

Time-course analysis of counts and degranulation of mast cells during early intestinal ischemia-reperfusion injury in mice

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Abstract. Findings of previous studies have revealed that intestinal mucosal mast cells (IMMCs) are involved in small intestinal ischemia-reperfusion injury (IIRI). However, time-course changes of mast cell counts and mast cell function in this process remain unclear. The present study aimed to observe the number of IMMCs and to investigate the correlation between their activation and small intestine injury at various time points during the period of small intestinal ischemia reperfusion (IIR). Healthy male Kunming mice were randomly divided into five groups, and were subjected to occlusion of the superior mesenteric artery (SMA) for 30 min and followed by reperfusion for 1, 3, 6 and 12 h. By contrast, the SMA was isolated but not clamped in the baseline group. Chiu's scores were assessed by light microscopy, tryptase protein and MCP7 protein expression in the intestine were quantified, and mast cell counts and levels of histamine and TNF- α in the intestine were measured. The results showed that IIR induced severe intestine injury within 12 h as demonstrated by Chiu's scores that was greatly increased as compared to the baseline group, accompanied by increased mast cell counts, histamine and TNF- α levels. However, the Chiu's scores were reduced in the IIR 12 h group compared with the IIR 1 h, IIR 3 h and IIR 6 h groups, with concomitant decreased mast cell counts, histamine and TNF- α levels. The tryptase and MCP7 protein expression was markedly increased in the IIR 1 h and IIR 3 h groups as compared with the baseline group, whereas this expression was gradually decreased at 6 and 12 h after reperfusion. The results of the present study suggest that IIR results in severe mucosal destruction within 6 h after reperfusion, associated with mast cell activation and substantial increases in the mast cell counts.

Introduction

Intestinal ischemia-reperfusion injury (IIRI) plays a critical role in the pathophysiology of numerous conditions, including mesenteric arterial occlusion, shock, cardiopulmonary bypass, trauma, liver transplantation and small bowel transplantation. IIRI is one of the major causes leading to systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF), which are correlated with high mortality rates of 32.1-90% (1-4). A number of factors may contribute to IIRI and the mechanisms by which intestinal ischemia induces reperfusion injury are extremely complicated (5-7). Mast cells are widely distributed in intestinal mucosa and are known as intestinal mucosal mast cells (IMMCs). Andoh *et al* (8) used Ws/Ws rats to investigate the role of mucosal mast cells (MMCs) in the development of IIRI, and results of those authors revealed that the damage was greatly attenuated in mast cell-deficient rats. Kalia *et al* (9) also reported that ketotifen (mast cell stabilizer) inhibits ischemia-reperfusion-induced leukocyte adhesion and prevents local and remote organs from damage in rats subjected to intestinal ischemia-reperfusion (IIR). Moreover, results of our previous study (10) demonstrated that cromolyn sodium (mast cell stabilizer) and ketotifen markedly increase the survival rates at 3 days after IIR in rats, and also alleviate local and remote organ injury. The findings strongly suggest that mast cells play a key role in IIRI.

Previous studies (10,11) have shown that IIR leads to changes in IMMC counts. Boros *et al* (11) investigated the changes of intestinal mast cells in rats following 15, 30, or 60 min ischemia and 30 min reperfusion, respectively, and the results clearly showed that mast cell counts are in part implicated in the severity of intestinal injury. However, those studies (10,11) only focused on one end point in rats subjected to small intestinal ischemia, and the changes of IMMC counts and their function at various time points during reperfusion after intestinal ischemia are poorly understood. Furthermore, the correlation between IIRI and mast cell counts is also not well documented. It is well known that the histological changes of the intestine induced by IIRI begin to recover at 6 h after reperfusion in rats subjected to 60 min ischemia that were suffering from severe damage at 3 h (12,13). Nevertheless, those studies did not investigate the changes of mast cell counts and mast cell activation in the process of IIRI. Therefore, it is imperative to elucidate the correlations and changes in mast

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cell counts and activation and intestinal injury in order to improve treatment of IIRI. In the present study, we observed the number and activation of IMMCs within 12 h of IIR.

