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Hydroxyethyl Starch Inhibits Intestinal Production of Cytokines and Activation of Transcription Factors in Endotoxaemic Rats

R Lv[1], W Zhou[2], J-J Yang[1], Y Jin[1] and J-G Xu[1]

[1]Department of Anaesthesiology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China; [2]Department of General Surgery, The Affiliated Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China

We studied the effect of hydroxyethyl starch (HES) on intestinal production of cytokines and activation of transcription factors in sepsis. Septic rats, induced by intraperitoneal lipopolysaccharide (LPS) (5 mg/kg), were treated with intravenous HES (16 ml/kg) or saline (64 ml/kg). Rat ileal tissues were collected at 2 h, 3 h or 6 h after LPS challenge. Levels of tumour necrosis factor alpha (TNF-α), interleukin (IL) 1β, IL-6, IL-8 and IL-10, cytokine mRNAs, activities of nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1), and the number of ileal myeloperoxidase (MPO)-positive cells were determined for each group. HES significantly reduced the LPS-induced increase in intestinal levels of TNF-α, IL-1β, IL-6, IL-8 and their corresponding mRNAs. HES also decreased the number of MPO-positive cells induced by LPS and inhibited activation of NF-κB and AP-1. The results suggest that in sepsis, HES may down-regulate intestinal pro-inflammatory cytokine production via suppression of NF-κB and AP-1 activation.

KEY WORDS: Hydroxyethyl starch; Lipopolysaccharide; Intestine; Inflammatory cytokines; Nuclear factor kappa-B; Activator protein-1

Introduction

Sepsis with multiple organ failure remains a leading cause of death in intensive care units, despite substantial research in this field over several decades. The patho-physiological changes that take place in the organs of critically ill patients are caused by alterations in cellular function in which cytokines play a central role. Levels of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL) 6 are elevated in the plasma of septic patients.[1,2] These cytokines work together to promote a massive inflammatory response, which can contribute to the death of the patient.

Hydroxyethyl starch (HES) is a colloidal, synthetically modified polymer of amylo-pectin derived from maize or sorghum. Clinically, HES is frequently used for volume replacement when attempting to maintain

or improve tissue perfusion in patients experiencing sepsis, trauma, shock or surgical stress.[3,4] In addition to the effect on maintenance of stability of haemodynamic parameters, recent studies have shown that HES may exert anti-inflammatory effects.[5,6] Previous studies in our laboratory demonstrated that during endotoxaemia HES could induce a down-regulation of inflammatory mediators in lung, heart and liver.[7,8] However, few studies have investigated the protective effect of HES on the inflammatory response in the gut, which is increasingly being recognized as an important source of cytokines.[9]

Given that the events that result in cytokine and chemokine expression involve activation of transcription factors, such as nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1),[10,11] we used a rat endotoxaemia model to determine the effects of HES on intestinal production of cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) and activation of transcription factors (NF-κB and AP-1).

Subjects and methods

ANIMALS

Adult male Sprague-Dawley rats (350 – 400 g body weight) were obtained from Shanghai Animal Centre (Shanghai, China) and kept in accordance with our Institutional Animal Care Committee guidelines. Animals were anaesthetized by intraperitoneal (IP) administration of urethane (1250 mg/kg body weight). The right jugular vein was cannulated with a polyethylene catheter for intravenous (IV) administration of solutions. The macrohaemodynamic parameters, mean arterial blood pressure (MAP) and heart rate (HR) were measured through the left carotid artery catheterized with a microtip transducer.

