Effect of Splenic Regulatory T-cell Apoptosis on the Postresuscitation Immune Dysfunction in a Porcine Model

Wei Gu1,2, Qian Zhang1, Chun-Sheng Li1,2
1Department of Emergency, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China
2Beijing Key Laboratory of Cardiopulmonary Cerebral Resuscitation, Beijing 100020, China

Abstract

Background: Postresuscitation immune dysfunction contributes to the low survival rate after successful resuscitation, but its mechanism remains poorly understood. The purpose of this study was to investigate whether splenic regulatory T-cell (Treg) apoptosis was involved in the postresuscitation immune dysfunction.

Methods: Thirty-eight pigs were randomly divided into sham-operated group (SHAM group, n = 8), 12 h post return of spontaneous circulation (ROSC) group, 24 h post-ROSC group, and 48 h post-ROSC group (n = 10 per group). A Wuzhishan miniature porcine model of 8-min ventricular fibrillation cardiac arrest (CA) was established. The apoptosis rates of Treg in the spleen were tested by flow cytometry; the expressions of forkhead/winged helix transcription factor (Foxp3) of Treg in the spleen were detected by real-time polymerase chain reaction; and the levels of interleukin-4 (IL-4), IL-10, and interferon gamma (IFN-γ) of Treg in the spleen were detected by enzyme-linked immunosorbent assay.

Results: The apoptosis rates of Treg in all post-ROSC groups were significantly lower than that of SHAM group (7.7% ± 1.9%, 7.1% ± 1.8%, 6.2% ± 0.4% vs. 13.1% ± 1.6%; P < 0.05); the expression levels of Foxp3 and IL-10 were also decreased with the increase of apoptosis rates of Treg. Helper T-cells CD4+ lymphocyte subsets were significantly lower in the post-ROSC groups compared with SHAM group (29.1% ± 2.2%, 24.3% ± 2.2%, 24.1% ± 2.5% vs. 43.8% ± 4.5%; P < 0.01) at 12, 24, and 48 h after ROSC. Compared with SHAM group, the levels of IFN-γ (161.0 ± 12.9, 167.7 ± 10.5, 191.2 ± 7.7 vs. 76.0 ± 9.4 ng/L) and IL-4 (27.7 ± 6.2, 35.9 ± 3.5, 50.6 ± 6.1 vs. 13.3 ± 2.3 ng/L) and the ratio of IFN-γ/IL-4 (8.6 ± 2.3, 4.9 ± 0.4, 4.5 ± 0.9 vs. 0.8 ± 0.2) were all greatly elevated in all post-ROSC groups (P < 0.05).

Conclusions: Apoptosis rate of Treg was significantly decreased after CA, and thus the proportion of Treg was increased and the inhibitory effects were enhanced, which further led to the decrease of the amount of CD4+ T-cells. In addition, the T helper type 2/T helper type 1 (Th2/Th1) cell drift of Treg in the spleen caused postresuscitation immune dysfunction.

Key words: Apoptosis; Cardiac Arrest; Foxp3; Immunity

Original Article

Introduction

Over the past 50 years, the rates of return of spontaneous circulation (ROSC) and survival after cardiopulmonary resuscitation (CPR) remain disappointing. In the United States, the survival rate until hospital admission after ROSC was 26.3% between 2005 and 2010 and only 9.6% of patients can be discharged from hospital.1,2 ROSC after cardiac arrest (CA) is a long unnatural pathophysiological state with complete and systemic ischemia that the whole body experiences, which often cause severe multiple organ failures.

During CPR and after ROSC, the body has experienced serious stress reaction. Not only the nerves, endocrine, and vasoactive substances are changed but also large amount of cytokines and inflammatory mediators are generated in response to the intense pathological stimuli, leading to systemic inflammatory response syndrome.3 Activation of neutrophils and macrophages occurs when sepsis is experienced a variety of inflammatory mediators such as interleukin-6 (IL-6), and tumor necrosis factor-α is produced; at the same time, massive amount of inflammatory factors, such as interferon gamma (IFN-γ) and IL-2, are released, causing the extreme disorder of immunity.
studies have found that ROSC patients often show “sepsis-like” changes, i.e., systemic inflammatory response and serious secondary infections. Our recent animal studies also revealed that in the early period after ROSC, the body experiences both severe immune suppression and excessive activation of inflammatory response and leads to postresuscitation immune inhibition, which is characterized by a shift from the anti-inflammatory (T helper type 2 [Th2]) state to the proinflammatory (T helper type 1 [Th1]) state.

