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ORIGINAL ARTICLE

Randomized Controlled Trial

MicroRNA profiling of the intestine during hypothermic circulatory arrest in swine

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Author contributions: Lin WB conducted the molecular studies, analyzed the data and drafted the manuscript; Chen GX, Liang MY and Yao JP participated in establishing the cardiopulmonary bypass model; Feng KN and Qin H participated in the design of the study and performed the statistical analysis; Wu ZK and Yang X conceived the study, participated in its design and coordination and helped to draft the manuscript; all authors read and approved the final manuscript.

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Abstract

AIM: To perform a profiling analysis of changes in intestinal microRNA (miRNA) expression during hypothermic circulatory arrest (HCA).

METHODS: A total of eight piglets were randomly divided into HCA and sham operation (SO) groups. Under general anesthesia, swine in the HCA group were subjected to hypothermic cardiopulmonary bypass at 24 ℃ followed by 80 min of circulatory arrest, and the reperfusion lasted for 180 min after cross-clamp removal. The counterparts in the SO group were only subjected to median sternotomy. Histopathological analysis was used to detect mucosal injury, and Pick-and-Mix custom miRNA real-time polymerase chain reaction (PCR) panels containing 306 unique primer sets were utilized to assay unpooled intestinal samples harvested from the two groups.

RESULTS: The intestinal mucosa of the animals that were subjected to 24 ℃ HCA exhibited representative ischemic reperfusion injury of grade 2 or 3 according to the Chiu score. Such intestinal mucosal injuries, with the subepithelial space and epithelial layer lifting away from the lamina propria, were accompanied by shortened and irregular villi. On the contrary, the intestinal mucosa remained normal in the sham-operated animals. In total, twenty-five miRNAs were differentially expressed between the two groups (15 upregulated and 10 downregulated in the HCA group). Among these, eight miRNAs (miR-122, miR-221-5p, miR-31, miR-421-5p, miR-4333, miR-499-3p, miR-542 and let-7d-3p) were significantly dysregulated (four higher and four lower). The expression of miR-122 was significantly (5.37-fold) increased in the HCA group *vs* the SO group, indicating that it may play a key role in

HCA-induced mucosal injury.

CONCLUSION: Exposure to HCA caused intestinal miRNA dysregulation and barrier dysfunction in swine. These altered miRNAs might be related to the protection or destruction of the intestinal barrier.

Key words: Reperfusion injury; Cardiopulmonary bypass; Animal model; Barrier function; Randomized controlled trial; MicroRNA

Core tip: Swine intestine was subjected to hypothermia, cardiopulmonary bypass and ischemia/reperfusion following hypothermic circulatory arrest (HCA). These factors caused barrier dysfunction, resulting in gastrointestinal complications. Histopathological and microRNA (miRNA) array analyses were used to investigate the effects of HCA on the gut barrier. HCA was found to disturb barrier function in the small intestine and influence the miRNA levels in swine. Our results contribute to the body of research examining gut barrier function following HCA *in vivo*.

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INTRODUCTION

Since Bigelow performed the first experiments on hypothermic circulatory arrest (HCA) in 1950, HCA has become a vital technique in surgery for aortic and congenital heart disease. HCA can decrease the metabolic rate of tissues throughout the body and provides a bloodless surgical field while increasing complications such as edema formation, coagulopathy and organ dysfunction. On the grounds that the brain is the organ that is the most sensitive to ischemia, many studies have focused on neurological impairment associated with HCA, while only a few studies have focused on other organs, such as the kidney, intestines and spinal cord.

The prognosis after cardiopulmonary bypass (CPB) is closely related to the degree of gastrointestinal complications[1]. Even simple CPB will impair gastrointestinal function[2]. Although the underlying mechanism remains unclear, possible explanations for these complications include gastrointestinal hypoperfusion[3,4], gut barrier dysfunction, preoperative immune function defects, the change in the blood capillary permeability of the intestinal wall, and the systemic inflammatory response. Furthermore, HCA may increase the risk associated with gut barrier dysfunction[5]. Due to HCA, the intestines suffer from ischemic reperfusion injury (IRI).

