was improved by glycyrrhizin versus control, regarding both clinical recovery and histopathological damages. This was associated with reduced cerebral $CD4^+$ and $CD8^+$ T-cell infiltration beginning 2 hours after CA. Conversely, granulocytes' attraction or loss of microglial cells or cerebral monocytes were not modified by glycyrrhizin after CA. These modifications were not related to the blood–brain barrier preservation with glycyrrhizin versus control. Interestingly, the specific blockade of the HMGB1 receptor for advanced glycation end products by FPS-ZM1 recapitulated the neuroprotective effects of glycyrrhizin.

CONCLUSIONS: Our findings support that the early inhibition of HMGB1-signaling pathway prevents cerebral chemoattraction of T cells and neurological sequelae after CA. Glycyrrhizin could become a clinically relevant therapeutic target in this situation.

Key Words: cardiac arrest ■ high-mobility group box 1 ■ inflammation ■ resuscitation

Sudden cardiac arrest (CA) is a leading cause of death and disabilities in the world. After resuscitation, most patients present dramatic neurological sequelae, multivisceral dysfunction, and innate immunity activation, sharing similarities with sepsis.[1] For instance, interleukin-6 blood levels were associated at hospital administration with mortality at 30 days in a prospective study in 171 patients resuscitated after CA.[2] A counterbalance between adaptive and innate immune responses was also proposed to predict neurological outcome at hospital admission using a set of blood transcripts.[3] Nevertheless, the exact mechanism triggering the immune response after CA is still poorly understood. Although cell necrosis is known to release intracellular contents activating proinflammatory receptors, the precise role of each putative mediator, including DNA fragments, ATP, and constitutive proteins,[4] still deserves further investigations.

Among these mediators, high-mobility group box 1 (HMGB1) is believed to be a cornerstone during

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CLINICAL PERSPECTIVE

What Is New?

- The inhibitor of high-mobility group box 1 glycyrrhizin improves outcome and attenuates neuroinflammation after cardiac arrest in rabbits.
- The protection afforded by glycyrrhizin is associated with an early reduction in T-cell infiltration in the brain.

What Are the Clinical Implications?

- The inhibition of the high-mobility group box 1–signaling pathways by glycyrrhizin could be promising to reduce neurological sequelae and inflammation after cardiac arrest, deserving further investigations for clinical translation.

Nonstandard Abbreviations and Acronyms

BBB	blood–brain barrier
CA	cardiac arrest
HMGB1	high-mobility group box 1
RAGE	receptor for advanced glycated end product
ROSC	resumption of spontaneous circulation

ischemia–reperfusion.[5] Its inhibition attenuates neuronal loss and neuroinflammation reduction effects after focal brain ischemia.[6–8] In rodent models of CA, similar benefits were observed with HMGB1-binding heptamer peptides or antibodies.[9,10] However, whether HMGB1 inhibition exerts its benefits through systemic or cerebral inflammation modulation still needs to be determined after CA.

Accordingly, our goal was to investigate the effect of HMGB1 inhibition on the immune response following CA at both tissular and systemic levels after CA. To achieve this goal, we used an established model of shockable CA in rabbits.[11–13] HMGB1 was targeted by an administration of glycyrrhizin, a clinically relevant inhibitor tested in patients with chronic hepatitis.[14,15] A single administration of glycyrrhizin does not provide a long-term hemodynamic effect that makes its use relevant for acute HMGB1 inhibition.[16] In this context, several sets of experiments were subsequently conducted and included: (1) a recovery study evaluating systemic inflammation and neurological outcome 72 hours after CA; (2) acute studies for the evaluation of the cerebral infiltration by immune cells and blood–brain barrier permeability in the first hours following resuscitation; and (3) a replicative study with the administration of a receptor for advanced glycation end products (RAGE) antagonist, as RAGE is a key receptor of HMGB1.[17]

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The animal instrumentation and ensuing experiments were conducted in accordance with official French regulations after approval by the local ethics committee (ComEth AnSES/ ENVA/UPEC no. 16; project 2017111414547261).

Animal Preparation and Induction of Cardiac Arrest

Male New Zealand rabbits (2.5–3.0 kg) were anesthetized using zolazepam, tiletamine, and pentobarbital (all 20–30 mg/kg IV). They received buprenorphine (30 μg/kg IV) for analgesia. Animals were intubated and mechanically ventilated. After the administration of rocuronium bromide (1 mg/kg IV), 2 electrodes were implanted upon the inner muscular wall or inserted into the esophagus, respectively. Body temperatures and ECG were monitored as well as systemic blood pressure through a catheter inserted into the ear artery. After a period of stabilization, an alternative current (12 V, 4 mA; 2.5 minutes) was delivered between the 2 electrodes to induce ventricular fibrillation. Concomitantly, mechanical ventilation was stopped. After 10 minutes of untreated fibrillation, cardiopulmonary resuscitation was performed using external cardiac massage (200 external chest compressions/min), electric defibrillation (10 J/kg), and intravenous administration of epinephrine (15 μg/kg IV). After resumption of spontaneous circulation (ROSC), epinephrine administration was allowed to achieve a target mean blood pressure of 70 mm Hg. Animals were maintained under normothermic conditions thanks to thermal pads. Animals were followed during 2 to 72 hours according to the investigated parameters, that is, during 72 hours for the assessment of neurological recovery after awakening or during 2 to 6 hours without awakening for the evaluation of cerebral infiltration by immune cells or blood–brain barrier (BBB) permeability after ROSC. In the recovery study (72 hours), rabbits received analgesics every day after CA (buprenorphine; 30 μg/kg IM).

Experimental Design of the Recovery Study

As illustrated in Figure 1, 27 animals were divided into 3 experimental groups after CA. The first group was submitted to animal preparation and subsequent follow-up with no CA (sham group, n=7). The 2 other groups were submitted to CA and randomly received either saline (control, n=10) or glycyrrhizin (4 mg/kg, IV; n=10) 5 minutes after ROSC. They received epinephrine, as previously described. Animals were allowed to awaken