Regulatory T-cell (Treg) is a group of naturally occurring regulatory/inhibitory T-cells in the body, with negative immunological regulation effects, which are characterized by immune suppression. In case of severe infections, the number of Treg increases substantially after being activated by the T-cell receptor signals, and through direct contact inhibits of activation and proliferation of CD4+ (helper T-cells) and CD8+ (cytotoxic T-cells), Treg can cause the cellular immune function impairment and lead to serious damage to tissues and organs ultimately. After ROSC, the body has experienced the most severe shock with an interrupted transportation of oxygen and metabolites. The accumulated oxygen debt leads to endothelial activation and systemic inflammation and is predictive of subsequent disorder of immune function and multiple organ failures. As an important regulatory T lymphocyte in immune regulation, Treg plays an important role in the regulation of immune function disorders and depression. However, the mechanism whether Treg is involved in the postresuscitation immunosuppression through the inhibition of CD4+ T-cells remains unclear. Our study was designed to confirm whether the effects of Treg apoptosis on the proliferation and cell drift of Th cells are involved in the postresuscitation immune dysfunction.

Methods

Animals and ethical approval

Thirty-eight inbred Wuzhishan miniature pigs (12–14 months of age, 30 ± 2 kg) purchased from the Chinese Academy of Agricultural Sciences (license number: SYXX [Beijing] 2008-050109) were used in this study. This study was carried out in strict accordance with the guideline for animal care and use established by the Capital Medical University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of Capital Medical University (Permit Number: 2010-D-013).

Animal preparation

Thirty-eight pigs were randomized into four groups through the stochastic indicator method: 12 h post-ROSC group (n = 10), 24 h post-ROSC group (n = 10), 48 h post-ROSC group (n = 10), and sham-operated group (SHAM group, n = 8). The same procedures except CA initiation were achieved in SHAM group, including induction of anesthesia, electrode positioning, mechanical respiration, and monitoring of physiological parameters. The animals were fasted overnight but had free access to water. Anesthesia was induced by intramuscular injection of midazolam and ketamine (0.5 mg/kg), following by an injection of propofol (1.0 mg/kg) into the ear vein, and an injection of pentobarbital (8 mg·kg⁻¹·h⁻¹) to maintain anesthesia. Animals were mechanically ventilated by a volume-controlled ventilator with a tidal volume of 8 ml/kg and FiO₂ of 21%, and ventilation rate of 12–20 breaths/min. End-tidal PCO₂ was monitored with an in-line infrared capnograph placed in the airway. Ventilation rate and tidal volume were adjusted to maintain normocapnia (35–45 mmHg, 1 mmHg = 0.133 kPa) by adjusting respiratory frequency. Standard lead II of the surface electrocardiograph was monitored continuously throughout the study. The left femoral artery was isolated by a layer, and a Swan-Ganz catheter (7F; Edwards Lifesciences, Irvine, CA, USA) was advanced from the left femoral vein and flow-directed into the pulmonary artery to measure cardiac output (CO). The right femoral artery was dissected to insert an angiographic catheter (5F; Terumo, Tokyo, Japan) into the aortic arch to measure mean aortic pressure (MAP). A 5 F pacing catheter was advanced from the right internal jugular vein into the right ventricle to induce ventricular fibrillation (VF). The temporary pacemaker conductor (PACEL™ Right Heart Curve, St. Jude Medical Inc., Sylmar, CA, USA) was connected into the right ventricle through the right sheathing canal and connected to an electrical stimulator (GY-600A; Kaifeng Huanan Equipment Co., Ltd., Kaifeng, China) programmed in the S1S2 mode (300/200 ms), 40 V, 8:1 proportion, and 10-ms step length to provide a continuous electrical stimulus until VF. VF was defined as an electrocardiogram showing waveforms corresponding to VF and a rapid decline in MAP toward zero. Ventilation was stopped while inducing VF. After 8 min of VF, manual CPR was carried out at a frequency of 100 compressions/min with mechanical ventilation at a FiO₂ of 100% and a compression-to-ventilation ratio of 30:2. The quality of chest compressions was controlled by a HeartStart MRx Monitor/Defibrillator with Q-CPR (Philips Medical Systems, Best, Holland, The Netherlands). After 2 min CPR, pigs were received central venous injection of epinephrine (0.02 mg/kg). ROSC was defined as 10 consecutive min of maintenance of systolic blood pressure at 50 mmHg. At the end of 30 min of CPR, animals remaining in VF, pulseless electrical activity or asystole were considered resuscitation failures. After successful resuscitation, mechanical ventilation was resumed with the same setting before the induction of VF. During CPR period, CPR time, defibrillation times, and defibrillation energy were recorded. Pigs were supplied by saline infusion, 5% glucose, and sodium chloride injection after operation according to postoperative central venous pressure and urine volume. At 6 h after ROSC, ventilator was removed and the pigs were placed in the feeding cage. Hemodynamic and oxygen metabolism parameters, including CO, MAP, oxygen delivery (DO₂), and oxygen consumption (VO₂), were measured and calculated continuously, and we recorded...
the values at baseline, 30 min, 2 h, and 6 h after ROSC. Animals in the post-ROSC groups were randomly sacrificed with intravenous injection of 10 ml of 10 mol/L potassium chloride intravenously at 12, 24, and 48 h after ROSC. Animals in SHAM group were sacrificed by the same method at 12 h after ROSC.

