C-peptide attenuates acute lung inflammation in a murine model of hemorrhagic shock and resuscitation by reducing gut injury

Raymond L.C. Kao, MD, Xuemei Xu, BSc, Anargyros Xenocostas, MD, Neil Parry, MD, Tina Mele, MD, PhD, Claudio M. Martin, MD, and Tao Rui, MD, PhD. Ontario, Canada

BACKGROUND: The study aims to evaluate whether C-peptide can reduce gut injury during hemorrhagic shock (HS) and resuscitation (R) therefore attenuate shock-induced inflammation and subsequent acute lung injury.

METHODS: Twelve-week-old male mice (C57/BL6) were hemorrhaged (mean arterial blood pressure maintained at 35 mm Hg for 60 minutes) and then resuscitated with Ringer's lactate, followed by red blood cell transfusion with (HS/R) or without C-peptide (HS/R + C-peptide). Mouse gut permeability, bacterial translocation into the circulatory system and intestinal pathology, circulating HMGB1, and acute lung injury were assessed at different times after R. The mice in the control group underwent sham procedures without HS.

RESULTS: Compared to the sham group, the mice in the HS/R group showed increased gut permeability (6.07 ± 3.41 µg of FD4/mL) and bacterial translocation into the circulatory system (10.05 ± 4.92, lipopolysaccharide [LPS] of pg/mL), and increased gut damage; conversely, mice in the HS/R + C-peptide group showed significantly reduced gut permeability (1.59 ± 1.39 µg of FD4/mL; p < 0.05) and bacterial translocation (4.53 ± 1.08 pg of LPS/mL; p < 0.05) with reduced intestine damage. In addition, mice in the HS/R group had increased circulating HMGB1 (21.64 ± 14.17 ng/mL), lung myeloperoxidase (23.73 ± 8.39 mU/g of tissue; p < 0.05), and pulmonary protein leakage (1.17 ± 0.42 Evans Blue/g tissue per minute). Mice in the HS/R + C-peptide group showed decreased HMGB1 (7.27 ± 1.93 ng/mL; p < 0.05), lung myeloperoxidase (23.73 ± 8.39 mU/g of tissue; p < 0.05), and pulmonary protein leakage (1.17 ± 0.42 Evans Blue/g tissue per minute; p < 0.05).

CONCLUSION: Our results indicate that C-peptide exerts beneficial effects to attenuate gut injury and dysfunction, therefore diminishing lung inflammation and subsequent injury in mice with HS and R. (J Trauma Acute Care Surg. 2017;83: 256–262. Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.)

KEY WORDS: Hemorrhagic shock and resuscitation; gut injury; acute lung inflammation; HMGB1; C-peptide.

Hemorrhagic shock (HS) due to unintentional traumatic injuries decreases oxygen supply to vital organs, provoking biochemical adaptation for acute cell survival.1,2 A systemic compensatory response to ensure sufficient blood supply to vital organs occurs after HS, which includes increases in heart rate and vasoconstriction in non-vital organs.3-6 Among patients who survive the initial traumatic injuries, the subsequent inflammatory response elicited during HS and R plays an important role in the fate of the injured patients.2,7 The gut is frequently affected in patients with HS/R.8 Hypoperfusion followed by reperfusion of the gut during R to correct the shock state could lead to gut barrier dysfunction, which further induces systemic inflammation and subsequent multiple organ failure.9

Insulin connecting peptide (C-peptide) is a 31-amino acid peptide that makes up a short segment of the proinsulin molecule.10,11 Since it was first described, its major biological role is to aid in the proper conformational folding and disulfide bond formation of proinsulin in pancreatic ß-cells. C-peptide is cleaved off of proinsulin prior to the secretion of insulin, resulting in the release of both insulin and C-peptide in equimolar amounts in the circulatory system. Originally, it was believed that circulating C-peptide did not play any biological roles; however, recent studies have demonstrated that the systemic administration of C-peptide can improve the vascular, neural, and renal function of diabetic rats.12 Moreover, it has been reported that C-peptide promotes arteriolar dilation and prevents polymorphonuclear-endothelial cell interactions in the mesenteric microcirculation.13,14 Additionally, some studies have shown that C-peptide attenuates ischemia/reperfusion-induced myocardial injury15 and ameliorates kidney injury after HS.16 The C-peptide also exerts beneficial effects in mice with endotoxic shock induced by the activation of PPAR-γ.17