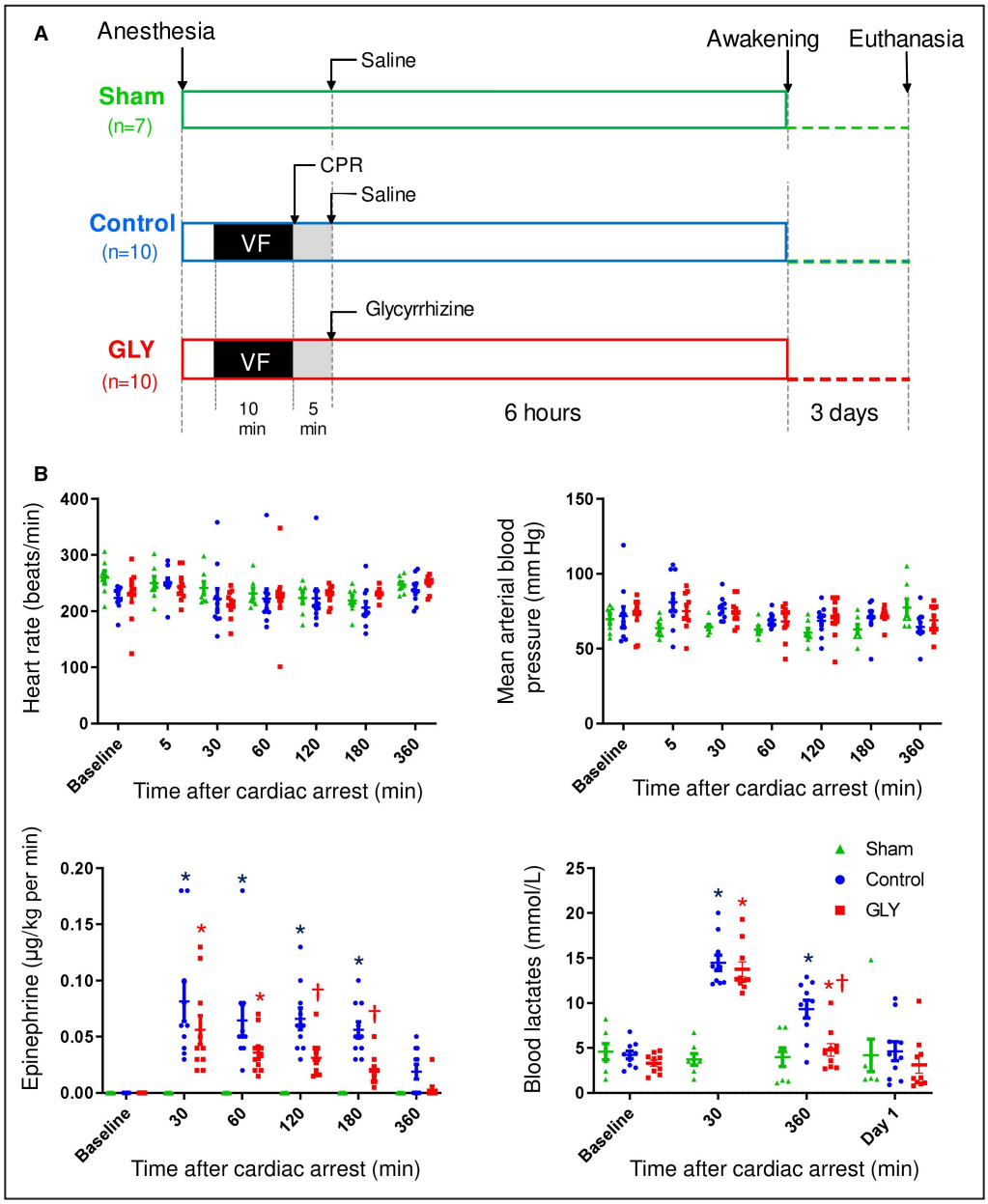

Figure 1. Experimental protocol and hemodynamic parameters in the recovery study.
A, Experimental protocol of the recovery study evaluating glycyrrhizin after cardiac arrest. The sham group was submitted to anesthesia and surgical instrumentation without cardiac arrest. The other groups were submitted to cardiac arrest with an administration of saline (control) or glycyrrhizin (4 mg/kg IV, glycyrrhizin), respectively. **B**, Heart rate, mean arterial pressure, epinephrine requirements, and lactate blood levels throughout protocol in the different groups. Data are shown as individual values and mean±SEM; **P*<0.05 vs corresponding sham; †*P*<0.05 vs corresponding control; n=7, 10, and 10 in sham, control and glycyrrhizin groups, respectively. CPR indicates cardiopulmonary resuscitation; GLY, glycyrrhizin; and VF, ventricular fibrillation.

beginning 6 hours after ROSC. Neurological dysfunction was evaluated blindly during 3 consecutive days using a clinical score (0%=normal, 100%=death; Table S1).[11,13,18] For ethical considerations, animals eliciting a neurologic dysfunction score >80% at 24 hours or 60% at 48 hours were prematurely euthanized. This concerned 2 and 2 animals at days 1 and 6, and 1 animal at 48 hours in the control and glycyrrhizin groups, respectively. All surviving animals were euthanized after 3 days of follow-up for histological analysis. Blood samples were withdrawn for blood gases and inflammatory response evaluations.

Blood levels of inflammatory mediators were evaluated using ELISA assays for HMGB1 (Abbexa,

Milton, United-Kingdom), interleukin-6 (R&D Systems, Minneapolis, MN), and interleukin-10 (Elabscience, Houston, TX). Blood samples were also prepared for flow cytometry and leukocyte counts, as described in additional material (Data S1, Figure S1).

At the end of the follow-up, rabbits were euthanized and perfused with 5% paraformaldehyde through both carotids. Brains were withdrawn and prepared for histological analysis. Degenerating neurons were identified as positive cells using the Fluorojade C staining (Merck Millipore, Burlington, MA). They were counted in 15 random nonoverlapping fields of the parasagittal cortex and hippocampus. Neutrophils and T cells were detected using immunohistochemistry with a monoclonal antibody against a cell surface antigen that is expressed by a subset of T cells and neutrophils (RPN3/57, diluted 1:100; BioRad, Hercules, CA).

Acute Experiments for the Evaluation of Cerebral Infiltration by Immune Cells

In another set of experiments, 25 rabbits were submitted to similar CA and experimental protocols and were euthanized 2 or 6 hours after ROSC (5 animals in each experimental condition, including the sham group). They were exsanguinated by intracarotid perfusion with ice-cold PBS, and the brain was withdrawn and prepared for flow cytometry and immune cell count as described in Data S1 and Figure S2. Results were expressed as a percentage of $CD45^+$ cells (Accuri C6, BD Biosciences, Le Pont de Claix, France).

Table. Rectal Temperatures and Biochemical Parameters in the Different Groups

Parameters and groups	Baseline	Time after ROSC		
		30 min	360 min	24 h
Rectal temperature (°C)				
Control	38.3±0.1	38.1±0.1*	38.7±0.2	…
Glycyrrhizin	38.4±0.2	37.9±0.2*	38.6±0.2	…
Sham	38.8±0.2	38.7±0.2	38.4±0.2	…
Arterial blood pH				
Control	7.44±0.02	7.05±0.04*	7.25±0.04*	7.38±0.03
Glycyrrhizin	7.38±0.03	7.06±0.03*	7.37±0.02*,†	7.42±0.02
Sham	7.40±0.02	7.41±0.03	7.44±0.03	7.33±0.03
Arterial blood PaO_2 (mm Hg)				
Control	251±12	134±16*	187±17	98±16
Glycyrrhizin	250±10	143±13*	241±24	75±10
Sham	226±33	286±39	286±39	83±7
Arterial blood $PaCO_2$ (mm Hg)				
Control	44±3	62±7*	47±2	39±3
Glycyrrhizin	47±2	58±3*	43±3	31±4
Sham	48±4	44±4	37±1	43±3
Bicarbonate blood level (mmol/L)				
Control	29.1±1.1	16.9±1.6*	20.5±1.9*	23.0±1.3
Glycyrrhizin	28.1±1.1	16.3±0.9*	24.2±1.1*	20.4±2.0
Sham	30.2±2.7	26.4±1.4	26.4±1.4	25.0±1.9
Creatinine blood level (µmol/L)				
Control	59±5	128±32	112±24	153±36
Glycyrrhizin	54±5	94±41	83±7	112±6
Sham	63±9	51±9	81±10	97±7
Glucose blood level (mg/dL)				
Control	1.9±0.2	3.8±0.1*	4.0±0.6*	1.6±0.1
Glycyrrhizin	2.0±0.1	3.8±0.2*	1.6±0.4†	1.6±0.1
Sham	1.9±0.1	1.5±0.1	1.5±0.1	1.7±0.1

Statistical comparisons were made for only group effect but not time effect. n=10 in each control and glycyrrhizin group; n=7 in sham group. ROSC indicates resumption of spontaneous circulation.