MicroRNAs (miRNAs) are small noncoding RNAs that are capable of silencing gene expression post-transcriptionally. In recent years, research on miRNA has been mainly focused on cancer, while miRNA expression following IRI has remained poorly understood. Growing evidence indicates that some miRNAs are related to IRI, such as miRNA expression in the liver[6], kidney[7], muscle[8] and flap[9]; however, there are no reports on miRNA expression in intestinal IRI following HCA.

We postulated that miRNA is also associated with intestinal IRI during HCA. The present study aims to identify the influence of HCA on microRNA expression in the intestine by using miRNA polymerase chain reaction (PCR) arrays in swine.

MATERIALS AND METHODS

Animals and study design

A total of 8 Wuzhishan pigs (6 to 8-wk-old, weight 9.7-13 kg, average 11.66 kg) were randomly assigned into HCA 24 ℃ and sham operation (SO) groups. The experiment was conducted in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996) and the Guidelines for Animal Experimentation, which were issued by the First Affiliated Hospital of Sun Yat-sen University.

Anesthesia

The animals were sedated with intramuscular ketamine hydrochloride and maintained on Ⅳ infusions of ketamine (40 mg/kg) and fentanyl (2 μg/kg per hour) after endotracheal intubation *via* tracheotomy. Following this step, an arterial pressure catheter was inserted into the right femoral artery for pressure monitoring and blood sampling. Then, an 8-Fr central venous catheter was inserted *via* the left femoral vein for fluid administration and central venous pressure monitoring.

CPB and sham operation

A median sternotomy was performed in both groups. Next, each of the piglets in the HCA group was administered heparin sodium (400 IU/kg). The ascending aorta was cannulated with a single 10F arterial cannula, and the superior and inferior vena cava were both cannulated with 12F cannulas. The CPB circuit consisted of a roller pump, a membrane oxygenator (SK3301, Medtronic Inc, Minneapolis, MN, United States), and a heat exchanger (Sarns Heater

Cooler, Ann Arbor, MI, United States). The circuit was primed with electrolyte solution, blood from a donor pig and heparin (3 mg/100 mL total fluid). A mean arterial pressure of 50 to 80 mmHg was maintained by keeping the CPB flow rate between 75 mL/kg per minute and 80 mL/kg per minute in the CPB group.

HCA

After initiation of CPB, the animals were cooled to a target nasopharyngeal temperature of 30 ℃ by a heat exchanger (Sarns, Ann Arbor, MI, United States). After the body temperature lowered to 30 ℃, the ascending aorta was cross-clamped, and the cardioplegia solution was administered. Topical ice slush was used to cool the surface of the heart throughout the HCA procedure. The heat exchanger operated until the body temperature was cooled down to 24 ℃. Then, the roller pumps were turned off, and HCA was performed for the next 80 min. Next, the aorta was declamped, and the animal was rewarmed to normothermia in 60 min using a temperature gradient of 8 ℃. For the hearts that encountered deleterious arrhythmia after de-clamping, direct-current defibrillation was used to restore the sinus rhythm. The animals were weaned off from CPB when the hemodynamic data became steady after partial perfusion, and the reperfusion lasted for 180 min following the removal of the cross-clamping. When the experiment was complete, midline laparotomy was performed to obtain specimens from the terminal ileum, which were preserved at -80 ℃.