In a mouse model of HS/R, we previously demonstrated that HS/R increases gut permeability, results in bacteria translocation, and elevates circulation levels of LPS;18 the increased LPS results in increased circulating HMGB1.19 HMGB1 interacts with its receptor, TLR4, and further induces acute lung inflammation or injury.20,21 Our previous study demonstrated that the inhibition of HMGB1 or the deletion of the HMGB1 receptor, TLR4, attenuates HS/R-induced acute lung injury.18 In the present study, we aimed to evaluate whether C-peptide provides protection to the gut of mice with HS/R and reduces
bacterial translocation, thus abolishing the increase in circulating HMGB1 and attenuating the HS/R-induced acute lung inflammation/injury.

**METHODS**

**Animals**

C57BL/6 mice were obtained from Charles River Canada (St. Constant, QC, Canada). The Western University Animal Care and Use Committee approved all aspects of this study (protocol 2011-028), and the methods complied with the recommendations outlined in the guide for the Care and Use of Laboratory Animals.

**Experimental Protocol**

This study contains four sets of experiments; the mice were randomly assigned into one of three groups in each set of experiments: the control group (sham); the HS/R group; and the HS/R with C-peptide treatment group. C-peptide treatment (1 mg/kg; Bachem Americas Inc., Torrance, CA) was initiated after completion of the shed blood infusion with one volume of the Ringer’s lactate (RL). In each set of experiments, six to eight mice were used in each group. The first set of experiments aimed to determine mouse gut permeability; the second set of experiments sought to determine mouse circulating LPS and to assess gut histology; the third set of experiments measured the mouse circulating HMGB1 and lung myeloperoxidase (MPO) activity; and the fourth set of experiments was conducted to determine mouse pulmonary protein leakage.

**Mouse Model of HS and R**

The mouse model of HS and R was induced according to our previous publication. Briefly, 10-week-old male mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (4 mg/kg). Both the right jugular vein and the right carotid artery were cannulated. Jugular vein cannulation was used for the administration of heparin (10 units in 0.1 mL of saline) and for R. Carotid artery cannulation was used for blood pressure monitoring and blood withdrawal. HS was initiated by blood withdrawal and by reducing the mean arterial blood pressure (MAP) to 35 mm Hg in 15 minutes. The blood was harvested into a 1-mL syringe with heparin to prevent coagulation. The MAP was kept at 35 mm Hg for an additional 60 minutes. The severity of shock among the mice was also evaluated by measuring blood lactate and hemoglobin with the YSI 2300 STAT PLUS Glucose/Lactate Analyzer (Yellow Springs, OH) and with the Hemoglobin Reagent Set (Pointe Scientific, Inc., Canton, MI), respectively. Subsequently, the mice were resuscitated with a transfusion of 1.5 volume of RL over the course of 10 minutes; the mice were monitored for an additional hour, followed by the transfusion of red blood cells derived from the shed blood diluted with one volume of RL. Subsequently, the catheters were removed, the blood vessels were ligated, and the incision was closed. The sham mice were given an equal amount of heparin and they underwent the same surgical procedures without blood withdrawal and R. Each mouse’s body temperature was kept with a heating pad set at 37°C during the surgery. The mice were given buprenorphine (1 mg/kg, subcutaneously) after recovery from the surgery, and they were monitored every hour until they were actively walking and able to access food and water. The key values that characterized the mouse model are listed in Table 1.

