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DISTURBANCES OF MESENTERIC LYMPH FLOW AND IN VIVO INTESTINAL LYMPHOCYTE TRAFFICKING DURING EARLY GUT INJURY INDUCED BY ISCHEMIA-REPERFUSION IN RATS

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ABSTRACT

We sought to define the role of mesenteric lymph in the generation of remote organ damage at the early stage of gut ischemia-reperfusion (I/R) injury. The measurement of mesenteric lymph flow was carried out by cannulation of mesenteric lymphatics. The distribution of in vivo intestinal lymphocyte trafficking was performed by ^{51}Cr labeled lymphocyte and measurement of ^{51}Cr-lymphocytes distribution by γ-counter. Endotoxin concentration was assayed using the limulus test kit and TNF-α level was detected by ELISA. After gut I/R injury, the volumes of lymph flow in mesenteric lymphatics per hour were sharply decreased by 72% and the number of intestinal lymphocytes per milliliter was decreased by 61%, which led to the intestinal lymphocyte output per hour significantly decreased by 90% (predominantly T cells), while the population of ^{51}Cr-lymphocytes in Peyer's patches, small intestine (except Peyer's patches), mesenteric nodes, large intestine, and stomach increased by 87%, 191%, 87%, 266%, 262%, respectively. Meanwhile, endotoxin and TNF-α levels in mesenteric lymph were significantly increased. These findings demonstrate the marked disorders of mesenteric lymph flow and in vivo intestinal lymphocytes migration and the accompanying increase of endotoxin and TNF-α levels in mesenteric lymph in the early stage of gut I/R injury.

Keywords: mesenteric lymph flow, intestinal lymphocyte trafficking, ischemia-reperfusion, gut injury

Gut ischemia-reperfusion (I/R) injury plays an important role in human pathophysiology in various clinical conditions including shock, infection, trauma, and organ transplantation and is associated with high morbidity and mortality (1). The gut I/R injury leading to a decrease in gut barrier function is the initial triggering event that contributes to the development of systemic inflammatory responses and multiple organ dysfunction syndrome (MODS) (2). However, understanding of the exact mechanisms by which gut I/R leads to intestinal barrier dysfunction and how gut injury is transduced into a gut-induced systemic response and remote organ damage remains incomplete.

Recently, the mesenteric lymph pathway has gained increasing attention as the potential bridge by which gut injury leads to other splanchnic organ dysfunction (3). Deitch et al have proposed the hypothesis that gut lymph and lymphatics are a source of factors leading to organ injury and dysfunction during gut failure (4,5). Shock, trauma, or sepsis-induced gut injury can

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result in the generation of cytokines and other pro-inflammatory mediators in the gut (6). Mesenteric lymph appears to be the route of delivery of the gut-derived toxic factors from the gut to remote organs (7). Toxic factors have been demonstrated in mesenteric lymph, but not in the systemic or portal circulation (7). Acute lung injury and haemopoietic failure have been shown to be caused by the toxic factors in mesenteric lymph (8,9). In addition, mesenteric lymph contains numerous activated lymphocytes (10). The functional integrity of the immune system is dependent upon the continual trafficking of these intestinal lymphocytes from lymph to blood. Furthermore, the migration of these lymphocytes from the bloodstream into secondary lymphoid tissues is orchestrated to ensure antigenic encounter and the triggering of effective immune responses (11,12). Tissue-specific recruitment of memory and effector lymphocytes may serve to increase the efficiency and robustness of regional immune responses and to allow functional immune specialization of particular tissues (13). However, what is the effect of gut I/R on the normal recruitment of intestinal lymphocytes? What is the relationship of in vivo intestinal lymphocyte trafficking to mesenteric lymph flow and gut-derived toxic factors in response to gut I/R?

