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• BRIEF REPORTS •

Effects of low molecular weight heparin on platelet surface P-selectin expression and serum interleukin-8 production in rats with trinitrobenzene sulphonic acid-induced colitis

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Abstract

AIM: To observe the effects of low molecular weight heparin (LMWH) on platelet surface P-selectin expression and serum interleukin-8 production in rats with trinitrobenzene sulphonic acid (TNBS) induced colitis.

METHODS: Colitis was induced in female Sprauge-Dawley rats by colonic administration of 2, 4, 6-TNBS. LMWH, a dalteparin (150 U/kg, 300 U/kg) was subcutaneously administrated one hour before induction of colitis and went on once a day for 6 days. Then a half dose was given for the next 7 days. Control animals received the same volume of normal saline once a day for 14 days after treated by TNBS. Animals were sacrificed at 24 h, days 7 and 14 after induction of colitis. The colon was excised for the evaluation of macroscopic and histological findings and TNF-α immunohistochemical assay. Platelet surface P-selectin expression was determined by radioimmunoassay and serum IL-8 production was assayed by ELISA method.

RESULTS: LMWH treatment in a dose of 300 U/kg for 14 days significantly improved colonic inflammation by histological examination. Serum IL-8 production in the 300 U/kg treatment group was more significantly decreased at day 14 than that at 24 h ($P<0.05$). However, platelet surface P-selectin expression and TNF-α staining in colonic tissue were not significantly different among the three groups.

CONCLUSION: LMWH has an anti-inflammatory effect on TNBS induced colitis in rats. The effect is possibly related to inhibition of proinflammatory cytokine IL-8, but not involved platelet surface P-selectin expression.

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD), both of which are chronic non-specific intestinal inflammation with unknown etiology. Several studies have shown that IBD exists a hypercoagulant state in active period of the disease and may have microvascular thrombosis in the wall of intestine[1-8]. An abnormal platelet activity has been reported in patients with UC and CD, and plays an important role in inflammation aggravation[9-12]. P-selectin is a membrane glycoprotein, which is expressed on activated platelets and endothelial cells, promotes leukocyte adhesion and migration as well as inflammatory cytokine production. IL-8 is a key proinflammatory cytokine and its production is increased in activated platelets. An up-regulation of platelet IL-8 receptors in patients with IBD has been reported[11].

Heparin is a glycoaminoglycan formed by sulphatedoliposaccharides and varies in the length of polymeric units and therefore has different molecular weights. Low molecular weight heparin (LMWH) is made by partial hydrolysis or enzymatic degradation of unfractionated heparin. Heparin and LMWH prevent the process of blood coagulation and have a natural anti-thrombin effect. In recent years several studies have shown that heparin and LMWH have an obvious anti-inflammatory activity in addition to its traditional anticoagulant effects[9,13,14]. In animal model heparin disaccharides inhibited TNF-α production by macrophages and decreased immune inflammation[15]. Heparin accelerated the healing process of mucosa in colitis in several clinical studies and had anti-inflammatory effects[16-21]. Therefore, administration of heparin can afford both anti-inflammatory and anticoagulant effects. The mechanisms of anti-inflammation of heparin are unknown. The limited studies demonstrated that it was possibly associated with the increase of several growth factors and the decrease of nitric oxide synthesis (NOS) and myeloperoxidase[9,13,14,22,23]. The effects of heparin on platelet surface P-selectin and IL-8 have not been studied. The present study was designed to observe the effects of LMWH on platelet surface P-selectin expression, serum IL-8 production, and TNF-α expression in mucosa of rats with trinitrobenzene sulphonic acid (TNBS)-induced colitis, and to clarify the anti-inflammatory mechanisms of LMWH in the treatment of colitis.

MATERIALS AND METHODS

Animals

Female Sprauge-Dawley rats weighing 200-250 g were used in the study. The animals were fasted for 24 h before the experiment and allowed food and water *ad libitum* after induction of colitis. The study was approved by the Ethic Committee of Wuhan University Medical School.