Detection of spleen CD4+ lymphocyte subsets by flow cytometry
Spleen was isolated and ground with a grinding rod steriley. Lymphocyte separation solution was added in a 60 mm Petri dish and covered with a 200 mesh gauze. The spleen was placed on it and ground to separate spleen cells. Isolated cells were collected and counted. Cells were washed with 1-ml fluorescence solution (0.15 mol/L phosphate buffer solution [PBS] containing 2% nonbinding surface, pH 7.4) twice, with centrifugation at 1000 round/min for 5 min after each wash. Two microliters PE/cy5-labeled monoclonal antibodies against CD4 and CD8 were added to one sample tube for each sample after the supernatant was discarded. It was mixed thoroughly and kept in a dark place for 30 min. It was washed with 1 ml fluorescence solution twice, and resuspended the cell pellets in 500 μl fluorescence storage solution (0.15 mol/L PBS, containing 2% glucose, 1% formaldehyde, 0.1% NaNO₃, and pH 7.4). The samples were subjected to flow cytometry (BD Biosciences, San Jose, CA, USA), the percentages of CD4+ and CD8+ were determined, and the ratio of CD4+/CD8+ were calculated.

Isolation and culture of splenic regulatory T-cell
Isolation by magnetic-activated cell sorting (MACS): spleen was dissected and cut into pieces. After grinding, the homogenate was filtered through a 400 mesh sieve. Cell suspension was centrifuged, and lymphocyte separation solution was then added to the cell pellets. After centrifugation, the white middle layer of cells was taken. PE-anti-CD25 and PE magnetic beads were added and washed twice with buffer. Then, anti-mouse IgG1-fluorescein isothiocyanate (FITC) isomer was labeled. After washed twice with buffer, anti-FITC beads were labeled. The mixture was loaded to mass spectrometry column, and CD25+ T-cells were harvested by positive selection and CD25+ T-cells with negative selection. The purity of double positive cells was detected by flow cytometry (BD Biosciences, San Jose, CA, USA), and antisense 5'-AGCGGATAGGGTGCGAT-3' and forward primer: 5'-GGTCGGAGTGAACGGATT-3' and Reverse primer: 5'-ATTTGATTTGGCGGGTA-3'. Total RNA was extracted from 75 to 150 mg of splenic tissue according to the protocol described for the BioEasy SYBR Green I Real-Time PCR Kit Manual (Bori Technology Co., Ltd., China). The reaction system components of real-time PCR were 12.5 μl, 2X SYBR Mix, 1 μl PCR forward primer (10 μmol/L), 1 μl PCR reverse primer (10 μmol/L). Preincubation was performed at 94°C for 5 min, followed by amplification in 30 cycles at 94°C for 45 s, 56°C (Foxp3), 72°C for 65 s, and finally, during slow heating up, 72°C for 10 min. After the amplification, melting curve analysis with a temperature gradient from 65°C to 95°C was recorded every 0.5°C (hold for 5 s). The optical density of the target genes was compared with that of GAPDH. Relative quantification is generally calculated with the 2-ΔΔCt formula by the comparative Ct method, the copy number of the target gene 2-ΔΔCt = 2-[(ΔCt target gene - ΔCt GAPDH gene)].

Quantitative real-time polymerase chain reaction assay for Foxp3 of splenic regulatory T-cell
Two milliliters of each IL-4 and IFN-γ samples were obtained from the supernatants of CD4+ CD25- Treg and CD4+ CD25+ T-cells cocultured for 68 h. Two milliliters of IL-10 sample were obtained from the supernatant of isolated Treg cells cultured for 12 h. The supernatants were centrifuged at 35,000 × g for 10 min at 4°C, and supernatants were collected and immediately frozen at −80°C for enzyme-linked immunosororbent assay (ELISA). IL-4 (Goat Anti-Pig, 6R019) and IFN-γ (Goat Anti-Pig, 6R082) were measured by ELISA according to the manufacturers’ instructions (Sunbio Biotech Co. Ltd., Beijing, China). The absorbance at 450 nm was measured after 30 min using an ELISA plate reader. Duplicate readings were obtained for all samples and the means were calculated.

T helper type 1- and T helper type 2-related cytokines analyses
Two milliliters of each IL-4 and IFN-γ samples were obtained from the supernatants of CD4+ CD25- Treg and CD4+ CD25+ T-cells cocultured for 68 h. Two milliliters of IL-10 sample were obtained from the supernatant of isolated Treg cells cultured for 12 h. The supernatants were centrifuged at 35,000 × g for 10 min at 4°C, and supernatants were collected and immediately frozen at −80°C for enzyme-linked immunosorbent assay (ELISA). IL-4 (Goat Anti-Pig, 6R019) and IFN-γ (Goat Anti-Pig, 6R082) were measured by ELISA according to the manufacturers’ instructions (Sunbio Biotech Co. Ltd., Beijing, China). The absorbance at 450 nm was measured after 30 min using an ELISA plate reader. Duplicate readings were obtained for all samples and the means were calculated.