Therefore, the objective of the present study was to observe the disturbances of mesenteric lymph flow and in vivo intestinal lymphocyte trafficking after gut I/R injury and to measure the levels of certain gut-derived toxic factors in the mesenteric lymph, which are relevant to the failure of gut function and a systemic response.

METHODS

Sixty healthy adult male Wistar rats weighing 250-300g were provided by the experimental animal center of Nanjing Medical University, China. The following experimental groups were included in this study: 1) normal control (sham-operation) group1 (n=10), and 2) ischemia-reperfusion (I/R) group1 (n=20) for flow and lymphocyte counting experiments, 3) normal control group2 (n=10), and 4) I/R group2 (n=20) for lymphocyte trafficking experiments. All procedures using animals were reviewed and approved by the Experimental Animal Review Board of Nanjing Medical University and were performed according to the criteria outlined by the National Ministry of Health.

Surgical Procedure

Rats in the I/R group underwent superior mesenteric artery (SMA) ligation for 45 min with a small clamp until complete ischemia was attained. Upon release of clamp, rats were allowed to recover from anesthesia for 6h. After reperfusion, rats were sacrificed under deep isoflurane anaesthesia. In the normal group, the SMA was isolated without clamping and was exposed to the same procedure as in rats with SMA occlusion.

Mesenteric Lymph Preparation

The procedures used to collect mesenteric lymph in rats have been described previously (14). Briefly, rats were anesthetized with amobarbital by intraperitoneal injection. A midline celiotomy incision was made and the mesenteric lymphatic vessel identified (adjacent to the SMA) by reflecting the loops of intestine to the left of the animal with a metal hook. A plastic tube, 1 mm in diameter, with beveled ends, was passed into the mesenteric lymphatic, and the lymph began to flow immediately through the tube. The lymph fluid was collected with visible confirmation of free flow into a microcentrifuge tube (*Fig.1*).

T and B Lymphocyte Analysis in Mesenteric Lymph

Intestinal lymphocytes were collected as described above. Cell viability was determined by 0.2% Evans blue staining. More than 95%

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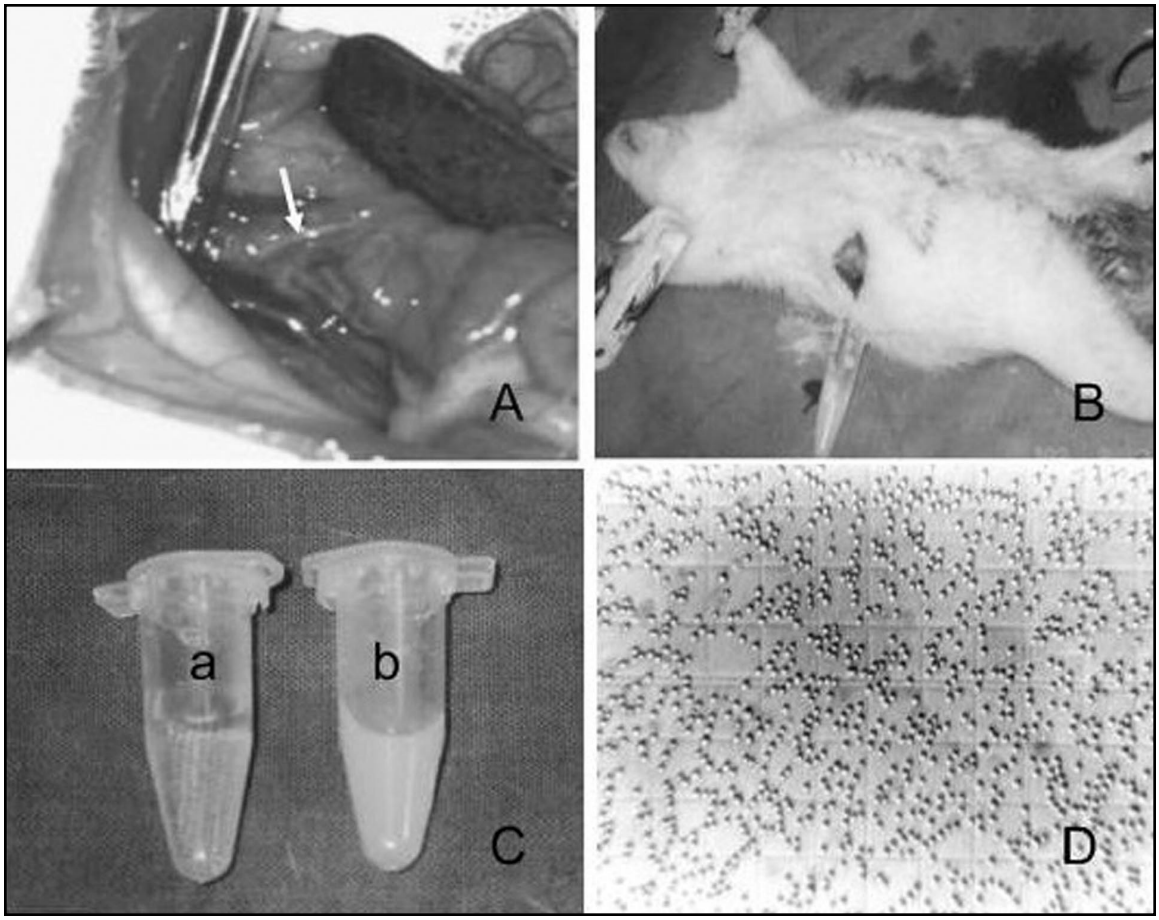