*$P<0.05$ vs sham.

†$P<0.05$ vs control.

Acute Experiments for the Evaluation of BBB Permeability

For the evaluation of BBB permeability, 8 additional rabbits were submitted to another set of experiments with similar CA protocols and Evans blue dye administration (4%, 3 mL/kg) 30 minutes after CA (n=4 in both the control and glycyrrhizin groups). Life support was maintained for 2 hours, after which animals were euthanized. Cerebral blood was rinsed with the administration of 250 mL of saline through each carotid. The brain was then sampled, and the cerebral cortex, hippocampus, and cerebellum were isolated and homogenized using formamide (1 mL/100 g) for 48 hours at 37°C. After centrifugation, the absorbance was measured at 620 nm. The corresponding concentration of Evans blue dye was calculated using a calibration curve. It was expressed as µg/g of brain tissue.

Replication Experiments With a RAGE Antagonist

Two additional groups of 6 animals were submitted to CA but received saline (control) or the RAGE antagonist FPS-ZM1 (1 mg/kg IV) after ROSC. Rabbits awaken 6 hours after ROSC for the evaluation of neurological outcome for 72 hours, as previously described for the glycyrrhizin study. At the end of the 72-hour follow-up, animals were euthanized and brain lesions were evaluated by fluorojade C staining.

Statistical Analysis

In the text, data are expressed as mean±SEM, unless otherwise stated. Time to ROSC and pre-ROSC epinephrine doses were compared between the control and glycyrrhizin groups only, since it was not relevant in sham conditions. Comparisons were made using a Student *t*-test for time to ROSC (normal distribution)

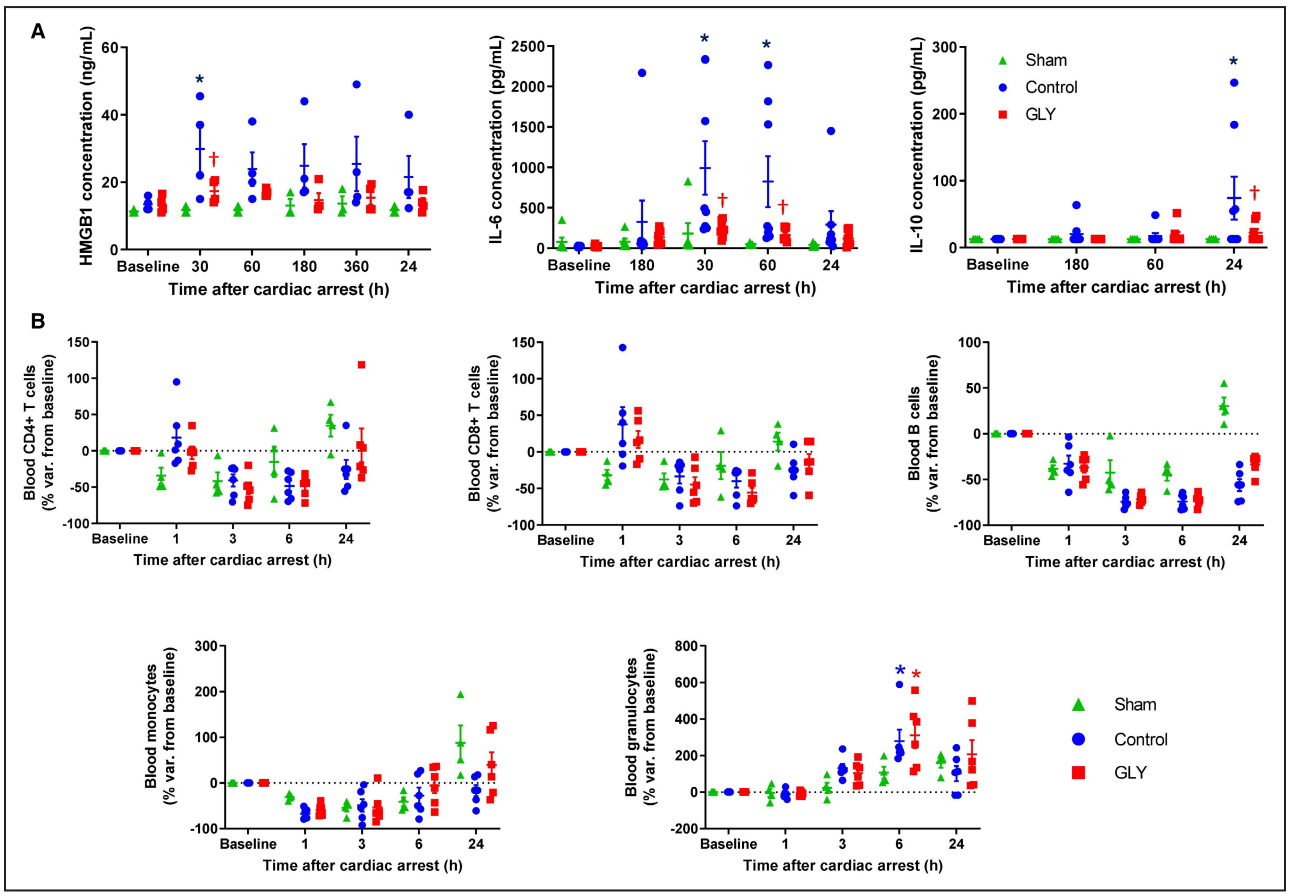

Figure 2. Humoral and cellular blood responses in the recovery study.
A, Blood levels of high mobility group box 1 (HMGB1), interleukin (interleukin)-6, and interleukin-10 and throughout follow-up in the different groups. **B**, Blood leukocyte counts using fluorescence-activated cell sorting (FACS) in the different groups. Counts are expressed as variation from baseline. T cells were classified as $CD4^+$ or $CD8^+$ cells. B cells, monocytes, and granulocytes were considered as $CD79a^+$, $CD11b^+CD14^+$, and $CD11b^+$ $CD14^-$ cells, respectively. Data are shown as individual values and mean±SEM; *P<0.05 vs corresponding sham; †P<0.05 vs corresponding control; n=7, 10, and 10 in sham, control, and glycyrrhizin groups, respectively. GLY indicates glycyrrhizin.