Materials and methods

Animals and experimental groups. Thirty-five male Kunming mice weighing 18–24 g (provided by Experimental Animal Center of Guangdong Province, China) were used in this study. The experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. The animals were housed with standard chow and free access to water and were subjected to a 12-h light-dark cycle (8:00 a.m.–8:00 p.m. light). Mice were randomly divided into five groups: baseline, IIR 1 h (intestinal ischemia for 30 min followed by 1 h reperfusion), IIR 3 h (intestinal ischemia for 30 min followed by 3 h reperfusion), IIR 6 h (intestinal ischemia for 30 min followed by 6 h reperfusion) and IIR 12 h (intestinal ischemia for 30 min followed by 12 h reperfusion) groups.

Experimental model of IIRI. Mice were fasted for 12 h and were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg). Following anesthesia, the mice were fixed in a supine position, the abdomen was incised and the superior mesenteric artery (SMA) was confirmed and isolated. Mice in the IIR 1, 3, 6 and 12 h groups suffered ischemia by occlusion of the SMA for 30 min and then the clamp was released and the mice were maintained for 1, 3, 6 and 12 h, respectively. In the baseline group, the same surgery was performed, with the exception of the clamping of the SMA, and the animals were maintained for 1 h. The mice were injected subcutaneously with 0.1 ml physiological saline after the clamp was released. Following completion of the experiments, the mice were sacrificed and the intestinal tissues were obtained for further study. The animals were maintained at 37°C by using a warm pad during the procedure.

Histopathological examination of intestine. A segment of 1.0 cm intestine (from 5 cm of the terminal ileum) was harvested and fixed in 10% formaldehyde. The small intestine tissues were paraffin-embedded and then stained with hematoxylin and eosin for light microscopy. Intestinal mucosal damage was evaluated by two pathologists, who were blinded initially to the experiment, using the criteria of Chiu's method (14) as follows: Grade 0, normal mucosa villi; Grade 1, development of subepithelial Gruenhagen's space at the tip of villus; Grade 2, extension of the subepithelial space with moderate epithelial lifting; Grade 3, large epithelial lifting, possibly with a few denuded villi; Grade 4, denuded villi with lamina propria and exposed capillaries; Grade 5, disintegration of the lamina propria, ulceration and hemorrhage.

Immunohistochemical detection of tryptase in intestine and IMMC counts. Sections (5 μ m) of small intestine were prepared from paraffin-embedded tissue according to previous instructions (9,10), with minor revisions. Briefly, endogenous peroxidase was quenched with 3% H₂O₂ in deionized water for 10 min after deparaffinization. Non-specific binding sites were blocked by incubating the sections in 10%

normal rabbit serum for 1 h. The sections were then incubated with polyclonal rat anti-mast cell tryptase (dilution 1:2,000) at 37°C for 20 min, followed by incubation with biotinylated mouse-anti-rat IgG for 10–15 min at room temperature. The horseradish peroxidase-conjugated streptavidin solution was added and incubated for 10–15 min at room temperature after three 5 min PBS rinses. The antibody binding sites were visualized by incubation with a diaminobenzidine-H₂O₂ solution. Sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in the cytoplasm were identified as positive staining for tryptase. The counts of tryptase-positive mast cells were calculated in five randomly selected areas by Image-Pro Plus 5.0 (Media Cybernetics, Inc., Rockville, MD, USA) software at a x400 magnification (15).