Fig. 1. Collection of mesenteric lymph and measurement of lymphocytes in rats. A. Mesenteric lymphatic is indicated as a white arrow. B. Mesenteric lymph begins to flow through a plastic cannula into collection tube. C. Tube b is filled with mesenteric lymph, tube a is filled with saline water as control. D. Intestinal lymphocytes are observed under light microscopy (x100).

of the cells were viable for measurement of lymphocyte subsets. CD3 T and CD20 B lymphocyte subsets were analyzed by flow cytometry.

Observation of Mesenteric Lymph Flow and Counting of Lymphocytes

In I/R group1, mesenteric lymph was collected for 1h in the 6th hour after reperfusion. In the normal control group1, the SMA in rats was not clamped, and mesenteric lymph was collected according to the same procedure as in rats with SMA occlusion. After collection stopped, mesenteric lymph flow was measured and lymphocytes were counted under a light microscope.

Determination of In Vivo Lymphocyte Trafficking

Mesenteric lymph was collected for 1 h at the 4th hour after reperfusion in I/R group2. The lymphocytes collected were labeled with ^{51}Cr and then infused into the femoral vein of rats at the beginning of the 6th hour after reperfusion. 1 h later, the rats were killed and

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the small intestine containing ^{51}Cr-intestinal lymphocytes as well as other vital organs were removed and counted with a γ-counter. Rats in normal control group2 were exposed to the same procedure as those in I/R group2.

Intestinal Lymphocyte Labeling with ^{51}Cr

1×10^7 lymphocytes/ml in RPMI 1640 (containing 20% fetal bovine serum) were incubated with 20 µCi/ml $Na_2[^{51}Cr]O_4$ for 1h at 37°C in water. After incubation, the tube was centrifuged at 500g for 5 min, supernatant decanted, and the remainder was added into a test tube containing 100% fetal bovine serum and 17% Nycodenz (Sigma Co. USA). This mix was then centrifuged at 1200g for 5 min. The ^{51}Cr labeled lymphocytes were collected from the layer between the fetal bovine serum and Nycodenz. $5x10^6$ ^{51}Cr-lymphocytes mixed with 0.4ml of RPMI-1640 were infused slowly into the femoral vein of rats. 1h later, rats were killed and the small intestine and other vital organs were removed and 1ml of blood sample was taken by heart puncture. The organs containing ^{51}Cr labeled lymphocytes were measured by γ-counter (TDC-601, Aloka Co, Japan) (15). Total counts of the 5×10^6 lymphocytes were measured and lymphocyte populations in the intestine and other organs were calculated as a background corrected percentage of counts per organ over the total counts.