Real-time PCR profiling of microRNAs

RNA was isolated from cryopreserved tissue using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, United States) and subsequently precipitated, washed and redissolved. The RNA purity was measured using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, United States). cDNA was generated from 20 μL of RNA using the buffer and the enzymes that were provided in the Qiagen kit (Exiqon, Denmark). Pick-and-Mix custom panels (Exiqon, Denmark) containing 306 primer sets uniquely designed for microRNAs were chosen for miRNA expression profiling. The cDNA was diluted × 110 and assayed in 10-μL PCR reactions according to the protocol for miRCURY LNA TM Universal RT microRNA PCR. PCR was performed using the ABI PRISM 7900 system (Applied Biosystems, Inc., Foster City, CA, United States). The miRNA expression was normalized relative to the expression of U6 using the ΔΔCt method. Then, the fold change was calculated by using the ratio of $miRNA_{HCA}/miRNA_{SO}$ or $miRNA_{SO}/miRNA_{HCA}$ (when $miRNA_{HCA}$ was down-regulated).

Histopathological analysis

Hematoxylin and eosin-stained sections from formalin-fixed and paraffin-embedded intestinal samples were assessed by two pathologists who were blinded to the study protocol according to Chiu's method[10]. All samples were observed under a stereomicroscope (DM 2500B, Leica, Germany) at × 200 magnification using an objective lens with an aperture of 22 mm. Photomicrographs were obtained using the Leica Application Suite (Leica Microsystems, Wetzlar, Germany) and edited with Adobe Photoshop 12.0 (Adobe Systems Inc., San Jose, United States).

Statistical analysis

Significant differences between the two groups were tested using Student's *t*-test, with a significance threshold of $P < 0.05$. miRNAs with expression changes of > 2-fold were considered to be differentially expressed. The statistical program SPSS 20.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis.

RESULTS

Morphological changes in the intestine following HCA

No significant differences were observed in the intestinal mucosa between the two groups based on observations with the naked eye. However, histological tissue evaluations revealed that the structures of the mucosal epithelial layer and the lamina propria were well preserved in the SO group (Figure 1A). In contrast, the intestinal mucosa of the animals that were subjected to HCA demonstrated representative IRI of grade 2 or 3 according to the Chiu score (Figure 1B). This result indicates that the gut barrier function was interrupted following 80 min of ischemia and 180 min of reperfusion despite exposure to 24 ℃ hypothermia.

MiRNA real-time PCR array

Relative to the SO group, miRNA profiling revealed that 25 miRNAs were differentially expressed ($P < 0.05$) in the intestine after 80 min of ischemia and 180 min of reperfusion (Figure 2 and Table 1). Of the 25 miRNAs, miR-122, -542-5p, -499-3p, -421, let-7d-3p, miR-31, -221-5p and -4333 were diversely expressed between the HCA and SO groups for $P < 0.05$ and a fold change > 2 levels of significance. In Table 2, we summarize six miRNAs that were previously implicated in IRI. Although these six miRNAs were not significantly expressed in the intestine, a lower miR-192 level was observed compared to the SO group (Table 2).

DISCUSSION

To date, no data are available regarding the intestinal miRNA expression profile during an HCA procedure. In the present study, we detected twenty-five dys-regulated miRNAs and injury to the mucosa barrier in the intestine following HCA. The factors that may have