Western blot analysis of intestinal MCP7. Total proteins were extracted from frozen intestine tissues using protein extraction kits for MCP7 measurement (KenGen Biotech Company, Nanjing, China). Protein concentration was measured by BCA Protein Assay reagent kit (KenGen Biotech Company). Protein (60 μ g) was loaded onto a 4–20% SDS-PAGE premade gel (Invitrogen, Carlsbad, CA, USA) for polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane pretreated with 100% methanol. Membranes loaded with proteins of interest were incubated with 5% skimmed milk, and then rat monoclonal anti-MCP7 antibody (1:500 dilution, Santa Cruz, USA) was added to the supernatant and the mixture was incubated on a rotating wheel at 4°C overnight. On the second day, membranes were washed with TBST three times and incubated with a second antibody conjugated to horseradish peroxidase (1:2,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Immunoblots were washed and then were incubated with an enhanced chemiluminescence detection system (KeyGen Biotech). After exposure to hyperfilm-ECL, the membranes were stripped and reprobed with β -actin antibody (1:2,000 dilution, Santa Cruz Biotechnology). Densitometry was analyzed using NIH ImageJ software (<http://rsb.info.nih.gov/ij/index.html>) and normalized by β -actin immunoreactivity to correct sample differences (16).

Detection of histamine and TNF- α levels in intestine. The other segment of intestine tissue was homogenized with frozen normal saline, then centrifuged at 1,500 \times g for 15 min. Supernatants were transferred into fresh tubes for detection of histamine and TNF- α . Intestinal protein was determined using a BCA Protein Assay Kit (KenGen Biotech Company). The concentrations of histamine and TNF- α were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems Inc., Minneapolis, MN, USA). The absorbance was read at 450 nm by a Biokinetics microplate reader Model EL340 (Biotek Instruments, Anaheim, CA, USA). The histamine and TNF- α levels were expressed as ng/ml and pg/ml, respectively. The concentrations of histamine and TNF- α in the intestine were calculated as ng/mg protein and pg/mg protein, respectively.

Statistical analysis. Data were expressed as the means \pm SD, and were analyzed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Repeated measurements were used for

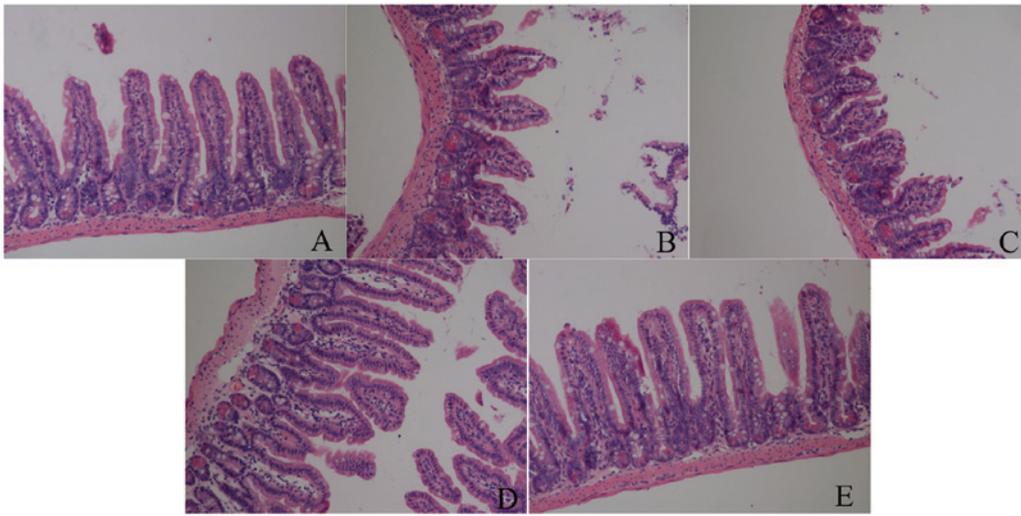


Figure 1. Histological analysis of small intestinal mucosa after H&E staining (x200). (A-E) Representative images of the baseline; 1 h after reperfusion (IIR 1H), 3 h after reperfusion (IIR 3H), 6 h after reperfusion (IIR 6H) and 12 h after reperfusion (IIR 12H) groups. H&E, hematoxylin and eosin.

intra-group comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Changes of intestinal mucosa under light microscopy and Chiu's scores. As shown in Fig. 1, there was no damage in the baseline group, which showed normal villus and glands. However, IIR induced intestinal structural destruction, particularly at 3 h after reperfusion, in which all the animals showed massive epithelial lifting down the sides of the villi, accompanied with some denuded villi and lamina propria in the IIR 1 h, IIR 3 h and IIR 6 h groups. Furthermore, the most severe injury was assessed in the IIR 3 h group, in which disintegration of the lamina propria and hemorrhage was observed. Nevertheless, there was less damage in the IIR 12 h group, which showed only an extension of the subepithelial space with lifting of the epithelial layer in the intestine.

