

1. Title Page

**Berberine Hydrochloride Prevents Post-Surgery Intestinal
Adhesion and Inflammation in Rats**

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2. Running Title Page

Running title: Berberine Prevents Intestinal Adhesion and Inflammation

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List of nonstandard abbreviations: Berberine, berberine Hydrochloride; ERK, extracellular signal-regulated protein kinase; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; JNK, c-Jun N-terminal kinases; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; Ox-LDL, Oxidized low-density lipoprotein; TAK1, transforming growth factor-activated kinase 1; TGF- β , transforming growth factor beta; TNF- α , tumor necrosis factor- α .

3. ABSTRACT

Intestinal adhesion, characterized by connection of the loops of the intestine with other abdominal organs by fibrous tissue bands, remains an inevitable event of abdominal operations and can cause a number of complications. Berberine hydrochloride (berberine), a natural plant alkaloid derived from Chinese herbal medicine, is characterized by diverse pharmacological effects, such as anticancer and lower elevated blood glucose. This study is designed to investigate its effects on adhesion and inflammation after abdominal surgeries and the underlying molecular mechanisms. Adhesion severity grades and collagen deposition were assessed 14 days after the surgery. We evaluated the levels of intercellular adhesion molecule-1(ICAM-1) and inflammatory cytokines IL-1 β , IL-6, TGF- β , TNF- α and examined TAK1/JNK and TAK1/NF- κ B signaling. The surgery group performed the most severe adhesions and berberine strikingly reduced the density and severity of adhesion. Results showed significant lower expression of IL-1 β , IL-6, TGF- β , TNF- α , ICAM-1, in berberine groups compared with the operation group. Activities of phosphorylated JNK and phosphorylated NF- κ B were inhibited in the berberine groups compared with the surgery group. Our novel findings identified berberine hydrochloride is a promising strategy to prevent adhesion by downregulating ICAM-1 and reduce inflammation by inhibiting the TAK1/JNK and TAK1/NF- κ B signaling after abdominal surgery, which brought out a good therapeutic approach for the development of clinical application for postoperative abdominal adhesion and inflammation.

4. Introduction

Intestinal adhesion, a common problem after the abdominal surgery (Kosaka et al., 2008), is bands of fibrous tissue that connect the loops of the intestine to other abdominal organs or abdominal wall (van Goor, 2007; Takagi et al., 2013), where slight bleeding cannot be completely prevented. Although surgical procedures and technologies have been improved to reduce adhesion formation, such as laparoscopic surgery (van't et al., 2002), minimally invasive surgery is not always applicable or available (van't et al., 2002; Ten Broek et al., 2013a). Thus, intraperitoneal adhesion formation after surgeries remains an inevitable event of most abdominal procedures (Wiseman et al., 1999). Studies have reported that the incidence of adhesions is over 90% (Chaturvedi et al., 2013; Takagi et al., 2013) and adhesions can also develop after abdominal bacterial infections, such as peritonitis (van Goor, 2007). Peritoneal adhesion is one of the leading causes of intestinal obstruction, up to 40% of all cases (Ellis, 1997), with 60–70% of the cases involving the small bowel (Townsend et al., 2011). Postsurgical adhesions are a principal factor that contributes to increased mortality and morbidities (Emans et al., 2009; Yigitler et al., 2012) such as life threatening ileus, chronic abdominopelvic discomfort, chronic pelvic pain, infertility, dyspareunia and also intestinal obstruction, accounting for approximately 75% of all cases (Miller et al., 2000; Ghosheh and Salameh, 2007; Izumi et al., 2007).

An increasing number of strategies to reduce adhesions have been clinically available, including fibrinolytic agents, anti-inflammatory drugs, and barrier/separation materials. However, only a few anti-adhesion agents can be applied effectively during laparoscopic surgery (Dupre et al., 2013), and few of the approaches work well to a satisfactory level

(Sahin et al., 2007; Takagi et al., 2013). Drugs such as corticosteroids inhibit fibrinolytic activity and may actually support adhesion formation (Ward and Panitch, 2011), which might contribute to an unacceptably high incidence of anastomotic leaks. Undoubtedly, there is an urgent need for an effective formula of anti-adhesion and anti-inflammation without similar clinical disadvantages.