Functional Evaluation of Vital Organs

Peripheral blood was taken from rats in each group before they were sacrificed to determine the oxygen partial pressure (PaO_2) with DMNI modular system (AVL, Graz, Austria), alanine aminotransferase (ALT), and creatinine level (AUOLYMPUS5400; Olympus, Tokyo, Japan), Plasma D-lactate concentration was measured by an enzymatic spectrophotometric assay using a centrifugal analyzer at 30°C (Hoffmann-LaRoche, Basel, Switzerland) as described earlier [16]. *d*-lactate, *d*-lactate dehydrogenase and NAD+ were purchased from Sigma Chemical Company (USA).

Measurement of Endotoxin Concentration and TNF-α Activity

Endotoxin concentration in plasma or mesenteric lymph was assayed using the limulus test kit (Yihua Clinical Technology Co, Shanghai, China). The assay depends on bacterial endotoxin to activate a proenzyme in the limulus amoebocyte lysate that catalyzes the cleavage of p-nitroanaline (pNA) from the colorless substrate. The pNA is assayed spectrophotometrically at 545 nm and provides a quantitative analysis of endotoxin content. TNF-α concentration in serum and mesenteric lymph was determined by using rat TNF-enzyme-linked immunoabsorbant assay (ELISA) kit (LIFEKEY Biotech, Co. USA) according to the manufacturer's protocol.

Morphological Changes of the Gut and Injury Score

Specimens from the small intestines (5cm from the distal end of ileum) were removed and fixed with 10% formaldehyde. Paraffin sections were stained with hematoxylin and eosin for histological evaluation in a single blinded fashion. For semiquantitative evaluation of lesions, 10 arbitrary microscopic fields were viewed in

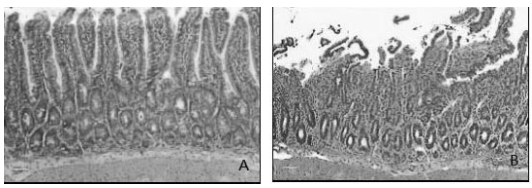

Fig. 2. Changes in gut morphology at the 6th hour after mesenteric reperfusion following gut ischemia in rats. Representative hematoxylin and eosin (H & E)-stained slides from the gut of rat subjected to ischemia-reperfusion (B) or sham operation (A) (HE, x100) were visualized and captured under a light microscope.

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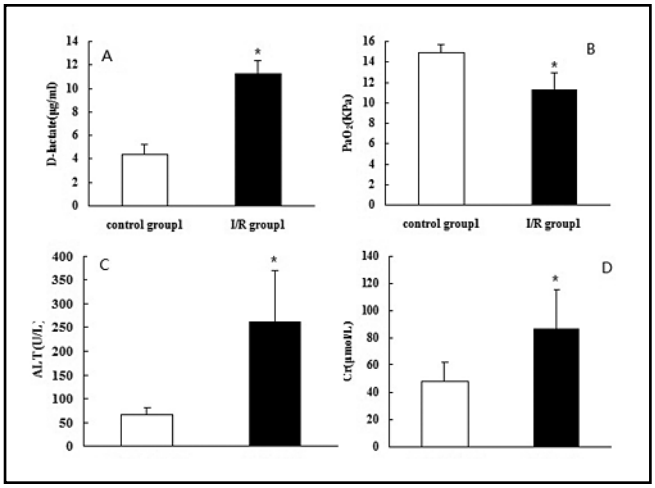

*Fig. 3. Changes in D-lactate (A), PaO_2 (B), ALT (C) and creatinine (D) level in peripheral blood at the 6th hour after mesenteric reperfusion following gut ischemia in rats. Gut ischemia-reperfusion injury was induced by 45 min of SMA occlusion, followed by 6 hours of reperfusion. *P<0.05 compared with control group1.*

each sample. The scoring system was based on area of the lesion: +, <1/3 total area; ++, 1/3-2/3 total area; +++, >2/3 total area.

