Increased Serum Endotoxin and Elevated CD14 and IL-1β Expression in a Rat Model of Cerebrogenic Multiple Organ Dysfunction Syndrome

Qu Chuanqiang[a,§], Guo Shougang[b,*], Guo Hongzhi[a,§], Wang Chunjuan[a,§], Ma Lin[a], He Yan[c], Wang Lei[c], Lou Jianwei[a] and Wang Xingbang[a]

[a]*Department of Neurology, Qilu Hospital Affiliated to Shandong University, Jinan, 250012, China;* [b]*Department of Neurology, Provincial Hospital Affiliated to Shandong University, Jinan, 250021, China;* [c]*Department of Neurology, Second Hospital Affiliated to Shandong University of Traditional Chinese Medicine, Jinan, 250012, China*

Abstract: *Objectives:* To study the mechanisms underlying cerebrogenic multiple organ dysfunction syndrome (CMODS) through investigation of endotoxin levels and the expression of endotoxin receptor CD14 and interleukin IL-1β mRNAs in a rat CMODS model.

Methods: Acute cerebral hemorrhage was induced in Wistar rats by focal intracerebral injection of collagenase into the caudate nucleus. Serum endotoxin levels were quantitated using a chromogenic limulus lysate method; CD14 endotoxin receptor mRNA and IL-1β mRNA levels in lung and intestine were determined by *in situ* hybridization.

Results: Serum endotoxin levels increased after 12 h, reaching a peak after 24 h, and declined to control levels at 72 h. The increase was statistically significant ($P<0.05$) compared to unoperated controls and the sham-operated group respectively. CD14 mRNA in lung and intestine increased after 12 h, peaked after 24-36 h, and then declined after 48 h. IL-1β mRNA levels were also increased in lung and intestine ($P<0.05$), peaking at 36 h and declining thereafter. Expression levels of both CD14 and IL-1β mRNAs correlated significantly with serum endotoxin levels ($P<0.01$). We conclude that acute cerebral hemorrhage results in endotoxemia and widespread increases in CD14 and IL-1β expression. We suggest that acute cerebrovascular challenge leads to a stress/shock response that compromizes the intestinal mucosal barrier. In turn, this allows endotoxin translocation into the body that provokes the release of pro-inflammatory lymphokines, leading to a systemic inflammatory response syndrome (SIRS) that culminates in multiple organ dysfunction.

Key Words: Multiple organ dysfunctio, endotoxins, CD14, IL-1β, gene expression.

INTRODUCTION

Multiple organ dysfunction syndrome (MODS), often known as multiple organ failure (MOF), was first defined by the American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference in 1991. The condition is defined as dysfunction affecting 2 or more body organs or systems occurring simultaneously or sequentially within 24 h of severe acute injury [1]. The development of the syndrome is dynamic, encompassing several sequential pathologic stages. These typically include a local primary response, an initial low-level systemic inflammatory response, followed by intense systemic inflammatory responses leading to immunosuppression, and culminating in multiple organ failure.

Cerebrogenic multiple organ dysfunction syndrome (CMODS) is caused by brain injury. Approximately 11-21% of CMODS cases are caused by acute cerebrovascular disease (ACVD). Compared with MODS cases of other etiologies, organ system damage in CMODS takes place more rapidly and with a more complex mechanism of disease progression; mortality rates are high (41-87%). We previously studied CMODS caused by cerebral ischemia [2]. In the present study we report the results of further investigations, using a rat model, into the pathologic mechanisms underlying the development of CMODS induced by cerebral hemorrhage.

*Address correspondence to this author at the Department of Neurology, Provincial Hospital Affiliated to Shandong University, Jinan, 250021, China; Fax: +86 0531 86735786; E-mail: wenzhong_sd@sina.com

[§]These authors contributed equally to this work.

MATERIALS AND METHODS

Experimental Animals and Materials

Adult male Wistar rats, 250-300 g in weight (n=56) were provided by the Experimental Animal Center of Shandong University. Collagenase VII (≥1,000-3,000 CDU/MG) 125 U/mg(Sigma Co.), the stereotactic apparatus (Type KOPE-975, USA), the dental drill (Type NSK-NE22L, Japan). These were divided into 7 groups: an unoperated control group (n=8); a sham-operated group (n=8), and the experimental group (n=40). The experimental group was subdivided into 5 (n=8) subgroups for analysis at different timepoints: 12, 24, 36, 48, and 72 h. Control sham-operated animals were analyzed 36 h after the procedure.