Berberine hydrochloride (berberine, 5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo [5,6-a] quinolizinium) (Fig.1), an isoquinoline alkaloid initially derived from *Rhizoma Coptidis*, *Hydrastis canadensis*, *Berberis aquifolium*, has been used in traditional Chinese medicine for many decades (Kong et al., 2004). Berberine extracts have had a long history of significant medicinal use of antimicrobial activity (Gray and Lachance, 1956; Feng et al., 2012) and anti-inflammatory effect (Cao et al., 2013; Mo et al., 2014). The predominant clinical uses of berberine include gastroenteritis, intestinal parasite infections, bacterial diarrhea, and ocular trachoma infections (Yao et al., 2013). These facts prompted us to propose that berberine may be used as a therapeutic strategy for preventing post-surgical intestinal adhesions due to its anti-inflammatory properties.

Adhesion molecules are key mediators of intestinal adhesion. Intercellular adhesion molecule 1 (ICAM-1) is a member of the immunoglobulin superfamily necessary for the adhesion of leucocytes to the capillary endothelium (Jung et al., 2012). ICAM-1 has been implicated in the development of intestinal adhesion and is proved to play an essential role in stabilizing cell-cell interactions (Sigal et al., 2000). ICAM-1 also participates in maintaining a pro-inflammatory environment conducive to leukocyte endothelial transmigration (Proud and Leigh, 2011).

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This study was set to investigate the anti-adhesion efficacy of berberine and delineating the underlying mechanisms in a rat model of intestinal adhesions induced by abrasive abdominal surgery.

5. *Material and Methods*

Animals. Fifty healthy male Sprague-Dawley rats (200–250 g body weight) were used for this study. Animals were housed under standard laboratory conditions at a constant room temperature ($22\pm 0.5^{\circ}\text{C}$) and relative humidity (65–70%) with a 12 h light and dark cycle. They were fed a commercial rat diet and water *ad libitum*. All the rats were acclimated to the laboratory environment for 7 days before the operation. They were deprived of food for 12 h before the experiment but had free access to water. All animal procedures were in accordance with the Declaration of Helsinki and were reviewed and approved by the Animal Care and Use Committee of the Harbin Medical University.

Experimental design. The rats were allocated randomly into five groups ($n = 10$): Sham control, Surgery, Berberine-1, Berberine-2, and Interceed groups. The animals were anesthetized intraperitoneally with sodium pentobarbital (3%) at a concentration of 0.13 ml/100 g body weight. The abdomen was then shaved and prepared with alcohol and iodine solution. After drying, a 3 cm anterior midline ventral laparotomy through the abdominal wall and peritoneum was performed under sterile conditions so as to gain access to the abdominal cavity. We isolated the cecum and induced intestinal adhesion by abrading the cecum with a toothbrush, such that a homogeneous surface of petechial hemorrhage was created (Supplemental Figure 1). After the cecum was returned to the abdomen, the midline incision was closed in two layers with 5-0 nylon (Townsend et al., 2011). For sham control animals, approximately 2.0 ml of normal saline was dropped into the abdominal cavity after the laparotomy surgery. For the Surgery group, approximately 2.0 ml of normal saline was dropped into the abdominal cavity

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immediately after the abrasive surgery. For the Berberine-1 group, berberine hydrochloride in normal saline (1.5mg/ml) was dropped into the abdominal cavity immediately after the abrasive surgery. For the Berberine-2 group, berberine hydrochloride in normal saline (0.75mg/ml) was dropped into the abdominal cavity immediately after the abrasive surgery. Interceed is an adhesion barrier used as a positive control in the current study, for the Interceed group, a 3.0×2.0-cm piece of Interceed was attached to the abdominal wall defect, anterior to the abraded cecum, and approximately 2.0 ml of normal saline was added onto the Interceed.

Reagents. Berberine hydrochloride (berberine) was obtained from Sigma-Aldrich (St Louis, USA). The Interceed Absorbable Adhesion Barrier was purchased from Ethicon, Inc (New Jersey, USA).

Evaluation of adhesion formation. On postoperative day 14, animals were anesthetized intraperitoneally with sodium pentobarbital (3%) at a concentration of 0.13 ml/100 g body weight. A laparotomy was undertaken to assess adhesion formation. The incision was performed at a position remote to the original laparotomy scar to prevent interfering any adhesions between the abdominal wall and viscera. The abdominal wall was then opened fully to examine the extent of adhesions. The adhesions were graded by the scoring system developed from comparison that have been previously validated (Townsend et al., 2011). This method, taking into account number, strength and distribution, provides a more objective measurement of adhesion formation. We adopted the macroscopic adhesion measurement score as our scoring system for assessing the severity of adhesion formation (Supplemental Table 1). Application of the scoring

system was performed by an observer blinded to the study design. Photographs of adhesions were taken for each animal to allow grading of the adhesions by another independent observer (Supplemental Figure 2).

