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Full Length Research Paper

Therapeutic hypothermia reduces intestinal ischemia/reperfusion injury after cardiac arrest in rats

Jun Zhu[1,2], Yue Fu[1,2], Jun Jiang[1,2], Jia-Kang Liang[1,2] and Zi-Tong Huang[1,2]*

[1]Department of Emergency Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China.
[2]Institute of Cardiopulmonary Cerebral Resuscitation, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China.

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To investigate the effects of therapeutic hypothermia (TH) on the morphology and function of intestine after cardiac arrest and resuscitation, 45 male rats were randomly assigned into three groups: (1) normothermia group, animals underwent ventricular fibrillation (VF) and cardiopulmonary resuscitation (CPR) with the rectal temperature maintained at 36.8 ± 0.2°C until 4 h after return of spontaneous circulation (ROSC); (2) hypothermia group, TH was induced with the aid of ice packs and an electrical fan because VF occurred and was maintained at 33.5 ± 0.5°C for 4 h after ROSC; (3) sham-operated group, animals underwent identical anesthetic and surgical procedures without VF, CPR or defibrillation. Five animals in each group were sacrificed at 4, 24 and 72 h post resuscitation. Serum diamine oxidase (DAO) and apoptosis rate of intestinal epithelial cells were tested by ELISA and flow cytometry, respectively. The concentration of FITC-Dextran that leaked out of enteric cavity was used to analyze the permeability of intestine. Histological changes were graded and compared among the three groups. Serum DAO concentrations in normothermia group reached the peak at 4 h post resuscitation, and then decreased at 24 and 72 h. In comparison with normothermia group, serum DAO concentrations were lower at 4 h in hypothermia group (0.97 ± 0.16 vs. 1.24 ± 0.29, $P < 0.05$). The amount of FITC-Dextran that passed the wall of small intestine in hypothermia group was significantly lower than that in normothermia group at 24 h after ROSC (7.81 ± 1.11 vs. 13.07 ± 3.07, $P < 0.05$). The amount of FITC-Dextran had no difference between normothermia and hypothermia groups at 4 and 72 h post resuscitation. The detached intestinal epithelial cells in hypothermia group showed significant lower frequency of apoptosis than those in normothermia group at 4 h (17.30 ± 2.56 vs. 25.63 ± 4.09, $P < 0.05$) and 24 h (9.38 ± 1.29 vs. 11.98 ± 1.78, $P < 0.05$). No obvious injury was observed in both normothermia and hypothermia groups at 4 h with grade of 0 to 1. The histopathological injury in normothermia group reached the peak at 24 h with grade of 2 to 3, which was significantly severe than that in hypothermia group with grade of 1 to 2. At 72 h post resuscitation, an almost complete restitution of the intestinal mucous could be observed both in hypothermia and normothermia groups. This study demonstrates that short term ischemia induced by cardiac arrest and resuscitation resulted in intestinal ischemia/reperfusion (IR) injury, which could be attenuated by therapeutic hypothermia.

Key words: Rat, intestine, cardiac arrest, cardiopulmonary resuscitation, therapeutic hypothermia.

INTRODUCTION

The interruption and restoration of blood flow, induced by cardiac arrest and cardiopulmonary resuscitation, causes the systemic ischemia/reperfusion (I/R) injury. Post-resuscitation dysfunctions of brain and heart are of most importance to researchers. However, till now, little attentions are paid to the injury of intestine, which is equally or even more sensitive to ischemia than heart

*Corresponding author. E-mail: zitonghuang@sohu.com. Tel: +86-20 81332410. Fax: +86-20 81332650.

and brain. The changes of intestinal morphology and function post resuscitation have not been clearly elaborated yet. Adrie et al. (2002) considered successful cardiopulmonary resuscitation after cardiac arrest as a “Sepsis-Like” syndrome, which include the release and activation of many inflammatory factors and endotoxemia. Over the last decades, although, sepsis as well as multiple organ dysfunction syndrome (MODS) has been the focus of extensive clinical and laboratory investigation, the exact mechanisms responsible for its occurrence and development remain to be fully elucidated. The intestinal barrier dysfunction and bacterial translocation have been considered as an initial factor of sepsis (Wells et al., 1988; Deitch, 1990). So we hypothesized that the intestinal IR injury may play an important role in the occurrence of post-resuscitation syndrome.