EXPERIMENTAL PROTOCOL

Endotoxaemia was induced by IP injection of lipopolysaccharide (LPS; 5 mg/kg) (*Escherichia coli* O111: B4; Sigma Chemical Co., USA). Control rats were injected with 0.9% NaCl solution (3 ml/kg, IP). To test the influence of different solutions on LPS-induced changes, groups of rats (six per group) were treated 1 h after LPS with HES (16 ml/kg) (hydroxyethyl starch, medium molecular weight, low degree of substitution; HAES-steril 200/0.5, 6%; Fresenius Kabi, Bad Homburg, Germany) or 0.9% isotonic NaCl solution (64 ml/kg) IV. The rate of infusion was 0.4 ml/min. Each ileum was collected in groups of rats 2 h after induction of endotoxaemia for the determination of TNF-α level and mRNA expression, and NF-κB and AP-1 activities; or 3 h after LPS for levels and mRNA expression of IL-1β, IL-6, IL-8 and IL-10. In order to exclude a contribution of cytokine expression from blood-borne elements, perfusion was performed in the following manner before removing the tissues: after thoracotomy, a cannula was placed in the left ventricle, and the right atrium was opened. A total of 300 ml of 0.9% NaCl solution was perfused through the cannula at a pressure of 120 cm H_2O. The intestinal samples were frozen in liquid nitrogen and stored at –70 °C until analysis.

DETERMINATION OF INTESTINAL INFLAMMATORY CYTOKINE LEVELS

The intestinal levels of inflammatory cytokines were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for rat cytokines according to each of the manufacturer's instructions (TNF-α from Diaclone Research, Besancon, France; IL-1β, IL-6, IL-8, and IL-10 from Biosource Europe SA, Nivelles, Belgium).

RNA ISOLATION AND cDNA SYNTHESIS

The intestinal tissue samples were homogenized in TRIzol reagent (Roche Molecular Biochemicals, Indianapolis, IN,

USA). Total RNA was extracted from the tissue according to the manufacturer's suggested protocol, and the concentration was determined from spectrophotometric optical density measurements (260 nm and 280 nm). Reverse transcriptase reactions were then carried out using a Reverse Transcription System Kit (Promega, Madison, WI, USA). Each reaction tube contained 1 µg of total RNA in a volume of 20 µl containing 5 mmol/l $MgCl_2$, 1 × Reverse Transcription Buffer, 1 mmol/l of each dNTP, 1 U/µl of RNase inhibitor, 15 U/µg of AMV Reverse Transcriptase, 0.025 µg/µl of $Oligo(dT)_{15}$ Primer, and DEPC-treated water to volume. Reverse transcriptase reactions were undertaken in a DNA Thermal Cycler (MiniCycler PTC 150, MJ Research Inc., Waltham, MA, USA) at 42 °C for 60 min and 95 °C for 5 min.

SEMIQUANTITATIVE POLYMERASE CHAIN REACTION

Polymerase chain reactions were performed using 0.5 U of Taq-polymerase (Promega), 0.005 µmol dNTP, and 50 pmol of each primer (Sangon Co., Shanghai, China), in a total volume of 25 µl in a DNA Thermal Cycler (MiniCycler PTC 150). The primers, cycle numbers, and amounts of cDNA used are shown in Table 1. Each PCR cycle consisted of 30 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C. The PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide. The gel was captured as a digital image and analysed using Scion Image software (Scion Corp., Frederick, MD, USA). The relative levels of cytokine mRNAs were normalized to β-actin transcript (535 base pair fragment) from the same PCR reaction.

PREPARATION OF NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Nuclear protein was extracted and quantified as previously described.[8] NF-κB or AP-1 consensus oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3' for

TABLE 1:
The primer sequences, amounts of cDNA and the number of cycles used for semiquantitative polymerase chain reaction analysis of RNA extracted from ileal samples of rats during the study

Gene	Primer sequences (5' → 3')	Fragment size (bp)	Amount of cDNA (µl)	Number of cycles
β-actin	CAGAGCAAGAGAGGCATCCT GGCAGCTCATAGCTCTTCTC	535	5	33/35
TNF-α	GTAGCCCACGTCGTAGCAAA CCCTTCTCCAGCTGGAAGAC	346	5	33
IL-1β	TGATGTTCCCATTAGACAGC GAGGTGCTGATGTACCAGTT	378	5	33
IL-6	AGCCAGAGTCATTCAGAGCA AGTTCTTAGAGAACAACATA	325	5	33
IL-8	ATGACTTCCAAGCTGGCCGTG TCTCAGCCCTCTTCAAAAACT	295	5	35
IL-10	TCCTTGGAAAACCTCGTTTG ATCATGGAAGGAGCAACCTG	412	5	35

bp, base pair; TNF-α, tumour necrosis factor alpha; IL, interleukin.