Samples. After 14 days the animals were euthanized and blood serum was collected for enzyme-linked immunosorbent assay measurement. The cecum samples were dissected for a series of experiments.

RNA extraction and Real-time RT-PCR analysis for mRNAs. IL-1 β , IL-6, TGF- β , TNF- α and ICAM-1 mRNA expression was analyzed. The animals were euthanized 14 days after the surgery and the cecum was immediately dissected, frozen in liquid nitrogen, and stored at -80°C . The tissue was carefully transferred into an RNase-free tube for extraction of RNA. Thawed tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen) and total RNA in the tissue was isolated. CDNA synthesis was performed by the Reverse Transcription Kit (Applied Biosystems, Cat. #4368814) according to the manufacturer's instructions. The SYBR Green PCR Master Mix Kit (Applied Biosystems, Cat. #4309155) was used for relative quantification of RNAs (Zhang et al., 2010). GAPDH was used as an internal control. Real-time RT-PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers used in our study were provided in Supplemental Table 2 (You et al., 2011; Werry et al., 2012).

Enzyme-linked immunosorbent assay measurement. Levels of IL-1 β , IL-6, TGF- β , ICAM-1, and TNF- α in serum and secreted from cecum were detected using rat IL-1 β ELISA kit, rat IL-6 ELISA kit, rat TGF- β ELISA kit, rat ICAM-1 ELISA kit (Wuhan

Boster Biological Technology., LTD), and rat TNF- α ELISA kit (Beijing 4A Biotech Co., Ltd), respectively.

Western blot analysis. The protein samples were extracted from rat cecum for immunoblotting analysis of p-TAK1, p-JNK, p-NF- κ B p65, TAK1, JNK, and NF- κ B p65. The protein content was determined using the BCA Protein Assay kit (Bio-Rad, Mississauga, ON, Canada). Equal amounts of protein (60 μ g) were loaded on a 10% SDS-PAGE gel. The lysates were resolved by electrophoresis (70 V for 30 min and 100 V for 1.2 h) and transferred onto nitrocellulose membranes. After blocking in 5% nonfat milk for 2 h at room temperature the membranes were treated with antibodies (Zhang et al., 2013). Membranes were blotted with antibodies against p-JNK (Cell Signaling), JNK (Cell Signaling), NF- κ B (Santa Cruz Biotechnology), p-NF- κ B (Cell Signaling), β -actin (Santa Cruz Biotechnology), p-TAK1 (Cell Signaling), and TAK1 (Cell Signaling), according to the manufacturer's instructions. Next day, the membrane was washed in PBST for three times (15 min/each) and incubated for 1 h with the fluorescence-conjugated anti-rabbit IgG secondary antibody (1:10000) in the blocking buffer. Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (Area \times OD) for each group and normalized by β -actin. The final results are expressed as fold changes by normalizing the data to the control values.

Tissue handling and immunohistochemistry assessment. The resected adhesion samples were fixed in a 10% formalin solution, and following dehydration, the samples were processed, embedded in paraffin using tissue processing equipment. The sections were stained with hematoxylin and eosin (HE) and Masson trichrome staining to assess

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the degree of fibrosis, inflammation and vascular proliferation, using a previously reported semi-quantitative scoring system. The fibrosis grading system was as follows: 0, nil; 1, minimal and loose; 2, moderate; and 3, florid and dense. Inflammation was scored as follows: 0, nil; 1, giant cells with occasional scattered lymphocytes and plasma cells; 2, giant cells with increased numbers of admixed lymphocytes, plasma cells, eosinophils and neutrophils; and 3, numerous admixed inflammatory cells with microabscesses present. Vascular proliferation was scored as follows: 0, no vascular proliferation; 1, mild proliferation; 2, moderate proliferation and 3, intense proliferation (Townsend et al., 2011).

For immunohistochemical analysis, cecum samples were fixed with 4% buffered paraformaldehyde embedded in paraffin, tissue slices were dehydrated in ascending series of ethanol, cleared in xylene. All sections were incubated with ICAM-1 antibody 4°C overnight. After incubation with second antibody immunoglobulin, the sections were stained with diaminobenzidine.