As a clinically effective therapy during cardiopulmonary resuscitation (CPR), therapeutic hypothermia (TH) has been recommended by the AHA Guideline Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science for several years, whereas very little is known about the effects of TH on the structure and function of intestine after cardiac arrest and resuscitation. In this preliminary study, we observed the changes of morphology and permeability in intestine and the effects of TH on them. We hypothesized that TH may reduce the intestinal IR injury after cardiac arrest.

MATERIALS AND METHODS

Preparation

All protocols were approved by the Intramural Animal Care and Use Committee of Sun Yat-sen University. Healthy male Sprague-Dawley rats, weighing 400 to 500 g, were provided by Experimental Animal Center, Guangzhou University of Traditional Chinese Medicine. All animals were fasted for 12 h before commencing the experiments but with free access to water. Anesthesia was induced by the intraperitoneal injection of pentobarbital sodium solution (Merk) with a dose of 45 mg/kg and additional dose of 10 mg/kg was administrated when necessary. The trachea was orally intubated with a 14-gauge cannula (Abbocath-T, Abbott Hospital Products Division; North Chicago). A 23-gauge polyethylene (PE) 50 catheter (Becton Dickinson, Franklin Lakes, NJ) was placed in the abdominal aorta from the left femoral artery for measurement of arterial pressure. Through the left external jugular vein, a 4-F PE catheter (model C-PMS-401J, Cook Critical Care; Bloomington, Ind) was advanced into the right atrium for electrical induction of VF. All the catheters were flushed intermittently with saline containing 5 IU/ml heparin. The rectal temperature and ECG through limb lead II was monitored continuously, and the temperature was maintained at 36.8 ± 0.2°C by a heating lamp before the induction of VF.

Experimental procedures

The rats were randomly assigned to three experimental groups (n = 15); group: (1) normothermia group, animals underwent VF and CPR with the rectal temperature maintained at 36.8 ± 0.2°C by a heating lamp until 4 h after return of spontaneous circulation (ROSC); (2) hypothermia group, mild hypothermia was induced with the aid of ice packs and an electrical fan (D'Cruz et al.,2002) because VF occurred and was maintained at 33.5°C ± 0.5°C to 4 h after ROSC; (3) sham-operated group, animals underwent identical anesthetic and surgical procedures without VF, CPR or defibrillation. VF was induced with electric current of 4.0 mA delivered to the right ventricular endocardium for 3 min. Eight minutes after the onset of untreated VF, cardiopulmonary resuscitation was attempted. Animals received ventilation and precordial chest compression (designed and fabricated by our group, Sun Yat-sen University). Precordial compression was at a rate of 200 min^{-1} with the depth of 1/3 thoracic anteroposterior diameter. The mechanical ventilation was begun at a precordial compression which was at a rate of 200 min^{-1} with the depth of 1/3 thoracic anteroposterior diameter. The mechanical ventilation was begun at an inspiration/expiration (I:E) ratio of 1:2. Defibrillation was implemented with 2-J biphasic waveform transthoracic shocks (M-Series, Zoll Medical Corporation; Chelmsford, Mass) after 6 min of CPR. If ROSC was not achieved within 15 s after shock, 1 more minute of compression and ventilation was adopted. ROSC was defined as the return of supraventricular rhythm with a MAP ≥ 60 mmHg for ≥5 min.

Five animals in each group were sacrificed at 4, 24 and 72 h post ROSC, respectively, for blood and histology tests.