Induction of TNBS-induced colitis

Colitis was induced by a method of hapten-induced colonic inflammation as previously described[24]. Rats were anaesthetized by intraperitoneal administration of 100g/L urethane. A small

volume of 2, 4, 6-TNBS (Sigma Company) was dissolved in 50% ethanol to a final concentration of 100 mg/mL, and 0.3 mL of TNBS solution was intracolonically administrated with a polypropylene catheter by inserting 8 cm via the anal canal. Rats in a group were given 150 U/kg LMWH (dalteparin made by Pharmacia & Upjohn Company) subcutaneously 1 h before induction of colitis, and went on once a day for 6 d. Then a half dose of LMWH was given for the next 7 d. Treatment of the LMWH 300 U/kg group was the same as the 150 U/kg group, but the dose of LMWH was as high as 300 U/kg. Control animals received the same volume of normal saline once a day instead of LMWH after treated by TNBS.

Animals in each group were sacrificed at 24 h, d 7 and 14 after induction of colitis. The colon was isolated and a segment of colon was excised for the evaluation of macroscopic and histological findings and also for TNF-α immunohistochemical assay. A blood sample was drawn from heart of the rats for determination of platelet surface P-selectin and serum IL-8 before the rats were sacrificed.

Macroscopic evaluation of colonic damage

Macroscopic evaluation of colonic damage was conducted by two authors (HH, LLG) and mucosal hyperemia, ulcers, inflammatory exudation and bleeding were recorded. The most damaged site of the colon was chosen as the part for histological studies. For control group the colon at 8 cm above anus was excised for histological studies.

Histological studies of colonic damage

For histological examination, formalin-fixed tissues were embedded in paraffin and 5 μm -thick sections were stained with hematoxylin and eosin, and evaluated under light microscope by a pathologist (GSG) blinded to the experimental protocol. Colonic damage was assessed by the grades described by Fedorak *et al*[25]. Mucosal ulceration: 0: no-ulceration; 1: focal ulceration; 2: multifocal-ulceration; 3: diffuse ulceration. Depth of injury was graded as follows: 0: no injury; 1: mucosal involvement only; 2: mucosal and submucosal involvement; 3: transmural involvement. The ulceration and depth of injury grades were scored and put together as a result ranging between the minimum of 0 and maximum of 6.

Determination of platelet surface P-selectin molecules

Platelet surface P-selectin molecules were detected by radioimmunoassay. The kit was purchased from the Institute of Thrombosis and Homeostasis of Suzhou University, China. Briefly, 2 mL of anti-coagulated blood was taken from the heart of rats and platelets were counted under microscope. The blood was fixed by 2g/L glutaraldehyde in PBS solution at room temperature for 30 min and stored at 4 ℃ for determination of platelet surface P-selectin. The fixed blood with 2.5×10^6 platelets was divided into 3 tubes and washed with 1 mL of 0.01mol/L PBS, pH 7.4, per tube and centrifuged at 2 500 rpm for 10 min. Supernatants were removed. 50 μL of SZ-51mab labeled with ^{125}I was added into the reaction tubes and mixed fully. 15 μL of SZ-51mab without ^{125}I was added into control tubes. Each tube was incubated at 37 ℃, washed 3 times with PBS, and centrifuged at 3 000 r/min for 10 min. Cpm of precipitations in each tube was measured by a γ reader.

Determination of serum IL-8 production

Serum IL-8 production was assayed by ELISA sandwich method. The IL-8 ELISA kit was purchased from the Institute of Immunology of the Fourth Military Medical University, China. Briefly, 96-well plates were precoated with monoclonal antibody specific to human IL-8. Serum samples, negative control and diluted IL-8 standard markers were added into the plates. The serum samples were detected according to the procedures described in the protocol. A_{410} value was read by the ELISA reader.

Determination of TNF-α in colonic mucosa

Expression of TNF-α in colonic mucosa was assessed by immunohistochemistry. The regents of TNF-α were purchased from Beijing Zhongshan Biotechnology, China. Positive expression of TNF-α was brown deposited granules in the plasma of neutrophils, monocytes and lymphocytes. The grades of expression of TNF-α in mucosa were classified as follows: 0: no staining; 1: a few maple granules, or a few fine brown granules, not exceeding the 1/4 total area of cytoplasm; 2: uniformity maple in the whole cytoplasm or wide brown granules in the cytoplasm, not exceeding the 1/2 area of cytoplasm; 3: the cytoplasm was full of brown granules with a lower density; 4: the total cytoplasm was full of crassitude dark brown granules, and covered whole nuclei of the cell. One hundred cells were counted under oil microscope, positive cells were recorded and positive cell rate was calculated. The scores of expression of TNF-α were calculated by sum of each grade multiplying its positive cell rate.