**Terminal Ileum Permeability Assay**

Gut permeability was assessed using a method described in our previous publication. Briefly, a 1-cm segment of ileum proximal to the cecum with intact superior mesenteric vessels was dissected 30 minutes after the shed blood transfusion was complete. The two ends of the isolated ileum segment were ligated with 2-0 silk sutures after injection of 0.2 mL of 0.1 M phosphate-buffered saline (PBS) (pH: 7.2) containing 25 mg/mL of fluorescein isothiocyanate-dextran (FD4; molecular weight: 4,000; Sigma-Aldrich Co., St. Louis, MO) into the lumen, and

**TABLE 1. Key Values for Mice in Study Groups (Mean ± SD)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Timing</th>
<th>Sham (n = 8)</th>
<th>HS/R (n = 8)</th>
<th>HS/R + C-Peptide (n = 8)</th>
<th>HS/R vs. Sham</th>
<th>HS/R + C-Peptide vs. Sham</th>
<th>HS/R + C-Peptide vs. HS/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>Baseline</td>
<td>26.82 ± 1.41</td>
<td>27.90 ± 1.89</td>
<td>26.67 ± 2.87</td>
<td>p = 0.994</td>
<td>p = 1.000</td>
<td>p = 0.795</td>
</tr>
<tr>
<td>24 h after HS/R</td>
<td>26.20 ± 1.16</td>
<td>25.84 ± 2.38</td>
<td>24.88 ± 2.86</td>
<td>p = 1.000</td>
<td>p = 0.761</td>
<td>p = 1.000</td>
<td>p = 1.000</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>Baseline</td>
<td>72.5 ± 4.24</td>
<td>74.6 ± 3.85</td>
<td>72.4 ± 3.58</td>
<td>p = 1.000</td>
<td>p = 1.000</td>
<td>p = 1.000</td>
</tr>
<tr>
<td>Postbleeding, min</td>
<td>15</td>
<td>72.3 ± 3.24</td>
<td>35.4 ± 0.52</td>
<td>35.3 ± 0.71</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 1.000</td>
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<tr>
<td></td>
<td>45</td>
<td>70.5 ± 3.25</td>
<td>35.9 ± 0.64</td>
<td>35.8 ± 0.46</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 1.000</td>
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<tr>
<td></td>
<td>75</td>
<td>71.0 ± 2.73</td>
<td>35.0 ± 0.53</td>
<td>35.1 ± 0.35</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 1.000</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>71.4 ± 4.03</td>
<td>67.1 ± 4.22</td>
<td>64.4 ± 5.34</td>
<td>p = 0.186</td>
<td>p = 0.014</td>
<td>p = 0.727</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>71.1 ± 3.31</td>
<td>43.5 ± 1.93</td>
<td>43.6 ± 1.85</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 1.000</td>
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<tr>
<td></td>
<td>135</td>
<td>71.6 ± 4.14</td>
<td>38.9 ± 0.99</td>
<td>39.3 ± 1.49</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 1.000</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>72.6 ± 4.98</td>
<td>73.1 ± 5.22</td>
<td>71.5 ± 5.04</td>
<td>p = 1.000</td>
<td>p = 1.000</td>
<td>p = 1.000</td>
</tr>
<tr>
<td>Blood lactate, mmol/L</td>
<td>Baseline</td>
<td>0.56 ± 0.89</td>
<td>0.54 ± 0.86</td>
<td>p = 0.598</td>
<td></td>
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<tr>
<td></td>
<td>1 h shock</td>
<td>2.25 ± 0.247</td>
<td>2.36 ± 0.493</td>
<td>p = 0.562</td>
<td></td>
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<tr>
<td></td>
<td>0.5 h after RBC</td>
<td>1.91 ± 0.38</td>
<td>1.53 ± 0.331</td>
<td>p = 0.048</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood hemoglobin, g/dL</td>
<td>Baseline</td>
<td>12.97 ± 1.20</td>
<td>12.42 ± 0.64</td>
<td>p = 0.275</td>
<td></td>
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<tr>
<td></td>
<td>1 h shock</td>
<td>10.06 ± 1.07</td>
<td>9.75 ± 0.80</td>
<td>p = 0.509</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.5 h after RBC</td>
<td>11.10 ± 1.07</td>
<td>10.97 ± 1.21</td>
<td>p = 0.824</td>
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<td></td>
<td></td>
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</tbody>
</table>

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blood samples were obtained 30 minutes after the FD4 injection. The anesthetized mice were sacrificed by cervical dislocation. Circulating FD4 levels were determined using a Victor-3 multilabel counter (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) at excitation and emission wavelengths of 480 nm and 520 nm, respectively. The standard curve for calculating the FD4 concentration in the samples was obtained by diluting various amounts of FD4 in PBS.