Statistical Analysis

All quantitative data were presented as mean ± SD and analyzed using SPSS11.0 software. Statistical analysis was performed using the unpaired Student's t-test. Difference was considered statistically significant when p<0.05. All data were expressed as mean ± standard deviation (SD).

RESULTS

As shown in *Fig. 2A*, the intestinal mucosa of saline-treated rats was intact and the villi presented in an orderly fashion. Samples displayed no abnormal epithelial cell morphology, and there was no evidence of congestion, edema, or infiltration of inflammatory cells. In contrast, the intestinal mucosal villi in the I/R injury gut were loosened and atrophic where the epithelial cells were necrotic. The mucosa was edematous and infiltrated with inflammatory cells (*Fig. 2B*). Semiquantitative evaluation showed that inflammatory lesions of the distal end of the ileum after gut I/R injury scored as ++.

After occluding the SMA for 45 min followed by reperfusion for 6h, plasma D-lactate, ALT, and creatinine levels were increased by 158% (11.28±1.10 vs 4.37±0.91 μg/ml, p<0.05, *Fig. 3A*), 291% (262±107 vs 67±15 U/L, p<0.05, *Fig.3 C*), (86.56±29.02 vs 48.67±13.43 μmol/L, p<0.05, *Fig. 3D*) respectively, when compared with normal control group1. PaO_2 in I/R group1 was decreased by 25% (11.222±1.674 vs 14.927±0.741 KPa, p<0.05, *Fig. 3B*) compared with normal control group1.

There were significant differences of endotoxin levels between I/R group1 and normal control group1 in plasma (0.470±0.157 vs 0.069±0.051 EU/mL, p<0.05) and mesenteric lymph (0.110±0.028 vs 0.043±0.022 EU/mL, p<0.05) as shown in *Fig. 4A*. Meanwhile, compared with normal control group1, TNF-α level in serum (28.75±10.46 g/ml) and mesenteric lymph (74.93±14.77 g/ml) in I/R group1 was significantly increased (p<0.05, *Fig. 4B*) compared with normal control group1 (TNF-α detection value was zero g/ml).

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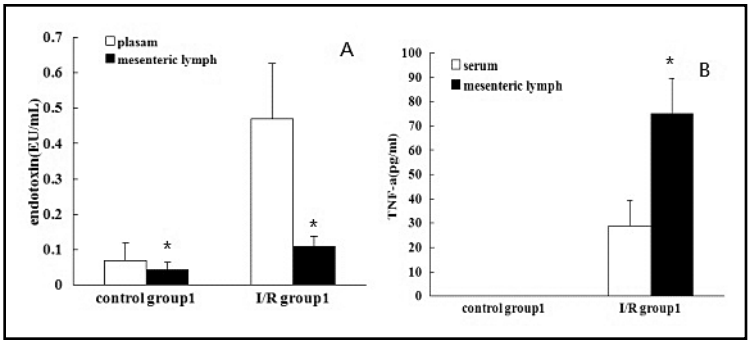

*Fig. 4. Changes in endotoxin (A) and TNF-α (B) levels in peripheral blood and mesenteric lymph at the 6th hour after mesenteric reperfusion following gut ischemia in rats. Gut ischemia-reperfusion injury was induced by 45 min of SMA occlusion, followed by 6 hours of reperfusion. *P<0.05 compared with control group1.*