Establishment of Animal Models

Animals were maintained at 22°C in constant humidity. Prior to operation rats were fasted for 12 h and deprived of water for 4 h. After anesthesia, rats secured onto a stereotac-

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tic apparatus such that the plane of incisor hook was lower than the biauricular line by 2.4 mm: at this inclination the anterior fontanelle and the posterior fontanelle lie approximately in the same horizontal plane. Scalp incision was along the sagittal suture to the exposed anterior fontanelle and the coronal suture. Using a dental drill, a perforation (diameter 1.5 mm) was introduced 0.2 mm from the front of the anterior fontanelle and 3 mm to right side of the midline. The perforation extended into the surface of the dura mater; there was no damage to underlying brain tissue. Guided by the stereotactic apparatus, a microinjector (diameter ca. 0.6 mm) was then vertically inserted to a depth of 6 mm into the drilled hole. Rats in the experimental group were injected with 2 μl of a mixture containing 0.8 U collagenase and and 3.5 IU heparin sodium. The injection needle was withdrawn after 10 min. Sham-operated rats were injected with the same volume of saline.

Vital Signs and Biochemical Indices

Post-operative health status was monitored, including consciousness, mental status and behavior. Rectal temperature and respiratory rates were recorded after anesthesia. At each time-point over the period 12-72 h, 5 ml of blood was withdrawn for evaluation of hepatic and renal function. Gross morphologic changes in lung and small intestine were examined; coronal frozen microtome sections were employed for histopathology.

Determination of Serum Endotoxin

Endotoxin levels were determined using the quantitative Limulus amebocyte lysate (LAL) test in conjunction with a chromogenic substrate. All reagents were purchased from Shanghai Yihua Clinical Medical Technology Co. (China).

In Situ Hybridization

CD14 and IL-1β mRNA levels were determined by hybridization to tissue sections using a kit supplied by Sigma-Aldrich (USA); relative mRNA levels were evaluated using the CMIA color medical image analysis system.

Diagnostic Basis of MODS Animal Models

Animal models were evaluated according to the diagnostic criteria for MODS experimental animals as proposed by Sheng Zhi-yong [3].

Statistical Analysis

Data were expressed as means ± standard deviation; SPSS 10.0 statistical software was employed. Multiple comparisons between groups employed the Student t-test (double sided, α=0.05); linear correlation analysis was used to correlation analysis between two variables (double sided, α=0.05).

RESULTS

Changes of Vital Signs and Biochemical Indices

Following focal intracerebral administration of collagenase plus heparin in the experimental group, respiration rate, heart rate, body temperature and white blood cell counts were all increased significantly versus both the the unoperated control group and the sham-operated group (P<0.01 and P<0.001 respectively). There were no significant differences in these parameters between the sham-operated (36 h) and unoperated control groups. Similarly, indices of hepatic and renal function, and myocardial enzyme levels, were all raised in the experimental group compared to unoperated and sham-operated animals (P<0.01 and P<0.001 respectively); there were no significant differences in these parameters between the control groups (Table **1**).

Pathological Organ Changes

No significant tissue changes were observed in sham-operated rats, with the possible exception of minor broadening of the alveolar septum and a low degree of pulmonary neutrophil infiltration. There were also low levels of hepatic edema. In contrast, marked changes took place in animals receiving collagenase plus heparin. In the lung a robust inflammatory response was observed after 12 h, peaking at 26 h, and was still evident after 48 h though the level of inflammation had declined. Lobar pneumonia was observed in some rats, and bronchopneumonia could still be observed

Table 1. Physiological Parameters at Different Time-Points Following Focal Intracerebral Collagenase in a Rat Model of CMODS

