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Article

Protection of intestinal immune barrier against ischemia/reperfusion injury in a swine model using anisodamine hydrobromide

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Intestinal ischemia/reperfusion (I/R) causes barrier impairment and bacterial influx. This study explored the protective effects of anisodamine hydrobromide (AH) on intestinal I/R injury caused by cardiopulmonary resuscitation (CPR) after cardiac arrest (CA). After successful CPR, minipigs were randomly divided into two groups (n = 8): saline and AH (4 mg/kg), and then treated with saline or AH via central venous injection, respectively. The same procedures without ventricular fibrillation initiation were conducted in the Sham group (n = 8). Levels of interferon gamma (IFN-γ) and interleukin 4 (IL-4) were measured at different time points (0, 0.5, 1, 2, 4, and 6 h) in serum and 6 h in gut associated lymphoid tissues (GALTs) after the return of spontaneous circulation (ROSC) to evaluate changes in the proportion of T-helper type 1 (Th1) and T-helper type 2 (Th2). Moreover, the positive culture rates of GALTs were examined to evaluate bacterial translocation. AH treatment markedly alleviated aberrant arterial blood gas and hemodynamics as well as intestinal macroscopic and morphological changes after CPR. Moreover, AH treatment significantly increased IFN-γ and decreased IL-4 in both serum and GALTs. Furthermore, AH treatment dramatically decreased positive bacterial growth in GALTs. AH treatment mitigated immunosuppression caused by intestinal I/R and protected the intestinal immune barrier against bacterial translocation, thereby reducing the risk of secondary intestinal infection.

Keywords: Cardiac arrest. Intestinal ischemia/reperfusion. Anisodamine hydrobromide. Bacterial translocation. T helper cell transformation.

INTRODUCTION

Cardiac arrest (CA) is a leading cause of death worldwide (Benjamin *et al*., 2019). Although the application of automated external defibrillator and high-quality cardiopulmonary resuscitation (CPR) has significantly improved the survival rate of patients with CA, the mortality remains quite high (Pijls *et al*., 2018; Sulzgruber *et al*., 2016). Additionally, patients who achieved the return of spontaneous circulation (ROSC) after resuscitation frequently die of post-CA syndrome within 72 hours (Mongardon *et al*., 2011). In particular, ischemia/reperfusion injury (IRI) is responsible for many systemic disorders with high morbidity and mortality (Bro-Jeppesen *et al*., 2014).

Studies on intestinal mucosal barrier damage caused by IRI mainly focused on mechanical barrier destruction. However, there are few studies on the immune barrier damage. The disruption of the intestinal mucosal barrier caused by intestinal IRI will ultimately result in the passage of viable bacteria and endotoxins from the gastrointestinal tract to mesenteric lymph nodes complex and distant organs, named as "bacterial translocation" (Camuesco *et al*., 2004), which may aggravate local injury and cause impaired function of remote organs by initiating inflammatory responses and subsequent immunosuppression (Leaphart, Tepas 3rd, 2007).

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Anisodamine is a belladonna alkaloid isolated from the Chinese medicinal herb, *Scopolia tangutica*, with antispasmodic and analgesic bioactivities. As a classic muscarinic antagonist, anisodamine has been extensively used clinically for decades to relieve smooth muscle spasms, gastrointestinal colic, biliary colic, acute microcirculation disorder, and organophosphate poisoning (Wang *et al*., 2014). Recent studies showed that anisodamine exerted protective effects against the damage caused by myocardial IRI in animals with CA by suppressing oxidative stress, inflammatory responses, and myocardial cell apoptosis (Li *et al*., 2019; Liu *et al*., 2013; Yao *et al*., 2018; Yin *et al*., 2011). This study was to investigate the protective effects of anisodamine hydrobromide (AH) on the intestinal immune barrier in a swine model, and further explore its underlying mechanisms.