Measurement of collagen content. Total collagen content was measured by biocolor Collagen Assay kit according to the manufacturer's instructions (Shan et al., 2009). Lysates (100ul) were stained with 1ml of biocolor dye reagent. The unbound dye solution was removed and 1ml of the alkali reagent was added. The absorbance was read at 540 nm. Total collagen (mg) was calculated according to a linear calibration curve from standards (Vitrogen 100, Angiotech Biomaterials, Palo Alto, CA) and normalized to the total protein (mg) of each lysate.

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Statistical analysis. Data are given as means \pm SEM. Comparisons of adhesion scores between different groups were made using the Kruskal-Wallis H and the Mann–Whitney U test by SPSS 19.0. One-way analysis of variance was applied for statistical comparisons between different experimental groups in Prism version 5 (GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

6. Results

The anti-adhesion formation properties of berberine. To determine the severity and incidence of adhesion, the adhesion formation was classified into six grades, using a predetermined adhesion measurement score (Townsend et al., 2011): grade 0, no adhesions; grade 1, loose filmy adhesions that could be separated by blunt dissection; grade 2, adhesions requiring <50% sharp dissection for separation; grade 3, adhesions requiring >50% sharp dissection for separation; grade 4, serosal injury; and grade 5, full-thickness injury, as described in Supplemental Table 1. Examples of photographic images at post-mortem evaluation of adhesions on day 14 after the surgery were displayed in Fig. 2. The results on adhesion severity and adhesion area were presented in Table 1. Statistically significant differences were found among the groups in terms of the incidence and severity of adhesion formation. Rats of the abrasive surgery group showed the most severe adhesions between the abraded cecum and the abdominal wall lesion, of which four had an adhesion score over 4 with full-thickness injury. The adhesion was remarkably alleviated by berberine at concentrations of 1.5mg/ml and 0.75mg/ml, compared with the non-treated surgery group. Moreover, berberine was as effective as the Interceed as a positive control agent.

Cellular mechanisms for the anti-adhesion properties of berberine. Histopathology revealed significant morphologic differences of myodegeneration and a sub-acute to chronic inflammatory response within the abraded muscle. Similar changes were observed in the sham (Fig.2A-C) and berberine treatment groups (Fig.2G-I), indicating that the changes were a response to the surgical procedure. The microscopic alterations

were evaluated for fibrin presence, inflammatory infiltrates (polymorphonuclear or mononuclear), giant cells presence, angiogenesis, fibroblast proliferation, hemorrhage, and necrosis. As shown in Fig. 2, in the surgery group (Fig.2D-F), the connective tissue and fibrosis were florid and dense, giant cells had increased numbers of admixed lymphocytes, plasma cells, eosinophils and neutrophils, and even numerous admixed inflammatory cells with microabscesses present. There was also intense vascular proliferation and congestion indicating the severe inflammatory process occurring in the surgery group compared with the sham group (Fig.2A-C). Berberine and Interceed treatment (Fig. 2G-L) reduced the inflammatory responses as indicated by the scattered lymphocytes and plasma cells, and the mild proliferation of the blood vessel. Results of Masson trichrome staining in the surgery group showed massive amounts of collagen production and deposition (blue), mainly in the lamina propria, submucosal areas, and muscularis propria in cecum. Berberine and Interceed groups remarkably attenuated fibrosis and collagen deposition of the cecum samples (Fig.3A-D). Measurement of collagen verified decreased mRNA level of collagen 1 (Col-1) (Fig.3E) and collagen 3(Col-3) (Fig3.F) in berberine and Interceed groups compared with the surgery group by real-time RT-PCR. Statistically significant differences of collagen content were discovered among the groups, the result showed collagen deposition increased in the surgery group and was attenuated with berberine and Interceed treatment (Fig.3G).

Molecular mechanisms for anti-adhesion properties of berberine. Intercellular adhesion molecule-1 (ICAM-1), also known as CD54, encodes a cell surface glycoprotein which is typically expressed in endothelial cells, immune cells and plays

