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Original Article

Carbon monoxide-releasing molecule-2 protects intestinal mucosal barrier function by reducing epithelial tight-junction damage in rats undergoing cardiopulmonary resuscitation

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ABSTRACT

Background: Ischemia-reperfusion injury (IRI) to the small intestine is associated with the development of systemic inflammation and multiple organ failure after cardiopulmonary resuscitation (CPR). It has been reported that exogenous carbon monoxide (CO) reduces IRI. This study aimed to assess the effects of carbon monoxide-releasing molecule-2 (CORM-2) on intestinal mucosal barrier function in rats undergoing CPR.

Methods: We established a rat model of asphyxiation-induced cardiac arrest (CA) and resuscitation to study intestinal IRI, and measured the serum levels of intestinal fatty acid-binding protein. Morphological changes were investigated using light and electron microscopes. The expression levels of claudin 3 (CLDN3), occludin (OCLN), zonula occludens 1 (ZO-1), tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), and nuclear factor kappa B (NF-κB) p65 were detected by western blotting.

Results: Compared with the sham-operated group, histological changes and transmission electron microscopy revealed severe intestinal mucosal injury in the CPR and inactive CORM-2 (iCORM-2) groups. In contrast, CORM-2 alleviated intestinal IRI. CORM-2, unlike iCORM-2, markedly decreased the Chiu's scores (2.38 ± 0.38 *vs.* 4.59 ± 0.34; $P < 0.05$) and serum intestinal fatty acid-binding protein level (306.10 ± 19.22 *vs.* 585.64 ± 119.84 pg/mL; $P < 0.05$) compared with the CPR group. In addition, CORM-2 upregulated the expression levels of tight junction proteins (CLDN3, OCLN, and ZO-1) ($P < 0.05$) and downregulated those of IL-10, TNF-α, and NF-κB p65 ($P < 0.05$) in the ileum tissue of rats that received CPR.

Conclusions: CORM-2 prevented intestinal mucosal damage as a result of IRI during CPR. The underlying protective mechanism was associated with inhibition of ischemia-reperfusion-induced changes in intestinal epithelial permeability and inflammation in intestinal tissue.

Introduction

A systemic ischemia-reperfusion process after cardiopulmonary resuscitation (CPR) can lead to multiple organ dysfunction and post-cardiac arrest (post-CA) syndrome, which is associated with a high mortality rate after successful initial resuscitation.[1] The small intestine is extremely susceptible to ischemia-reperfusion injury (IRI) and is exposed to a large number of pathogenic bacteria in the intestinal cavity.[2] Therefore, the impairment of small intestine is a major site for the development of multiple organ failure, due to the release of endotoxins or bacterial translocation. The intestinal tract is also vulnerable to IRI, owing to the lack of autonomic mechanisms in the regulation of blood flow.[3] Intestines must tolerate markedly reduced perfusion for a certain time because a physiological compensatory mechanism was activated to provide and stabilize perfusion in the majority of vital organs (e.g., brain, heart, and lungs) during CPR.[4] Thus, the gut plays a pivotal role in the development of systemic inflammation and multiple organ failure after CPR; further

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research on the prevention of IRI to the gut during CPR is warranted.

The normal intestinal mucosal barrier is composed of mechanical, chemical, immune, and biological barriers; among those, the most critical are the mechanical and immune barriers.[5] Tight junctions (TJs) create a paracellular barrier in epithelial and endothelial cells, protecting them from the external environment; importantly, damage to the TJs may lead to increased intestinal permeability.[6] As a consequence of the leaky gut, enteric bacteria, endotoxins, and macromolecular substances may enter the blood through paracellular pathways.[7] As 70–80% of immune cells are located in the gut, translocated bacteria, endotoxins, and other antigens can stimulate the lymphatic tissue in the intestinal mucosa. Subsequently, the body produces an immune response which can initiate the systemic inflammatory response syndrome.[8] Growing evidence indicates that IRI affects the mass and function of gut-associated lymphoid tissues, which actively participate in IRI.[9] The damage to the intestinal immune barrier caused by IRI can partly explain the immunosuppression induced by the anti-inflammatory reaction in the later stages of shock. Therefore, it is essential to protect the intestinal mucosal barrier function against IRI. This approach may effectively prevent the onset of multiple organ dysfunction after CPR. However, currently, there is no effective treatment regimen for the prevention of intestinal barrier dysfunction during CPR.

Traditionally, carbon monoxide (CO) at high concentrations was considered a chemical asphyxiant. However, emerging evidence has confirmed that CO is produced naturally by the human body as a signaling molecule. At low concentrations, exogenous CO has demonstrated several cytoprotective functions: anti-apoptosis; anti-inflammatory; vascular modulation; maintenance of homeostasis; and modulation of cell differentiation.[10,11] CO-releasing molecules (CORMs) are being developed as potential therapeutic agents to locally deliver CO to cells and tissues, thereby overcoming limitations of CO gas inhalation protocols. Experimental research in animal models has shown the therapeutic potential of CORMs. The biological effects of CORMs have also been observed in preclinical trials via the genetic modulation of heme oxygenase-1 (HO-1).[12] However, further research should be carried out to determine whether CO has a protective effect on the intestinal mucosal barrier against IRI during CPR.

In the present study, a rat model of intestinal IRI after cardiac arrest (CA) and CPR was established. We pretreated rats with carbon monoxide-releasing molecule-2 (CORM-2), investigated pathological changes in the colonic mucosa, including the intestinal barrier and inflammatory response, and further investigated the changes in intestinal TJ proteins in different groups. The study aimed to assess the effects of CORM-2 on intestinal mucosal barrier function in cardiac arrest/cardiopulmonary resuscitation (CA/CPR) rats.