NF-κB and 5'-CGCTTGATGAGTCAGCCGGAA-3' for AP-1) was end-labelled with [γ-^{32}P] ATP (Free Biotech, Beijing, China). The electrophoretic mobility shift and supershift assays were performed according to our previous study.[8]

IMMUNOHISTOCHEMICAL ANALYSIS

Cellular aspects of intestinal inflammation were assessed immunohistochemically by staining for granulocytes (myeloperoxidase [MPO]-positive cells) 6 h after injection of LPS using an additional group of rats. Segments of ileum were rinsed with 0.9% NaCl and fixed in 10% buffered formaldehyde solution at room temperature. After dehydration, intestinal tissue was embedded in paraffin and sectioned (5 µm). Sections were pre-incubated with normal rabbit serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to prevent non-specific binding and then incubated for 90 min at room temperature with anti-rat MPO antibody (Santa Cruz Biotechnology Inc.; 1:200). Secondary reagents were made with the Vector Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). NovaRed® (Vector Laboratories Inc.) was used for colour development.

STATISTICAL ANALYSIS

All data were expressed as means ± SD and compared by analysis of variance (ANOVA) and Student's *t*-test. Differences in values were considered significant if $P < 0.05$.

Results

EFFECTS OF HES ON MACROHAEMODYNAMICS

In all groups, MAP and HR were measured at baseline (0 min), 15 min, 30 min, 1 h, 2 h and 3 h after LPS injection. The data showed that MAP and HR were comparable among the animal groups at baseline (data not shown). LPS exposure did not induce significant changes in MAP and HR over time. Accordingly, there were no significant differences between the experimental groups at the different time points studied.

EFFECTS OF HES ON INTESTINAL LEVELS OF INFLAMMATORY CYTOKINES

Endotoxaemia in rats was associated with significant increases in intestinal levels of TNF-α (Fig. 1A), IL-1β (Fig. 1B), IL-6 (Fig. 1C) and IL-8 (Fig. 1D) 2 h or 3 h after LPS injection. In contrast, the endotoxin-induced increases in these cytokines in the ileum were significantly reduced in rats treated with HES. The levels of TNF-α, IL-1β, IL-6 and IL-8 did not differ between rats treated with saline control and HES alone.

Similarly, LPS significantly increased the intestinal level of IL-10 (Fig. 1E). HES infusion only slightly up-regulated intestinal IL-10 levels in endotoxaemic rats compared with LPS treatment alone.

EFFECTS OF HES ON INTESTINAL EXPRESSION OF CYTOKINE mRNA

Baseline levels of all of the cytokine mRNAs except IL-1β mRNA were detected in the intestines of control rats (Fig. 2). At 2 h or 3 h after injection of LPS, TNF-α (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C), IL-8 (Fig. 2D) and IL-10 (Fig. 2E) mRNA levels were markedly increased. Infusion of HES in endotoxaemic rats significantly reduced the LPS-induced increases in TNF-α, IL-1β, IL-6 and IL-8 mRNA expression and slightly increased IL-10 mRNA expression.