Diamine oxidase (DAO) ELISA

The samples of blood were drawn from the portal vein. After centrifugation at 1500 rpm for 5 min, the serum was harvested and DAO was assayed using commercial ELISA kit according to the directions of the manufacturer (Wuhan Boster Biological Technology., LTD, China).

Intestinal permeability

Quantification of intestinal permeability was evaluated with the method described by Yasuda et al. (2007). In brief, after the related mesentery was ligated, 8 cm of terminal ileum was removed. The distance between the distal cut end and ileocecal junction was 5 cm. Intestinal lumen was cautiously rinsed with saline, and then one side of the intestine was ligated. Before another side was ligated, 0.5 ml of 40 g/L FITC-Dextran (average mol wt 3000-5000, Sigma) was injected into the intestinal lumen. The closed intestinal pouch was put in 20 ml saline at 37°C and placed on the shaker for 60 min. Care was taken to avoid mechanical damage to the specimens. The amount of FITC-Dextran that leaked out of the intestinal pouch was assayed with fluorospectrophotometer (Hitachi F3010, Tokyo, Japan). The permeability of intestine was assessed by the contents of FITC-Dextran.

Apoptosis of intestinal epithelial cells

The apoptosis rates of intestinal epithelial cells were assayed by flow cytometry. The detached epithelial cells were collected according to the method described by Ikeda et al. (1998). The resected segments of the distal ileum were opened and shaken vigorously in 20 ml of phosphate buffered saline (PBS), then centrifuged at 1000 rpm/min for 10 min. After resuspended in PBS, the detached epithelial cells were stained by Anneix V and PI and analyzed immediately by flow cytometry (Becton Dickinson FACSCalibur, USA).

Table 1. DAO levels in the portal vein blood (10^3 U/L).

Group	4 h	24 h	72 h
Normothermia group	1.24±0.29$^{\Delta}$	0.79±0.17$^{\Delta}$	0.58±0.09
Hypothermia group	0.97±0.16*$^{\Delta}$	0.72±0.10$^{\Delta}$	0.61±0.09
Sham-operated group	0.51±0.07	0.57±0.08	0.54±0.06

*P < 0.05 as compared with normothermia group, $^{\Delta}P$ < 0.05 as compared with sham-operated group. Values are expressed as mean ± SEM (n = 5).

Histopathology

For histological examination under light microscope, a sample of 1 cm resected from the terminal ileum was fixed for at least 24 h in 10% neutral buffered formalin, and then was embedded in paraffin. After continued paraffin section and hematoxylin/eosin (HE) staining, the tissue sections were examined with light microscope at ×200 magnification. Intestinal mucosal lesions were graded from 0 to 5 according to the scale defined by Chiu et al. (1970) as follows: grade 0, normal mucosa; grade 1, subepithelial space at villus tips; grade 2, extension of subepithelial space with a moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of villus, few tips denuded; grade 4, denuded villi and dilated capillaries; grade 5, disintegration of lamina propria, hemorrhage and ulceration. All sections were evaluated microscopically in a blinded fashion for degree of histological damage.

Statistics

Statistical analysis was performed using SPSS 13.0 software. Numeration data from each group was described as mean ± SEM and compared by using one-way analysis of variance. Histological grading comparisons between hypothermia and normothermia groups were evaluated by Mann-Whitney Rank Sum Test. P < 0.05 were defined as significant difference.

RESULTS

Normothermia and hypothermia groups had no significant differences in baselines of weight, blood pressure and partial pressure of end-tidal carbon dioxide.

DAO ELISA

Table 1 shows DAO levels in portal vein blood. Serum DAO concentrations in normothermia group reached its peak at 4 h post resuscitation, and then decreased at 24 and 72 h, but were still higher than those in sham-operated group at 24 h. In comparison with normothermia group, serum DAO concentrations were lower at 4 h in hypothermia group (0.97 ± 0.16 vs. 1.24 ± 0.29, P < 0.05). There was no difference of DAO in the three groups at 72 h.