Methods

Preparation of animals and establishment of a rat model of intestinal IRI after CA/CPR

Thirty-two adult healthy male Sprague–Dawley rats (age: 12–16 weeks; weight: 348 ± 20 g) were purchased from the Experimental Animal Center of Ningxia Medical University, Yinchuan, China (License No. SCXK (Ning) 2015–0001). All animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University (No. 2019–022). Rats were housed in the Animal Center of the Brain Laboratory of Ningxia Medical University at a constant temperature (20–22 ˚C) and under a 12-h light/dark cycle. After fasting overnight, with the exception of free access to water, the rats were anesthetized through intraperitoneal injection of 2% sodium pentobarbital (45 mg/kg). Subsequently, they were placed on a surgical board in the supine position. The rats were mechanically air-ventilated with a tidal volume of 20 mL/kg at a rate of 100 breaths/min. The left femoral artery was cannulated with polyethylene (PE)−50 catheters into the aorta for the monitoring of arterial pressure. A PE-50 catheter was advanced from the right carotid artery into the left ventricle for hemodynamic monitoring. The right external jugular vein was cannulated using a 4-F PE catheter, which was advanced into the right atrium for the injection of 4% potassium chloride and 10% epinephrine. Surface electrocardiography was performed with electrodes placed on the skin. Following general anesthesia, 4% potassium chloride (0.12 mL/100 g) was rapidly injected through the right jugular vein; the tracheal intubation was clamped at the same time, resulting in CA (mean arterial pressure [MAP] ≤20 mmHg) lasting for 4 min. After CA, CPR was initiated (duration: 3 min) with chest compression at a rate of 100/min and mechanical ventilation with tidal volumes of 20 mL/kg. The compression-to-ventilation ratio was 5:4. At the beginning of CPR, 10% epinephrine (0.04 mg/kg) was administered through the right jugular vein. Return of spontaneous circulation (ROSC) was defined as return of a supraventricular rhythm with a MAP ≥60 mmHg lasting for ≥5 min. Moreover, the hemodynamics were monitored continuously for 4 h after successful resuscitation. In the absence of ROSC after 5 min of CPR, failure of CRP was declared.

Reagents

Tricarbonyldichlororuthenium (II) dimer ($(Ru(CO)_3Cl_2)_2$ or CORM-2) was purchased from Sigma–Aldrich (St. Louis, MO, USA), and 1 mg/mL CORM-2 solution was freshly produced prior to use by dissolving CORM-2 powder into normal saline containing 2% dimethyl sulfoxide (DMSO). The inactive CORM-2 (iCORM-2) solution was prepared by adding CORM-2 into DMSO and incubating the mixture in a 5% carbon dioxide-containing humidified atmosphere at 37 ˚C for 24 h to release the CO.

Experimental procedure

Rats were randomly assigned into the following four groups (n = 8 per group): (1) sham-operated group (rats were instrumented with a catheter without undergoing CA/CPR and intraperitoneally injected with an equal volume of normal saline); (2) CPR group (aortic catheters were placed into adult rats and CA/CPR was performed); (3) CORM-2 group; and (4) iCORM-2 group. All rats in the CPR group, CORM-2 group, and iCORM-2 group received CA/CPR. In the CORM-2 group, the rats re-

ceived CORM-2 (4 mg/kg, dissolved in 2% DMSO and diluted in normal saline) through intraperitoneal injection 12 h before commencing CA/CPR. In the iCORM-2 group, the rats received iCORM-2 (4 mg/kg, diluted in normal saline) at the same time point. In the CPR groups, animals received 0.9% saline at the same time point. The hemodynamic data were monitored for 4 h after CA/CPR (or catheterization) in each group. All animals were euthanized by barbiturate overdose (intravenous injection of 150 mg/kg pentobarbital sodium) for tissue collection. For each rat, the last 5 cm of the small intestine (ileum) were excised, and blood samples were collected at 4 h after operation.

Determination of the serum concentration of intestinal fatty-acid binding protein (I-FABP)

To detect the serum concentration of I-FABP, standard or sample (100 μL) was added into each well of the microplate and incubated for 90 min at 37 ˚C. After discarding the liquid, biotinylated working solution (100 μL; Elabscience Biotechnology, Wuhan, China) was added into each well, and the microplate was incubated for 1 h at 37 ˚C. The primary antibody solution (dilution: 1:100; Elabscience Biotechnology, Wuhan, China) was decanted and the wells were washed thrice with phosphate-buffered saline. Subsequently, horseradish peroxidase solution (100 μL; Elabscience Biotechnology) was added into each well, and the microplate was incubated for 30 min at 37 ˚C. Finally, the substrate solution and the stop solution were added into each well, and the optical density value was immediately measured at the wavelength of 450 nm.

Histopathologic evaluation

The ileum was excised 4 h after ROSC, and the ileum tissue was fixed in 4% paraformaldehyde, partially embedded into paraffin, cut into sections (thickness: 6 mm), and stained with hematoxylin and eosin (H&E). Each ileum section was analyzed using an optical microscope (OLYMPUS, Tokyo, Japan) by a researcher who was blinded to the experimental conditions. The morphological integrity of the intestinal wall was classified using a modified protocol presented by Chiu *et al.*[13]: Grade 0, normal mucosa; Grade 1, development of a sub-epithelial space at the tips of the villi; Grade 2, more extended sub-epithelial space at the tips of the villi and development of Gruenhagen's space at the tips of the villi; Grade 3, massive epithelial lifting down the sides of the villi, villus necrosis; Grade 4, villi are denuded of the epithelial layer; and Grade 5, loss of the villi and mucosal ulceration and necrosis with invasion of the muscularis propria.