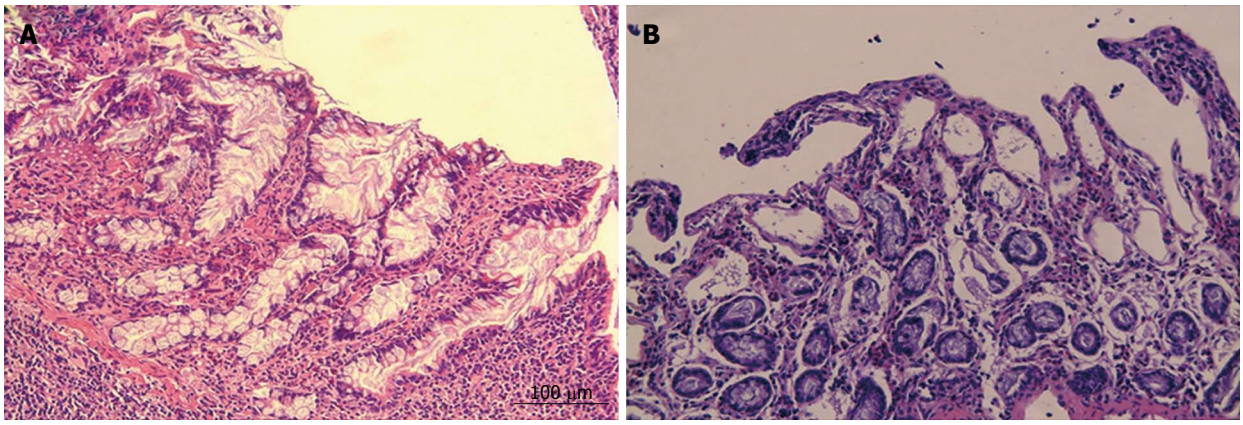

Figure 1 Histopathological changes in the mucosa in each group. A: Epithelial layer and the lamina propria of the intestinal mucosa in the SO group were normal; B: Shortened and irregular villi as well as subepithelial space were observed; some lifting of the epithelial layer of the lamina propria was observed in mucosa exposed to HCA (magnification × 200).