**Circulating LPS**

Mouse blood samples were collected into heparinized tubes 2 hours after shed blood transfusion R. Blood plasma was obtained after centrifugation (500g for 5 minutes, 4°C). The circulating levels of LPS were determined using a chromogenic limulus amebocyte lysate endotoxin assay kit (Lonza, Walkersville, MD) according to the manufacturer’s instructions. Briefly, an equal volume (50 μL) of plasma or diluted standard (E. coli 0111:B4 endotoxin) was mixed with 50 μL of limulus amebocyte lysate, and it was then added to a 96-well plate and incubated at 37°C for 10 minutes, followed by the addition of 100 μL of substrate solution and incubating it for an additional 6 minutes. Subsequently, stop reagent was added to stop the reaction and absorbance was measured at a wavelength of 405 nm with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The LPS concentration of the plasma was determined according to the standard curve.18

**Histological Evaluation of the Ileum Injury**

After the blood samples were obtained, the mice were sacrificed and the mouse ileum was harvested and fixed with 10% formalin. The samples were processed for paraffin embedding and cut at 4 μm; they were then stained with hematoxylin and eosin (H&E). The ileum mucosa injury was evaluated blindly using a standardized histologic injury scoring system described by Zhang et al.,24 as follows: grade 0, no injury; grade 1, vacuolization at the top of the villi; grade 2, vacuolization at the upper one third of villi; grade 3, vacuolization at the upper two thirds of villi; grade 4, presence of a subepithelial space; and grade 5, mucosal ulceration.

**Circulating HMGB1**

Mouse blood samples were collected into heparinized tubes 24 hours after the shed blood R. The HMGB1 concentration in plasma was determined using an HMGB1 enzyme-linked immunosorbent assay detection kit (IBL International, Hamburg, Germany), according to the instructions provided by the manufacturers.19

**Lung MPO Activity**

The MPO activity in the lung tissue was measured as an index of lung inflammation, as previously described.24 Briefly, after the 24-hour blood samples were collected, the mice were sacrificed, and the mouse lungs were harvested, homogenized, and sonicated in detergent buffer. The prepared samples were used in reactions to spectrophotometrically determine the MPO activity (650 nm) by measuring the hydrogen peroxide-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine.24

**Pulmonary Protein Leakage**

Evans blue (EB) dye was used to assess pulmonary protein leakage, as previously described.25 Briefly, mice were sacrificed with intravenous sodium pentobarbital (100 mg/kg) 30 minutes after an EB (0.4%) injection. The mouse blood was aspirated via cardiac puncture of the right ventricle and the pulmonary circulation was flushed with cold. The lungs were excised, rinsed in PBS, blotted dry, snap frozen in liquid nitrogen, and stored at −80°C. The frozen tissue was homogenized in PBS (4°C) and incubated in formamide (60°C; 16 hours). After centrifugation (7,000g) for 25 minutes at 4°C, the light absorption of the supernatant at 620 nm (A620) and 740 nm (A740) was recorded. The tissue EB content (in μg of EB/g of lung tissue/minute) was calculated by correcting A620 for the presence of heme pigments: A620 (corrected) = A620 − (1.426 × A740 + 0.030) and comparing this value to a standard curve of EB in formamide/PBS.

**Statistical Analysis**

The data are expressed as mean ± SD. Statistical analysis was performed with one-way analysis of variance followed by a Bonferroni correction for multiple comparisons; t-test for two group comparison. All analyses were performed with SAS 9.3 (SAS Institute Inc. Cary, NC, NC). All tests presented are two sided, and a p less than 0.05 is considered as significant. We use the F-test to the equality of variance. If the p value is greater than 0.05, then we do not reject the null hypothesis that the two variances are equal, and we use the p value from the pooled analysis. However, we reject the null hypothesis that the two variances are equal if the p value is 0.05 or lower and the Satterthwaite method is used for unequal variances.