IIR led to marked increases in the Chiu's scores 12 h after the clamp was released as compared with the baseline group (all $P < 0.05$ vs. baseline group). As shown in Fig. 2, the Chiu's scores peaked at 3 h after reperfusion and then decreased gradually, and the Chiu's scores were markedly lowered at 12 h after reperfusion as compared with the IIR 1 h, IIR 3 h and IIR 6 h groups ($P < 0.05$).

Immunohistochemical detection of tryptase and IMMC counts in the intestine. IIR induced the IMMC counts to increase significantly up to 6 h after reperfusion, particularly at 1 h, compared with the baseline group, and the counts were slightly decreased by 6 h after reperfusion. The counts in the IIR 1 h, IIR 3 h and IIR 6 h groups were comparable, while the counts were decreased to the baseline level at 12 h after reperfusion in the IIR 12 h group (Fig. 3).

Consistent with the IMMC counts, the tryptase protein expression in the IIR 1 h group was higher than that in the baseline group, and was slightly but not significantly decreased in the IIR 3 h and IIR 6 h groups. Furthermore, the tryptase

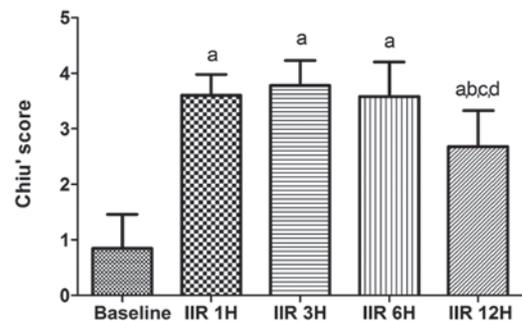


Figure 2. Changes of Chiu's score in the intestine. Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; IIR 12H, 12 h after reperfusion. Results are expressed as the means \pm SD (n=7 per group). ^a $P < 0.05$, compared with baseline group; ^b $P < 0.05$, compared with IIR 1H group; ^c $P < 0.05$, compared with IIR 3H group; ^d $P < 0.05$, compared with IIR 6H group.

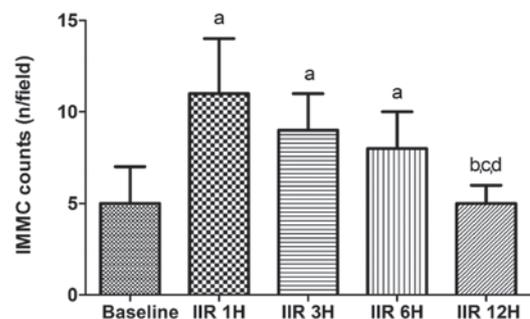


Figure 3. Changes of IMMC counts in the intestine. Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; IIR 12H, 12 h after reperfusion. Results are expressed as the means \pm S.D. n=7 per group. ^a $P < 0.05$, compared with baseline group; ^b $P < 0.05$, compared with IIR 1H group; ^c $P < 0.05$, compared with IIR 3H group; ^d $P < 0.05$, compared with IIR 6H group.

protein expression in the IIR 12 h group was markedly reduced compared with the IIR 1 h, IIR 3 h and IIR 6 h groups, and was decreased to baseline levels (Figs. 4 and 5).