Statistical analysis
The experimental data were analyzed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± standard deviation (SD), and Student’s t-test and one-way analysis of variance (ANOVA) were used for comparisons between two groups. Correlation studies were
calculated by Pearson correlation coefficient. A two-tailed value of $P < 0.05$ was considered statistically significant.

**RESULTS**

Six of the thirty pigs died in the post-ROSC groups during CPR (two in each group). The HR, MAP, CO, DO2, and VO2 were not statistically different at baseline, 0.5, 2, and 6 h after ROSC among the post-ROSC groups ($P = 0.483, P = 0.508, P = 0.808, P = 0.232, and P = 0.329$, respectively) [Table 1]. Furthermore, the number of shocks, total defibrillation energy, and CPR time were not statistically different among the post-ROSC groups ($P = 0.680, P = 0.702,$ and $P = 0.939$, respectively) [Table 2].

**Percentage of CD4+ lymphocyte subsets in the spleen**

The percentage of CD4+ lymphocyte subsets in the spleen was significantly lower in post-ROSC group (29.1% ± 2.2%, 24.3% ± 2.2%, and 24.1% ± 2.5%) compared with SHAM group (43.8% ± 4.5%) at 12, 24, and 48 h post-ROSC ($P = 0.009, P = 0.003,$ and $P = 0.002$, respectively). The CD4+/CD8− was also significantly lower in post-ROSC group (2.3 ± 0.7, 2.1 ± 0.6, 2.1 ± 0.4) compared with SHAM group (2.9 ± 0.8) at 12, 24, and 48 h post-ROSC ($P = 0.008, P = 0.003, P = 0.003$) [Table 2].

**Purity of CD4+ CD25+ regulatory T-cell**

The mononuclear cells of spleen were separated after two times of MACS; average purity of CD4+ CD25+ Treg and CD4+ CD25− Treg were 93.2% ± 3.9% and 89.3% ± 3.1%, respectively.

**Apoptosis rate, expression of Foxp3, and level of interleukin-10 of regulatory T-cell in the spleen**

The apoptosis rate of Treg in the spleen was markedly decreased in post-ROSC groups (7.7% ± 1.9%, 7.1% ± 1.8%, and 6.2% ± 0.4%) compared with SHAM group (13.1% ± 1.6%) at 12, 24, and 48 h post-ROSC ($P = 0.032, P = 0.047,$ and $P = 0.078$, respectively). Compared with SHAM group (23.0% ± 2.7%), the expression of Foxp3 of Treg in the spleen significantly increased in the 12, 24, and 48 h post-ROSC groups (47.2% ± 5.1%, 49.1% ± 5.8%, and 57.1% ± 3.3%) ($P = 0.003, P = 0.002,$ and $P = 0.001$, respectively); the level of IL-10 in the 12, 24, and 48 h post-ROSC groups (114.3 ± 14.1, 162.4 ± 13.8, and 147.6 ± 29.6 ng/L) were markedly increased compared to that of SHAM group (25.5 ± 5.0 ng/L) ($P = 0.002, P = 0.001,$ and $P = 0.006$, respectively) [Table 3]. Correlation analysis: the expression of Foxp3, level of IL-10, and apoptosis rate of Treg are negatively correlated (the correlation coefficient $r = -0.83, P = 0.04; r = -0.71, P = 0.01$, respectively).

**Levels of interferon gamma, interleukin-4, and interferon gamma/interleukin-4 of regulatory T-cell in the spleen**

The results showed that the level IFN-γ of Treg in the spleen was markedly increased in post-ROSC groups compared with SHAM group at 12, 24, and 48 h post-ROSC (161.0 ± 12.9, 167.7 ± 10.5, and 191.2 ± 7.7 vs. 7.6 ± 0.9 ng/L) ($P = 0.001, P = 0.001,$ and $P = 0.001$, respectively); the level IL-4 was markedly increased in the post-ROSC groups compared with SHAM group at 12, 24, and 48 h post-ROSC (27.7 ± 6.2, 35.9 ± 3.5, and 50.6 ± 6.1 vs. 13.3 ± 2.3 ng/L) ($P = 0.001, P = 0.004,$ and $P = 0.002$, respectively). Furthermore, IFN-γ/IL-4 was markedly increased in the post-ROSC groups compared with SHAM group at 12, 24, and 48 h post-ROSC (8.6 ± 2.3, 4.9 ± 0.4, and 4.5 ± 0.9 vs. 8.8 ± 0.2) ($P = 0.014, P = 0.003,$ and $P = 0.004$, respectively) [Figure 1].