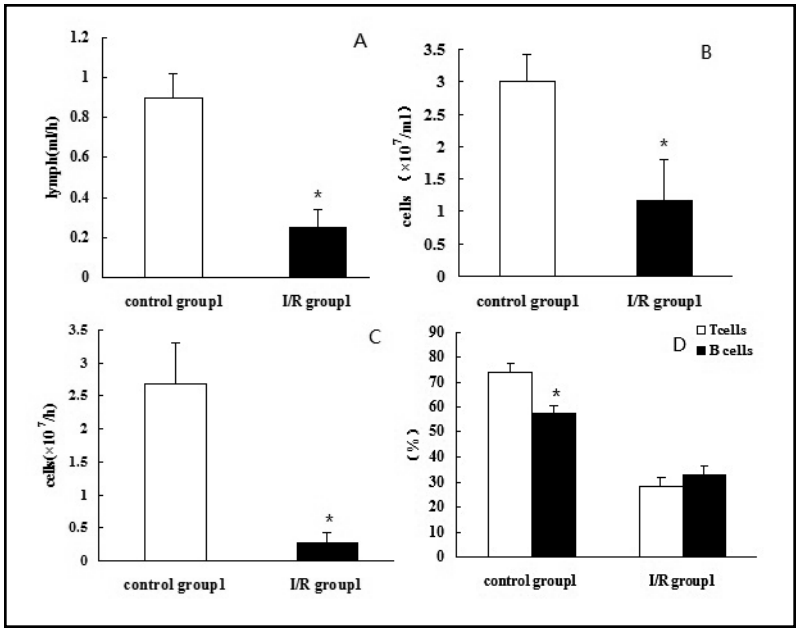

*Fig. 5. Changes in volume of mesenteric lymph and lymphocyte output at the 6th hour after mesenteric reperfusion following gut ischemia in rats. A. Volume of mesenteric lymph at the 6th hour after mesenteric reperfusion (ml/h). B. Number of lymphocytes per milliliter of mesenteric lymph (10^7/ml). C. Lymphocyte output at the 6th hour after mesenteric reperfusion in mesenteric lymph (10^7/h). D. Percentage of T and B lymphocyte subsets in lymphocyte output at the 6th hour after mesenteric reperfusion (%). Gut ischemia-reperfusion injury was induced by 45 min of SMA occlusion, followed by 6 hours of reperfusion. *P<0.05 compared with control group1.*

The output (predominantly T cell type (57.40±3.21% vs 73.80±3.96%, I/R group1vs normal control group1 respectively, p<0.05, *Fig. 5D*) of intestinal lymphocytes in mesenteric lymph at the 6th hour after mesenteric reperfusion following gut ischemia was significantly decreased in I/R group1 compared with normal control group1 (0.28±0.15x10^7/h vs 2.69±0.61x10^7/h, p<0.05, *Fig. 5C*), which was related to the decreases of mesenteric lymph volume at the same period of time (0.25±0.09 vs 0.90±0.12 ml/h,

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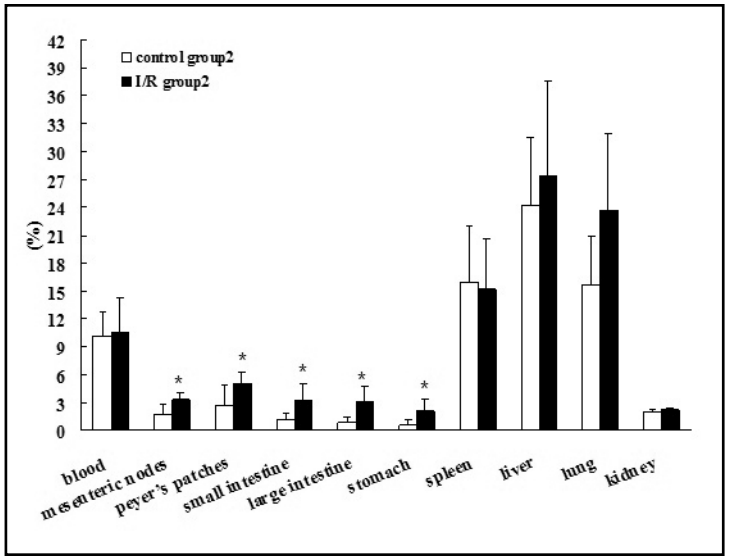