**RESULTS**

**Characterizing a Mouse Model of HS/R With or Without C-peptide**

As shown in Table 1, there are no significant differences in the mouse body weight at baseline. No differences between mice in the HS/R and HS/R plus C-peptide groups at 24 hours post-HS/R were noted. As compared with the sham mice, the MAP of mice in HS/R or HS/R with C-peptide groups dropped to the 35 mm Hg range 15 minutes after the initiation of the blood withdrawal. The mouse MAPs in the HS/R and HS/R with C-peptide groups elevated after RL R, but they remained lower than those of mice in the sham group until completion of the shed blood R (p < 0.05 and p < 0.01). The MAP, levels of blood lactate, and hemoglobin were used to compare the shock severity in mice. There was no severity difference between the HS/R and HS/R with C-peptide groups (Table 1).

**C-Peptide Protects Against HS/R-induced Gut Injury**

HS/R resulted in intestinal barrier dysfunction, as indicated by increased circulating levels of FD4 and LPS. The circulating levels of FD4 and LPS were determined 30 minutes and 2 hours after R, respectively. Both FD4 and LPS were increased in mice with HS/R. The results were consistent with our previous findings;16 however, when C-peptide (1 mg/kg) was given to mice after the shed blood transfusion, the HS/R-induced intestinal barrier dysfunction was prevented, because these mice did not show increases in circulating FD4 and LPS (Fig. 1). The C-peptide administration also attenuates the structural intestinal
injury in those mice that were subjected to HS/R. The HS/R mice had increased histological injury scores 2 hours after the R, as compared with those of sham mice. The C-peptide treatment significantly decreased the intestinal injury score in mice with HS/R (Fig. 2).

**C-Peptide Attenuates an HS/R-Induced Increase in Circulating HMGB1**

We previously reported that mice with HS/R showed increased plasma levels of HMGB1, a cytokine that mediates HS/R-induced acute lung inflammation.18 Thus, we studied the effect of C-peptide on circulating HMGB1. As shown in Figure 3, levels of circulating HMGB1 were increased 24 hours after R. The increased circulating HMGB1 levels in mice with HS/R were attenuated by C-peptide (1 mg/kg).

**C-Peptide Attenuates HS/R-Induced Acute Lung Inflammation and Subsequent Injury**

HMGB1 is a cytokine that has been reported to mediate acute lung injury in HS.18,21 As C-peptide treatment decreased HS/R-induced HMGB1 levels, we investigated the role of C-peptide on HS/R-induced acute lung inflammation and injury. As shown in Figure 4, mice with HS/R incurred acute lung inflammation and injury, as indicated by increased lung MPO activity and pulmonary protein leakage. However, C-peptide–treated mice with HS/R demonstrated less lung inflammation (MPO activity) and injury (decreased pulmonary protein leakage) when compared with those mice without C-peptide treatment (Fig. 4).

**DISCUSSION**

The gut is one of the most vulnerable organs affected by HS, particularly due to the systemic adaptive response that maintains perfusion for vital organs during this state.26,27 The gut tissue is progressively injured during ischemia; therapeutic...
interventions that increase reperfusion can paradoxically cause further damage. Previous studies demonstrated that oxidative stress, increased endoplasmic reticulum stress, inducible nitric oxide synthase expression, and inflammatory response after ischemia/reperfusion are involved in gut injury, leading to the disruption of tight junctions and the death of epithelial cells of the gut mucosa. The intestinal injury and gut barrier dysfunction result in bacterial translocation into the circulatory system and nearby organs, and they consequently induce sepsis and multiple organ failure. Therefore, therapeutic interventions that assist in maintaining gut homeostasis may be important in the treatment of HS/R.