Intestinal permeability

As shown in Table 2, the amount of FITC-Dextran that passed the wall of the small intestine in hypothermia group was significantly lower than that in normothermia group at 24 h after ROSC (7.81 ± 1.11 vs. 13.07 ± 3.07, P < 0.05). The amount of FITC-Dextran had no difference between normothermia and hypothermia groups at 4 and 72 h post resuscitation. The two resuscitation groups had higher concentration of FITC-Dextran than sham-operated group at 4 h after ROSC, which was not observed at the time point of 72 h.

Apoptosis of intestinal epithelial cells

The detached intestinal epithelial cells in hypothermia group showed significant lower frequency of apoptosis than those in normothermia group at 4 h (17.30 ± 2.56 vs. 25.63 ± 4.09, P < 0.05) and 24 h (9.38 ± 1.29 vs. 11.98 ± 1.78, P < 0.05) post resuscitation. Both hypothermia and normothermia groups had higher apoptosis rate than the sham-operated group at 4 and 24 h. All the three groups had no difference in the frequency of apoptosis at 72 h post resuscitation (Table 3).

Histopathology

Rats in sham-operated group had normal morphology of the small intestinal epithelium and villus. No obvious injury was observed in both normothermia and hypothermia groups at 4 h with grade of 0 to 1. The histopathological injury in normothermia group reached the peak at 24 h with grade of 2 to 3, which was more significantly severe than that in hypothermia group with grade of 1 to 2 (Figure 1). At 72 h post resuscitation, an almost complete restitution of the intestinal mucous could be observed both in hypothermia and normothermia groups.

DISCUSSION

Despite the important role of the intestinal injury in cardiopulmonary resuscitation, few investigations have focused on this topic and whether TH has the potential of attenuating the injury. This study shows that TH alleviates intestinal IR injury after cardiac arrest and successful

Table 2. FITC that leaked out of the damaged intestine (mg/L).

Group	4 h	24 h	72 h
Normothermia group	6.75±0.89$^{\Delta}$	13.07±3.07$^{\Delta}$	1.85±0.36
Hypothermia group	5.45±0.70$^{\Delta}$	7.81±1.11$^{*\Delta}$	1.65±0.33
Sham-operated group	1.25±0.21	1.12±0.21	1.28±0.19

$^{*}P < 0.05$ as compared with normothermia group, $^{\Delta}P < 0.05$ as compared with sham-operated group. Values are expressed as mean ± SEM (n = 5).

Table 3. Apoptosis rate of the intestinal epithelial cells (%).

Group	4 h	24 h	72 h
normothermia group	25.63±4.09$^{\Delta}$	11.98±1.78*	8.66±1.21
hypothermia group	17.30±2.56$^{*\Delta}$	9.38±1.29$^{*\Delta}$	7.93±1.28
sham-operated group	5.63±0.88	6.47±1.01	6.93±1.25

$^{*}P < 0.05$ as compared with normothermia group, $^{\Delta}P < 0.05$ as compared with sham-operated group. Values are expressed as mean ± SEM (n = 5).

cardiopulmonary resuscitation in rats.

Post-cardiac arrest syndrome is considered to be one of the components contributing to the poor outcome of CPR (Neumar et al., 2008). Since limited information is available on the underlying mechanism of post-cardiac arrest syndrome and the post-cardiac arrest syndrome shares many common features with sepsis, the concerned hypothesis of sepsis is often used for reference (Adrie et al., 2002; Geppert et al., 2000). Till now, in the pathophysiological process of sepsis/SIRS, gut hypothesis proposed by Deitch (1989) is the most popular. According to Deitch, the combination of visceral hypoperfusion, mucosal ischemia and the subsequent reperfusion changes the structure and function of the intestinal epithelial cells, increases the permeability of the intestinal mucosal barrier, and leads to the translocation of bacteria and its endotoxins. Besides, gut ischemia secondarily results in the formation of oxygen radicals, the release of inflammatory mediators and the activation of leukocytes. Considering that the destruction of intestinal barrier initiates the development of sepsis, we presume that intestinal IR injury play an important role in the pathogenesis of post-cardiac arrest syndrome.