MATERIAL AND METHODS

Chemicals and Reagents

Interleukin 4 (IL-4) and interferon gamma (IFN-γ) assay kits were purchased from Sunbio Biotech Co. Ltd. (Beijing, China). AH was purchased from the National Institutes for Food and Drug Control (Beijing, China) with more than 99% purity. AH was dissolved in saline for treatment.

Experimental Procedures and Treatment

Twenty-four male minipigs (12-14 months, 30 ± 2 kg) were purchased from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All animals were housed in a dedicated, specific pathogen-free environment. Minipigs had free access to food and water during the experimental period. All procedures were performed following the Animal Care Guidelines of the Institutional Animal Care and Use Committee of Capital Medical University. Minipigs were fasted overnight but allowed free access to water before the experiment.

After an intramuscular injection with 0.5 mg/kg midazolam (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China), anesthesia was induced by ear vein injection of propofol (1.0 mg/kg) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) and maintained with intravenous infusion of pentobarbital (8 mg/kg/h) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China). Heart rate and electrocardiogram measurements were monitored using a four-channel physical recorder (BL-420F Data Acquisition& Analysis System; TME Technology Co. Ltd, Chengdu, China). A cuffed 6.5 mm cannula was advanced into the trachea. Animals were mechanically ventilated with a volume-controlled ventilator (Servo 900C; Siemens, Germany) with a fraction of inspiration O_2 (FiO_2) of 0.35 and a respiratory frequency of 12 breaths/min using a tidal volume of 15 mL/kg. An angiographic catheter was inserted from the femoral artery into the aortic arch to measure the aortic pressure. The electrocardiogram and all hemodynamic parameters were monitored using a patient monitoring system (M1165; Hewlett-Packard, Palo Alto, CA, USA).

After anesthesia, animals were allowed to equilibrate for 30 min to achieve a stable resting level, followed by CA induction. The temporary pacemaker conductor was inserted into the right ventricle through the right sheathing canal and connected to an electrical stimulator (GY-600A; Kaifeng Huanan Equipment Co., Ltd., 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 the occurrence of ventricular fibrillation (VF) (Wang *et al*., 2010) and the mean aortic pressure was suddenly declined to zero.

Ventilation was withheld for the entire eight-minute duration of VF arrest. Then manual CPR was conducted at a frequency of 100 compressions/min with mechanical ventilation at 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) (Wang *et al*., 2010). If the spontaneous circulation was not restored, defibrillation was attempted once using a diphase 150 J.

ROSC was defined by a systolic blood pressure above 50 mmHg lasting at least 10 min. If spontaneous circulation was not restored within 30 min, the animal was considered dead (Valenzuela *et al*., 1997). After successful CPR, minipigs were randomly divided into

two groups (n = 8): saline and AH (4 mg/kg), and then treated with saline or AH via central venous injection, respectively. The dosage of AH was based on our preliminary study and clinical recommendation on humans. The same procedures without VF initiation were conducted in the Sham group (n = 8), including the induction of anesthesia, electrode positioning, mechanical ventilation, and eight min-suspension of ventilation.

Arterial Blood Gas and Hemodynamics Measurement

Arterial blood gas was measured at baseline, 0, 1, 2, 4, and 6 h after ROSC using a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory, Lexington, MAs). Hemodynamics were measured at baseline, 0, 1, 2, 4, and 6 h after ROSC using a monitoring system (M1165; Hewlett-Packard, Palo Alto, CA, USA).

Pathological Analysis

Intestines were excised and the mucosa was scraped carefully to remove the digest, followed by vigorous washing with sterile phosphate-buffered saline (PBS). Tissue fragments were sectioned and stained with H&E (Sigma-Aldrich, St. Louis, MO, USA). The pathological and ultrastructural changes of GALT specimens were observed by light microscope and transmission electron microscopy (TEM), respectively.

Cytokine Measurement

Blood samples were collected at baseline, 0, 0.5, 1, 2, 4 and 6 h after ROSC. GALTs were collected under aseptic operation 6 h after ROSC. IL-4 and IFN-γ in the serum and GALTs were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. The absorbance at 450 nm was measured using a microplate reader.