*Fig. 6. Changes in in vivo trafficking of ^{51}Cr labeled intestinal lymphocytes at the 6th hour after mesenteric reperfusion following gut ischemia in rats. *P<0.05 compared with control group2.*

p<0.05, *Fig. 5A*) and the number of intestinal lymphocytes per milliliter (1.16±0.63x10^7/ml vs 3.00±0.42x10^7/ml, p<0.05, *Fig. 5B*).

Compared with normal control group2, distribution of intestinal ^{51}Cr-lymphocytes in Peyer's patches (2.69±2.19% vs 5.04±1.23%, p<0.05), small intestine (except Peyer's patches 1.11±0.75% vs 3.23±1.69%, p<0.05), mesenteric nodes (1.75±1.17% vs 3.28±0.79%, p<0.05), large intestine (0.80±0.55% vs 3.04±1.74%, p<0.05), and stomach (0.58±0.57% vs 2.10±1.24%, p<0.05) was significantly increased in I/R group2. There were no significant differences between normal control group2 and I/R group2 in blood (10.10±2.67% vs 10.58±3.70%, p>0.05), spleen (15.90±6.01% vs 15.19±5.46%, p>0.05), liver (24.21±7.34% vs 27.32±10.28%, p>0.05), lung (15.65±5.20% vs 23.70±8.25%, p>0.05) or kidney (2.00±0.18% vs 2.18±0.21%, p>0.05, *Fig. 6*).

DISCUSSION

The gut in I/R injury is of interest not only because its functions are damaged, but also it is a potential factor in MODS associated with reperfusion injury (17,18). In our studies, the impairments of the gut mucosa after I/R injury were obvious including small intestinal epithelial cell degeneration, necrosis, and even sloughing off when compared with the normal control group1 *(Fig. 2)*. D-lactate is produced by bacteria of the gastrointestinal tract and is absorbed in the small intestine and colon, and plasma and is a sensitive marker for gut barrier failure (16). After gut I/R injury, plasma D-lactate concentration was 2.6 fold higher than that in normal control group1. This finding indicates that gut I/R disrupts the gut barrier function and changes permeability. Meanwhile, functional damage of the other vital organs such as lung, liver, and kidney developed and plasma ALT and creatinine levels increased by 291% and 78%, respectively, and blood PaO_2 pressure decreased by 25% (*Fig. 3*).

When the gut barrier is injured, gut-derived endotoxin may enter into the extraintestinal tissues and provoke cytokines that potentiate the development of MODS

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(19,20). The accepted concept is that gut-derived endotoxin is the major source of that in the systemic circulation (21). In I/R group1, the endotoxin level in plasma and intestinal lymph was significantly increased by 581% and 156%, respectively, compared with normal control group1. Within I/R group1, the endotoxin level in mesenteric lymph was 23% of that in plasma. This finding shows that gut-derived endotoxin is an important source of that in the circulation, but probably is not the only source (*Fig. 4A*). Increased levels of endotoxin in the blood circulation is associated with the coordinated activation of a cascade of cytokines (22). Mesenteric lymph is the key conduit for factors leaving the gut leading to organ injury and dysfunction (4). When gut damage releases a large amount of inflammatory cytokines, including rapidly produced TNF-α, inflammatory cells accumulate and intestinal inflammatory damage occurs (23). TNF-α is an important mediator involved in activating the cascade of inflammatory reactions of I/R (24). Our results showed that the TNF-α level in serum and mesenteric lymph in I/R group1 was significantly higher than that in normal control group1. Moreover, the level of TNF-α in mesenteric lymph was 2.6-fold higher than TNF- alteration in serum during gut I/R injury (*Fig. 4B*). This finding indicates that gut-derived TNF-α plays a more crucial role during the development of the gut I/R injury.

