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Original research

Evaluation of ligustrazine on the prevention of experimentally induced abdominal adhesions in rats

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HIGHLIGHTS

- The effect of ligustrazine on preventing adhesion formation was evaluated.
- Ligustrazine remarkably alleviated the adhesions.
- Ligustrazine could inhibite inflammation and regulate TGF/Smad signaling pathway.
- There is a potential new therapeutic use for ligustrazine in adhesion prevention.

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ABSTRACT

Objective: To determine the effects of ligustrazine on the prevention of postoperative intra-abdominal adhesions in rats.
Materials and methods: Abdominal adhesions were induced by scraping the cecum of rats. Various dosages of ligustrazine were administered for 10 days after surgery. Grades of abdominal adhesions were ranked by macroscopic observation on the 11th day after surgery. Meanwhile, the levels of IL-1β, IL-6, TNF-a in blood serum and TGF-β_1, CTGF in peritoneal fluid were determined by ELISA assay. In the cell experiment, the secretion of FN and CTGF undergoing stimulation with TGF β_1 in the culture fluid of rat peritoneal mesothelial cells (RPMC) were measured by ELISA assay. And the expression of Smad7 and p-smad 2/3 in RPMC were evaluated by western blot analysis.
Results: Ligustrazine remarkably alleviated the adhesions at the dose of 30 mg/kg and 60 mg/kg compared with the non-treated surgery group ($p < 0.05$). Ligustrazine decreased the levels of IL-1β, IL-6, TNF-α in blood serum and the expression of TGF-β_1, CTGF in the peritoneal fluid in dose-dependent manner. In the cell experiment, ligustrazine markedly attenuated TGF-β_1 induced upregulation of FN and CTGF in RPMC. Meanwhile, ligustrazine significantly inhibited the expression of pSmad 2/3 and increased the level of Smad7 in the RPMC.
Conclusions: Ligustrazine is a potent postoperative adhesion preventer by inhibiting inflammation and regulating the TGF/Smad signaling pathway.

1. Introduction

The formation of abdominal adhesions is a common and serious postsurgical complication, with up to 90% of the abdominal surgeries [1]. Adhesions can result from mechanical peritoneal damage, tissue ischemia or the presence of foreign materials. Abdominal adhesions can induce bowel obstruction, infarction, chronic pelvic pain and technical difficulty in cases of reoperation.

The formation of adhesions is associated with inflammatory response and extracellular matrix deposition in response to injury. The inflammatory response, which is generally induced by tissue injury or infection, plays an important role in adhesion formation. The degree of adhesion is determined by the extent of peritoneal inflammation [2]. Many anti-inflammatory agents have been

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shown to prevent adhesion formation [3,4].

In addition to inflammatory response, many researches have confirmed that TGF-β/Smad signal pathway plays an important role in the development of fibrosis [5,6]. TGF-β_1 induces both epithelial–mesenchymal transition (EMT) [7] and extracellular matrix (ECM) deposition [8]. Fibrosis positively correlated with the activation of TGF-β_1/Smad signal pathway [9]. The activation of TGF-β_1 dependent Smad2/3 pathway can promotes the secretion of extracellular matrix (ECM) proteins [10,11]. These suggest an important contribution of TGF-β1/Smad signaling in the pathogenesis of abdominal adhesions. Although the pathophysiology of adhesion formation is revealed, an effective solution of this problem has still not be found. So new therapies are in great needed.

Ligustrazine ($C_8H_{12}N_2$) is an important chemical component isolated from the dried root of *Ligusticum chuanxiong hort* (umbelliferae). Ligustrazine has been routinely used to treat patients with coronary vascular diseases for many years in China. Moreover, ligustrazine has exhibited the ability to attenuate inflammatory responses in rats with liver injury, asthma et al. [12,13]. Recently, it has been discovered that ligustrazine has good antifibrosis effect. Ligustrazine can evidently resist renal interstitial fibrosis induced by unilateral ureteral obstruction in rats [12,14]. Moreover, it has been shown to inhibit the proliferation of vascular smooth muscle cells after arterial injury and to prevent intimal hyperplasia of the deendothelial artery [15,16]. But the pharmacological mechanisms underlying the effects are largely unknown and the effectiveness of ligustrazine on preventing adhesion formation has not been reported.

The purposes of this study is to assess the effectiveness of ligustrazine on the prevention of abdominal adhesion formation and to characterize its potential mechanism(s) of action.

2. Material and methods

2.1. Animal experiment

2.1.1. Animal model of abdominal adhesions

60 Wistar rats of both genders aged 10 weeks (200–250 g) were used in the study. The rats were purchased from Experimental animal center of Henan Province. The rats were treated under the guidelines of Institutional Animal Care and Use Committee (IACUC). The study was approved by the ethics committee of Xinxiang Medical University (permit number: 20141201, date: 22/03/2013).