Parameter	Unoperated Group	Sham-Operated Group	12 h	24 h	36 h	48 h	72 h
WBC	8.63±0.26	8.35±0.70	11.18±1.11 $^{*\#}$	16.23±0.57 $^{**\#\#}$	9.06±0.78	10.15±0.43	9.35±1.57
Respiration rate	93.50±2.84	78.16±4.35	43.66±2.87 $^{**\#\#}$	63.83±5.23 $^{**\#}$	68.66±1.20 **	43.83±5.39 $^{**\#\#}$	76.33±3.190 *
^{o}C	37.45±0.36	38.15±0.20	35.35±0.17 $^{*\#}$	36.18±0.15 *	34.71±0.23 $^{*\#}$	35.16±0.27 $^{*\#}$	36.70±0.45
ALT	117.16±16.75	101.33±3.63	294.83±118.62	125.33±9.30	244.16±48.16	292.16±78.05 $^{\#}$	269.63±62.35
AST	189.16±17.89	209.50±23.50	291.31±96.81	228.16±61.45	719.83±259.90 $^{**\#}$	800.33±138.0 $^{**\#\#}$	362.00±100.76
Cr	56.00±2.10	65.68±1.49	84.43±9.41 **	65.55±3.04	65.01±3.79	74.96±2.71 $^{*\#}$	60.10±3.67
BUN	7.43±0.22	7.49±0.38	14.31±1.23 $^{**\#\#}$	7.28±0.29	8.44±0.35	13.69±0.36 $^{*\#}$	9.10±0.70

Figures are means ± SD. Parameters in the sham-operated group were determined after 36 h. Statistical significances compared with normal control group were: *, P<0.01; **, P<0.001; compared with sham-operation group, $^{\#}P$<0.01; $^{\#\#}P$<0.001.

after 72 h. In the intestine, edema and submucosal thickening was present after 12 h and after 24 h this extended to the mucosa. Edema and congestion between the muscular layer and mucosa with mild infiltration of inflammatory cells was observed after 36 h and 48 h; this persisted at 72 h.

Serum Endotoxin Levels

Levels of serum endotoxins were significantly increased in the experimental group at 12 h, and peaked at 24 h. The increase was statististically significant versus unoperated controls and the sham group (P<0.05 in both cases). Levels were decreased after 48 h, but remained elevated compared with both control groups (P<0.01). Thereafter levels declined further but remained elevated compared with the unoperated group (P<0.01); at this time-point there was no significant difference versus the sham-operated group (Fig. **1**).

Organ Levels of CD14 and IL-1β mRNA

Only low levels of CD14 mRNA were detected in the lung and intestine of unoperated controls. However, there was a significant elevation in the sham-operated group versus unoperated controls (P<0.01). In the experimental group, CD14 mRNA in lung and intestine began to increase after 12 h, reaching a peak after 24-36 h. The elevation after 12-48 h was statistically significant versus both control groups. The most significant increase took place in lungs and intestine (P<0.05); this decreased after 48 h (Fig. **2**).

For IL-1β, low levels of mRNA were found in the lung and intestine of unoperated controls; In experimental animals IL-1β mRNA in lung and intestine was increased after 12 h, reaching a peak after 24-36 h. The increase after 12-48 was statistically significant (P<0.05) versus both control groups. Levels declined after 48 h (Fig. **3**).

Correlation between CD14 and IL-1β mRNA and Serum Endotoxin Levels

In both lung and intestine there was a significant correlation between the increase in serum endotoxin levels and the rise in CD14 mRNA levels (r values were 0.756, 0.571, 0.814 and 0.482 respectively, all P<0.01). The most significant correlation (P<0.01) was between lung CD14 mRNA and endotoxin levels. For IL-1β mRNA, correlations with serum endotoxin were also significant in lungs and intestine (r values were 0.705, 0.854, 0.693 and 0.412 respectively, all P<0.01). When the increases in CD14 and IL-1β mRNAs were compared there was also a significant correlation in both lungs and intestine (r values were 0.611, 0.653, 0.832 and 0.577 respectively, all P<0.01).

DISCUSSION

The pathology of multiple organ dysfunction syndrome (MODS) is thought to have a sequential etiology. This involves a stress response induced by primary injury, leading to visceral ischemia. In turn this prompts the release of inflammatory mediators, resulting in systemic inflammation (systemic inflammatory response syndrome, SIRS), and culminating in multiple organ failure and death. The gastrointestinal tract is thought to play a pivotal role in this sequence of pathologic changes in view of the particular sensitivity of this system to stress and ischemia.