Transmission electron microscopy (TEM)

The ileum tissues were harvested and cut into slices (1 mm^3), fixed in 3% glutaraldehyde for 2 h at 4 ˚C, post-fixed in 1% osmium tetroxide, dehydrated, and embedded into epoxy resin. According to the standard principle of three-dimensional localization, tissue sections (thickness: 5 mm) were randomly cut, mounted on copper mesh, double stained with lead citrate and uranyl acetate, and observed using an H-7650 TEM (HITACHI, Tokyo, Japan).

Detection of differential expression of TJ proteins

Protein was extracted from the ileum tissue of each rat and placed in lysis buffer to detect the expression levels of claudin 3 (CLDN3), zonula occludens 1 (ZO-1), and occludin (OCLN). Protein samples (30 μg/sample) were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. After blocking the nonspecific binding sites with 5% nonfat milk for 60 min, the membranes were incubated overnight at 4 ˚C with the primary antibodies, namely ZO-1/tight junction protein 1 (ZO-1/TJP1; dilution: 1:500; Thermo Fisher Scientific, Waltham, MA, USA), OCLN (dilution: 1:200; Thermo Fisher Scientific), and anti-CLDN3 (dilution: 1:200; Abcam, Cambridge, UK). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 60 min at room temperature. The binding was visualized using the enhanced chemiluminescence (ECL) Plus reagent (Cell Signaling Technology). The relative protein expression levels were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). All experiments were performed in triplicate.

Cytokine assays

The ileum tissues were harvested and homogenized in phosphate-buffered saline. The concentrations of cytokines in the ileum tissues were detected by western blotting. Anti-tumor necrosis factor-alpha (TNF-α), anti-nuclear factor kappa B (NF-κB) p65, and anti-interleukin-10 (IL-10) antibodies were obtained from Abcam. The images were captured and analyzed with the KODAK Image Station 4000R (IS4000R) system (KODAK, Beijing, China).

Statistical analysis

Statistical analysis was performed using the SPSS 22.0 software (IBM, Armonk, NY, USA). The data were presented as the mean ± standard deviation. Normally distributed data were analyzed with the Shapiro–Wilk test or Fisher's exact test, as appropriate. Outcomes were compared among the four groups using one-way analysis of variance (ANOVA) *post-hoc* tests. Intragroup analysis of outcome change (over time) was performed using repeated measures ANOVA. *P*-values < 0.05 denoted statistically significant differences.

Results

Hemodynamic data

The present study monitored hemodynamic changes in rats at various time points [Figure 1]. There were no significant differences found in the baseline MAP between the sham-operated and CPR groups. In the CPR, CORM-2, and iCORM-2 groups, the MAP at each time point after resuscitation was markedly lower than that recorded at baseline, and this phenomenon was observed until 4 h after resuscitation ($P < 0.05$) [Table 1]. The MAP at each time point after resuscitation in all resuscitation groups was significantly lower than that noted at the corresponding time point in the sham-operated group ($P < 0.05$) [Table 1].