Identification of Microbial Species in GALTs

GALTs were homogenized to harvest tissue lysate. The lysate was then inoculated onto fresh blood agar and Chinese blue agar plates (Scientific Biotech, Taipei, Taiwan). The plates were incubated at 37 °C overnight. The colony number was counted, and bacterial identification was performed.

Real-time PCR

Total RNA from GALTs was extracted using TRIzol reagents (Sigma, St. Louis, MO, USA). Complementary DNA (cDNA) was synthesized from total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). Quantitative PCR reactions were performed using SYBR™ Green Master Mix (Thermo Fisher, Waltham, MA, USA) on an Applied Biosystems 7500 system. Relative mRNA levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method. *β-actin* was used as the internal reference. The primer sequences are the following: for *IFN-γ*, forward: agcatggatgtgatcaagca; reverse: tgcaggcaggatgacaatta, NCBI reference sequence: NM_213948.1; for *IL-4*, forward: tctcacctcccaactgatcc; reverse: aaggtttccttctccgtcgt, GenBank: HQ236500.1; for β-actin, forward: gacatccgcaaggacctcta; reverse: acacggagtacttgcgctct, GenBank: DQ845171.1.

Western Blot Analysis

Protein was extracted from GALTs using the RIPA buffer (Thermo Fisher, Waltham, MA, USA). The protein concentration was determined by the bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with primary antibodies (anti-IFN-γ goat pAb AF985, 1:1000, Bio-Techne Corporation; anti-IL-4 goat pAb AF654, 1:1000, Bio-Techne Corporation; anti-GAPDH goat pAb ab157156, 1:3000, Abcam) at 4 °C overnight. The membrane was washed three times and incubated with the secondary antibody (donkey anti-goat IgG H&L (HRP), ab6885, 1:5000, Abcam) at room temperature for 1 h. Chemiluminescent signals were detected using the

ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Data were expressed as mean ± SD and analyzed by SPSS 17.0 (SPSS Inc, Chicago, IL, USA). The Kolmogorov-Smirnov test was used for normality test. Measurement data between two groups were compared using the Student's t-test; measurement data among multiple groups were compared using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered significant difference.

RESULTS

AH Ameliorated the Intestinal Injury Caused by Ischemia/Reperfusion

CA was induced in minipigs, followed by manual CPR. There was no statistical difference in experimental animals' characteristics and baseline hemodynamic parameters in the three groups before CA (Table I). The resuscitation duration and defibrillation times were similar between saline and AH groups (Table II). After resuscitation, compared to the Sham group, the bowel showed congestion, edema, and erosion and intraperitoneal ascites became hemorrhagic in the Saline group. However, AH treatment markedly mitigated the intestinal damage.

TABLE I - Characteristics of minipigs in three groups

	SHAM group (n = 8)	Saline group (n = 8)	AH group (n = 8)
Weight (kg)	23.75±0.96	24.13±1.16	24.0±1.21
Heart rate (bpm)	101.75±5.85	101.38.75±6.63	101.63.5±5.53
MAP (mmHg)	99.50±5.94	99.70±5.38	99.45±5.93
CO (L/min)	3.60±0.22	3.57±0.29	3.68±0.28
DO2 (ml/min)	432±7	438±18	437±15
VO2 (ml/min)	113±4	115±6	114±6
OER (%)	26.10±0.65	26.83±1.9	26.28±1.05

MAP: mean arterial pressure; CO: cardiac output; DO2: oxygen delivery; VO2: oxygen consumption; OER: oxygen extraction ratio

TABLE II - CPR parameters

	Saline group (n = 8)	AH group (n = 8)
CPR duration (min)	5.60±1.13	5.88±1.59
Number of defibrillation times	1.38±0.52	1.43±0.56

Effects of AH on Arterial Blood Gas

After CPR and successful ROSC, pCO2 and lactic acid values significantly increased, whereas pO2 and pH decreased ($p < 0.01$). Compared with the Saline group, AH treatment significantly increased pH and pO2 values 1 h after ROSC. It decreased pCO2 and lactic acid values 2 h after ROSC ($p < 0.05$) (Table III). These data suggested that AH treatment ameliorated hypoxia and acid accumulation caused by I/R.