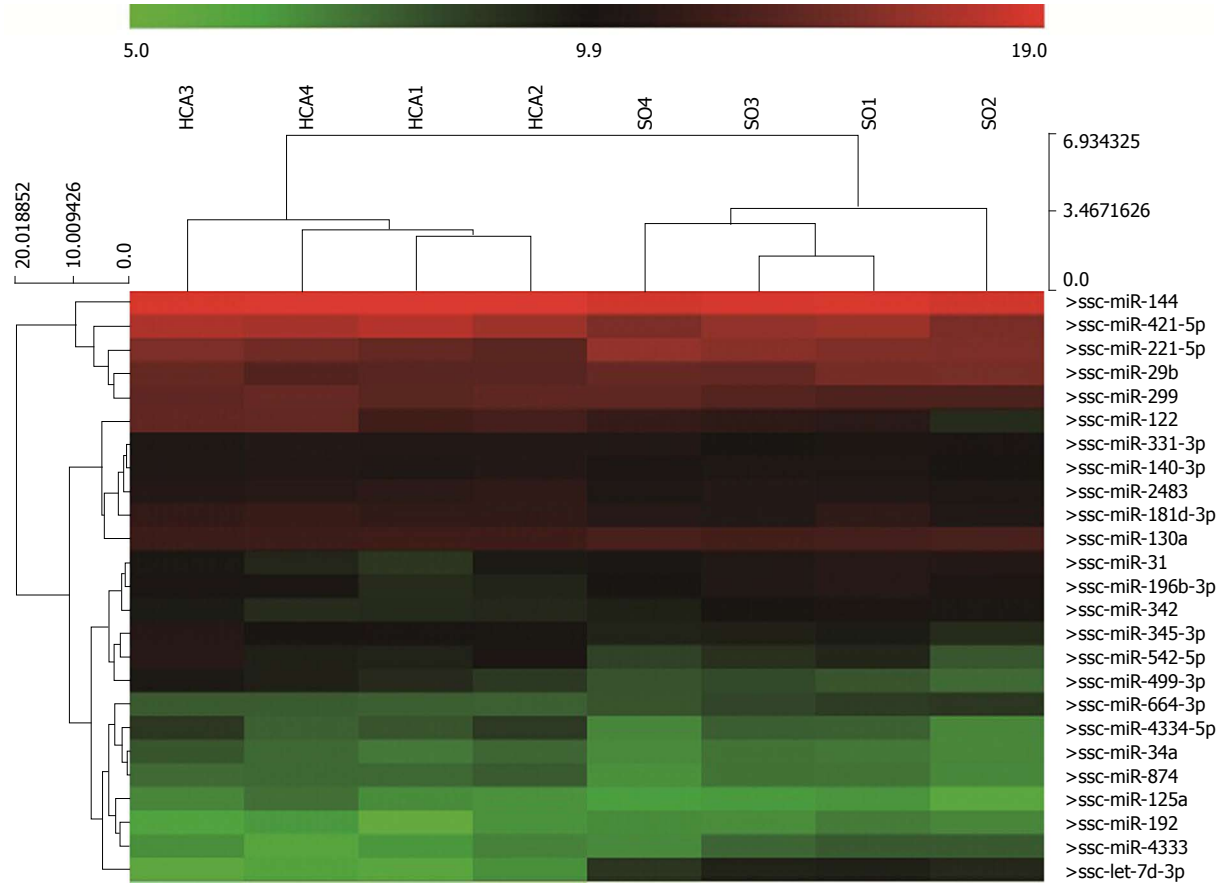

Figure 2 Heat maps of the differentially expressed microRNAs. Twenty-five dysregulated microRNAs were detected between the HCA and SO groups ($P < 0.05$) in this study. HCA: Hypothermic circulatory arrest; SO: Sham operation.

influenced miRNA expression in this study included hypothermia, CPB and circulatory arrest. Firstly, hypothermia may induce miRNA dysregulation. For example, higher miR-21 expression levels have been detected in skeletal muscle and liver at -3℃ compared to 5℃ *in vivo*[11]. Interestingly, in another tissue miRNA study performed in a traumatic brain injury rat model, miR-874 was markedly differentially expressed under hypothermia conditions (33℃) compared to normothermia (37℃)[12]. Specifically, temperature-dependent miRNA modulation was mediated by RNA-binding motif protein 3[13]. These studies were not performed at 24℃; nevertheless, they reflected the effect of hypothermia on miRNA. Therefore, the modulated miRNAs in our study (let-7d-3p, miR-29b, miR-125a, miR-130a, miR-874) may be temperature-dependent miRNAs that are affected by HCA. Secondly, changes in the miR-499 levels

Table 1 MicroRNAs that were dysregulated in the hypothermic circulatory arrest group *vs* the sham operation group

	Up-regulation	FC	*P* value	Down-regulation	FC	*P* value
1	miR-122	5.37	0.0193	let-7d-3p	8.73	0.0000
2	miR-542-5p	2.64	0.0273	miR-31	2.33	0.0141
3	miR-499-3p	2.47	0.0026	miR-221-5p	2.18	0.0248
4	miR-421-5p	2.41	0.0141	miR-4333	2.01	0.0219
5	miR-4334-5p	1.95	0.0344	miR-29b	1.89	0.0213
6	miR-345-3p	1.73	0.0212	miR-196b-3p	1.79	0.0440
7	miR-181d-3p	1.63	0.0211	miR-192	1.63	0.0494
8	miR-2483	1.59	0.0074	miR-664-3p	1.55	0.0085
9	miR-125a	1.52	0.0487	miR-342	1.49	0.0266
10	miR-874	1.48	0.0226	miR-130a	1.40	0.0006
11	miR-299	1.47	0.0440			
12	miR-34a	1.41	0.0489			
13	miR-331-3p	1.38	0.0392			
14	miR-140-3p	1.36	0.0232			
15	miR-144	1.35	0.0166			

Among the 25 aberrantly expressed miRNAs, only eight miRNAs showed significant changes. miR-4333 expression was found to overlap with that of the well-conserved SNORA53 based on information retrieved from miRBase 20.