The intestinal tract is considered to be the biggest storage warehouse for lipopolysaccharide endotoxins, and damage to the tract is likely to permit endotoxin influx from the gut into the circulation. Several severe diseases are known to produce dysfunction of the intestinal reticuloendothelial system, resulting in loss of function of the intestinal mucosal barrier,

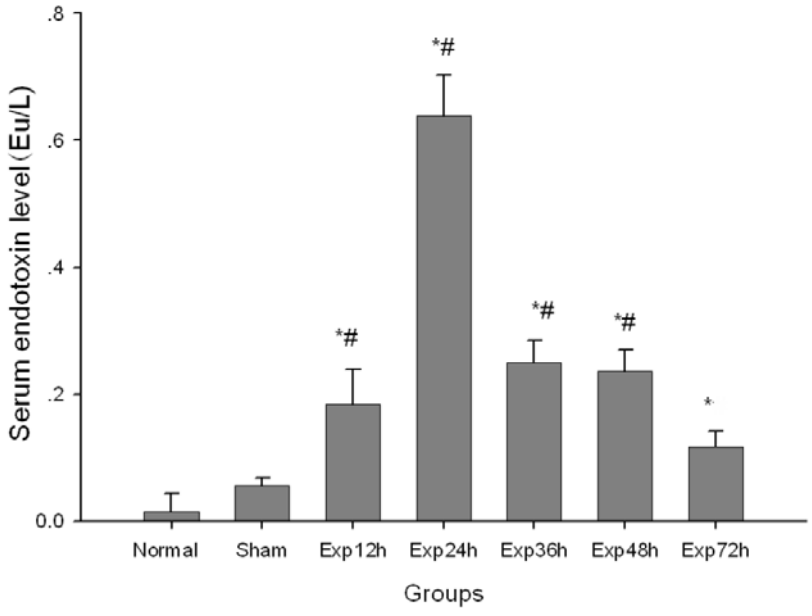

Fig. (1). Serum endotoxin levels in a rat model of CMODS. Acute cerebral hemorrhage was induced in Wistar rats by focal intracerebral injection of collagenase+heparin; endotoxin levels were determined at different experimental time-points (Exp 12-72 h) using a chromogenic Limulus amebocyte lysate method. Peak endotoxin levels were at 24 h following operation. Normal, unoperated animals; sham, operated animals receiving saline (36 h post-operative). Statistical significances were: *, P<0.05 compared to the unoperated controls; #, P<0.05 compared to sham-operated controls.

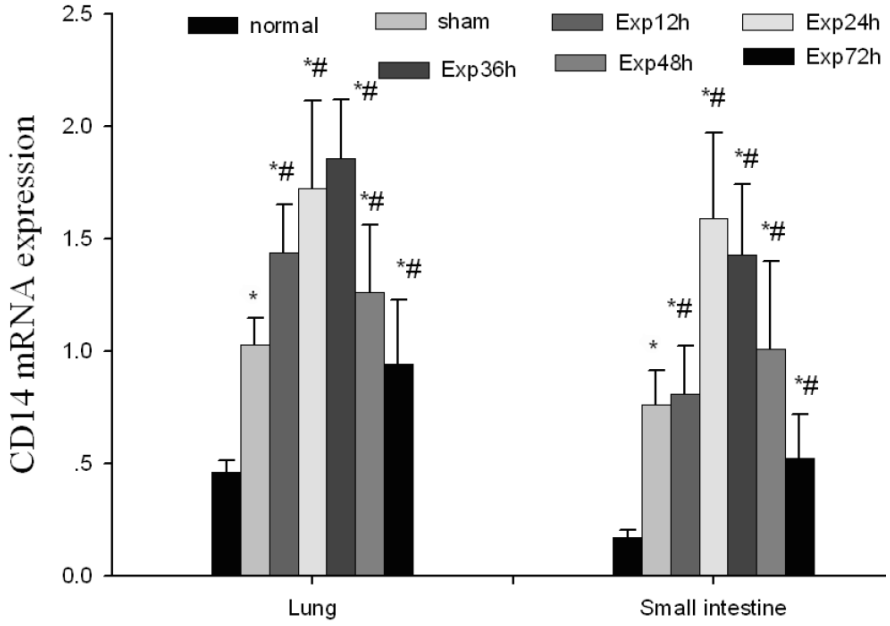

Fig. (2). CD14 expression in a rat CMODS model. Following acute induction of cerebral hemorrhage with collagenase+heparin, lung and small intestine were removed at different different experimental time-points (Exp 12-72 h) and mRNA levels determined by quantitative *in situ* hybridization on tissue sections. Peak CD14 expression was at 36 h following operation. Normal, unoperated animals; sham, operated animals receiving saline (36 h post-operative). Statistical significances were: *, $P<0.05$ compared to the unoperated controls; #, $P<0.05$ compared to sham-operated controls.

and permitting endotoxin translocation. Intact bacteria may enter the circulation if barrier dysfunction is more severe, leading to endotoxemia and pyemia. Such entry in turn can aggravate the damage to the intestinal mucosal barrier, setting in train a vicious cycle of increasing inflammation and reduced gut barrier function. The intestinal tract has been considered to be the 'promoting organ' in the pathogenesis of MODS.