**DISCUSSION**

In our study, the activation of Foxp3 was detected in piglet spleen after ROSC, which led to the decrease of Treg

**Table 1:** Hemodynamic and oxygen metabolism characteristics of the post-ROSC groups at baseline, 0.5, 2, and 6 h after ROSC ($n = 8$)

| Groups        | HR (beat/min) | MAP (mmHg) | CO (L/min) | DO2 (ml/min) | VO2 (ml/min) |
|---------------|---------------|------------|------------|--------------|--------------|
| 12 h post-ROSC| 84.8 ± 4.9    | 103.3 ± 8.0| 2.8 ± 0.5  | 422.6 ± 9.5  | 114.1 ± 3.2  |
| Baseline      | 125.1 ± 9.2   | 112.7 ± 6.6| 2.5 ± 0.5  | 388.8 ± 8.3  | 101.3 ± 7.0  |
| ROSC 0.5 h    | 119.6 ± 7.1   | 106.2 ± 7.9| 2.3 ± 0.1  | 259.1 ± 7.3  | 68.5 ± 2.1   |
| ROSC 2 h      | 110.0 ± 8.4   | 95.6 ± 8.3 | 2.2 ± 0.4  | 271.3 ± 3.2  | 81.5 ± 2.2   |
| ROSC 6 h      | 79.5 ± 3.6    | 107.8 ± 8.5| 2.7 ± 0.3  | 417.6 ± 6.7  | 116.0 ± 3.3  |
| 24 h post-ROSC| 121.7 ± 8.2   | 99.6 ± 7.1 | 2.4 ± 0.3  | 367.8 ± 3.3  | 98.7 ± 5.9   |
| Baseline      | 115.7 ± 9.2   | 104.8 ± 7.1| 2.3 ± 0.5  | 251.7 ± 4.3  | 72.5 ± 2.1   |
| ROSC 0.5 h    | 105.0 ± 8.4   | 110.7 ± 5.2| 2.0 ± 0.4  | 262.3 ± 9.2  | 79.5 ± 3.2   |
| ROSC 2 h      | 83.1 ± 5.1    | 105.6 ± 11.0| 2.6 ± 0.5  | 429.8 ± 7.1  | 115.8 ± 6.9  |
| ROSC 6 h      | 129.1 ± 8.5   | 120.1 ± 7.6| 2.4 ± 0.2  | 387.1 ± 10.1 | 102.9 ± 5.2  |
| Baseline      | 126.6 ± 7.9   | 116.8 ± 6.9| 2.3 ± 0.3  | 253.6 ± 6.7  | 73.5 ± 3.9   |
| ROSC 0.5 h    | 108.3 ± 7.4   | 105.6 ± 5.4| 2.1 ± 0.4  | 269.6 ± 4.3  | 85.5 ± 4.3   |

Data are reported as mean ± SD. ROSC: Restoration of spontaneous circulation; HR: Heart rate; MAP: Mean aortic pressure; CO: Cardiac output; DO2: Oxygen delivery; VO2: Oxygen consumption; SD: Standard deviation. No significant differences were found among the post-ROSC groups at baseline, 0.5, 2, and 6 h after ROSC.
apoptosis in the spleen and the increase of Treg number. Meanwhile, expressions of the inflammatory factor IFN-γ, as the main representative of Th1 cell response, and the anti-inflammatory factor IL-4, as the main representative of Th2 cell response, were both significantly increased. In addition, the ratio of Th1/Th2 (IFN-γ/IL-4) was obviously out of balance, with a cell drift of Th2 to Th1. These results are reflected in the facts that body experiences ischemia/reperfusion injuries and severe stress reactions after CPR, leading to systemic disorders of inflammatory response and cellular immune functions. This is very similar to sepsis in many ways. Therefore, it is recognized that the mechanism of immunosuppression of postresuscitation syndrome has many features in common with sepsis.

Treg, a mature T-cell subset with regulatory functions, plays a major role in the negative regulation of the immune system. Treg can both suppress the inappropriate immune response and define the range, extent, and action time of systemic immune responses, and thus inhibit the proliferation and immune activity of effector cells.[14] When the body is subjected to external shock (severe trauma, and infection), Treg will not produce proliferative response to stimulations of specific antigens, and also the cytokine productions were significantly inhibited, which was reflected in the cell apoptosis and immunological unresponsiveness.[15] A steady increase in the number and proportion of Treg will aggravate the immune reaction, particularly in sepsis, which are closely related to the spread of infection and death in sepsis patients. In our study, it was found that after CA, apoptosis rate of spleen Treg in piglet was significantly reduced and that the number of Treg was increased considerably, which is consistent with the immunosuppressive status in sepsis. This is a reflection of the serious systemic stress response after CA. The remarkable increase of Treg leads to the inhibited lymphocyte proliferation reaction, which may be one of the main reasons for postresuscitation immune dysfunction.

Foxp3, a member of forkhead transcription factor family, is considered to be the molecular marker for the Treg. It is specifically expressed in CD4⁺ CD25⁺ Treg and can determine the function of CD4⁺ CD25⁺ Treg. Its expression is a sign that Treg has immunosuppressive effects.[16] As a transcriptional regulator, Foxp3 can regulate the activity of Treg through direct regulation of various genes. It plays a key role in the regulation of immunological self-stability.[17] In addition, secretory cytokine (IL-10) is the main factor released responsible for the immunosuppression effect of CD4⁺ CD25⁺ Treg, which can suppress effector T-cells. Therefore, the expression of Foxp3 and IL-10 level can be used as the main indicators of functional activation of Treg. In this study, it was found that the expression of Foxp3 and IL-10 level in porcine splenic Treg in post-ROSC groups were significantly increased. Our previous studies also showed a great number of apoptotic T lymphocytes in porcine spleen in post-ROSC groups.[18] All these data suggested that after ROSC, both the apoptosis of T lymphocytes and abnormal