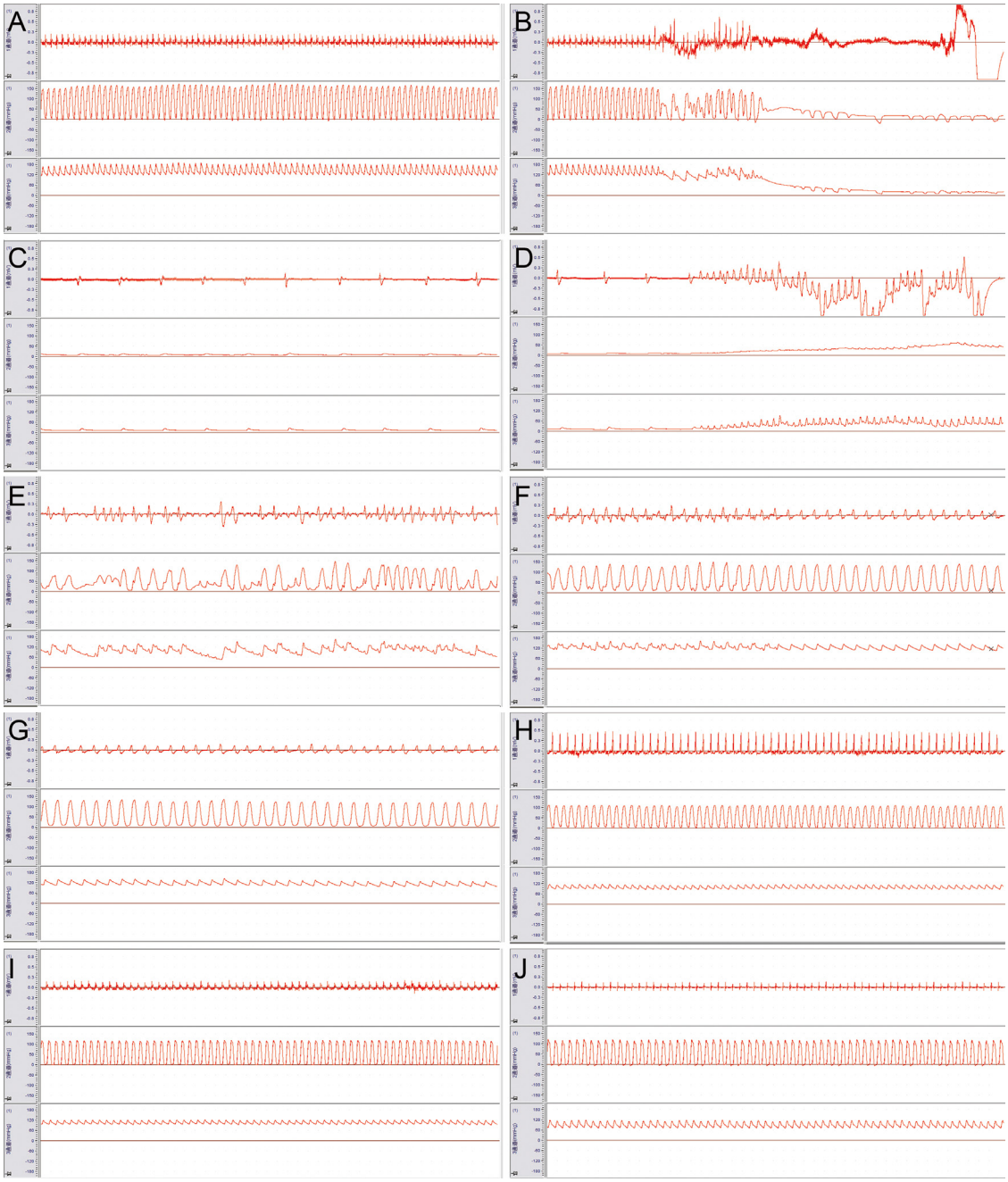

Figure 1. Time course of MAP during CPR. Sprague–Dawley rats were randomly divided into four groups and subjected to asphyxia-induced CA (duration: 4 min) followed by CPR. Baseline data for the MAP of rats under general anesthesia (A). Following the clamping of the tracheal intubation, a MAP <20 mmHg for 4 min (B, C) revealed the induction of CA. After 3 min of CPR (D, E), a supraventricular rhythm with a MAP >60 mmHg (F) indicated successful ROSC. Post-resuscitation MAP was monitored at 1 h (G), 2 h (H), 3 h (I), or 4 h (J).
CA: Cardiac arrest; CPR: Cardiopulmonary resuscitation; MAP: Mean arterial blood pressure; ROSC: Return of spontaneous circulation.

The MAP at each time point after resuscitation in the CORM-2 group was noticeably higher than that observed in the CPR and iCORM-2 groups ($P < 0.05$) [Table 1].

Effects of CORM-2 on the alleviation of damage to ileum tissues in rats after CPR

Figure 2 shows the histological changes in the intestinal tissues of rats in each group. Rats in the sham-operated group exhibited an intact ileal mucosa, neat intestinal villi, deep crypts, and a clear and complete gland structure. The ileum of rats in the CPR group was characterized by arrangement of the villi in a disorderly fashion, mucosal swelling, villi edema, partial villi destruction, and extensive necrosis in the intestinal epithelial cells. The CORM-2 group showed mild mucosal edema, a small amount of necrosis in the intestinal epithelial cells, and partial destruction of the villi. CORM-2, unlike iCORM-2, reduced intestinal epithelial damage; this effect improved morphological changes in the intestinal mucosa during CA/CPR. Compared with the sham-operated group, Chiu's scores for intestinal mucosal injury in all resuscitation groups were elevated

Table 1
Mean arterial pressure in different time point before and after resuscitation.

Time point	Sham-operated group (n = 8)	CPR group (n = 8)	CORM group (n = 8)	iCORM group (n = 8)	F-value	P-value
Baseline	136.21 ± 6.22	133.03 ± 4.68	135.49 ± 4.8	135.57 ± 4.79	0.588	0.628
After resuscitation (or catheterization)						
0.5 h	136.46 ± 5.73	93.01 ± 5.15*,†	110.08 ± 3.84*,†,‡	96.80 ± 3.79*,†,§	139.983	0.000
1 h	136.78 ± 4.68	96.48 ± 4.93*,†	113.03 ± 4.18*,†,‡	101.13 ± 5.45*,†,§	111.329	0.000
2 h	136.29 ± 5.15	101.24 ± 5.34*,†	114.23 ± 4.78*,†,‡	102.29 ± 5.87*,†,§	75.485	0.000
3 h	138.88 ± 3.98	105.02 ± 6.09*,†	117.28 ± 5.13*,†,‡	103.86 ± 5.56*,†,§	76.733	0.000
4 h	139.02 ± 4.82	105.40 ± 5.71*,†	119.17 ± 4.80*,†,‡	105.46 ± 5.05*,†,§	77.379	0.000

Data are presented as mean ± standard deviation.
Each rat underwent monitoring of blood pressure using the femoral artery at different time points, including catheterization at 0 h, 0.5 h, 1 h, 2 h, 3 h, and 4 h after resuscitation (or catheterization).
CPR: Cardiopulmonary resuscitation; CORM-2: Carbon monoxide-releasing molecule-2; iCORM: Inactive CORM; MAP: Mean arterial pressure.
* Inter-group comparison: $P < 0.05$ compared with the sham-operated group.
† Intra-group comparison: $P < 0.05$ compared with baseline.
‡ $P < 0.05$ compared with the CPR group.
§ $P < 0.05$ compared with the CORM-2 group.