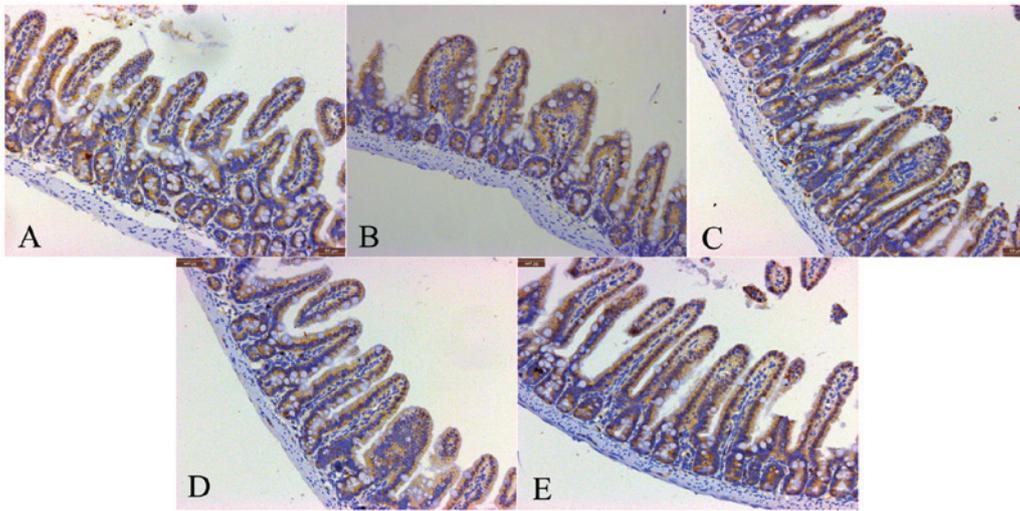


Figure 4. Immunohistochemical detection of tryptase in small intestine (magnification, x200). (A-E) Representative images of the baseline 1 h after reperfusion (IIR 1H), 3 h after reperfusion (IIR 3H), 6 h after reperfusion (IIR 6H) and 12 h after reperfusion (IIR 12H) groups.

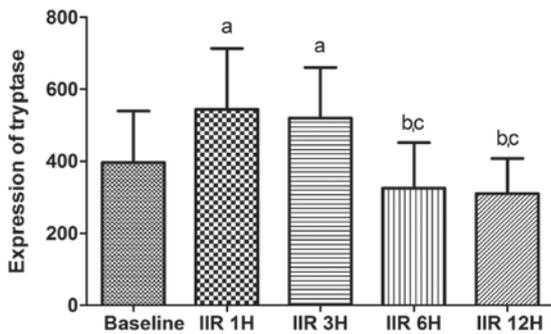


Figure 5. Changes of expression of tryptase in the intestine. Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; and IIR 12H, 12 h after reperfusion. Results are expressed as the means \pm SD (n=7 per group). ^aP<0.05, compared with the baseline group; ^bP<0.05, compared with the IIR 1H group; ^cP<0.05, compared with IIR 3H group.

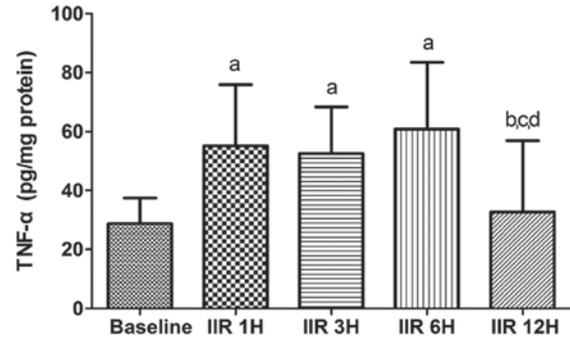


Figure 7. Changes of TNF- α in the intestine. Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; IIR 12H, 12 h after reperfusion. Results are expressed as the means \pm SD (n=7 per group). ^aP<0.05, compared with the baseline group; ^bP<0.05, compared with the IIR 1H group; ^cP<0.05, compared with the IIR 3H group; ^dP<0.05, compared with the IIR 6H group.