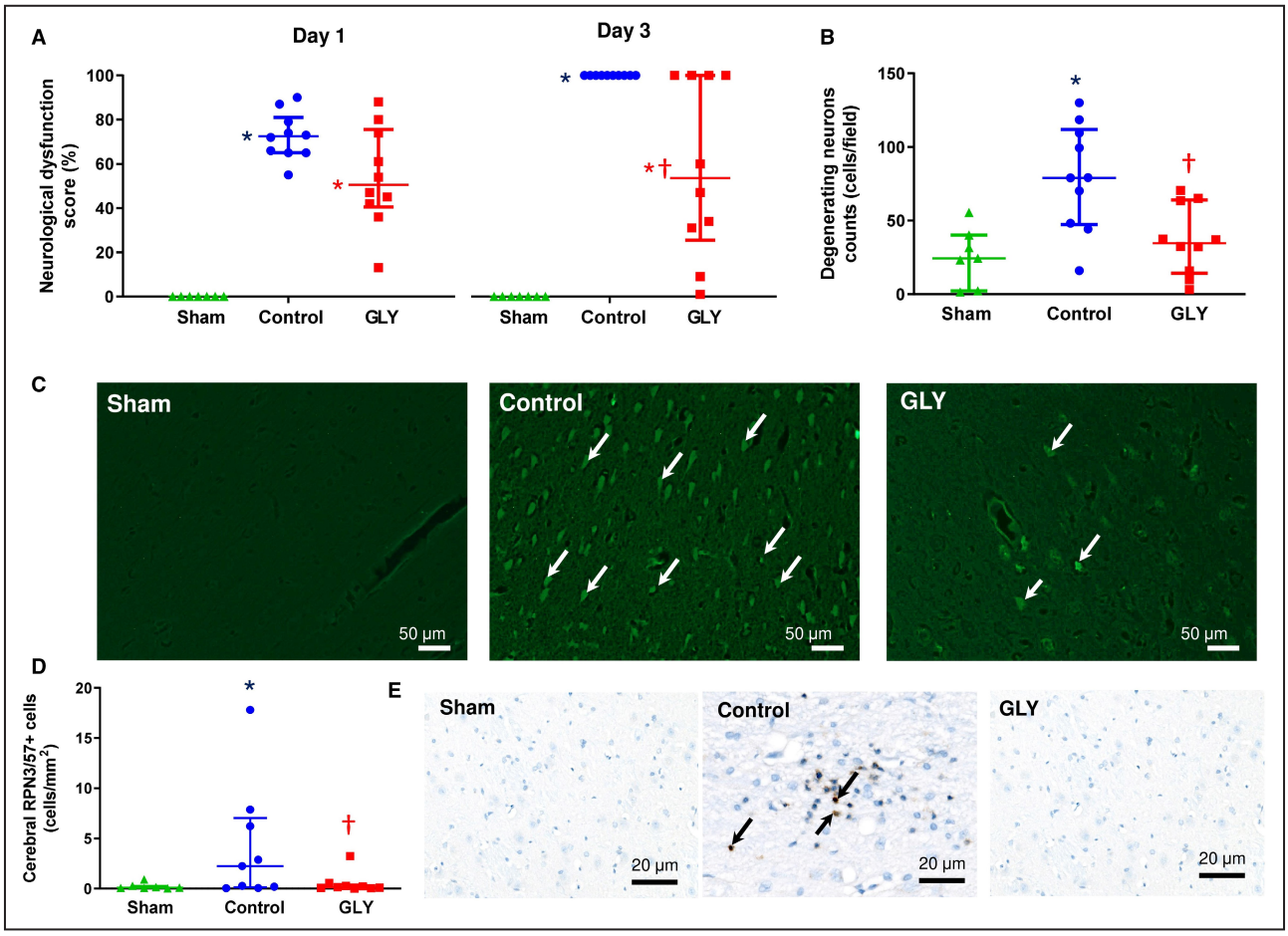

Figure 3. Neurological recovery in the recovery study.
A, Neurological dysfunction at days 1 and 3 following resuscitation in the different groups (0% = lack of dysfunction; 100% = death). **B**, Number of degenerating neurons per histological field in the different groups, as defined by fluorojade C staining cells. Symbols and lines represent individual data and mean of the group, respectively. **C**, Typical histological appearance of the fluorojade-C staining in a field from the cerebral cortex, showing no or few degenerating neurons in the sham and glycyrrhizin groups, as compared with frequent degenerating neurons in the control group. **D**, Mean number of RPN3/57+ cells (neutrophils and T cells) per mm^2 into the cerebral parenchyma in each group. **E**, Morphological appearance of the RPN3/57immunohistochemical staining in rabbits from the sham, control, and glycyrrhizin groups. RPN3/57+ cells are shown by arrows. Data are shown as individual values and median±interquartile range; *P<0.05 vs corresponding sham; †P<0.05 vs corresponding control; n=7, 10, and 10 in the sham, control, and glycyrrhizin groups, respectively. GLY indicates glycyrrhizin.

and Mann–Whitney test for epinephrine doses before ROSC (skewed distribution using a Kolmogorov–Smirnov analysis). Other variables were compared among the different groups using a 2-way ANOVA for repeated measures, except for neurological and histological scores. The ANOVA considered time, group, and time–group interaction effects. If necessary, post hoc analyses were performed to assess the group effect at each time point using Holm Sidak's correction. Neurological dysfunction and histological scores were compared between groups using a nonparametric Kruskal–Wallis test. Significant differences were determined at $P \leq 0.05$. Statistical analyses were conducted using Prism 9.4.0 (GraphPad Software, San Diego, CA).

RESULTS

A total of 72 animals were included in the present study, that is, n=27 in the recovery study, n=25 for the acute evaluation of immune cells in the brain, n=8 in the acute study evaluating BBB permeability, and n=12 in the replication study with FPS-ZM1.

Glycyrryzhin Attenuates Shock After Cardiac Arrest

Twenty-seven animals were successfully included in the recovery study assessing the effect of glycyrrhizin (ie, n=7 in sham and n=10 in both the control and glycyrrhizin groups, respectively). In the control and glycyrrhizin groups, rabbits achieved ROSC after