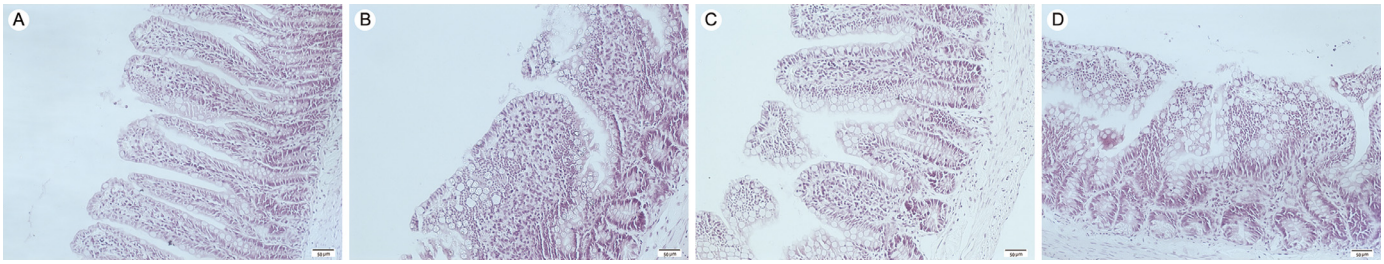

Figure 2. Histopathological morphology of rats in each group (magnification, 200 ×). Following CA/CPR, the small intestine of rats was excised for H&E staining. Typical images of H&E staining of intestinal sections from sham-operated rats (A), CPR (B) rats or CPR rats receiving treatment with CORM-2 (C) or treatment with iCORM-2 (D). Severe inflammation (ulceration, hemorrhage, and epithelial loss) was found in CPR rats (B) and CPR rats treated with iCORM-2 (D). Treatment with CORM-2 alleviated the changes in CPR rats (C).
CORM-2: Carbon monoxide-releasing molecule-2; CA/CPR: Cardiac arrest/cardiopulmonary resuscitation; H&E: Hematoxylin and eosin; iCORM-2: Inactive carbon monoxide-releasing molecule-2.

Table 2
Serum concentrations of I-FABP and Chiu's scores.

Parameter	Sham-operated group(n = 8)	CPR group(n = 8)	CORM-2 group(n = 8)	iCORM-2 group(n = 8)	F-value	P-value
I-FABP (pg/mL)	230.19 ± 37.01	585.64 ± 119.84*	306.10 ± 19.22†	414.12 ± 36.13*,†,‡	43.689	0.000
Chiu's scores	0.40 ± 0.13	4.59 ± 0.34*	2.38 ± 0.38*,†	3.64 ± 0.53*,†,‡	187.588	0.000

Data are presented as mean ± standard deviation.
CORM-2 decreased intestinal injury and the serum levels of I-FABP in CA/CPR rats. Semi-quantitative intestinal injury was evaluated by two investigators using the Chiu's scores of mucosal injury. Blood samples were drawn from the portal vein 4 h after resuscitation. The serum levels of I-FABP were measured using ELISA kits. CPR led to histopathological injury in ileum tissues, and treatment with CORM-2 decreased injury in ileum tissues. iCORM-2 did not have a significant effect on injury in the ileum tissues of CPR rats.
CA: Cardiac arrest; CPR: Cardiopulmonary resuscitation; CA/CPR: Cardiac arrest/cardiopulmonary resuscitation; CORM-2: Carbon monoxide-releasing molecule-2; ELISA: Enzyme-linked immunosorbent assay; iCORM-2: Inactive carbon monoxide-releasing molecule-2; I-FABP: Intestinal fatty-acid binding protein.
* $P < 0.05$ compared with the sham-operated group.
† $P < 0.05$ compared with the CPR group.
‡ $P < 0.05$ compared with the CORM-2 group.

(0.40 ± 0.13 *vs.* 4.59 ± 0.34, 2.38 ± 0.38, and 3.64 ± 0.53; $P < 0.05$) [Table 2]. In the CORM-2 group, Chiu's score was significantly lower than that recorded in the CPR and iCORM-2 groups (2.38 ± 0.38 *vs.* 4.59 ± 0.34 and 3.64 ± 0.53; $P < 0.05$) [Table 2].

TEM revealed ultrastructural changes in the intestinal mucosal tissues in all groups. As illustrated in Figure 3, in the sham-operated group, the epithelial cells were closely arranged, and the microvilli on the surface of epithelial cells were arranged in neat rows. The TJs and desmosomes were clear and complete, and the paracellular spaces were narrow. In the CPR group, the microvilli were sparse with an irregular length and arrangement. The TJs and desmosomes were obscured or disappeared, mitochondria were swollen and vacuolated, and the paracellular spaces were wider. CORM-2 improved the ultrastructural integrity of the intestinal mucosa in the CA/CPR group compared with the CPR and iCORM-2 groups. However, there was no significant improvement observed following treatment with iCORM-2.

Serum levels of I-FABP

I-FABP serves as a biomarker of enterocyte damage. The results showed that the levels of I-FABP were significantly elevated in the CPR and iCORM-2 groups compared with the sham-operated group (585.64 ± 119.84 and 414.12 ± 36.13 pg/mL *vs.*

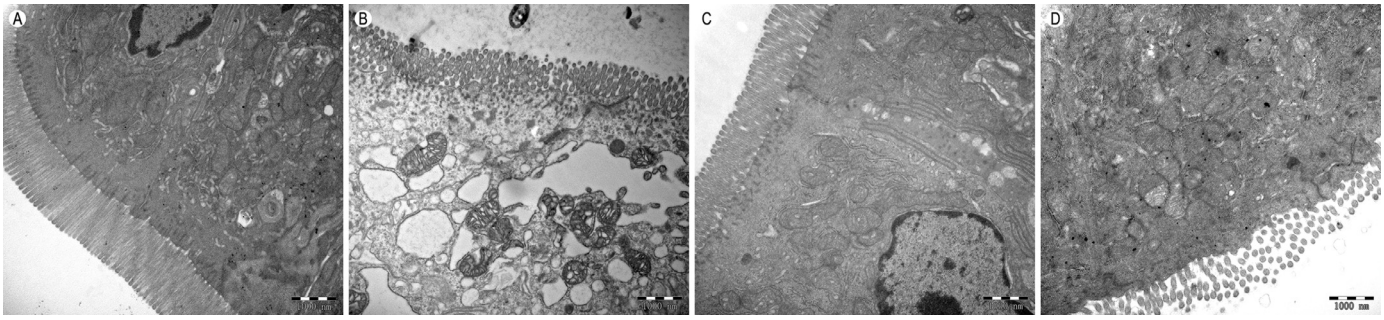

Figure 3. Ultrastructural changes in the ileum of rats (magnification, 20,000 ×). Following CA/CPR, the small intestine of rats was excised and analyzed using electron microscopy with lead-uranyl acetate. Sham-operated rats (A) showed a normal ultrastructure of the intestinal epithelium. Typical images from CPR rats (B) and rats treated with iCORM-2 (D) revealed widened intercellular space, swollen mitochondria, and absent microvilli. Ultrastructural changes were alleviated in the CORM-2 (C) group *vs.* the other groups.
CORM-2: Carbon monoxide-releasing molecule-2; CA/CPR: Cardiac arrest/cardiopulmonary resuscitation; iCORM-2: Inactive carbon monoxide-releasing molecule-2.