TABLE III - Comparison of arterial blood gas at different time points after ROSC

Group	Parameter	Baseline	0h	1h	2h	4h	6h
Saline	PH	7.38±0.3	6.98±0.2**	7.03±0.2	7.05±0.2	7.14±0.15	7.2±0.2
	pO2 (mm Hg)	88.0±5.0	50.6±1.4**	56.3±1.6	60.3±1.9	64.9±2.4	76.9±3.2
	pCO2 (mm Hg)	41.3±2.5	66.9±6.9**	57.7±5.5	52.1±5.0	49.0±4.5	46.8±3.6
	Lactic acid (mmol/L)	2.4±0.3	8.8±0.8**	7.3±0.73	6.8±0.6	6.1±0.5	4.8±0.45
AH	PH	7.34±0.3	7.07±0.23**	7.13±0.3#	7.20±0.4#	7.28±0.44#	7.32±0.32#
	pO2 (mmHg)	89.8±5.7	51.5±1.5**	62.6±1.9#	68.0±2.6#	72.1±3.8#	83.3±3.9#
	pCO2 (mmHg)	41.0±2.7	59.1±5.2**	54.5±5.6	49.0±5.4#	43.3±4.4#	42.1±3.5#
	Lactic acid (mmol/L)	2.3±0.2	8.3±0.7**	7.0±0.7	6.1±0.6#	4.9±0.5#	3.5±0.4#

**$p < 0.01$ vs. baseline; #$p < 0.05$ vs. Saline group.

Effects of AH on Hemodynamics

After ROSC, heart rate (HR) significantly increased, whereas mean arterial pressure (MAP) and cardiac output (CO) decreased, compared with the baseline level ($p < 0.01$). AH treatment significantly decreased HR, and increased CO as well as MAP values 2 h after ROSC ($p < 0.05$) (Table IV), suggesting that AH treatment facilitated hemodynamics stabilization in CA-resuscitated animals.

TABLE IV - The comparison of hemodynamics at different time points after ROSC

Group	Parameter	Baseline	0 h	1 h	2 h	4 h	6 h
Saline	HR (bpm)	105.4±6.6	158.6±9.8**	141.3±9.1	137.8±8.9	126.2±8.3	119.0±7.8
	CO (L/min)	3.69±0.28	0.92±0.16**	1.47±0.30	1.88±0.34	2.40±0.49	2.77±0.51
	MAP (mmHg)	100.7±10.6	54.5±5.6**	77.4±7.2	81.9±8.0	85.8±8.9	92.6±9.9
AH	HR (bpm)	105.6±5.3	155.4±9.2**	134.5±8.6	131.5±7.9#	118.8±6.8#	111.2±5.8#
	CO (L/min)	3.65±0.29	1.06±0.22**	1.75±0.28	2.53±0.43#	3.10±0.57#	3.44±0.60#
	MAP (mmHg)	101.7±10.9	58.4±5.7**	78.5±7.1	89.2±8.8#	92.0±9.4#	97.4±10.2#

HR: heart rate; CO: cardiac output; MAP: mean aortic pressure.

**$p < 0.01$ vs. baseline; #$p < 0.05$ vs. Saline group.

AH Treatment Inhibited the Transformation of Th1 to Th2

IFN-γ and IL-4 levels were measured in serum and GALTs. Results showed that IL-4 secretion significantly increased in the serum 2 h after ROSC and in GALTs 6 h after ROSC, but IFN-γ level significantly decreased in the serum 2 h after ROSC and in GALTs 6 h after ROSC. However, AH treatment significantly increased the IFN-γ level, decreased the IL-4 level, and increased the ratio of IFN-γ to IL-4 in both the serum and GALTs (Figures 1 and 2). Moreover, compared with the Saline group, AH treatment significantly increased gene and protein expression of IFN-γ, but decreased gene and protein expression of IL-4 (Figure 2). These data indicated that the I/R process promoted Th1 to Th2 transformation, but was largely inhibited by AH treatment.