It was reported that ileum was more susceptible to IR injury than the rest parts of the gut (Chan, 2002). So, in this study, we focused on the ileum IR injury and observed that the most severe morphological injury of the intestine happened at 24 h and the intestinal mucosal barrier was rehabilitated at 72 h after ROSC.

The inchoate detectable sign of intestinal ischemia was considered to be the increased permeability of capillary and mucosal, and then the morphological injury could be located in mucosal epithelial cells (Haglund, 1994). As an endocellular enzyme, DAO exists in the intestinal villus tip cells and have high activity in jejunum and ileum. Both plasma and mucosal levels of DAO activity were regarded as the sensitive markers of mucosal damage and were positively correlated with the degree of mucosal damage in previous publications (Luk et al., 1980; Bragg et al., 1991). Our results showed that the serum level of DAO peaked at 4 h after ROSC in both the normothermia and the hypothermia group, whereas the permeability and the histological damage of the intestine culminated at 24 h post resuscitation. It confirms the previous hypothesis that DAO is an early marker that predicts the occurrence of intestinal injury. By the detection of circulating DAO and the intestinal mucosal permeability, we preliminarily demonstrated the protective effect of TH on intestine subjected to VF and ensuing ROSC.

Apoptosis, as a major mode of intestinal epithelial cell death, was reported by Ikeda et al. (1998) to play an important role in the damage of intestinal mucous epithelial barrier after a short term of intestinal IR. Contrary to Ikeda's ischemia model of superior mesenteric artery occlusion (SMAO) for at least 15 min, we adapted a whole-body IR model in this study, in which blood flow was completely occluded for 8 min and followed by 6 min of low flow. Our results showed that a systemic ischemia within 15 min may result in apparent intestinal IR injury as well. This study also demonstrated that the culmination of apoptosis occurred at an early stage and previous to the occurrence of obviously visible intestinal injury. Moreover, the apoptosis rate in the hypothermia group was significantly lower than that in the normothermia group at the early stage after ROSC, indicating that the inhibition of apoptosis may be one of the protective mechanisms of TH.