in the myocardium during CPB were reported[14]; however, no change about miR-499 was reported in the intestine. miR-499 is an acute myocardial infarction marker that is overexpressed in cardiac IRI. In a study by Reddy *et al*[15], the authors stated that miR-499 is cardiac cell-specific in swine and cannot be detected in other organs; however, only the stomach was included in this previous study, and the intestine was not analyzed. Our data revealed differential miR-499 expression between the normal and experimental intestines, indicating that miR-499 may be a marker of IRI in the intestine, distinct from the heart. However, with the present study design, we cannot differentiate among the hypothermia-induced, CPB-induced and IRI-induced miRNA changes. Therefore, additional studies are needed to assess the individual impact of these factors. Thirdly, few studies examining alterations in the expression of miRNAs in the intestine subjected to IRI have been reported, although miRNAs were found to be altered in other organs subjected to IRI. Nevertheless, the dysregulation of several miRNAs was found in other organs subjected to IRI[9,16-18], including the liver (miR-146), kidney (miR-10a, miR-30d), and skin flap (miR-21, miR-96, miR-193-3p, miR-210). However, the miRNA expression found in our study was quite different than in other IRI studies. The decreased levels of miR-192 that we detected were consistent with a previous report on renal IRI[18]. This phenomenon also suggests that IRI is not the only factor related to intestinal miRNA changes following HCA. Consequently, this finding suggests that the superior mesenteric artery ligation model may not be suitable to mimic the intestinal pathophysiology changes in cardiac surgery.

The histological change of the intestinal mucosa is one of the key factors used to evaluate gut barrier function. Our results suggest that the gut barrier function was compromised in animals subjected to 24 ℃ HCA. Altered levels of several miRNAs were associated with barrier dysfunction, indicating that they may play a protective/detrimental role in HCA. Cytokines, such as IL-6 and IL-10, are key inflammation regulators of CPB-induced intestinal dysfunction mediated by the NF-kB pathway. Let-7d[19,20] and miR-421[21] were hypothesized to promote gut barrier conservation by reducing the cytokine level. However, in the present study, the let-7d expression level decreased, while the miR-421 level increased in response to HCA. Therefore, although high miR-421 levels may benefit the intestinal mucosa, reduced let-7d-3p levels are likely to negatively affect the intestinal mucosa.

Our morphological results also confirmed that IRI caused by HCA leads to barrier dysfunction. Hypoxia inducible factor (HIF) is activated by IRI[22], resulting in intestinal mucosa barrier dysfunction[23]. HIF is down-regulated by factor inhibiting HIF (FIH-1)[24], a target gene of miR-31[25], suggesting that the decreased miR-31 expression observed in our study may protect the intestinal barrier. The CPB procedure activates several pathways, including oxidative stress, which dysregulates senescence-associated miR-542 expression[26] and inhibits cell proliferation *in vitro*[27]. Ultimately, high miR-542 levels might injure the mucosa in our model. miR-122 showed significantly (5.37-fold) increased expression in the HCA group *vs* the SO group. During myocardial IRI, the activity of myocardial p38-MAPK may lead to the down-regulation of miR-122[28,29], suggesting that miR-122 may also show similar changes during IRI in other organs. On the contrary, miR-122 was shown to be up-regulated in intestinal IRI in our study, which may be due to a different activated sub-family of p38-MAPK or organ differences. In a recent study by Ye *et al*[30], it was shown that TNF-alpha may lead to increased miR-122 levels in the intestine, suggesting that miR-122 may be involved in TNF-alpha induced intestinal barrier dysfunction. However, *NOD2* and *TNF-alpha* are two gut function-associated target genes of miR-122. Lower NOD2 levels protect intestinal epithelial cells by increasing anti-inflammatory cytokines[31], and reduced TNF-alpha levels alleviate intestinal IRI[32,33]. In contrast, elevated miR-122 levels are postulated to protect barrier function in our HCA model.

IRI can lead to increased miR-221 expression through the high-mobility group box 1 protein pathway[34,35]; however, the miR-221 level was decreased in HCA, suggesting that hypothermia or CPB influence the expression of miR-221 in HCA. Low miR-221 expression is related to high TNF mRNA expression[36,37]; therefore, miR-221 down-regulation may be detrimental to intestinal epithelial cells *via* activation of the NF-kB pathway[38].