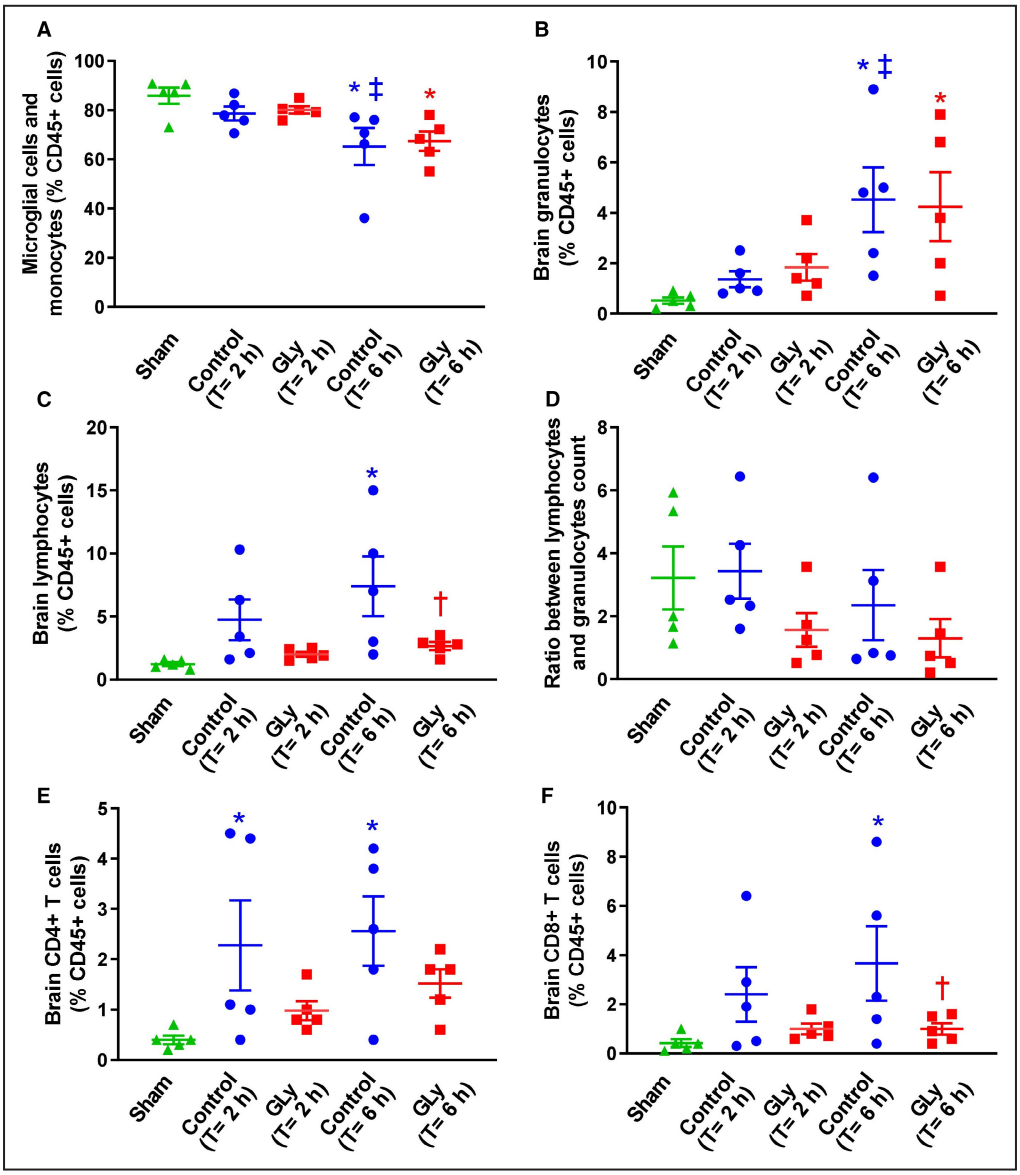

Figure 4. Neuroinflammation and brain immune cell infiltration.
Counts of inflammatory cells using "fluorescence-activated cell sorting" (FACS) in the brains of animals submitted to a sham procedure (no cardiac arrest) or to cardiac arrest with brain sampling 2 or 6 hours after resuscitation. In the latter case, animals received either saline (control groups) or glycyrrhizin (glycyrrhizin groups) after cardiac arrest. Five animals were enrolled in each condition. **A**, Percentage of microglial and monocytes among CD45$^+$ cells. They were considered as CD11b$^+$ and SSC_{low} cells during FACS analysis. **B**, Percentage of granulocytes among CD45$^+$ cells. They were considered as CD11$^+$ and high side scatter (SSC_{high}) cells. **C**, Percentage of lymphocytes among CD45$^+$ cells. **D**, Ratio between the number of lymphocytes and granulocytes in the brain. **E**, Percentage of CD4$^+$ cells among CD45$^+$ cells. **F**, Percentage of CD8$^+$ cells among CD45$^+$ cells. Data are shown as individual values and mean±SEM; *P<0.05 vs corresponding sham; †P<0.05 vs corresponding Control (T=6h); ‡P<0.05 vs corresponding Control (T=2h); n=5 in each of the 5 groups. GLY indicates glycyrrhizin.

a median time of 2.9 minutes (interquartile range, 2.0–5.0) and 2.6 minutes (2.0–3.6) after the onset of cardiopulmonary resuscitation, respectively. As illustrated by Figure 1B, the amount of epinephrine preventing hypotension was significantly lower in the glycyrrhizin group versus control after ROSC. Blood lactates were also reduced 360 minutes after CA in glycyrrhizin versus control (4.2±0.7 versus 9.3±1.0 mmol/L, respectively). This was associated with higher blood pH at 360 minutes after ROSC (Table), evidencing improved tissue perfusion and attenuated shock with glycyrrhizin.

Glycyrryzhin Attenuates Blood Humoral but Not Cellular Inflammatory Response

As illustrated in Figure 2A, HMGB1 blood levels increased rapidly after CA in both thre glycyrrhizin and control groups as compared with sham. This increase was dramatically reduced in glycyrrhizin versus control. The post- CA increase in interleukin-6 blood levels was also abolished in glycyrrhizin versus control. In the same line, the rise in interleukin-10 blood levels was prevented by glycyrrhizin at 24 hours after resuscitation. However, glycyrrhizin had no effect on the immune blood cell count as compared with control after CA (Figure 2B).

Glycyrryzhin Provides Robust Neuroprotection After Cardiac Arrest

As illustrated in Figure 3A, the blindly assessed neurological dysfunction score was attenuated in glycyrrhizin as compared with control (eg, median values of 51% [interquartile range, 10–76] versus 73% [65–81] at day 1 after CA, respectively). Importantly, the final survival rate, taking into account euthanasia for ethical purpose after day 1, achieved 60% versus 0% in glycyrrhizin versus control. This effect was corroborated by a significant reduction of brain damages assessed by fluorojade C staining (Figure 3B and 3C). Importantly, we also observed a strong decrease in RPN3/57+ leukocytes in the brains of glycyrrhizin versus control animals (Figure 3D and 3E), suggesting attenuated neuroinflammation.

Glycyrryzhin Reduces Early Cerebral Infiltration by T Cells

We further investigated the early immune cell migration into the brain after CA. Animals were submitted to a sham procedure or to CA with brain collection 2 or 6 hours after resuscitation for flow cytometry analyses (n=5 in each of the 5 groups). As shown in Figure 4A, a decrease in microglial cells and monocyte count was similarly observed in both the control and glycyrrhizin groups, as compared with sham. Brain granulocyte counts increased similarly in the control and glycyrrhizin groups as compared with sham (Figure 4B). Conversely, brain lymphocyte count increased in control versus sham but not in glycyrrhizin versus sham after CA (Figure 4C). The rise in brain lymphocyte-to-granulocyte ratio observed in control was also abolished by glycyrrhizin (Figure 4D). As illustrated in Figure 4E and 4F, the $CD4^+$ and $CD8^+$ T-cell subpopulation also demonstrated higher levels of infiltration in control versus glycyrrhizin and sham.