### Table 2: Resuscitation outcome of the post-ROSC groups (n = 8)

| Outcome    | Post-ROSC | 12 h | 24 h | 48 h | F   | P     |
|------------|-----------|------|------|------|-----|-------|
| CPR time   | (min)     | 16.6 ± 1.5 | 15.2 ± 1.7 | 14.4 ± 2.3 | 0.394 | 0.680 |
| Number of shocks | 4.4 ± 0.5 | 3.9 ± 0.5 | 4.1 ± 0.5 | 0.360 | 0.702 |
| Defibrillation energy (J) | 937.5 ± 101.7 | 999.0 ± 136.3 | 987.5 ± 150.5 | 0.636 | 0.939 |

Data are reported as mean ± SD. No significant difference among the post-ROSC groups. CPR: Cardiopulmonary resuscitation; ROSC: Restoration of spontaneous circulation; SHAM: Sham-operated; SD: Standard deviation.

### Table 3: Comparison of apoptosis of Treg and T lymphocyte subsets in the SHAM group and post-ROSC groups at 12, 24, 48 h after ROSC (n = 8)

| Groups       | CD4⁺ (%) | CD4⁺/CD8⁺ (%) | Apoptosis rate (%) | Foxp3 (%) | IL-10 (ng/L) |
|--------------|----------|---------------|--------------------|-----------|--------------|
| SHAM group   | 43.8 ± 4.5 | 2.9 ± 0.8    | 13.1 ± 1.6         | 23.0 ± 2.7 | 25.5 ± 5.0   |
| 12 h post-ROSC | 29.1 ± 2.2* | 2.3 ± 0.7*        | 7.7 ± 1.9†         | 47.2 ± 5.1* | 114.3 ± 14.1* |
| 24 h post-ROSC | 24.3 ± 2.2* | 2.1 ± 0.6*        | 7.1 ± 1.8†         | 49.1 ± 5.8* | 162.4 ± 13.8* |
| 48 h post-ROSC | 24.1 ± 2.5* | 2.1 ± 0.4*        | 6.2 ± 0.4          | 57.1 ± 3.3* | 147.6 ± 29.6* |

Data are reported as mean ± SD. *P<0.01, †P<0.05 vs. SHAM group. Treg: Regulatory T-cell; SHAM: Sham-operated; ROSC: Return of spontaneous circulation; IL-10: Interleukin-10; SD: Standard deviation.

### Figure 1: ELISA analysis of IL-4 and IFN-γ levels of Treg in the spleen in SHAM group and post-ROSC group (a-c). Data reported as mean ± standard deviation. *P < 0.01, †P < 0.05 vs. SHAM group. Treg: Regulatory T-cell; SHAM: Sham-operated; ROSC: Return of spontaneous circulation; ELISA: Enzyme-linked immunosorbent assay; IL: Interleukin; IFN-γ: Interferon gamma.
activation of Treg will occur, which is reflected as the increased proportion of Treg and enhanced inhibitory effects and is associated with immune dysfunction.

T lymphocyte is the largest group of cells with the most important immunological function in the immune system, which is responsible for the maintenance of the normal immune function. T lymphocytes are mainly divided into two subsets, namely CD4+ and CD8+, in which CD4+ is helper T lymphocytes, with auxiliary function to switch T lymphocytes into effector cells. The ratio of CD4+/CD8+ can reflect the immune function of the body, and the decreased ratio of CD4+/CD8+ indicates the inhibition of cellular immunological functions. Our previous study found that after ROSC, the CD4+/CD8+ ratio of peripheral blood is significantly reduced, and the drift of anti-inflammatory factor Th2 to the proinflammatory factor Th1 was observed. In this study, it was found that after resuscitation, the number of CD4+ lymphocyte subsets and the ratio of CD4+/CD8+ were significantly reduced in piglet spleen, which was consistent with the previous results of peripheral blood test. It was reflected in the serious stress-induced neuroendocrine dysfunction after CA. It leads to the substantial secretion of inflammatory mediators and cytokines, which play significant inhibitory effects on the proliferation, differentiation, and function of lymphocytes. Treg has two major characteristics of immunological unresponsiveness and immunosuppression. The nonspecific immunosuppression on CD4+/CD8+ T-cells is mainly achieved through mechanism of cell contacts and the secretion of inhibitory cytokines. We speculated that the apoptosis rate of spleen Treg was reduced after CA, resulting in the significantly increased number of Treg and the enhanced function. Thus, the nonspecific inhibitory effects of CD4+/CD8+ T-cells were increased remarkably and the CD4+ lymphocyte subsets in peripheral blood and spleen were decreased significantly, which may be one of the mechanisms of postresuscitation immune dysfunction.