Statistical analysis

Data were expressed as mean±SD. Data in different groups were analyzed by ANOVA and post multiple tests (Tukey-Kramer or Student-Newman-Keuls). A P value less than 0.05 was considered statistically significant.

RESULTS

Macroscopic evaluation of TNBS-induced colitis

Control animals subjected to intracolonic administration of TNBS in 500ml/l ethanol showed colonic mucosal injury with ulceration. Signs of the mucosal damage were monitored for 14 d. As early as 24 h after TNBS treatment, colonic mucosa was shown to have hyperemia, congestion, erosion, hemorrhagic ulcerations in the injured site. The damage was still maintained at d 7 and appeared to be multiple ulcerations and partial epithelial necrosis. On d 14, the colonic ulceration still existed. In both doses of LMWH treated groups, mucosal hyperemia, congestion and ulcerations in the injured site were slighter than those in control group. However, mucosal hemorrhage in both LMWH treated groups was severer than that in control group at 24 h, but was resolved on d 7 and 14, respectively.

Histological examination of TNBS-induced colitis

In control group a large number of neutrophils, monocytes and eosinophils infiltrated in mucosa and submucosa at 24 h and the damage reached peak on d 7. As shown in Table 1, the histological grades according to Fedorak *et al*[25] were greatly higher on d 7 compared with those at 24 h (P<0.01). On d 14, the damage of colon was mild, but still had ulceration, chronic inflammatory cell infiltration, vesculitis and granulation tissue formation. In contrast, in both 150 U/kg and 300 U/kg LMWH treated groups colonic damage was mild. Table 1 shows that the histological injury grades in the 300 U/kg LMWH group were decreased at 24 h and on d 7 after treatment, and much obviously on day 14 compared with the control group (P<0.01).

Expression of TNF-α in colonic mucosa of TNBS-induced colitis

As shown in Table 2, the scores of expression of TNF-α in mucosa were greatly higher in the first 24 h and decreased on d 7 and 14 after induction of colitis, but there were no

significant differences among the three groups except a difference at 24 h between 300 U/kg LMWH treatment group and 150 U/kg LMWH treatment group.

Table 1 Effect of low molecular weight heparin (dalteparin) in Fedorak grades on mucosa of TNBS-induced colitis in rats

	n	24 h	Day 7	Day 14
Control group	5	1.33±0.54	4.33±0.81[b]	3.67±2.13
150 U/kg LMWH group	4	1.00±0.00	3.33±2.08	1.67±1.15
300 U/kg LMWH group	5	0.57±0.33	2.00±1.33	0.66±0.32[d]

[b]$P<0.01$ *vs* control group at 24 h; [d]$P<0.01$ *vs* control group on day 14.

Table 2 Effect of low molecular weight heparin (dalteparin) on scores of mucosal TNF-α expression in rats with TNBS- induced colitis

	n	24 h	d 7	d 14
Control group	5	217±44[a]	48±11	50±18
150 U/kg LMWH group	4	264±42[a]	46±21	48±16
300 U/kg LMWH group	5	198±38[a,b]	26±18	16±8

[a]$P<0.001$ *vs* control group on day 7 and 14; [b]$P<0.05$ *vs* 150 U/kg group at 24 h.

Platelet surface P-selectin expression and serum interleukin 8 production

Expression of platelet surface P-selectin was increased on d 7 and 14 in all three groups as shown in Table 3, but reached a significant level only in control group. There were no significant differences among these three groups at the three time points, 24 h, d 7 and 14. Production of serum interleukin 8 was higher at 24 h in the three groups, but significantly decreased on day 14 compared with that at 24 h in 300 U/kg LMWH treated group as shown in Table 4.