Glycyrryzhin Does Not Modify BBB Permeability After CA

BBB permeability was evaluated after Evans blue dye administration after CA in the control and glycyrrhizin groups (n=4 animals in each group). As illustrated in Figure 5A and 5B, the dye leakage was similar among groups, demonstrating a lack of effect of glycyrrhizin on BBB permeability.

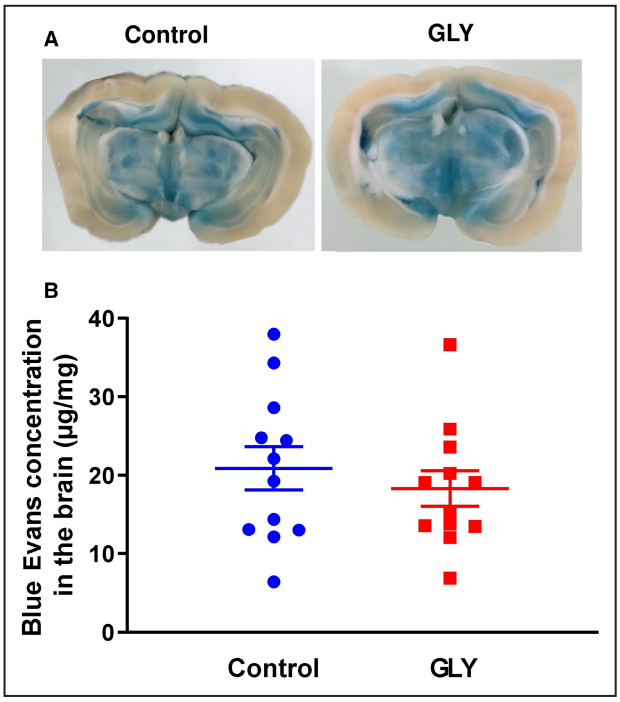

Figure 5. Assessment of blood–brain barrier integrity following cardiac arrest.
A, Morphological appearance of brain slices from 2 animals submitted to cardiac arrest and Evans blue dye administration for blood–brain barrier permeability evaluation after cardiac arrest. Animals received either saline (control, left panel) or glycyrrhizin (GLY, right panel) after cardiac arrest. Blue areas represent Evans blue vascular leakage. **B**, Mean values of the Evans blue cerebral concentrations in 4 animals of each group submitted to the above-mentioned procedure. Data are shown as individual values and mean±SEM; n=4 rabbits in each group with 3 samples for each rabbit. GLY indicates glycyrrhizin.

Blockade of RAGE With FPS-ZM1 Improves Neurological Outcome

We conducted a replicative study to investigate the effect of a direct blockade of RAGE by FPS-ZM1 to support the deleterious effect of HMGB1 with targets other than glycyrrhizin. Animals were submitted to CA with either vehicle or FPS-ZM1 administration after resuscitation (control and FPS-ZM1 groups, respectively; n=6 animals per group). As illustrated in Figure 6A and 6B, the neurological dysfunction score and the number of degenerating neurons were significantly reduced in FPS-ZM1 versus control, showing the neuroprotective effect of direct RAGE inhibition. Importantly, we also observed a decrease in RPN3/57+ leukocytes in the brain of FPS-ZM1 versus control animals (Figure 6C), despite the fact that it did not achieve statistical significance (P=0.09).

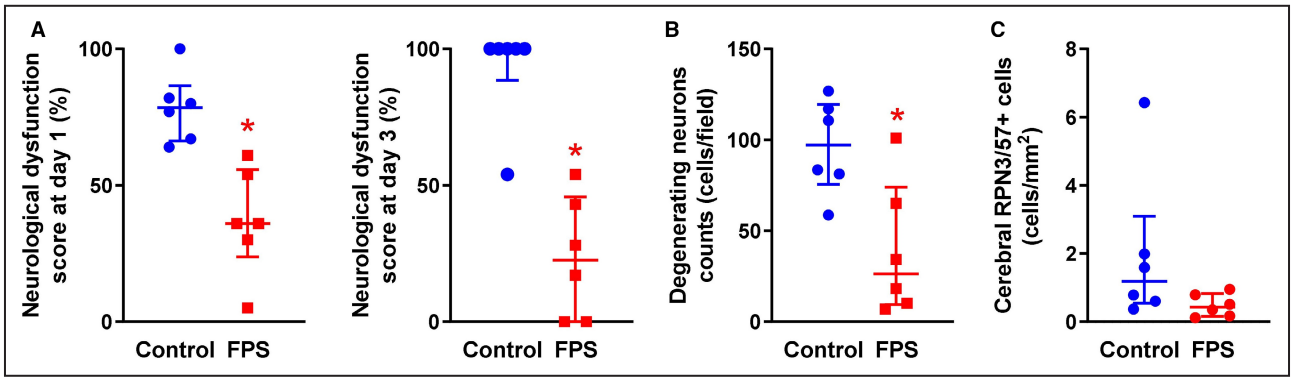

Figure 6. Neurological recovery in animals receiving an inhibitor of the receptor for advanced glycation end-product (FPS-ZM1) after cardiac arrest.

A, Neurological dysfunction at days 1 and 3 following resuscitation in the different groups (0% = lack of dysfunction; 100% = death). Open circles and lines represent individual values and mean of the group, respectively. The control group received saline after cardiac arrest. The FPS-ZM1 group received 1 mg/kg of FPS-ZM1 after cardiac arrest, as an inhibitor of the receptor for advanced glycation end-product. **B**, Mean number of degenerating neurons per histological field in the different groups, as defined by fluorojade C staining cells. **C**, Mean number of RPN3/57+ cells (neutrophils and T-cells) per mm^2 into the cerebral parenchyma in each group. Data are shown as individual values and median±interquartile range; *, $P<0.05$ vs corresponding control; n=6 in each group.

DISCUSSION

In the present study, we demonstrated that glycyrrhizin reduced neurological injury and brain inflammation with inhibition of T-cell chemoattraction after CA. This effect was associated with reduced HMGB1, interleukin-6, and interleukin-10 blood levels, while blood cell count or BBB permeability was not modified by glycyrrhizin. The direct blockade of the HMGB1 receptor RAGE recapitulated the effects of glycyrrhizin by also showing an improved neurological outcome.