Activated CD4+ T-cells can differentiate into two kinds of cells of different functions, namely, Th1 and Th2 cells. Th1 and Th2 cells play important immune effects. Th1 cells are primarily responsible for cell-mediated immunity to mediate protective immune responses to activate macrophages and to kill pathogens; Th2 cells are responsible for humoral immunity to mediate nonprotective immune responses. In normal conditions, Th1/Th2 remains balanced. While the imbalance of Th1/Th2 can occur in pathological conditions, which is known as the Th1/Th2 drift. Th1/Th2 drift will directly affect the immunological functions. The ischemia/reperfusion injury can cause systemic inflammatory responses in case of CA. Moreover, both inflammatory response and anti-inflammatory response are initiated and the anti-inflammatory/proinflammatory response is in a shift, which is in charge of the prognosis of inflammatory response. It was confirmed that after CPR, IFN-γ and IL-4 and the ratio of IFN-γ/IL-4 in spleen in resuscitation groups were significantly increased compared with SHAM, showing that the imbalance of Th1/Th2 in Treg and the transformation to Th1 occurred in the spleen. The possible mechanism involved may be that the inhibitory effects of Treg were enhanced and Th2 was selectively inhibited. CD4+ T-cells differentiated and drifted into Th1. This Th1/Th2 drift caused by decreased apoptosis rate of Treg may be one of the mechanisms of postresuscitation immune dysfunction.

In summary, the results of this study showed that the proportion of Treg was increased and the inhibitory effect was enhanced due to the significantly decreased apoptosis rate of splenic Treg after CA, which led to the decrease of the amount of CD4+ lymphocytes. And then, the cell drift from Th2 to Th1 was mediated and the cellular immune function was suppressed. Hence, we presumed theoretically that the recovery of Th2-mediated anti-inflammatory response was stimulated by the induced apoptosis of Treg and thereby improving the postresuscitation immune dysfunction.

Financial support and sponsorship

This research was supported by the grants from the National Natural Science Foundation of China (No. 81372025), Beijing Municipal Administration of Hospitals Incubating Program (No. Px2016022), and Basic and Clinical Research Cooperation Project of Capital Medical University (No. 15JL42).

Conflicts of interest

There are no conflicts of interest.

References

1. Neumar RW, Nolan JP, Adrie C, Aibiki M, Berg RA, Böttiger BW, et al. Post-cardiac arrest syndrome: Epidemiology, pathophysiology, treatment, and prognostication. A consensus statement from the International Liaison Committee on Resuscitation (American Heart Association, Australian and New Zealand Council on Resuscitation, European Resuscitation Council, Heart and Stroke Foundation of Canada, InterAmerican Heart Foundation, Resuscitation Council of Asia, and the Resuscitation Council of Southern Africa); the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; and the Stroke Council. Circulation 2008;118:2452-83. doi: 10.1161/CIRCULATIONAHA.108.190652.

2. Pan J, Zhu JY, Kee HS, Zhang Q, Lu YQ. A review of compression, ventilation, defibrillation, drug treatment, and targeted temperature management in cardiopulmonary resuscitation. Chin Med J 2015;128:550-4. doi: 10.4103/0366-6999.151115.

3. Huang LF, Yao YM, Li JF, Dong N, Liu C, Yu Y, et al. Reduction of plasma gelsolin levels correlates with development of multiple organ dysfunction syndrome and fatal outcome in burn patients. PLoS One 2011;6:e25748. doi: 10.1371/journal.pone.0025748.

4. Tan M, Zhu JC, Du J, Zhang LM, Yin HH. Effects of probiotics on serum levels of Th1/Th2 cytokine and clinical outcomes in severe traumatic brain-injured patients: A prospective randomized pilot study. Crit Care 2011;15:R290. doi: 10.1186/cc10579.

5. Xu L, Zhang Q, Zhang QS, Li Q, Han JY, Sun P. Improved survival and neurological outcomes after cardiopulmonary resuscitation in toll-like receptor 4-mutant mice. Chin Med J 2015;128:2646-51. doi: 10.4103/0366-6999.166024.