The mesenteric lymphatic system forms an important pathway to return the lymph fluid from the gut tissue spaces back to the blood stream. Lymph contains a large number of lymphocytes. The circulating lymphocytes enter secondary lymphoid tissues and subsequently return to the circulation through the efferent lymphatic. In I/R group1, the volumes of lymph flow in the mesenteric efferent lymphatic at the 6th hour after mesenteric reperfusion gut ischemia were sharply decreased by 72% and the number of intestinal lymphocytes per milliliter was decreased by 61%, accounting for the significant decrease in the intestinal lymphocyte output per hour by 90% (largely in T cells) compared with normal control group1. To account for this observation, we speculate that because of mesenteric lymph flow reduction, the major route by which gut-derived toxic factors (including endotoxin and TNF-α) reach the systemic circulation is blocked after mesenteric reperfusion, which serves to protect against gut I/R induced organ damage. On the other hand, T lymphocyte selective migration back through the gut tissues is also blocked as reflected in the decreased number of T lymphocytes exiting in mesenteric lymphatic, thereby interfering in normal lymphocyte trafficking and materially impairing immune function. In this way, the harmful effects are mediated and there is protection of the tissue (especially the gut) damage after reperfusion.

Recent data demonstrated an important role for lymphocytes, particularly T cells but also B cells, in I/R injury (25). Shigematsu et al (26) demonstrated that T-cell adhesion was significantly increased after 6 h of reperfusion. This finding suggests that T cells can have a pathogenic effect during gut I/R injury even in the absence of adhering to vascular endothelium. In our studies, the lymphocytes in mesenteric lymphatics were labeled with ^{51}Cr, infused into the blood, circulation, and 1 h later, the distribution of ^{51}Cr-intestinal lymphocytes in vital organs was observed. In I/R group2, the population of ^{51}Cr-intestinal lymphocytes in Peyer's patches, small intestine (except Peyer's patches), mesenteric nodes, large intestine, and stomach was increased compared with normal control group2 ($P<0.05$). There were no significant changes in the population of ^{51}Cr-intestinal lymphocytes in blood, spleen, liver, lung and kidney between both groups ($p>0.05$) (*Fig. 6*). These results show that after mesenteric reperfusion, the population of ^{51}Cr-labeled lymphocytes in the gastrointestinal tract was sharply increased. We speculate that during the gut I/R injury, the increased population of ^{51}Cr-intestinal lymphocytes in the

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gastrointestinal tract may be considered as a compensatory response for the limitation of intestinal lymphocyte recirculation back through the gastrointestinal tract due to the reduced number of intestinal lymphocytes trafficking in mesenteric lymphatics, which may serve to protect the gut from I/R induced organ damage.

According to the above, we speculate that during the development of gut I/R injury, there are two kinds of factors in the mesenteric lymph circulation: 1) Harmful factors including increased gut-derived toxic factors and the decreased lymphocyte output in mesenteric lymph; and 2) Beneficial factors including the reduced mesenteric lymph flow preventing the major route by which gut-derived toxic factors reach the systemic circulation and the increased population of ^{51}Cr-intestinal lymphocytes in the gastro-intestinal tract that promote efficiency of the intestinal immune responses. Disturbances in the balance between these two factors determine the results of the gut I/R injury. When the harmful factors exceed the beneficial factors, the gut I/R injury will develop into the systemic inflammatory response syndrome and even MODS. When the beneficial factors exceed the harmful factors, the gut I/R induced organ damage will improve. Thus, therapeutic strategies that promote the beneficial factors will ameliorate gut I/R induced organ damage. This hypothesis needs substantiation by further experimental and clinical evidence.

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