EFFECTS OF HES ON INTESTINAL ACTIVATION OF NF-κB AND AP-1

Compared with treatment with saline control and HES alone, LPS (5 mg/kg IP) significantly increased the activity of NF-κB

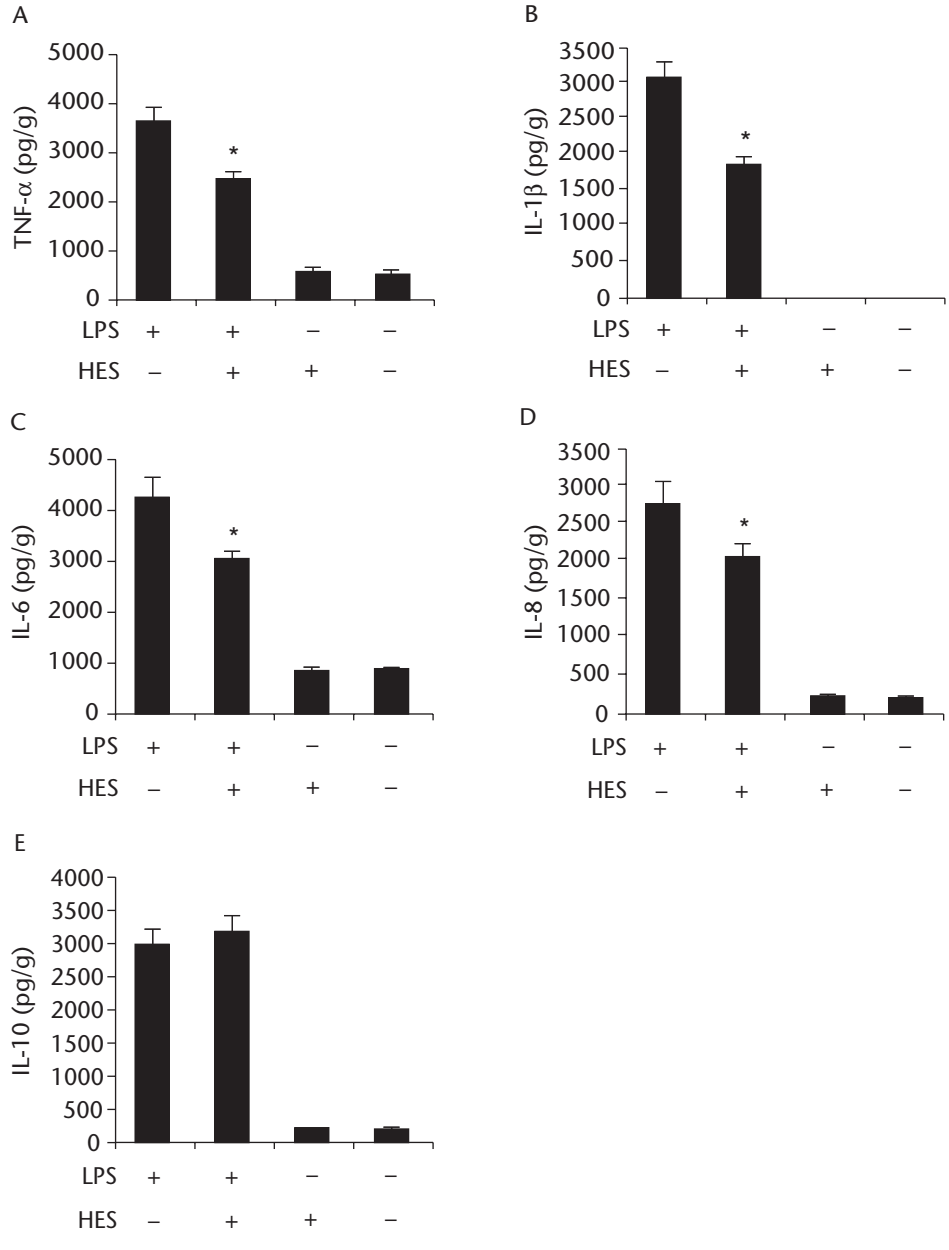

FIGURE 1: Intestinal cytokine levels in rats treated with LPS alone, LPS and HES, HES alone, and saline control 2 h or 3 h after injection of LPS. The mean ± SD values of six rats per group are shown for TNF-α (A), IL-1β (B), IL-6 (C), IL-8 (D) and IL-10 (E). LPS, lipopolysaccharide; HES, hydroxyethyl starch; TNF-α, tumour necrosis factor alpha; IL, interleukin. *$P < 0.05$ versus LPS alone