TH has been widely investigated in both global and

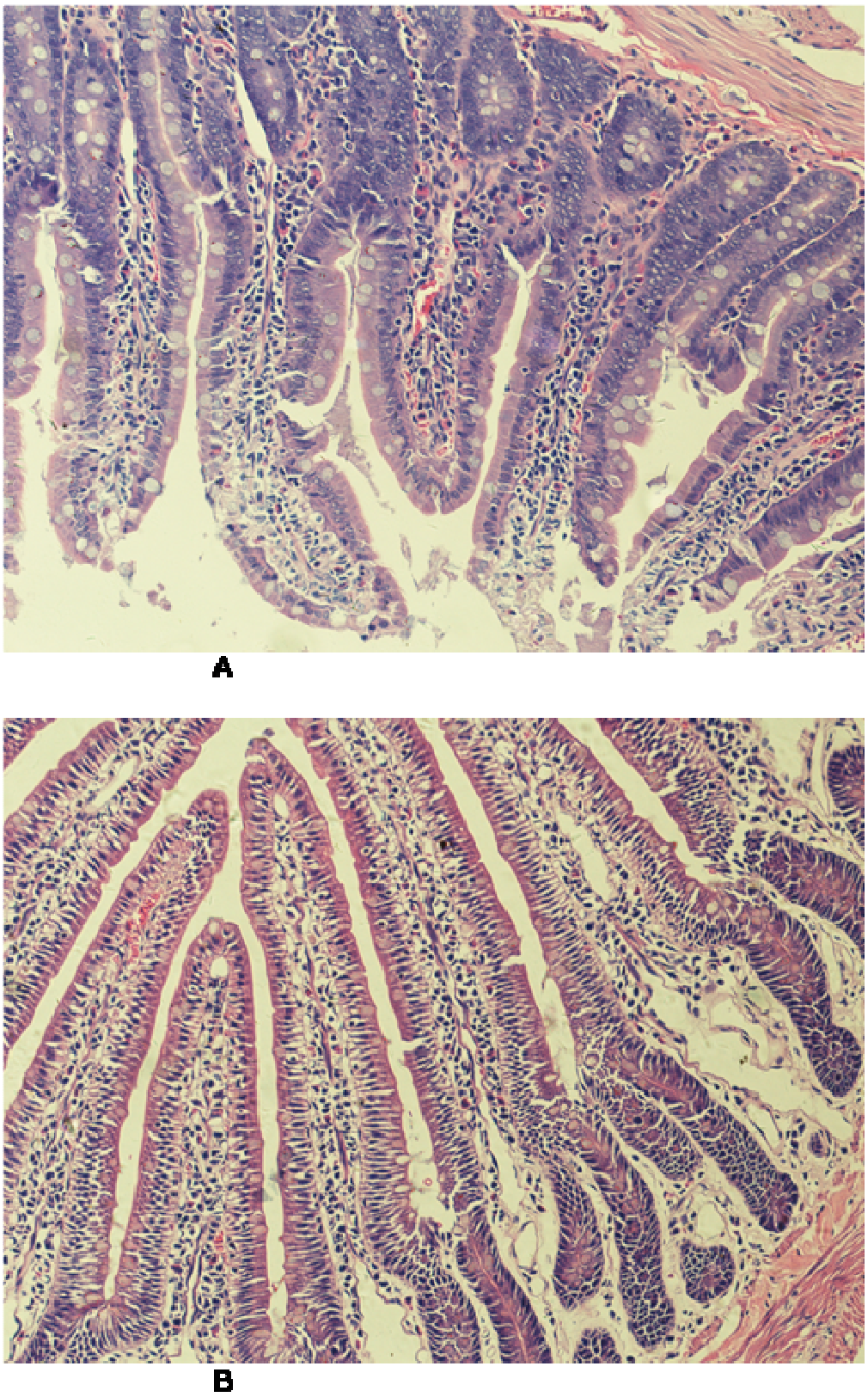

Figure 1. Representative photomicrographs of histological sections of ileum. (A) Mucosal damage seen in normothermia group at 24 h post resuscitation (grade 3). (B) Mucosal damage seen in hypothermia group at 24 h post resuscitation (grade 1) (hematoxylin-erosin staining, ×200).

local ischemic brain injury and found to improve survival rate, as well as reduce neurological deficits in different neurological insults, such as cerebrovascular accident, subarachnoid hemorrhage, head injury and anoxic brain damage (Hartemink et al., 2004; Clifton et al., 2009; Sagalyn et al., 2009). Moreover, moderate hypothermia is considered to provide protection against intestinal IR injury of SMAO and other intestinal ischemia cases (Attuwaybi et al., 2003; Stefanutti et al., 2008). However, to the best of our knowledge, the effects of TH on the structure and function of intestine after cardiac arrest and resuscitation have not been reported. This study validates the beneficial influence of TH on the intestine in the period of cardiopulmonary resuscitation.

In conclusion, this study demonstrated that the intestinal IR injury can be induced by the short-term systemic ischemia following cardiac arrest and cardiopulmonary resuscitation, which may be attenuated by TH.

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ABBREVIATIONS

TH, Therapeutic hypothermia; **VF,** ventricular fibrillation; **CPR,** cardiopulmonary resuscitation; **ROSC,** return of spontaneous circulation; **DAO,** diamine oxidase; **IR,** intestinal ischemia/reperfusion.

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