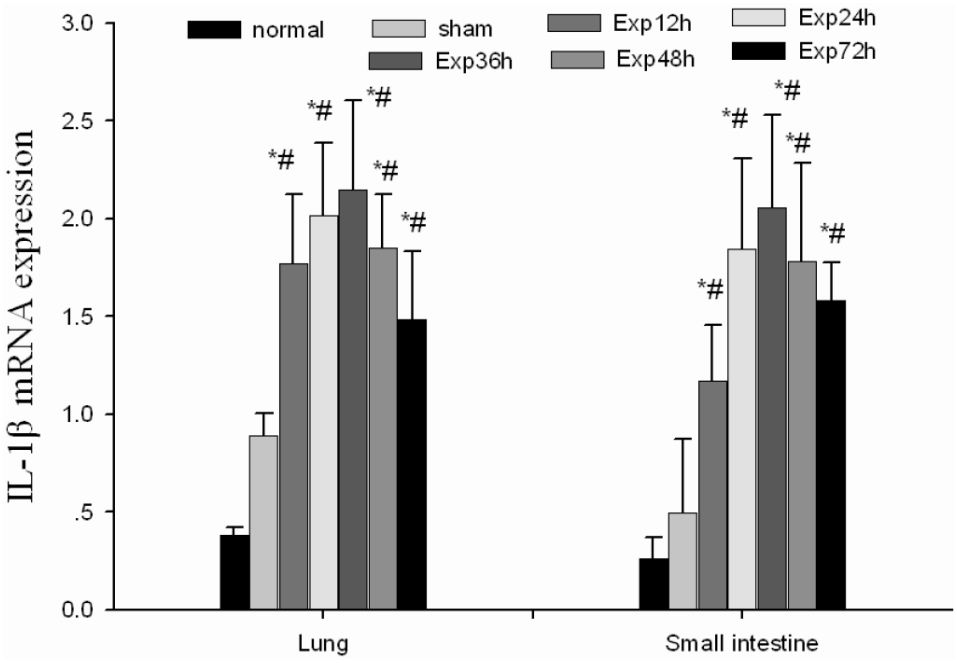

Fig. (3). IL-1β expression in a rat CMODS model. Following acute induction of cerebral hemorrhage with collagenase+heparin, lung and small intestine were removed at different time-points and IL-1β mRNA levels were determined by quantitative *in situ* hybridization on tissue sections. Peak IL-1β expression was at 24 h in small intestine and at 36 h in lung. Normal, unoperated animals; sham, operated animals receiving saline (36 h post-operative). Statistical significances were: *, $P<0.05$ compared to the unoperated controls; #, $P<0.05$ compared to sham-operated controls.

The exact mechanisms by which endotoxins initiate systemic inflammatory cascades are not known. It is possible that they stimulate local and systemic humoral responses (including the activation of complement and coagulation pathways) and cellular pathways (including macrophage and neutrophil activation) that together enhance transcapillary fluid exchange and promote a "waterfall" effect on the intestinal vasculature. Such mechanisms are thought likely to induce SIRS through the amplification of inflammatory mediators. However, systemic damage to diverse organ systems can be expected, and this is likely to be an important trigger for the development of MODS.

Endotoxemia and abnormal expression of IL-6, -8 and -10 can take place as a result of severe cerebral trauma [4]. Marked changes in serum levels of soluble tumor necrosis factor-α receptor (sTNFR-α), IL-1ra and IL-6 seen after subarachnoid hemorrhage (SAH) are related to the onset of SIRS and peripheral organ dysfunction, suggesting that inflammatory mediators induced by SAH play a role in SIRS and MODS. There was, however, no correlation between cerebrospinal fluid (CSF) levels of these cytokines and development of the syndrome [5, 6]. Cytokine production is clearly stimulated by serum endotoxin, but these reports indicate that cytokines are largely unable to cross the blood-brain barrier to enter the CSF. This suggests that inflammatory mediators in serum and cerebrospinal fluid have separate origins. A marked inflammatory response occurs in acute cerebral hemorrhage [7, 8]; again no rise in CSF levels of inflammatory markers was reported. In addition, Maier *et al*. found that the levels of soluble tumor necrosis factor receptors (sTNFRs) following traumatic brain injury were significantly higher in serum than in CSF, consistent with an imbalance between pro-inflammatory and anti-inflammatory responses in the central nervous system [9].