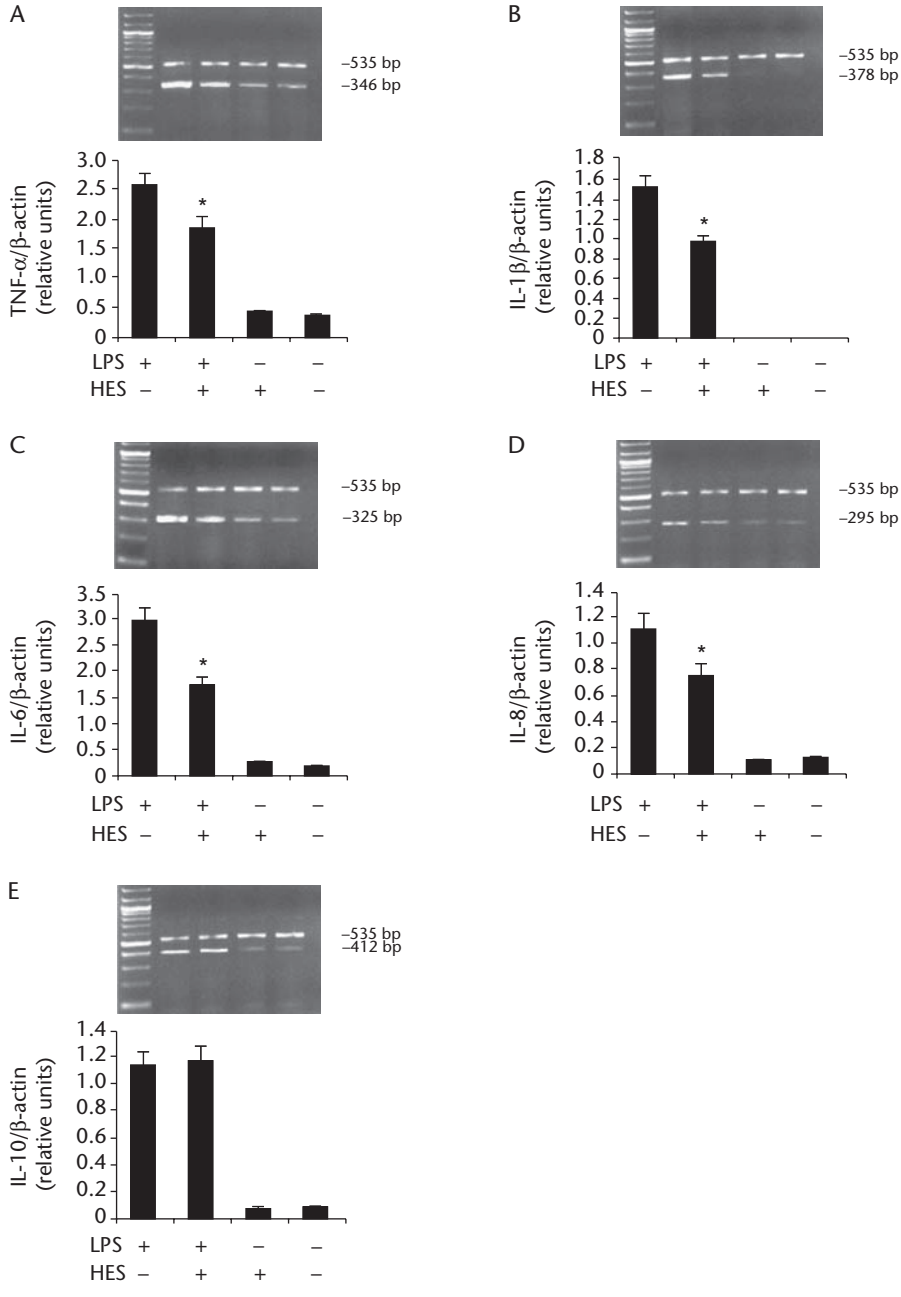

FIGURE 2: Intestinal expression of cytokine mRNA in rats treated with LPS alone, LPS and HES, HES alone, and saline control 2 h or 3 h after injection of LPS. The mRNA levels of TNF-α, IL-1β, IL-6, IL-8 and IL-10 are expressed as relative units normalized to the expression of β-actin (535 bp fragment). The mean ± SD values of six rats per group are shown for TNF-α mRNA (A), IL-1β mRNA (B), IL-6 mRNA (C), IL-8 mRNA (D) and IL-10 mRNA (E). LPS, lipopolysaccharide; HES, hydroxyethyl starch; TNF-α, tumour necrosis factor alpha; IL, interleukin; bp, base pair. *$P < 0.05$ versus LPS alone

and AP-1 in the rat intestine 2 h after injection (Fig. 3). HES administration markedly decreased the activities of these two transcription factors in endotoxaemic rats. The changes in the intestinal activities of NF-κB and AP-1 followed similar patterns to those of the intestinal pro-inflammatory cytokines TNF-α (Fig. 1A), IL-1β (Fig. 1B), IL-6 (Fig. 1C) and IL-8 (Fig. 1D).

IMMUNOHISTOCHEMICAL ANALYSIS

No lesions were observed in the intestinal mucosa and the villi were regularly arranged in control rats and in rats treated with HES alone (Figs 4A and 4B). At 6 h after LPS injection, the morphological structure of the ileum had changed, with disruption of the villi and erosion (Fig. 4C). Mucosal ulcers appeared and a marked neutrophil infiltration was observed, characterized by an increase in the number of MPO-positive cells. HES treatment reduced the LPS-induced neutrophil infiltration in endotoxaemic rats but did not markedly improve the villi structure (Fig. 4D).

Discussion

It has been suggested that the cellular dysfunction observed early after onset of sepsis may be a consequence of up-regulated pro-inflammatory cytokines.[12] A number of studies have also examined the role of the gut in producing pro-inflammatory cytokines during sepsis or following other adverse circulatory conditions.[9,13] HES, a synthetic colloid, may have an anti-inflammatory effect under several pathological conditions.[14] Previous studies in our laboratory demonstrated that during endotoxaemia HES could down-regulate inflammatory mediators in lung, heart and liver.[7,8] It remains unknown whether or not HES may have anti-inflammatory effects in the gut during sepsis. The present study