C-peptide is not only a biomarker for the secretion of insulin by β-cells, but it also exerts additional anti-inflammatory effects. The administration of C-peptide to mice treated with an endotoxin (LPS) increases the mouse survival rate by decreasing tumor necrosis factor (TNF-α) and other inflammatory mediators (MCP-1, MIP-1α). It has been reported that C-peptide protects the myocardium from ischemia/reperfusion-induced injury. In addition, Luppi et al. have demonstrated that C-peptide attenuates the endothelial cell expression of VCAM-1, thereby diminishing HS/R-induced lung inflammation. Finally, it has been demonstrated that C-peptide exerts nephroprotective effects after ischemia/reperfusion-induced injury by modulating activator protein-1 (AP-1) signaling. Our study provided evidence to indicate that C-peptide directly protects the gut from the dysfunction/injury (Figs. 1 and 2), and it also attenuates the acute lung inflammation and subsequent (Fig. 4) lung injury induced by HS/R (Fig. 4).

An earlier study by Deitch’s group indicated that injury to the gastrointestinal system played an important role in acute lung injury after traumatic shock. We, and others, have also demonstrated that injury to the gut is the initial step in the induction of a systemic inflammatory response, and in subsequent acute lung inflammation and injury. The associated gut barrier dysfunction and subsequent bacterial invasion can result in the bacteria entering into the bloodstream, as well as to the induction of HMGB1. In addition, gut barrier dysfunction can further lead to the local induction of inflammatory cytokine production (e.g., TNF-α). More importantly, a recent study indicates that HMGB1 produced by the gut can also contribute to acute lung injury after HS. It appears that the induction of HMGB1 after the initial gut barrier dysfunction is a trigger that induces acute lung injury in HS/R. In the lung, HMGB1 further activates proinflammatory transcription factor, nuclear factor kappa B (NFκB); which leads to induction of proinflammatory cytokines, such as IL-6 and IL-1β, and promotes neutrophil infiltration and acute lung injury.

HMGB1 has been implicated as an inflammatory mediator that is involved in various organ injuries/dysfunctions under sepsis, shock, and ischemia/reperfusion. In this study, we demonstrated that circulating HMGB1 is increased in mice with HS/R, and that C-peptide reduced the circulating levels of HMGB1 (Fig. 3). This effect could be attributable to the following factors: (1) C-peptide directly protects the gut from injury,
thus resulting in a reduced systemic inflammatory response due to less bacterial translocation; (2) C-peptide protects the gut by improving the microcirculation, inhibiting HMGB1 production by a specific organ, such as the gut; 3) C-peptide may inhibit macrophage HMGB1 production, which is an important source of HMGB1 in systemic inflammatory responses. Since the gut is an important organ during the inflammatory response (including during lung inflammation/injury) in HS, the protective effect of C-peptide on the gut may be a useful agent in the treatment of HS, and it could also prevent subsequent multiple organ dysfunction.

CONCLUSION AND LIMITATIONS

Figure 5 schematically summarizes our working hypothesis of the mechanisms involved in the ability of C-peptide to attenuate acute lung inflammation and injury induced by HS and R. Our study indicates that the administration of C-peptide to mice with HS/R protects the mouse gut from HS/R-induced injury and dysfunction, diminishes bacteria translocation, blunts systemic increases of the inflammatory cytokine HMGB1, and consequently attenuates acute lung inflammation and injury.

There are several limitations of this study. First, our study used a rodent model to evaluate the effect of C-peptide on HS/R-induced acute lung inflammation/injury. Although C-peptide showed beneficial effects, more studies are needed to ensure that the effect of C-peptide can be translated into clinical practice when treating patients with HS, because a mouse model cannot be assumed to accurately represent the entire complexity of the human response. Second, the present study only observed HMGB1 as a systemic inflammatory mediator. Other inflammatory biomarkers, such as white blood cell count and body temperature, may be changed in our models.

AUTHORSHIP

R.K., X.X., C.M., T.R. were involved in the study design and conception of the article. X.X. and T.R. conducted the animal experiments, tissue harvesting, experimental analysis. R.K., A.X., N.P., T.M., C.M., T.R. contribute to the writing of the article and preparation of the figures. All authors read and approved the final article.

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