230.19 ± 37.01 pg/mL; $P < 0.05$) [Table 2]. However, treatment with CORM-2 markedly decreased the serum levels of I-FABP in the CORM-2 group compared with the CPR group (306.10 ± 19.22 *vs*. 585.64 ± 119.84 pg/mL; $P < 0.05$) [Table 2]. Taken together, the data suggested that treatment with CORM-2 could reduce intestinal IRI in the CA/CPR group.

Effects of CORM-2 on the expression levels of CLDN3, OCLN, and ZO-1 in ileum tissue

We sought to investigate the mechanisms underlying the CO-mediated alleviation of increased intestinal permeability in the CA/CPR group. For this purpose, western blotting was used to detect the expression levels of CLDN3, ZO-1, and OCLN – markers of intestinal barrier function and permeability. Figure 4 displays that the expression levels of CLDN3, OCLN, and ZO-1 in the intestinal mucosa were reduced in the CPR, CORM-2, and iCORM-2 groups ($P < 0.05$) compared with those noted in the sham-operated group. However, these expression levels ($P < 0.05$) were markedly elevated in the CORM-2 group compared with those observed in rats that received CPR or those that underwent CPR + iCORM-2. The above-mentioned findings showed that CORM-2, unlike iCORM-2, increased the levels of TJ proteins in rats during CPR.

Effects of CORM-2 on the expression levels of TNF-α, IL-10, and NF-κB p65 in ileum tissue

We also examined whether CO can inhibit intestinal inflammation in rats that received CA/CPR. The expression levels of TNF-α, IL-10, and NF-κB p65 were detected using western blotting [Figure 5]. The expression levels of TNF-α and IL-10 in the CPR and iCORM-2 groups were significantly elevated compared with those recorded in the sham-operated group ($P < 0.05$). The expression levels of TNF-α were decreased in the CORM-2 group *vs.* the CPR group ($P < 0.05$). The expression levels of IL-10 were increased in the CORM-2 group compared with the sham-operated group; however, the difference was not statistically significant ($P > 0.05$). Compared with the sham-operated group, the expression levels of NF-κB p65 were significantly elevated in the CPR and iCORM-2 groups ($P < 0.05$). However, the expression levels of NF-κB in the CORM-2 group did not change significantly ($P > 0.05$). Collectively, CO plays an anti-inflammatory role in intestinal injury. This effect of CO is associated with a reduction in the release of pro-inflammatory factors.

Discussion

According to the definition established by the American Heart Association and the American College of Cardiology, sudden cardiac arrest is the sudden cessation of cardiac activity, so that a victim becomes unresponsive, with no normal breathing and no signs of circulation.[14] CPR is an emergency procedure that combines chest compressions often with artificial ventilation in an effort to manually preserve brain function until further measures are taken to restore spontaneous blood circulation and breathing in a person who is in CA.[15,16] The intestine is the organ where ischemia occurs first, and blood perfusion recovery is mainly delayed during CPR.[17] Intestinal ischemia can induce a systemic inflammatory response, and gut-origin infection related to bacterial translocation may cause multiple organ failure after resuscitation.[8] In the present study, we demonstrated that CORM-2 exerted protective effects against post-resuscitation IRI by attenuating inflammation response and upregulating the expression levels of intestinal TJ proteins. Additionally, we found that the MAP of rats treated with CORM-2 at each time point after CPR was higher than that measured in the other CPR groups. Therefore, our results suggested that pre-treatment with CORM-2 through intraperitoneal injection 12 h before CPR can stabilize the hemodynamics. It has been reported that CORMs possess a vasodilatory effect that involves the activation of soluble guanylyl cyclase and the large-conductance Ca^{2+}-activated K^+ channel (BKCa channel).[10,18] However, the changes in MAP induced by CORM-2 in rats undergoing CPR after ROSC were discordant with the vasodilatory effect of CORMs. The increased MAP noted in rats treated with CORM-2 could be attributed to two factors. First, according to our previous study, CORM-2 increased cardiac output and the left ventricular ejection fraction, and exerted a positive effect on ischemic myocardial mitochondrial function and ultrastructural changes to protect the cardiac tissues of lipopolysaccharide-stimulated rats.[19] This beneficial effect of CORM-2 on cardiac function may increase the MAP in rats. Second, the protective effect of CORM-2 on the vascular en-