6. Adrie C, Adib-Conquy M, Laurent I, Monchi M, Vinsonneau C, Fitting C, et al. Successful cardiopulmonary resuscitation after cardiac arrest in patients with severe traumatic brain injury (STABLE Study): An international, multicentre, phase 2b, randomized, controlled trial. The Lancet 2016;388:1552-61.
arrest as a “sepsis-like” syndrome. Circulation 2002;106:562-8. doi: 10.1161/01.CIR.0000023939.80661.c
7. Adrie C, Laurent I, Monchi M, Carion A, Dhaenau JF, Spaulding C. Postresuscitation disease after cardiac arrest: A sepsis-like syndrome? Curr Opin Crit Care 2004;10:208-12. doi: 10.1097/01.ccc.0000023891.80661.7.
8. Gu W, Li CS, Yin WP, Hou XM, Zhang J, Zhang D, et al. Expression imbalance of transcription factors GATA-3 and T-bet in post-resuscitation myocardial immune dysfunction in a porcine model of cardiac arrest. Resuscitation 2013;84:848-53. doi: 10.1016/j.resuscitation.2012.11.023.
9. Gu W, Li C, Yin W, Hou X, Zhang D. Effects of Shen-Fu injection on the expression of T-cell-specific transcription factors T-bet/Gata-3 in porcine postresuscitation lung injury. Evid Based Complement Alternat Med 2013;2013:464650. doi: 10.1155/2013/464650.
10. Condotta SA, Cabrera-Perez J, Badovinac VP, Griffith TS. T-cell-mediated immunity and the role of TRAIL in sepsis-induced immunosuppression. Crit Rev Immunol 2013;33:23-40. doi: 10.1615/CritRevImmunol.2013006721.
11. Okeke EB, Okwor I, Mou Z, Jia P, Uzonna JE. CD4+ CD25+ regulatory T cells attenuate lipopolysaccharide-induced systemic inflammatory responses and promotes survival in murine Escherichia coli infection. Shock 2013;40:65-73. doi: 10.1097/SHK.0b013e3182896e65b.
12. Gu W, Hou X, Li C. Effect of different resuscitation strategies on post-resuscitation brain damage in a porcine model of prolonged cardiac arrest. Chin Med J 2014;127:3432-7. doi: 10.3760/ cma.j.issn.0366-6999.20140743.
13. Yin W, Guo Z, Li C. Comparison of epinephrine and Shen-Fu injection on resuscitation outcomes in a porcine model of prolonged cardiac arrest. Chin Med J 2014;127:724-8. doi: 10.3760/ cma.j.issn.0366-6999.20132255.
14. Nascimento DC, Alves-Filho JC, Sônego F, Fukada SY, Pereira MS, Benjamim C, et al. Role of regulatory T cells in long-term immune dysfunction associated with severe sepsis. Crit Care Med 2010;38:1718-25. doi: 10.1097/CCM.0b013e3181e78a0.
15. Wisnosi N, Chung CS, Chen Y, Huang X, Ayala A. The contribution of CD4+ CD25+ T-regulatory-cells to immune suppression in sepsis. Shock 2007;27:251-7. doi: 10.1097/01.shk.0000239780.33398.e4.
16. Li Z, Li D, Tsun A, Li B. FOXP3+ regulatory T cells and their functional regulation. Cell Mol Immunol 2015;12:558-65. doi: 10.1038/cmi.2015.10.
17. Lu Y, Guo Y, Xu L, Li Y, Cao L. Foxp3 regulates ratio of Treg and NKT cells in a mouse model of asthma. Mol Cell Biochem 2015;403:25-31. doi: 10.1007/s11010-015-2333-2.
18. Jost NH, Abel S, Hutzler M, Sparwasser T, Zimmermann A, Roers A, et al. Regulatory T cells and T-cell-derived IL-10 interfere with effective anti-cytomegalovirus immune response. Immunol Cell Biol 2014;92:860-71. doi: 10.1038/icb.2014.62.
19. Miller AC, Rashid RM, Elamin EM. The “T” in trauma: The helper T-cell response and the role of immunomodulation in trauma and burn patients. J Trauma 2007;63:1407-17. doi: 10.1097/ TA.0b013e31815b839e.
20. Gu W, Zhang Q, Yin W, Li C. Caspase-3-mediated splenic lymphocyte apoptosis in a porcine model of cardiac arrest. Am J Emerg Med 2014;32:1027-32. doi: 10.1016/j.ajem.2014.06.004.
21. Hekimian G, Baugnon T, Thuong M, Monchi M, Dabbane H, Jaby D, et al. Cortisol levels and adrenal reserve after successful cardiac arrest resuscitation. Shock 2004;22:116-9. doi: 10.1097/01.shk.0000132489.79498.e7.
22. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787-93. doi: 10.1038/383787a0.
23. Romagnani S. Type 1 T helper and type 2 T helper cells: Functions, regulation and role in protection and disease. Int J Clin Lab Res 1991;21:152-8. doi: 10.1007/BF02591635.
24. Carson WF 4th, Ito T, Schaller M, Cavassani KA, Chensue SW, Kunkel SL. Dysregulated cytokine expression by CD4+ T cells from post-septic mice modulates both Th1 and Th2-mediated granulomatous lung inflammation. PLoS One 2011;6:e20385. doi: 10.1371/journal.pone.0020385.
25. Adams JA. Endothelium and cardiopulmonary resuscitation. Crit Care Med 2006;34 Suppl:S458-65. doi: 10.1097/01.CCM.0000246012.68479.e5.
26. Karimova A, Pinsky DJ. The endothelial response to oxygen deprivation: Biology and clinical implications. Intensive Care Med 2001;27:19-31. doi: 10.1007/s001340000790.
27. Cerchiari EL, Safar P, Klein E, Diven W. Visceral, hematologic and bacteriologic changes and neurologic outcome after cardiac arrest in dogs. The visceral post-resuscitation syndrome. Resuscitation 1993;25:119-36. doi: 10.1016/0300-9572(93)00090-D.