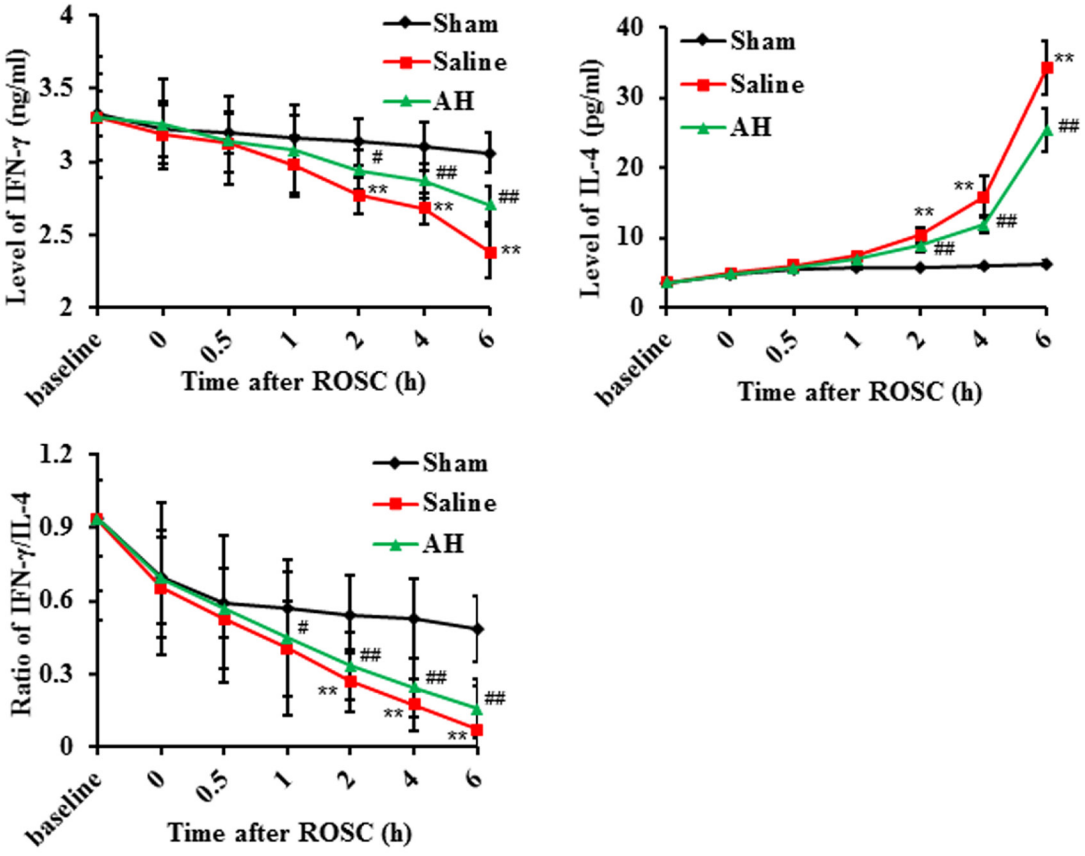

FIGURE 1 - AH treatment inhibited Th1-Th2 transformation in the blood. Blood samples were collected at baseline, 0, 0.5, 1, 2, 4 and 6 h after ROSC. Compared with the Saline group, AH treatment significantly increased IFN-γ level, decreased IL-4 level and increased the ratio of IFN-γ to IL-4 in serum. Data were expressed as mean ± SD (n = 8). $^{**}p < 0.01$ vs. Sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Saline group.