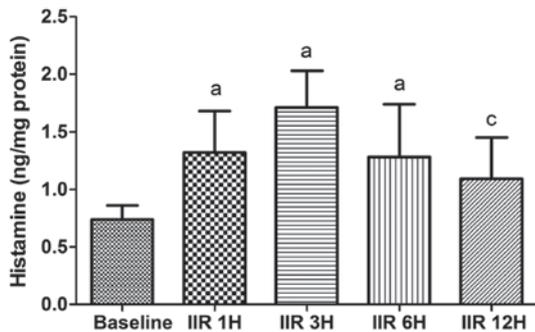


Figure 6. Changes of histamine concentration in the intestine. Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; IIR 12H, 12 h after reperfusion. Results are expressed as the means \pm SD (n=7 per group). ^aP<0.05, compared with the baseline group; ^cP<0.05, compared with the IIR 3H group.

Histamine contents in the small intestine. Compared with the baseline group, the histamine contents in the small intestine were significantly higher in the IIR 1 h, IIR 3 h and IIR 6 h

groups (P<0.05). Moreover, the histamine contents reached a maximum level at 3 h after reperfusion, as shown in Fig. 5, but this level was not significantly increased as compared with the IIR 1 h and IIR 6 h groups. The histamine content in the IIR 12 h group was significantly lower than that in the IIR 3 h group (P<0.05; Fig. 6).

TNF- α levels in small intestine. As shown in Fig. 6, IIR-induced TNF- α levels markedly increased in the IIR 1 h, IIR 3 h and IIR 6 h groups as compared with the baseline group (P<0.05). After reaching a plateau level, the contents of TNF- α in the intestine were markedly decreased to the baseline level (Fig. 7).

Expression of MCP7 in intestine. MCP7 is a subtype of tryptase. As shown in Fig. 7, the expression of MCP7 was gradually increased in the 6 h following the clamp release, and reached maximum levels in the IIR 3 h and IIR 6 h groups as compared to the baseline group. Notably, the expression of MCP7 was markedly decreased to baseline levels at 12 h after reperfusion (Fig. 8).

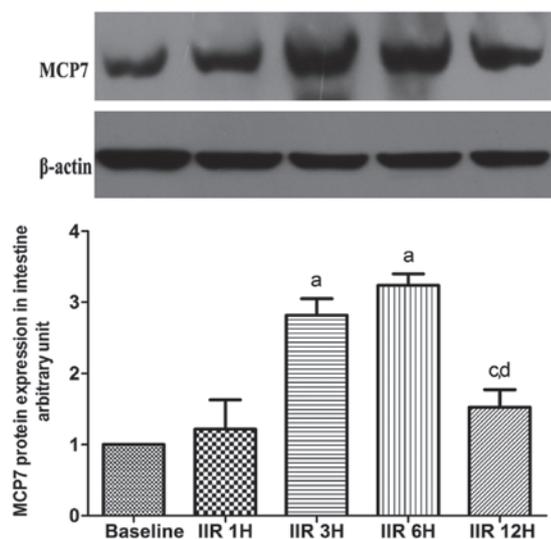


Figure 8. Western blot analysis of expression of small intestine MCP7 (means \pm SD, n=7). Baseline, baseline group; IIR 1H, 1 h after reperfusion; IIR 3H, 3 h after reperfusion; IIR 6H, 6 h after reperfusion; IIR 12H, 12 h after reperfusion. ^aP<0.05, compared with the baseline group; ^bP<0.05, compared with the IIR 1H group; ^cP<0.05, compared with the IIR 3H group; ^dP<0.05, compared with IIR 6H group.

Discussion

The damage to small intestinal mucosa and the recovery after IIR depend on the duration of ischemia and the animal species involved. In the present study, we observed that IIR led to the greatest destruction of pathological structure at 6 h after reperfusion. As shown in Fig. 1, massive collapsed epithelia and villi, as well as denuded villi and lamina propria were observed, and the structure of intestinal mucosa, as assessed by light microscopy, appeared to recover to normal at 12 h after release of the clamp, which is in agreement with the study of Chang *et al* (12), in which they reported that the small intestinal mucosal injury in rats that were subjected to 60 min of hemorrhagic shock was severe at the onset after resuscitation, and the structure of small intestinal mucosa began to recover gradually at 6 h after resuscitation and almost normal intestinal mucosa was observed at 24 h after reperfusion.