All animals were randomly divided into six groups (10 per group consist of 5 female and 5 male), including the normal group (A), model group (B) and four treated groups (C, D, E and F). Anesthesia was done using an intraperitoneal injection of ketamine 80 mg/kg and Xylazin 10 mg/kg. Then all groups underwent abdominal surgery except the normal group. The animals of normal group were given only laparotomies. The caecum serosa was gently rubbed with dry gauze at 2.5 × 2.5 cm [17]. 4 ml/kg saline was injected to the rats' abdominal cavity for group A and B, while 10 mg/kg Dexamethasone, 15 mg/kg, 30 mg/kg, 60 mg/kg ligustrazine for group C, D, E, F. The drugs were administered for 10 days after surgery.

2.1.2. Measurement of adhesions degree

Animals in each group were anesthetized with an intraperitoneal injection of ketamine 80 mg/kg and Xylazin 10 mg/kg on the post-surgical day 11. The adhesions were defined by Nair's method (Table 1) [18]. The adhesion scores were determined by two investigators blind to the division of the groups.

2.1.3. ELISA assay of IL-1β, IL-6 and TNF-α level in blood

The blood samples drawn from inferior vena cava were centrifugated at 150 × g for 30 min. The serum were collected and preserved at −20 °C. Levels of IL-1β, IL-6, and TNF-α in serum were detected using rat IL-1β, IL-6, (GenStar BioSolutions Co.,Ltd) and TNF-α ELISA kit (Beijing BoLingKeWei Biological Technology Co. Ltd), respectively.

2.1.4. Detection of TGF-β_1 and CTGF in peritoneal fluid

The peritoneal fluid was collected from the rats' abdominal cavity. The contents of TGF-β_1 and CTGF in peritoneal fluid were measured by rat TGF-β_1 ELISA kit (GenStar BioSolutions Co.,Ltd) and rat CTGF ELISA kit (Shanghai Kaibo Biotechnology Inc) according to the manufacturers' instruction.

2.2. Cell experiment

2.2.1. Isolation and primary culture of rat peritoneal mesothelial cells (RPMCs)

Male Wistar rats weighing 150–180 g were provided by Experimental animal center of Henan Province, China. Animals were received an injection of 20 ml 0.25% trypsin (in 0.02% EDTA solution) intraperitoneally. Two hours later, the abdominal fluid was collected, then it was centrifuged at 150 × g for 15 min. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium (Power, Gibco, USA) containing 10% fetal bovine serum and cultured in flasks under 5% CO_2 at 37 °C. The RPMCs were identified by immunostaining with anti-cytokeratin antibody and anti-vimentin antibody (Shanghai Kemin Biotechnology Inc).

2.2.2. TGF-β_1 stimulation

1×10^5 RPMCs were inoculated into a 6-pore plate. When 70–80% confluence was achieved, the medium was changed to serum-free DMEM/F12 culture fluid for 24 h to achieve growth synchronization. They were divided into the following groups: (1) the control group (cells cultured in serum-free medium and collected at 24 h) (2) TGF-β_1 stimulation group (5 ng/ml) (3) TGF-β_1 stimulation + ligustrazine (15 mg/L) (4) TGF-β_1 stimulation + ligustrazine (30 mg/l) (5) TGF-β_1 stimulation + ligustrazine (60 mg/L). For TGF-β_1 treatments, cells were treated with 5 ng/ml TGF-β_1 (Peprotech, USA) for 48 h to induce cellular fibrogenesis and cells were co-treated with different concentrations of ligustrazine and TGF-β_1 in last 24 h. Three wells were allocated for each treatment.

2.2.3. Detection of FN and CTGF in supernatant

The levels of FN and CTGF protein in the cell culture supernatant were measured using an ELISA kit (Shanghai Kaibo Biotechnology Inc) according to the manufacturer's instructions.

2.2.4. Western blot analysis

Proteins were extracted from RPMCs by RIPA buffer containing 1 mM PMSF. The proteins were transferred onto nitrocellulose membrane after separating in PAGE. Following washing and blocking, the membrane was incubated overnight at 4 °C with primary antibody against p-Smad2/3 (Santa Cruze, 1: 500 dilution), Smad7 (Santa Cruze, 1: 500) and Smad2/3 (Santa Cruze, 1: 500 dilution). Then the membrane was incubated with secondary antibody at 37 °C for 1 h after washing. Bands were visualized by ECL western blotting kit (Beyotime Institute of Biotechnology, china). Target proteins levels were normalized by β-action.

2.3. Statistical analysis

Datas were expressed as mean ± standard deviation. SPSS 19.0 package was used for statistical processing. One-way analysis of variance (ANOVA) was used to evaluate the differences of six groups. The Kruskal–Wallis test was used to identify the