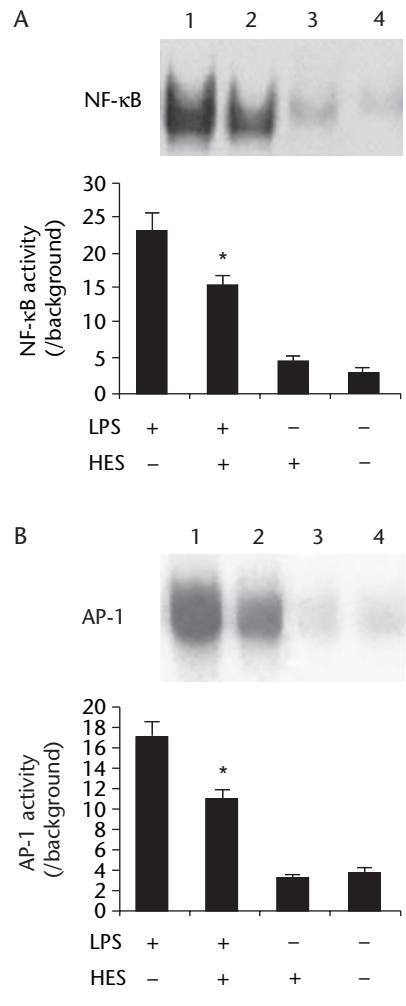

FIGURE 3: Intestinal activities of NF-κB and AP-1 in rats treated with LPS alone, LPS and HES, HES alone, and saline control 2 h after injection of LPS. The activity of the NF-κB (A) or AP-1 (B) complex was determined by the mean band intensity measured by an electrophoretic mobility shift assay. The mean ± SD values of six rats per group are shown for NF-κB and AP-1. NF-κB, nuclear factor kappa-B; AP-1, activator protein-1; LPS, lipopolysaccharide; HES, hydroxyethyl starch. *$P < 0.05$ versus LPS alone

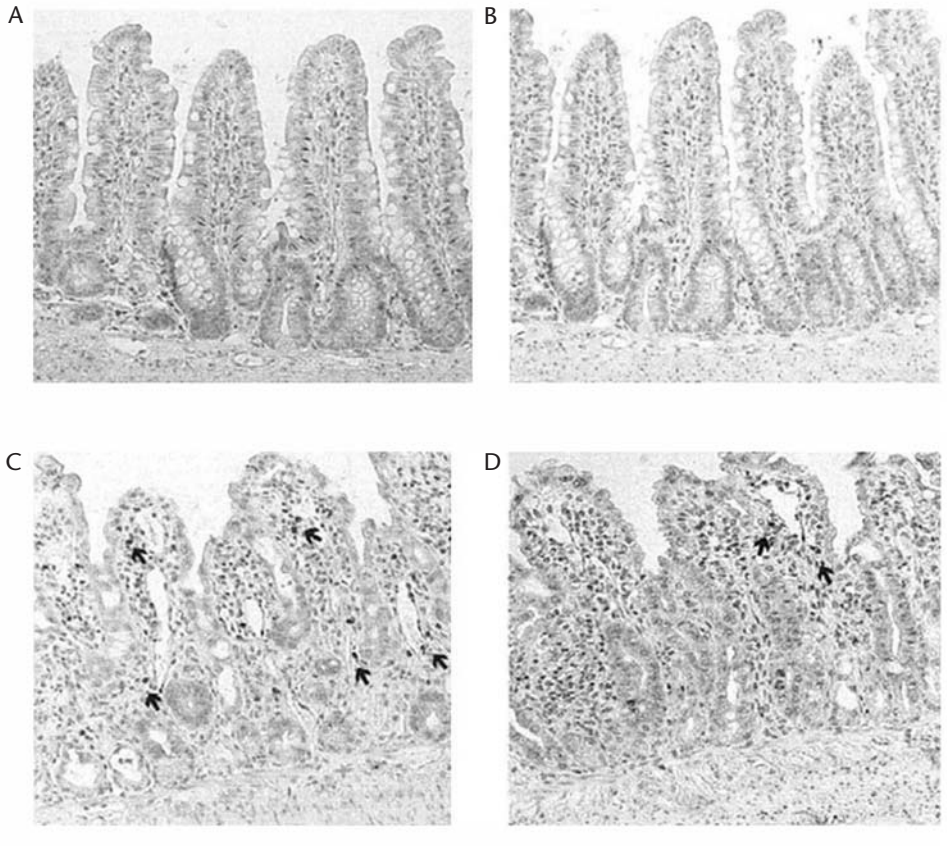

FIGURE 4: Myeloperoxidase (MPO)-positive cells in the ileum 6 h after injection of LPS. The photomicrographs were obtained from rats treated with saline control (A), HES alone (B), LPS alone (C), and LPS and HES (D). The arrowheads represent MPO-positive cells (magnification × 100). LPS, lipopolysaccharide; HES, hydroxyethyl starch

was therefore carried out to determine whether HES could exert anti-inflammatory effects via the down-regulation of pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-8) and/or the up-regulation of anti-inflammatory cytokines (e.g. IL-10) in the intestinal tissues of a rat sepsis model. To increase the clinical relevance of the experimental protocol, a post-treatment mode of therapy, beginning 1 h after exposure, was chosen. The ratio of 1:4 between the colloid and crystalloid solutions (16 ml/kg and 64 ml/kg) was used to account for the higher acute volume effect of the colloid solution. By doing this, we most closely simulated a typical clinical resuscitation protocol.