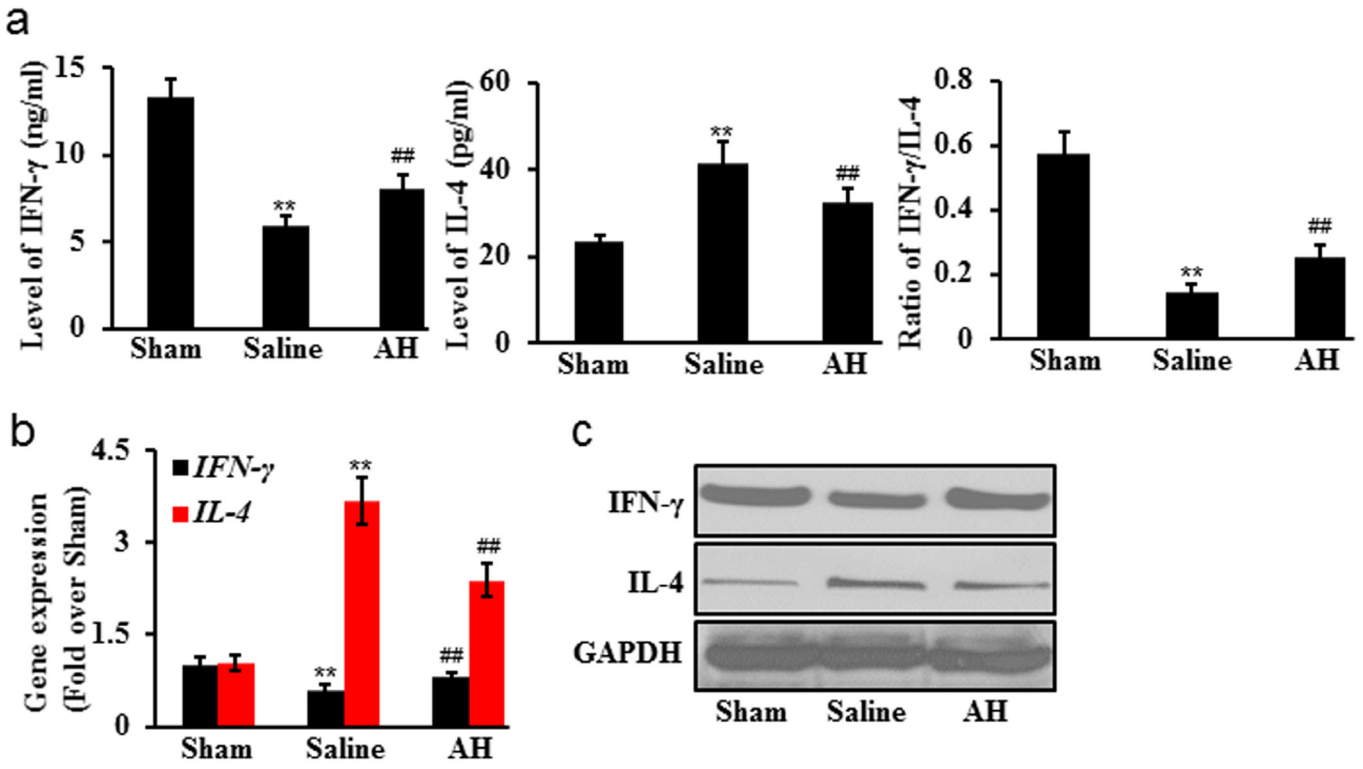

FIGURE 2 - AH treatment inhibited Th1-Th2 transformation in GALTs. The GALTs were collected under aseptic operation 6 h after ROSC. Compared with the Saline group, AH treatment significantly increased IFN-γ levels, decreased IL-4 levels and increased the ratio of IFN-γ to IL-4 in GALTs (a). Moreover, AH treatment significantly increased the gene and protein expression of IFN-γ, but decreased the gene and protein expression of IL-4 (b, c). Data were expressed as mean ± SD (n = 8). $^{**}p < 0.01$ vs. Sham group. $^{\#\#}p < 0.01$ vs. Saline group.