IMMCs, widely distributed in the intestinal lamina propria, are adjacent to blood vessel and nerve fiber cells. IMMCs interact with neuropeptides and cytokines through the immune and neural pathways, which play an important role in regulating the physical function of the gastrointestinal tract, as well as the development of inflammatory bowel diseases (17,18). A study has previously demonstrated that the ratio of IMMCs to intestinal mucosal lamina propria cells is ~2-3% under normal conditions. However, this ratio can increase by 10-fold in subjects suffering from intestinal diseases (19). Several lines of evidence have demonstrated that mast cells play a critical role in small IIR by using mast cell-deficient rats or by inhibiting mast cells (8-10).

Mast cells are important pro-inflammatory cells. Previous studies (20,21) have reported that the number of mast cells were increased in lungs, intestines or other organs subjected to ischemia-reperfusion injury. In the present study, the find-

ings showed that 30 min of ischemia followed by 1, 3 or 6 h of reperfusion induced a significant increase in the number of IMMCs by analyzing time-course changes of mast cell counts. Moreover, the IMMC counts were higher at 1 h after reperfusion, and then the IMMC counts gradually decreased to baseline at 12 h after reperfusion, which is consistent with the Chiu's scores, although severe intestinal mucosal destruction was found at 6 h after initiation of reperfusion.

Mast cells are generated in the bone marrow, and then distributed to tissues and organs, and play various roles in regulating physiological function (22). A previous study (23) has reported that bone marrow-derived mast cells (BMMCs) migrate to the injury sites to serve their function. MCP7 is a subtype of tryptase and is only expressed in immature BMMCs (24). Accordingly, the expression of MCP7 can partly identify the number of BMMCs that have migrated to injured tissues (25). The findings in the present study have shown that the MCP7 expression was substantially increased at 3 h after reperfusion, slowly decreased at 6 h and had almost reached baseline level at 12 h after reperfusion. The findings suggest that small intestinal ischemia-reperfusion may result in BMMC migration to the injured intestine tissue, which further aggravates small intestine injury by releasing numerous chemokines and cytokines.

Levels of histamine and tryptase are characteristic markers of mast cell activation and degranulation. Previous studies (26,27) have demonstrated that histamine and tryptase, which are involved in tissue injury, was able to increase microvascular permeability, induce inflammatory cell infiltration, and amplify the effects of mast cells. Several studies (9,10,28) have confirmed that the application of mast cell membrane stabilizers or antihistamines were capable of inhibiting mast cell degranulation and attenuate IIRI. In the present study, we observed that the levels of histamine and tryptase in the small intestine were markedly and rapidly increased after IIR, and peaked within 3 h after reperfusion, and then gradually decreased to the baseline level at 12 h. Furthermore, these changes were consistent with the pathological damage. The results indicated that the best time to target mast cells is at ~3 h after reperfusion.

Inflammatory reactions mediated by cytokines are one of the main mechanisms of IIRI. Among the many mediators that contribute to IIR, TNF- α is one of the key mediators and initiates the cascade effect (29). A number of cells, such as endothelial cells as well as inflammatory cells, are capable of producing TNF- α when they are subjected to injury. Mast cells can also produce and release TNF- α . Furthermore, Bischoff *et al* (30) speculated that the concentration of TNF- α in the small intestine is largely from IMMC degranulation. The findings in the present study showed that the levels of TNF- α in intestine were greatly increased at 6 h after reperfusion, and then decreased gradually at 12 h after reperfusion. Of note, the results were in agreement with the changes of mast cell counts and intestinal injury scores. Therefore, the results suggest that mast cell degranulation leads to small intestinal ischemia reperfusion injury.

In conclusion, small intestinal ischemia reperfusion results in substantial increases in the mast cell counts within 6 h after reperfusion, which contributes to the small intestine mucosal destruction by degranulation.

Acknowledgements

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