Our results indicated that endotoxaemia in rats was associated with increased concentrations of TNF-α, IL-1β, IL-6 and IL-8 in the small intestine, and that treatment with HES could significantly reduce the LPS-induced increases in these pro-inflammatory cytokines. This suggested that during endotoxaemia, HES participated in the down-regulation of pro-inflammatory

cytokine production in the small intestine and could therefore have an anti-inflammatory effect in sepsis. In addition, from the cellular aspect of intestinal inflammation, HES treatment decreased the number of MPO-positive cells, which are characteristic of neutrophil infiltration. The reduction in MPO-positive cells might be attributed to the decreased level of IL-8, which plays an essential role in inducing neutrophil infiltration and activation in various types of acute inflammation. In contrast, the effect of HES on up-regulation of intestinal IL-10 was only moderate, implying that the inhibition of intestinal pro-inflammatory cytokines was not due to increased intestinal IL-10 levels. In order to exclude the influence of blood on the levels of cytokines in the intestine, perfusion with 300 ml of saline was performed before intestinal samples were collected. In addition, the concentrations of cytokine mRNAs in the intestinal tissues were determined. The fact that the changes observed in cytokine mRNA expression were consistent with those of the cytokine levels demonstrated that the increased intestinal TNF-α, IL-1β, IL-6 and IL-8 levels after injection of LPS were the result of stimulated local production of cytokines, and HES could reduce this local production through inhibition of mRNA expression.

Our findings corresponded well with the results of experimental studies on haemorrhagic shock, which demonstrated that HES could reduce the haemorrhage-induced increase in plasma IL-6 concentrations.[15] The authors could not clarify the underlying mechanism by which infusion of HES could down-regulate the proinflammatory factor, however. In sepsis, NF-κB appears to be a particularly important transcription factor, activated extracellularly and binding to the promoter region of 'inflammatory' genes to increase their rate of transcription.[16] Several studies have also demonstrated that LPS may activate NF-κB in rat intestine.[17,18] Based on these findings, we hypothesized that HES might exert its anti-inflammatory effects in gut during endotoxaemia and down-regulate pro-inflammatory mediators, TNF-α, IL-1β, IL-6 and IL-8, through the NF-κB signalling pathway. Our results demonstrated that HES reduced NF-κB activity and decreased TNF-α, IL-1β, IL-6 and IL-8 levels in the small intestine, thus supporting our hypothesis. In addition to NF-κB, AP-1 designates another class of transcription factor that mediates inflammatory responses.[11] In this study, HES significantly suppressed LPS-induced activation of AP-1, suggesting that other transcription factors, like AP-1, are also involved in the anti-inflammatory actions of HES.

Currently, HES is often used for volume support during septic disorders. Our results illustrated that in the septic rat model in which sepsis was secondary to LPS, HES may play a beneficial role by down-regulating pro-inflammatory mediators in parallel with the inhibition of NF-κB and AP-1 activities in the intestine. These anti-inflammatory actions of HES may have important clinical implications, particularly as an adjunct to fluid resuscitation, and may shed more light on the wider clinical application of HES in sepsis management.

Conflicts of interest

No conflicts of interest were declared in relation to this article.

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Address for correspondence
Professor J-G Xu
Department of Anaesthesiology, Jinling Hospital, 305 East Zhongshan Road,
Nanjing 210002, China.
E-mail: nulvran@yahoo.com.cn