AH Mitigated Pathological Changes in GALTs

To determine effects of AH on morphological changes associated with I/R. GALTs were sectioned and observed under a light microscope. Upon resuscitation, the GALTs showed obvious signs of inflammation, evidenced by enlarged lymph nodes, inflammatory cell infiltration, thickened cortex, and an aggrandized number of lymph nodes in the cortex. However, AH treatment considerably diminished lymph nodes in size and number, compared with the Saline group (Figure 3a). Moreover, ultrastructural changes of GALTs were examined by TEM. As shown in Figure 3b, obvious nucleoli in lymphocytes, nuclear heterochromatin, swollen mitochondria, and even rupture of some mitochondrial membranes were observed in GALTs. These abnormal changes were largely mitigated after treatment with AH.

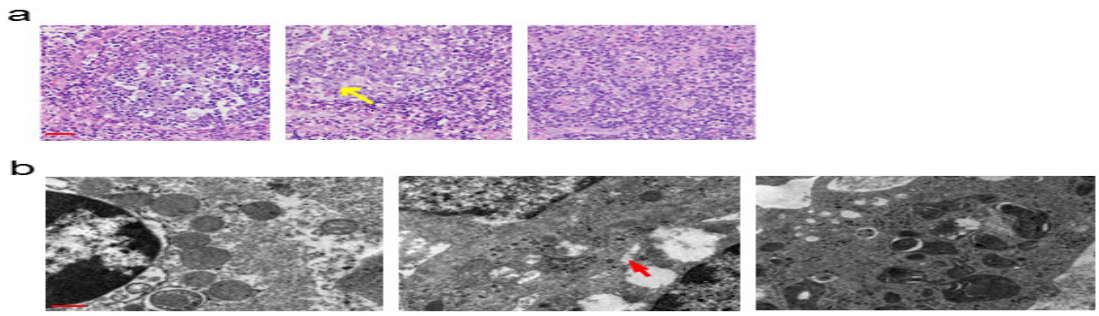

FIGURE 3 - AH treatment mitigated the pathological changes in GALTs. GALTs were sectioned and observed under the light microscope (magnification ×200, scale bar = 20 μm). The yellow arrow showed the phagocytosis in the reaction center (a). Ultrastructure was observed by TEM (magnification ×200, scale bar = 0.5 μm). The red arrow showed the damage of mitochondria (b).

AH Treatment Suppressed Translocation of Intestine Bacterial

Microbiological growth in GALTs cultures was analyzed to assess bacterial translocation status. As shown in Table V, few colonies existed in the Shame group. When animals were subjected to I/R, Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella* were observed, which were in line with the dominant strains among normal intestinal flora. However, AH treatment markedly reduced the percentage of positive cultures, compared with the Saline group (62.5% *vs.* 87.5%), suggesting a decrease in the incidence of bacterial translocation caused by I/R.

TABLE V - Bacterial culture

Group	Positive rate (%)	Bacterial species
Sham	0	-
Saline	87.5(7/8)	*Escherichia coli*, *Klebsiella acidophilus*, *Serratia marcescens*, *Pseudomonas aeruginosa*
AH	62.5(5/8)	*Escherichia coli*, *Klebsiella acidophilus*, *Serratia marcescens*, *Pseudomonas aeruginosa*

DISCUSSION

CA leads to severe systemic ischemia and hypoxia, accompanied by acidosis, causing energy metabolism disorders, protein degeneration, damage or even death of tissue cells. Although blood supply is restored after a period of ischemia, the damage to tissues and organs persists and becomes more serious. This process is known as IRI, which can occur in various organs, such as heart, brain, liver, kidney, and lung. In particular, reperfusion in the gastrointestinal mucosa is an important cause of multiple organ dysfunction syndrome, myocardial failure, diverticulitis, and sepsis (Pastores, Katz, Kvetan, 1996). Here, the protective effects of AH on intestinal damage of animals caused by resuscitation after the onset of CA were investigated.