Table 3 Effects of low molecular weight heparin (dalteparin) on platelet surface P-selectin expression (moleculars/per platelet) in rats with TNBS-induced colitis

	n	24 h	d 7	d 14
Control group	5	177.50±88.60	657.23±300.90[a]	767.50±359.11[b]
150 U/kg LMWH group	4	193.67±59.98	521.95±200.10	534.61±16.13
300 U/kg LMWH group	5	211.72±72.23	598.23±233.70	653.90±286.70

[a]$P<0.05$ *vs* control group at 24 h; [b]$P<0.01$ *vs* control group at 24 h.

Table 4 Effects of low molecular weight heparin (dalteparin) on production of serum IL-8 (pg/ml) in rats with TNBS-induced colitis

	n	24 h	d 7	d 14
Control group	5	7.50±3.50	4.51±2.59	4.53±3.37
150 U/kg LMWH group	4	8.38±4.01	5.04±2.01	4.44±2.88
300 U/kg LMWH group	5	8.11±3.87	3.09±1.28	2.06±1.03[a]

[a]$P<0.05$ *vs* 300 U/kg LMWH group at 24 h.

DISCUSSION

In this study we observed an anti-inflammatory effect of LMWH (dalteparin) in rats with TNBS-induced colitis. This effect was demonstrated by improvement of colonic inflammation with macroscopic and histological alterations in a dose of 300 U/kg of heparin treatment for 14 d. The result was similar to that of Fries *et al*[26], in which they showed that heparin could prevent TNBS-induced colitis, but steroids could not. Our data showed that serum IL-8 production in 300 U/kg LMWH treated group was significantly lower on d 14 than that at 24 h, but we did not find this variation in other groups. Expression of platelet surface P-selectin in control group was significantly increased consecutively at 24 h, d 7 and 14, but expression of platelet surface P-selectin did not increase in LMWH treatment group. We also did not find differences of expression of TNF-α in colonic mucosa between LMWH treatment group and control group. Our results suggested that LMWH (dalteparin) in a high dose had anti-inflammatory effects. The effects were possibly related to the decrease of proinflammatory cytokine IL-8, but not related to platelet surface P-selectin.

We observed dose and time- dependent effects of LMWH in rats with TNBS-induced colitis. Three hundreds U/kg LMWH treatment group showed more histological improvement of colitis, lower TNF-α expression in mucosa and serum IL-8 production than 150 U/kg LMWH treated group. With a continuing LMWH treatment these effects were gradually demonstrated on d 7 and 14. Our result was slightly different from that of Dotan *et al*[18]. They showed that a single dose of 80 μg/kg of LMWH (enoxaparin) was more optimal for amelioration of dinitrobenzene sulphonic acid- and iodoacetamide-induced colitis in rats than a dose of 200 μg/kg and 40 μg/kg of enoxaparin. The mechanism is not known, but may be related to optimal interactions between LMWH fragments and their receptors.

Chowers *et al*[27] found that disaccharides derived from heparin sulfate and heparin could suppress IL-8 and IL-1β production in intestinal epithelial cells *in vivo*. Our result was similar to that of the treatment of LMWH (dalteparin), serum IL-8 was much decreased. Salas *et al*[28] also showed that heparin pretreatment significantly attenuated leukocyte rolling, adhesion, and migration in *ex vivo* but did not affect the expression of cell adhesion molecules or vascular permeability elicited by TNF-α. The effects of heparin involved attenuation of a CD11b dependent adherent mechanism. Nelson *et al*[29] found that *in vitro* heparin tetrasaccharides reduced binding of neutrophils to COS cells expressing P-selectin but not to COS cells expressing E-selectin.

As for the side effects of haparin in the treatment of IBD, intestinal bleeding was mentioned in several studies[30]. Our study also showed more severe intestinal bleeding in LMWH treatment group than in control group at 24 h by macroscopic and histological observations. Thus, we should be cautious of using LMWH for clinical treatment of IBD.

In conclusion, our data indicate that LMWH has an anti-inflammatory effect in TNBS- induced colitis. The mechanism is possibly related to inhibition of proinflammatory cytokine, IL-8, but not to platelet surface P-selectin production.

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