Organ dysfunction is common in severe brain injury, and 89% of patients with brain injury suffer at least one organ dysfunction, and this correlated with negative outcome [10]. Goldstein has proposed that the high morbidity and mortality seen in myocardial and cerebral ischemia is related to an inflammatory response which in turn leads to multiple organ failure and death [11]. These reports indicate that brain injury can result in endotoxemia, systemic inflammatory responses, gastrointestinal dysfunction, as well as in MODS.

The endotoxin receptor CD14 is differentially expressed in lung and intestine. We report here that pulmonary CD14 expression was detected in unoperated control animals; lung expression was above the levels seen in the small intestine. The induction of CD14 expression by lipopolysaccharide (LPS) endotoxin is complex. LPS induces cellular cytokine production, but the time-courses can differ: heightened production of TNF-α was seen first, only later followed by IL-1 and IL-6 [12]. The induction of cell-surface CD14 by gram-negative bacteria or LPS is regulated by IL-6 produced *via* an autocrine pathway [13]. Exogenous TNF-α can up-regulate CD14 expression *in vivo* [14] and the time course of CD14 expression induced by LPS was also altered following TNF-α administration. TNF-α also activates neutrophil infiltration into the lung interstitium [15, 16] and enhances the expression of CD14 mRNA in infiltrating neutrophils.

Our results demonstrate that acute cerebrovascular injury leads to influx of endotoxins into the circulation. This is presumed to be due to impaired barrier function in the intestinal mucosa. In turn, this influx leads to organ dysfunction culminating in MODS. The intestinal tract thus appears to plays a pivotal role in the pathway leading to CMODS. Our previous studies [2] reported intestinal endotoxin translocation and elevated serum endotoxin levels in an animal model of MODS initiated by cerebral ischemia. We also reported pathological changes in the intestinal mucosa of the cerebrogenic MODS model, including the destruction of intestinal mucosal barrier function. We report here that the rise in serum endotoxin levels is accompanied by marked changes in several physiological parameters, suggesting that organ damage can be widespread. Blood endotoxin levels increased 12 h after acute cerebral hemorrhage, and this was followed by the induction of CD14 endotoxin receptor mRNA expression levels in lung and intestine.

Interleukin 1 (IL-1), also known as lymphocyte activating factor, encompasses 2 distinct structural sub-types, IL-1α and IL-1β. IL-1β is an inflammatory mediator with multiple biological functions, and regulates both inflammatory response and immune responses [17] and is thought to play a central role in MODS [18]. We report here that the expression of IL-1β mRNA in lung and intestine was strongly increased following intracerebral focal collagenase and cerebral hemorrhage. Endotoxins are known to induce the production of IL-1β [19]. We have found that the induction of IL-1β mRNA in lung and intestine correlated significantly with the increase in serum endotoxin levels and the induction of endotoxin receptor CD14 mRNA expression.

The tight correlation between these different factors, and the relative time-courses of their induction, suggests a sequential mechanism for MODS induced by cerebral hemorrhage. We propose that acute cerebrovascular disease elicites a stress/shock response that damages the integrity of the intestinal mucosal barrier. This permits endotoxin translocation into the circulation, and endotoxemia then provokes the release of inflammatory factors leading to systemic inflammatory response syndrome (SIRS) and internal organ dysfunction, culminating in MODS. Nevertheless, the mechanism by which cerebrovascular damage compromises intestinal function is not known. It is also not known if endotoxemia following intestinal damage is the only pathway involved in the etiology of CMODS. Further investigations will be required to determine if other pathways operate in parallel with endotoxemia and SIRS to provoke multiple organ failure in response to brain injury.

ACKNOWLEDGEMENTS

This work was supported by a grant from Shandong Provincial youth Foundation of Medical Science and Technology of China (Y2007C043) and from Shandong Province 'Eleventh Five Year' health science and technology development plan item (2007HW079).

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Received: 27 April, 2009 **Revised: 01 July, 2009** **Accepted: 01 July, 2009**