Once gastrointestinal dysfunction occurs due to splanchnic ischemia and hypoxia, the mucosal barrier will be damaged, which can subsequently lead to disruption of the micro-ecological balance, secondary

translocation of bacteria, and release of endotoxin into the blood. Mesenteric lymph nodes (MLN) and GALTs are important components of the intestinal mucosal barrier and are pivotal for immune responses. Our results showed that after VF and successful resuscitation, the lymph nodes in GALTs were enlarged and increased in number, suggesting that I/R induced an obvious inflammatory responses.

Studies have demonstrated that T lymphocytes, especially the CD4 subpopulation, act as an important mediator in the pathogenesis of organ IRI. Immunosuppressants, such as FK506 can alleviate organ IRI damage. Blocking T cell co-stimulatory factors and inhibiting T-cell activation reduced IRI in organs (Ishikawa *et al*., 2005; Sharkey, Butcher, 1994; Yang *et al*., 2005). $CD4^+$ T lymphocytes can differentiate into regulatory T-cells and various effector T-cells such as Th1, Th2, and Th17 in response to the stimulation of different cytokines (Afzali *et al*., 2007). Th1-type lymphocytes produce pro-inflammatory cytokines including IFN-γ and IL-2, which mainly contribute to cell-mediated immune responses. In contrast, Th2-type lymphocytes secrete anti-inflammatory cytokines, such as IL-4 and IL-10, responsible for host defense against the invasion of exogenous pathogens (Bretscher, 2014; Paul, Zhu, 2010). The balance between Th1 and Th2 cells plays a critical part in maintaining the normal immune function. Abnormal Th1/Th2 ratios were found in the spleen, lung, and myocardial tissues in previous studies on CA models undergoing ROSC (Gu *et al*., 2013; Gu *et al*., 2015; Gu, Zhang, Li, 2016). Our study demonstrated that after resuscitation, IFN-γ levels in serum exhibited a continuous decline, while the IL-4 level in the serum was markedly elevated. In addition, IFN-γ significantly decreased whereas IL-4 increased in GALTs 6 h after resuscitation. These data were consistent with previous observations in animal models with CPR (Adrie *et al*., 2002; Callaway *et al*., 2008). As a result, AH treatment significantly increased the ratio of IFN-γ to IL-4, indicating that AH treatment mitigated the transformation of Th1 to Th2.

During ischemia/reperfusion, the gastrointestinal mucosal barrier is disrupted, promoting intestinal-derived bacteria and toxins to invade the body, and causing damage to the gastrointestinal tract and distal organs (Kaye, Parnell, Ahlers, 2002). In the event of ischemia and hypoxia, epithelial cell membrane and intercellular connection breaks, the epithelium begins to fall from the villus terminus, and the entire mucosa falls off to form an ulcer, leading to increased intestinal permeability and further bacterial translocation (Secchi *et al*., 2000). Immunosuppression and subsequent bacterial translocation significantly contribute to higher risk of infection in patients during hospitalization after ROSC. Our results showed that after intestinal IRI, there were various bacteria identified in the GALTs, mainly comprised of *E. coli* and other Gram-negative strains, which were consistent with dominant bacteria in the intestinal flora. This suggested that bacteria translocated to the mesenteric lymph node after intestinal IRI were mainly derived from the intestine. However, AH treatment reduced the positive cultures and mitigated the incidence of bacterial translocation.

Some study limitations should be mentioned. First, although AH treatment demonstrated apparent effects, its activities need to be confirmed by other animal models. Second, the exact mechanisms of AH against IRI should be further explored. Finally, it is uncertain whether the data from the animal model accurately reflect molecular changes in humans.

CONCLUSIONS

The intestinal mucosal immune barrier is damaged during IRI. This study demonstrated that AH treatment inhibited Th1-Th2 transformation in the blood and GALTs, which reduced the incidence of bacterial translocation and lessened the risk of secondary infection caused by IRI.

CONFLICT OF INTEREST

None

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