To our knowledge, the detrimental effect of HMGB1 is yet poorly understood after CA, as compared with focal ischemia–reperfusion and stroke.[5] Here, we observed a rapid increase in HMGB1 blood levels after resuscitation with peak concentrations starting as early as 0.5 hours after resuscitation and sustained elevation during 24 hours. These results are in agreement with a previous study showing a quick increase in HMGB1 blood level after resuscitation in patients.[19] In contrast, HMGB1 blood levels rose more gradually after regional myocardial ischemia or stroke with maximal concentration after 24 hours.[20] The kinetic of HMGB1 blood release is therefore likely different after CA versus stroke or myocardial ischemia. Glycyrrhizin prevented this release early after CA, suggesting that only a minor part of HMGB1 is passively released by the initial cell death after ischemia, while a secondary active secretion occurs massively and rapidly.[21] Glycyrrhizin could then blunt a sort of "HMGB1 perpetuation circle" by immune cells, as also supported by the dramatic decrease in interleukin-6 blood levels with glycyrrhizin. It is consistent with the speculated action mechanism of glycyrrhizin on HGMB1, that is, the direct chelation with subsequent inhibition of secondary release and immune response stimulation.[12,13]

Importantly, the effect of glycyrrhizin on neurological recovery and cerebral infiltration by immune cells was not associated with any mitigation in blood immune cell count. For instance, blood granulocyte increase, which is a well-known part of the sepsis-like syndrome after CA, was not modified. More importantly, glycyrrhizin did not modify BBB permeability, which could have indirectly explained the reduction in cerebral leukocyte infiltration. It is another important difference with stroke in which HMGB1 contributes to BBB alteration through matrix metallopeptidase 9 activation,[22] which breaks down the extracellular matrix. One could then speculate that glycyrrhizin attenuates HMGB-1–induced neuroinflammation through cerebral chemoattraction inhibition rather than a systemic blunting of cellular immune response or BBB preservation. The chemoattractive properties of HMGB1 are indeed well known in other conditions, especially for the fully reduced form of HMGB1, which is directly released by necrotic cells. Conversely, one would argue that glycyrrhizin rather modifies the time course of BBB permeability since we evaluated Evans blue penetration only during the first 2 hours following resuscitation. The latter technique could also be considered as nonoptimal for BBB evaluation. However, leukocyte infiltration was already evidenced 2 hours after CA in the same conditions, again suggesting that glycyrrhizin's effect on cerebral infiltration by immune cells is not related to BBB permeability modification.

Interestingly, HMGB1 also exerted specific effects on lymphocyte cerebral infiltration, while cerebral migration of granulocytes was not attenuated by glycyrrhizin. In stroke models, glycyrrhizin also directly

reduced cerebral infiltration by T cells.[23] This effect was lost in T- or B-cell–deficient severe combined immunodeficiency mice, while restoring the T-cell population rescued the protective effect of glycyrrhizin on cerebral infarction.[23] Conversely, activation of T cells by HMGB1 was directly shown in cultured splenocytes.[23] Therefore, T-cell reduction seems to be a determinant for the neuroprotective effect of glycyrrhizin after both stroke and CA. In nonischemic conditions, HMGB1 also stimulates T-cell activation and polarization to the T-helper 1 phenotype, enhancing the secretion of interleukin-2/interferon-γ and reducing that of interleukin-10, respectively.[24] In our conditions of CA, the effect of glycyrrhizin is then likely attributable to the early blunting of HMGB1 production, which can inhibit all the secondary maladaptive cerebral immune response. We can also hypothesize that the suppression of T-cell infiltration at the early phase following CA (ie, 2 or 6 hours) could contribute to attenuating the brain damage via suppression of other immune cells at later stages. The role of other immune cells, including dendritic cells, monocytes, and macrophages, are indeed known on the longer terms, for example, after 3 days in mice submitted to experimental CA.[25] It opens promising perspective for anti-inflammatory strategies targeting HMGB1 pathways if delivered during the right window after CA to mitigate neuroinflammation. The replication study with FPS-ZM1 further suggests that RAGE direct inhibition could recapitulate the effect of glycyrrhizin and further supports our conclusions.

Our study presents several limitations. First, we did not evaluate other important cytokines (eg, interleukin-1α, interleukin-8, interleukin-18) because of the lack of appropriate antibodies for ELISA techniques in rabbits. For the same reasons, we could not obtain relevant data for cerebral cytokine concentration. However, the relevance of the rabbit model, as compared with rodent models, is that the size of the animal allows a better hemodynamic and respiratory management with blood gas and hemodynamic follow-up. It is of importance since, for example, blood pressure targets or alteration in blood O_2 and CO_2 partial pressures dramatically modify the ultimate outcome per se after CA. Second, glycyrrhizin is sometimes considered as a hypertensive drug because of mineralocorticoid receptor activation.[16] However, this effect appears only during chronic exposure.[16] We also confirmed the lack of proper hemodynamic effect of glycyrrhizin in preliminary experiments in sham animals (Table S2). Third, we evaluated only T cells but not B cells in the brain, which would have also been relevant. The rationale was the well-known link between HMGB1 and T cells in other conditions, as well as the deleterious effect of T cells in stroke models. Fourth, we did not perform immune cell counting through fluorescence-activated cell sorting but only immunohistochemistry for the replication study with FPS-ZM1, as we focused only on the ultimate neurological outcome for this study. Finally, we used only male rabbits in the present study, which does not allow evaluation of a potential gender effect.

In conclusion, we demonstrated that glycyrrhizin improved neurological outcome after CA in rabbits. Glycyrrhizin reduced HMGB1 secretion, secondary to its initial release by dying cells after resuscitation. It further blunted the chemoattractive properties of HMGB1 and reduced the cerebral infiltration by T cells, as major promotors of neurological sequelae after ischemia–reperfusion. This could open promising perspectives for the administration of glycyrrhizin in patients with CA if administered early enough after resuscitation.

ARTICLE INFORMATION

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Disclosures

Drs Tissier and Kohlhauer are stakeholders of a company dedicated to liquid ventilation in critical care conditions (not related to the present work). The remaining authors have no disclosures to report.

Supplemental Material

Data S1
Tables S1–S2
Figures S1–S2

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Supplemental Material

Data S1.

Supplemental Methods

Fluorescence-activated cell sorting from blood samples

Blood samples were sampled at different time points in the different groups. Erythrocytes were lysed for 15 min, and the debris were washed away, in accordance to the manufacturer recommendations (RBC Lysing Buffer, Tebubio, Le Perray-en-Yvelines, France). Cells were then fixed in 0.5% paraformaldehyde/PBS for overnight conservation. Then, cells were incubated with Fcblock, and stained using the following monoclonal antibodies: allophycocyanin (APC)-labelled anti-T-cells marker (KEN5, 5 µL, Santa Cruz Biotechnology, Dallas, TX, USA), phycoerythrin (PE)-labelled anti-CD4 (0.75 µL, Lifespan Biosciences, Seattle, USA), fluorescein isothiocyanate (FITC)-labelled anti-CD8 (5 µL, Lifespan Biosciences, Seattle, USA), APC-labelled anti-CD11b (clone M1/70, 1 µL, EXBIO praha, Vestec, Czechoslovakia), APC-labelled anti-CD79a (clone HM57, 5 µL, EXBIO praha, Vestec, Czechoslovakia). To perform surface antigen staining, cell suspensions were incubated on ice with appropriate mix of antibodies for 30 min. Then, cells were washed three times with a phosphate buffered saline solution containing bovine serum albumin (0.5%) before resuspension in running buffer for flow cytometry. Intracellular staining with CD79-a, for B-lymphocytes identification, was performed with Thermofisher Fix & Perm cell permeabilization kit ® (Waltham, Massachusetts, USA), according to the manufacturer's instructions. Four-color flow cytometry was conducted with the BD Biosciences AccuriC6 device. Gating strategy is illustrated in Supplemental Figure 1.