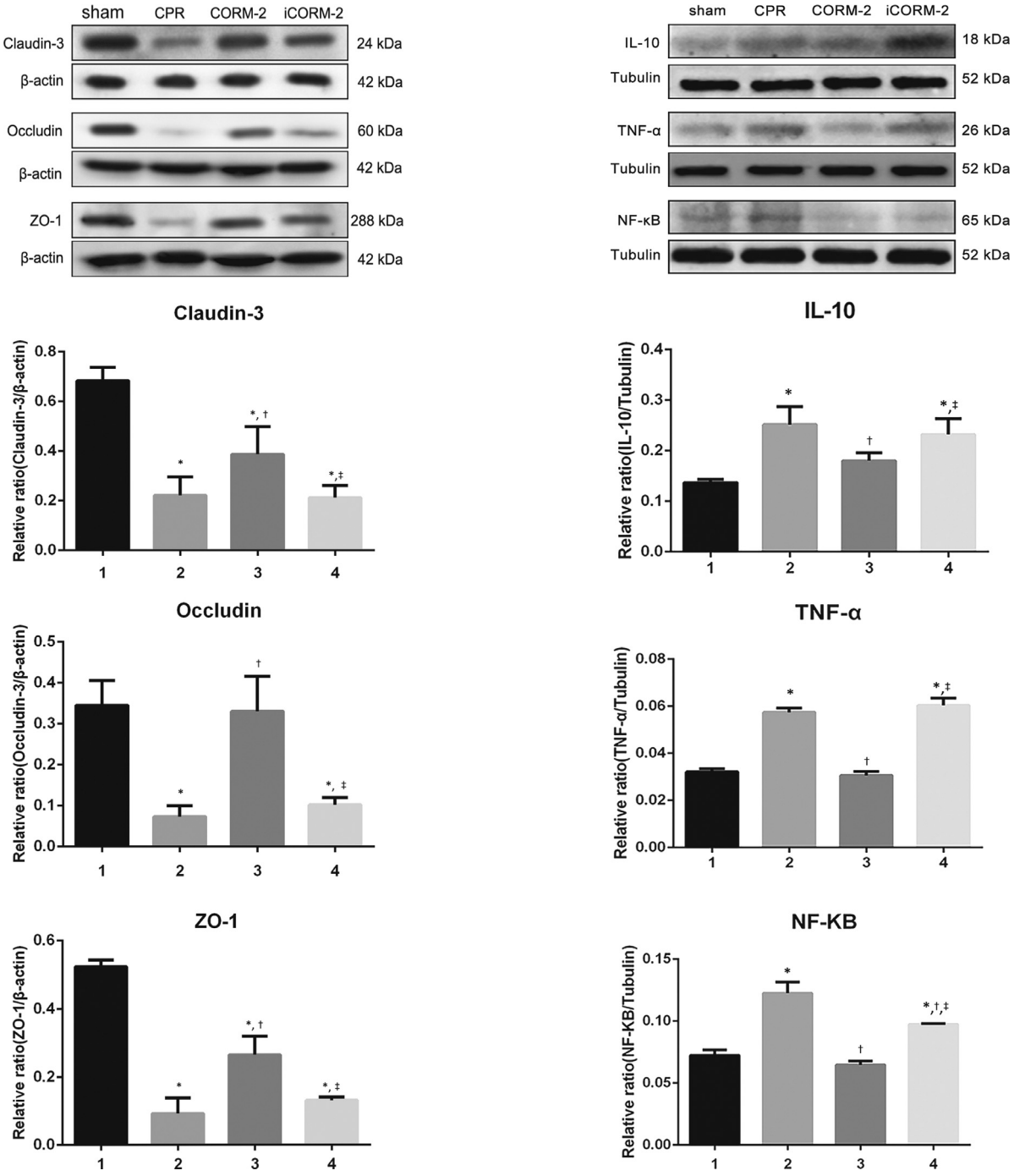

Figure 4. Relative expression levels of CLDN3, ZO-1, and OCLN in the ileum tissue of rats. Ileum samples were collected and homogenized. The tissue lysates were subjected to western blotting using specific antibodies for CLDN3, ZO-1, and OCLN; β-actin was used as endogenous control. The band intensity was quantified using densitometry. The ratios of CLDN3/β-actin, ZO-1/β-actin, and OCLN/β-actin in different groups were presented as the mean ± standard deviation. (1) Sham-operated group; (2) CPR group; (3) CORM-2 group; and (4) iCORM-2 group (n = 8 per group). $^{*}P < 0.05$ compared with the sham-operated group; †$P < 0.05$ compared with the CPR group; ‡$P < 0.05$ compared with the CORM-2 group. CORM-2: Carbon monoxide-releasing molecule-2; CPR: Cardiopulmonary resuscitation; CLDN3: Claudin 3; iCORM-2: Inactive carbon monoxide-releasing molecule-2; OCLN: Occludin; ZO-1: Zonula occludens 1.

Figure 5. Relative expression levels of TNF-α, IL-10, and NF-κB p65 in the ileum tissues of rats. Lysates of ileum obtained from the different groups of rats were subjected to western blotting with anti-TNF-α, anti-IL-10, and anti-NF-κB p65 antibodies; tubulin was used as endogenous control. The ratios of TNF-α/tubulin, IL-10/tubulin, and NF-κB p65/tubulin were calculated based on the densities of the bands. (1) Sham-operated group; (2) CPR group; (3) CORM-2 group; and (4) iCORM-2 group (n = 8 per group). $^{*}P < 0.05$ compared with the sham-operated group; †$P < 0.05$ compared with the CPR group; ‡$P < 0.05$ compared with the CORM-2 group. CORM-2: Carbon monoxide-releasing molecule-2; CPR: Cardiopulmonary resuscitation; iCORM-2: Inactive carbon monoxide-releasing molecule-2; IL-10: Interleukin-10; NF-κB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor-alpha.

dothelium during IRI may partly explain the elevation of MAP in CA/CPR rats. This is because healthy blood vessels are maintained by moderate levels of CO via crosstalk with the endothelial nitric oxide synthase/nitric oxide pathway.[20,21] Hence, the administration of CORM-2 during CA/CPR exerts a therapeutic effect against hemodynamic deterioration induced by the CA.

