Original Article

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

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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. 7.6 ± 0.9 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

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.[1] 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

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the immune system and resulting in serious damage to tissues and organs eventually.[3-5] Studies have found that ROSC patients often show "sepsis-like" changes, i.e., systemic inflammatory response and serious secondary infections.[6,7] 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[8,9] 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.[8]

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.[10] 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.[5,7] As an important regulatory T lymphocyte in immune regulation, Treg plays an important role in the regulation of immune function disorders and depression.[11] 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: SYXK [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 $mg \cdot kg^{-1} \cdot h^{-1}$) to maintain anesthesia. Animals were mechanically ventilated by a volume-controlled ventilator with a tidal volume of 8 ml/kg and FiO_2 of 21%, and ventilation rate of 12–20 breaths/min. End-tidal PCO_2 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).[12]

Experimental protocol

The temporary pacemaker conductor (PACEL™ Right Heart Curve, St. Jude Medical Inc., Sylmar, CA, USA) was inserted 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.[13]

After 8 min of VF, manual CPR was carried out at a frequency of 100 compressions/min with mechanical ventilation at a FiO_2 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.[12] 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 (DO_2), and oxygen consumption (VO_2), 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 sterilely. 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% NaN_3, 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 $CD4^+/CD8^+$ was 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. RPMI 1640 medium containing 20% (v/v) FBS was added to 48-well culture plate and was incubated in a CO_2 incubator. At the 3rd day, $CD4^+$ $CD25^+$ Treg was isolated and cultured for 12 h. Then, using culture medium, the cell concentrations of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^-$ T were adjusted to 1:1, and cultured. Concanavalin A (5 mg/L) was used to stimulate the cells, and then cells were cultured in a CO_2 incubator at 37°C. After 68 h of incubation, cells were centrifuged and the supernatants were saved and stored at −70°C for test.

Test of apoptosis of splenic regulatory T-cell

After isolation, cells were cultured for 12 h. The suspension of $CD4^+$ $CD25^+$ Treg (1×10^9/L) was washed twice with PBS. One-hundred microliters binding buffer and 10-μl APC-labeled Annexin V (20 mg/L) were added and incubated in darkness at room temperature for 30 min. Ten microliters 7-amino-actinomycin D (7-AAD) were then added and incubated in darkness for 5 min. After that, 400-μl binding buffer was added. 7-AAD negative and Annexin V positive cells were selected by flow cytometry as apoptotic cells, and cell apoptosis rate was detected.

Quantitative real-time polymerase chain reaction assay for *Foxp3* of splenic regulatory T-cell

Primers was designed and synthesized according to the sequence of *Foxp3* gene, and the primer sequences of the expected polymerase chain reaction (PCR) products were as follows: for *Foxp3*, sense 5'-CTGACAAGGGTTCCTGCTGTATTG-3' and antisense 5'-AGCGGATGAGGGTGGCGTAT-3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Forward primer: 5'-GGTCGGAGTGAACGGATTT-3' and Reverse primer: 5'-ATTTGATGTTGGCGGGAT-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, 2XSYBR 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 $2^{-\Delta\Delta Ct}$ formula by the comparative Ct method, the copy number of the target gene $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ target gene} - \Delta Ct \text{ GAPDH gene})}$.

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, DO_2, and VO_2 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 3].

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. 0.8 ± 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)	DO_2 (ml/min)	VO_2 (ml/min)
12 h post-ROSC					
Baseline	84.8 ± 4.9	103.3 ± 8.0	2.8 ± 0.5	422.6 ± 9.5	114.1 ± 3.2
ROSC 0.5 h	125.1 ± 9.2	112.7 ± 6.6	2.5 ± 0.5	388.8 ± 8.3	101.3 ± 7.0
ROSC 2 h	119.6 ± 7.1	106.2 ± 7.9	2.3 ± 0.1	259.1 ± 7.3	68.5 ± 2.1
ROSC 6 h	110.0 ± 8.4	95.6 ± 8.3	2.2 ± 0.4	271.3 ± 3.2	81.5 ± 2.2
24 h post-ROSC					
Baseline	79.5 ± 3.6	107.8 ± 8.5	2.7 ± 0.3	417.6 ± 6.7	116.0 ± 3.3
ROSC 0.5 h	121.7 ± 8.2	99.6 ± 7.1	2.4 ± 0.3	367.8 ± 3.3	98.7 ± 5.9
ROSC 2 h	115.7 ± 9.2	104.8 ± 7.1	2.3 ± 0.5	251.71 ± 4.3	72.5 ± 2.1
ROSC 6 h	105.0 ± 8.4	110.7 ± 5.2	2.0 ± 0.4	262.3 ± 9.2	79.5 ± 3.2
48 h post-ROSC					
Baseline	83.1 ± 5.1	105.6 ± 11.0	2.6 ± 0.5	429.8 ± 7.1	115.8 ± 6.9
ROSC 0.5 h	129.1 ± 8.5	120.1 ± 7.6	2.4 ± 0.2	387.1 ± 10.1	102.9 ± 5.2
ROSC 2 h	126.6 ± 7.9	116.8 ± 6.9	2.3 ± 0.3	253.6 ± 6.7	73.5 ± 3.9
ROSC 6 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; DO_2: Oxygen delivery; VO_2: 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 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			F	P
	12 h	24 h	48 h		
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.

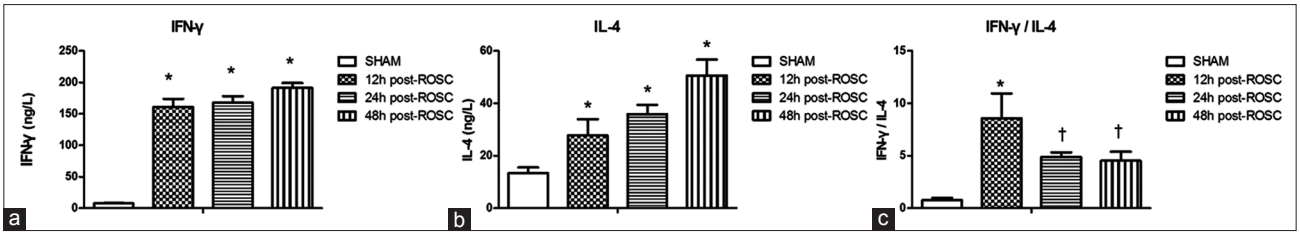

Figure 1: ELISA analysis of IL-4 and IFN-γ levels of Treg in the spleen in SHAM group and post-ROSC group (a-c). Data rereported as mean ± standard deviation. *P < 0.01, †P < 0.05 vs. SHAM group. Treg: Regulatory T-cell; SHAM: Sham-operated; ROSC: Return of spontaneous circulation; ELISA: Enzyme-linked 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.[19] 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.[8-9] 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.[8,20] 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.[21] 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.[14] 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, Th1and 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.[22,23] In normal conditions, Th1/Th2 remains balanced. While the imbalance of Thl/Th2 can occur in pathological conditions, which is known as the Th1/Th2 drift. Th1/Th2 drift will directly affect the immunological functions.[4,24] The ischemia/reperfusion injury can cause systemic inflammatory responses in case of CA.[25] 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.[26,27] 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 Thl/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.

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Conflicts of interest

There are no conflicts of interest.

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