Table 2 ischemic reperfusion injury-associated miRNA expression in hypothermic circulatory arrest and sham operation groups

miRNA	miR-10a-3p	miR-10a-5p	miR-21	miR-193a-3p	miR-210	miR-205
FC	-1.23	-1.16	-1.11	-1.11	-1.41	-1.17
P value	0.3752	0.5034	0.6219	0.7919	0.0952	0.6146

Six previously reported ischemic reperfusion injury-associated miRNAs showed nonsignificant changes.

There are several advantages of the swine model, such as steady hemodynamics, tolerance of long duration circulatory arrest and similarity to human beings. Furthermore, the age of six to eight weeks in swine likely corresponds to the relevant age in human infants for congenital heart disease. This model more closely resembles the real disease state in human beings, while animals such as rats, canines and rabbits have failed to match closely with real disease states. Therefore, we selected the swine as a model of HCA. Little is known about mucosa injuries caused by circulatory arrest. Karhausen reported that 45 min of circulatory arrest can lead to obvious rat intestinal mucosa IRI[39]. Although the duration of ischemia was longer in our study, minor microscopic changes were found. A possible explanation might be that the rat intestine is more sensitive to ischemia or temperature changes than it is to the duration of the ischemia.

One limitation of this study is that we did not set up groups of cardiopulmonary hypothermia without circulatory arrest or circulatory arrest at normal temperatures. Therefore, although intestinal miRNA could be influenced by HCA, we were not able to detect the most significant factor among hypothermia, CPB and circulatory arrest during HCA. However, the animal model that we aim to establish can be used to simulate the clinical surgical process. Therefore, circulatory arrest following atmospheric temperature and superior mesenteric artery ligation without CPB may not provide useful information. Another limitation of our study is that we did not link the dysregulated miRNAs with significant changes in pathological characteristics. Although many candidate genes are potentially targeted by the 7 identified miRNAs, only a few of these target genes were validated in other studies. We assessed the mutual target genes of these miRNAs, but the results we obtained are not ideal for determining the connection between miRNAs and the gut barrier, suggesting that the direct relationship between the genes and the pathological characteristics shown in our figures is difficult to identify. We hope that the initial data provided by our study may inspire other researchers to determine the connection between these miRNAs and the gut barrier.

In summary, intestinal barrier dysfunction and miRNA deregulation occurred when the swine were subjected to 24 ℃ HCA. Among these miRNAs, altered miR-122, miR-31 and miR-421-5p levels may protect barrier function, while altered miR-542-5p, let-7d-3p and miR-221-5p levels may negatively affect barrier function. Furthermore, miR-499 may be a marker of IRI in the intestine.

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COMMENTS

Background

Although the incidence of gastrointestinal complications is low following cardiopulmonary bypass (CPB) during cardiac surgery, the complications may be serious. Hypothermic circulatory arrest (HCA), a special technique in CPB, may increase the risk associated with gut barrier dysfunction.

Research frontiers

Due to HCA, ischemic reperfusion injury (IRI) occurs in the intestine. The study aimed to determine the influence of reperfusion injury during HCA on microRNA expression in the intestine.

Innovations and breakthroughs

The injury to the mucosal barrier in the intestine following HCA was validated in swine, which are large animals. Furthermore, miRNA dysregulation was observed following the HCA procedure.

Applications

A better understanding of the miRNAs involved in HCA related to intestinal mucosal injury may be helpful in developing strategies to protect organ function.

Terminology

CPB is a technique that enables blood circulation throughout the body when the heart is stopped during cardiac surgery. HCA is a brief period during CPB in which the systemic circulation is shut down while maintaining heart and brain perfusion.

Peer-review

This manuscript concerns HCA-regulated miRNA expression in swine intestine. By conducting a miRNA array experiment, the authors targeted some miRNAs which might be significant in HCA or I/R-induced tissue injury. Overall, the research design is very decent with adequate animal experiment, miRNA sample preparation, and array profiling.

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