To our knowledge, the intestinal villi are extremely susceptible to ischemic damage, and necrosis of these villi is one of the earliest histological changes that occur during intestinal ischemia. The results of the current study confirmed that CORM-2 attenuated IRI in a rat model of CA/CPR. We found that I-FABP, as a marker for intestinal damage, was localized in epithelial cells of the small intestine, and the serum levels of I-FABP were decreased in the CORM-2 group compared with the CPR group. Moreover, pathological changes in intestinal mucosa, observed by light microscopy, showed that treatment with CORM-2 mitigated villi edema, goblet cell swelling, and inflammatory cell infiltration, and reduced Chiu's score. Additionally, intraperitoneal injection of CORM-2 resulted in protection against intestinal IRI in rats. The TEM observations indicated that CO could preserve the microvilli on the surface of epithelial cells. Notably, the key role of CORM-2 in protecting the intestinal mechanical barrier is the maintenance of paracellular spaces and TJs, playing a significant role in selective paracellular permeability.[22]

In this study, we confirmed that CORM-2 could maintain the intestinal epithelial barrier integrity; thus, we used western blotting to detect the expression levels of CLDN3, ZO-1, and OCLN in ileum tissues. The results showed that pre-treatment with CORM-2 significantly upregulated the expression levels of CLDN3, ZO-1, and OCLN. In addition, CORM-2 could promote the transcription and translation of TJ proteins in rats undergoing CA/CPR. The present findings confirmed that the protective effect of CORM-2 against CA/CPR-induced intestinal permeability is associated with the modulation of the expression of TJ proteins. Moreover, there was no significant change detected in the expression levels of OCLN between the sham-operated group and CORM-2 group. OCLN is highly expressed at cell–cell contact sites and plays an important role in the assembly and maintenance of TJ proteins.[6] Consequently, CORM-2 is involved in reducing intestinal TJ injury induced by ischemia reperfusion in rats undergoing CA/CPR.

The mechanism through which CO regulates the assembly of TJ proteins after CA/CPR remains elusive. In recent years, growing evidence suggested that intestinal mucosal damage may be triggered by the release of pro-inflammatory cytokines via activation of the gut mucosal immune system.[23,24] TNF-α plays a key role in the chain reaction of proinflammatory factors, triggering structural damage in TJ proteins. The protective effect realizing the maintenance of TJ proteins is exerted via downregulation of the myosin light chain kinase (MLCK) and phosphorylated myosin light chain. The MLCK is a kinase that leads to fast transient changes in paracellular permeability by phosphorylation of the myosin II regulatory light chain.[25] Activation of the mitogen-activated protein kinase (MAPK) and NF-κB pathways causes the release of proinflammatory cytokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8), and TNF-α, which may lead to the inflammatory response.[26] The present study revealed that IRI may increase the expression levels of TNF-α and NF-κB; this result is consistent with findings of previous studies. However, the administration of CORM-2 reduced the expression levels of TNF-α and NF-κB in ileum tissue. Therefore, the alleviation of IRI-induced downregulation of TJ protein expression in CA/CPR rats may be related to the reduction of TNF-α and NF-κB production by CORM-2. However, the protective effects of CORM-2 on small intestinal TJs can also be attributed to stabilization of hemodynamic changes for the alleviation of IRI.[27,28] In addition, our results showed that iCORM-2 had only a slight inhibitory effect on NF-κB, whereas it did not influence TNF-α in rats undergoing CPR. This is because the CORM-2 molecule has a long-lasting CO-releasing monomer form, and it is difficult to completely inactivate CORM-2 in DMSO.[29]

The results of the current research demonstrated that CORM-2 exerts anti-inflammatory effects. To the best of our knowledge, the anti-inflammatory properties of CO are associated with the inhibition of proinflammatory cytokines and activation of anti-inflammatory cytokines. IL-10 is a pleiotropic cytokine that controls inflammatory processes by suppressing the production of proinflammatory cytokines.[30] The results of the present research revealed that CORM-2 reduced the expression levels of IL-10 in ileum tissue. This result is inconsistent with previously reported findings, indicating that treatment with CORM could increase the expression levels of IL-10 in rats with severe autoimmune diseases.[31,32] However, our results are in agreement with those of a recent study demonstrating that pretreatment with CORM-2 could significantly decrease the serum concentrations of transforming growth factor beta-3 (TGF-β3), IL-10, TNF-α, interferon gamma (IFN-γ), and granulocyte-macrophage colony stimulating factor (GM-CSF) in rats subjected to IRI compared with the control group.[33] Although we did not identify the mechanism through which CORM-2 downregulated the expression levels of IL-10, we found that the anti-inflammatory properties of CO were not mediated by IL-10, partially through the direct inhibition of the expression levels of NF-κB during CPR. Therefore, the mechanism underlying the CO-induced decrease of IL-10 in intestinal IRI requires further investigation.

This study was characterized by several limitations. First, we tested only three cytokines from the ischemia-reperfusion injury and the signal transduction pathway. Use of exogenous inhibitors or agonists is necessary to determine the mechanism through which the TNF-α/NF-κB pathway affects the permeability of intestinal mucosa in CPR rats. Second, the intestinal mucosa is a complex multilayer system, consisting of an external "physical" barrier and an inner "functional" immunological barrier.[34] Thus, *in vitro* experiments are warranted to elucidate the influence of CORM-2 and the mechanism involved in the development of intestinal ischemia-reperfusion injury after CA. Finally, the prediction of CA is impossible; hence, further investigation is warranted to elucidate the effect of treatment with CORM-2 on the intestines following CA/CPR.

Conclusions

CO exerts a protective effect on the intestinal mucosal barrier in rats undergoing CPR. Treatment with CORM-2 upregulated the expression levels of TJ proteins (e.g., CLDN3, OCLN, and ZO-1) in the intestinal mucosa, leading to a reduction in permeability. Furthermore, CORM-2 exhibited anti-inflammatory effects by downregulating the levels of TNF-α and NF-κB.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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