Fluorescence-activated cell sorting from cerebral samples

After animal euthanasia, the brain was withdrawn and an hemisphere was mechanically dissociated by chopping the tissue with a razor blade. Then the tissue was mixed with a digestion buffer (mix of collagenase I-A at 1mg/ml and DNase I at 100 U/ml) and the resulting cell suspension was incubated during 45 min at 37°C, under slow continuous rotation. The

suspension was then filtered through a 40 µm cell strainer and washed with 15 ml of a HBSS solution containing 3% of fetal bovine serum and 100 U/ml of DNase I. Microglia and leucocytes were isolated thanks to a density gradient centrifugation with Percoll. For that, the cell pellet was resuspended in 10 ml of 80% SIP and over layered with 10 ml of 38% SIP, followed by another 10 ml of 21% SIP. The gradient was finally covered with 5 ml of HBSS containing 3% FBS. The gradient was centrifuged at 480xg for 30 min at room temperature. The interphase containing the cells of interest was then collected into a new tube and washed twice with 30 ml o HBSS containing 3% of FBS. The resulting cell pellet was dispatched into fresh tubes and resuspended in an appropriate amount of FACS Buffer (BioRad, Hercules, CA, USA) for antibody labelling. Prior to antibody adjunction, cells were incubated with Fcblock at 4°C for 5 min. Then, cells were incubated on ice with the appropriate mix of antibody during 30 min. The monoclonal antibody panels for brain immune cells identification used in our study consisted of: the pan-leukocyte marker anti-CD45 labelled with FITC, APC-labelled anti-T-cells marker (KEN5, 5 µL, Santa Cruz Biotechnology, Dallas, TX, USA), PE-labelled anti-CD4 (0.75 µL, Lifespan Biosciences, Seattle, USA), PE-labelled anti-CD8 (1 µL), and APC-labelled anti-CD11b (clone M1/70, 1 µL, EXBIO praha, Vestec, Czechoslovakia). Microglial and monocytes were considered as CD11b+ and SSC_{low} cells during FACS analysis. Granulocytes were considered as $CD11^{+}$ and SCC_{high} cells. T cells were classified as CD4+ or CD8+.

Table S1. Rabbit neurological deficit grading scale.

	Score
Level of consciousness	
Normal	0
Clouded	5
Stuporous	10
Comatose	25
Maximum score	*(25)*
Breathing	
Normal	0
Abnormal	5
Maximum score	*(5)*
Lack of cranial nerves reflexes	
Vision	1
Light reflex	1
Oculocephalic	1
Corneal	1
Facial sensation	1
Auditory	1
Gag reflex	1
Maximum possible score	*(7)*
Motor and sensory function	
Lack of flexor response to pain (Front)	2
Lack of flexor response to pain (Rear)	2
Lack of righting reflex	10
Maximum possible score	*(14)*
Gait	
Normal	0
Minimal ataxia	5
Moderate ataxia	10
Able to stand	15
Unable to stand	20
No purposeful movement	25
Maximum possible score	*(25)*
Behavior	
Lack of grooming	4
Lack of eating/drinking	10
Lack of exploration	10
Maximum score	*(24)*
Maximum possible score (total)	**100**

Table S2. Preliminary results.

Effect of glycyrrhizin (4 mg/kg i.v.) in four anesthetized rabbits compared to four control animals receiving saline. Rabbits were anesthetized and instrumented as described in the manuscript. They did not undergo any cardiac arrest, since the goal was to evaluate a potential effect of a single-administration of glycyrrhizin.

	Baseline	Time after glycyrrhizin or saline administration (min)			
		30 min	60 min	180 min	360 min
Heart rate (beats/min)					
Control (saline)	255±33	257±31	261±23	240±20	262±15
Glycyrrhizin (4 mg/kg i.v.)	235±19	221±27	214±34*	240±25	268±24
Mean blood pressure (mmHg)					
Control (saline)	56±8	61±7	63±4	62±11	70±3
Glycyrrhizin (4 mg/kg i.v.)	57±5	68±19	74±3	63±13	77±8
Rectal temperature (°C)					
Control (saline)	38.6±0.8	38.7±0.6	38.7±0.6	38.2±0.7	38.3±0.4
Glycyrrhizin (4 mg/kg i.v.)	38.7±0.2	38.7±0.3	38.6±0.2	38.2±0.1	38.4±0.3
Blood pH					
Control (saline)	7.37±0.01	7.37±0.01	7.37±0.01	7.39±0.02	7.37±0.01
Glycyrrhizin (4 mg/kg i.v.)	7.44±0.07	7.44±0.08	7.42±0.06	7.31±0.06	7.44±0.07
Blood glucose (g/L)					
Control (saline)	1.8±0.2	1.7±0.2	1.6±0.1	1.7±0.1	1.8±0.2
Glycyrrhizin (4 mg/kg i.v.)	2.2±0.3	1.8±0.7	1.4±0.1	1.7±0.5	2.2±0.3

Figure S1. Gating strategy of the Fluorescence-activated cell sorting from blood samples.

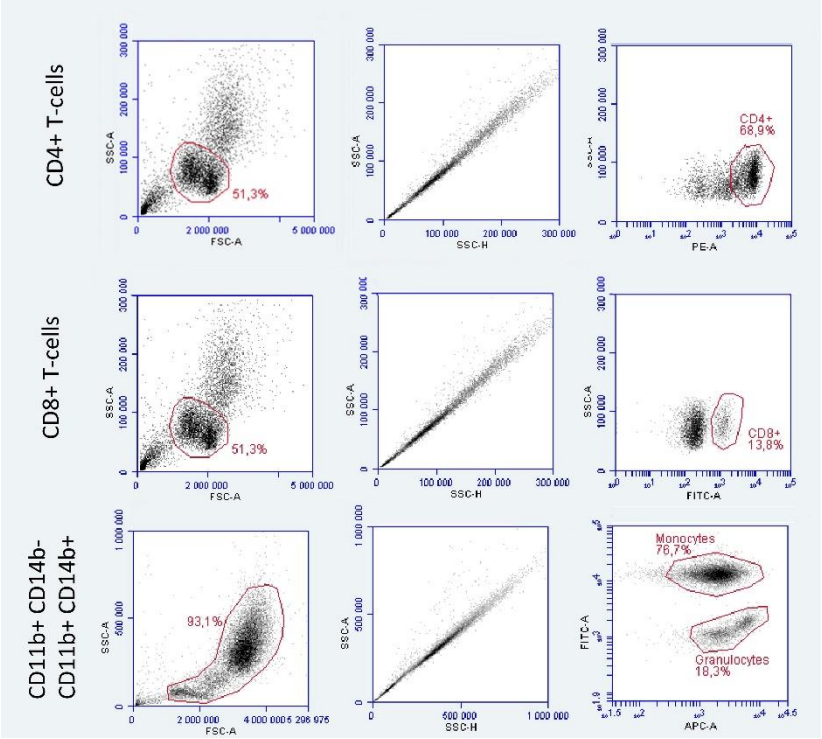

Figure S2. Gating strategy of the Fluorescence-activated cell sorting from brain samples.