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important role in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. ICAM-1 also participates in a positive-feedback loop to maintain a pro-inflammatory environment conducive to leukocyte endothelial transmigration (Rothlein et al., 2011). We measured both circulating and tissue ICAM-1 levels, and found that serum ICAM-1 was significantly elevated (~6.5-fold) in the surgery group and were reduced in the berberine-1 (~20.8% of the surgery group), berberine-2 (~31.2% of the surgery group) and Interceed (~37.1% of the surgery group) groups (Fig. 4A). Similarly differential changes of cecum ICAM-1 levels were also consistently observed, ICAM expression was significantly up-regulated in the surgery group, from 347.7 ± 63.6 ng/ml in sham group to 1028.7 ± 112.6 ng/ml, and the effect was nearly eliminated after treatment with berberine-1 (367.6 ± 77.1 ng/ml), berberine-2 (471.9 ± 76.7 ng/ml) or Interceed (511.7 ± 76.5 ng/ml) (Fig. 4B). In the immunohistochemical analysis, expression of ICAM-1 in the surgery group increased remarkably, berberine reduced ICAM-1 and ameliorated adhesion (Fig.4C-F). To further investigated the role of ICAM-1 in mediating berberine's anti-adhesion effect, we applied intestinal HT29 cell line which was reported to have higher expression of ICAM-1 (Gallicchio et al., 2008). Treatment with berberine or ICAM-1 siRNA led to a decrease of ICAM-1 expression. ICAM-1 relative protein expression and mRNA level in cell, as well as ICAM-1 concentration in cultural supernatants were all significantly decreased in berberine high dose group and ICAM-1 siRNA group. The cell adhesion assay exhibited obvious reduction of relative adherent cells with berberine treatment and ICAM-1 siRNA transfection compared to the negative control (NC) group (Supplemental Figure 3).

The pathogenesis of inflammation is a result of complex interactions between circulating inflammatory factors. Recent studies have revealed essential roles of cytokines, the chemical messengers between immune cells, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-1 β , TGF- β , in mediating inflammatory and immune responses (Holmdahl and Ivarsson, 1999; Kosaka et al., 2008; Kim et al., 2011; Morin et al., 2012). We observed that secretion of inflammatory cytokines IL-1 β , TGF- β , TNF- α and IL-6 was significantly decreased from 54.8 ± 8.4 ng/ml, 102.8 ± 14.6 ng/ml, 106.1 ± 23.6 ng/ml and 496.3 ± 50.1 ng/ml, to 28.1 ± 1.1 ng/ml, 26.9 ± 4.6 ng/ml, 48.0 ± 2.4 ng/ml and 84.3 ± 3.5 ng/ml in the berberine-1 groups compared with the surgery group, respectively (Fig. 5).

Consistently, the qPCR results of IL-1 β , TGF- β , TNF- α and IL-6 exhibited higher expression levels in cecum tissue in the surgery group compared with the sham animals. Treatment with berberine substantially reversed the overexpression of the cytokines (Fig. 6).

Signaling mechanisms for the anti-adhesion properties of berberine. TAK1/JNK and TAK1/NF- κ B signaling pathways have been known to play a critical role in the inflammatory response (Gonzalez-Guerrero et al., 2013). Our data suggest that these two signaling pathways were also involved in the post-surgical intestinal adhesion. As illustrated in Fig. 7, our Western blot results revealed that TAK1 was highly activated in the surgery group as reflected by the increased phosphorylated form of TAK1 and this activation was suppressed by berberine. Expectedly, the activities of JNK and NF- κ B

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signaling were decreased by berberine as indicated by the decreased phosphorylation of
JNK and NF- κ B.

7. Discussion

Here we have presented results from our experimental investigation on the efficacy of berberine on adhesion formation in a rat model of intestinal adhesions developed following abrasive abdominal surgery. Our data unraveled berberine as an anti-adhesion agent capable of inhibiting post-abdominal surgery adhesion formation and inflammatory responses. Blockade of ICAM-1 secretion, downregulation of expression of various inflammatory molecules, and inhibition of TAK1/JNK and TAK1/NF- κ B signaling may account for the anti-adhesion properties of berberine, as summarized schematically in Fig. 8.

Intestinal adhesion after abdominal surgery is a continuous state of fibrosis and inflammation that concomitantly facilitates the progression of a variety of complications, such as chronic abdominopelvic discomfort, pain and infertility. Berberine has recently become a useful anti-inflammatory drug for cardiovascular, endocrine and intestinal disorders, owing to its antitumor and antilipase effects (Jeong et al., 2009; Jiang et al., 2011; Meng et al., 2012; Yao et al., 2013). Here, we identified berberine as an anti-adhesion agent which effectively protects against post-abdominal surgery adhesion and inflammation, with several lines of evidence including reduced adhesion scores from direct assessment of adhesions, histopathology measurement of lymphocytes and vascular proliferation, decreased circulating concentration of ICAM-1, downregulated expression levels and secretion of various proinflammatory cytokines in both serum and cecum, and suppression of TAK1, JNK and NF- κ B signaling.

ICAM-1 is a key pro-adhesive protein that is markedly elevated in inflammatory diseases. Berberine effectively decreased ICAM-1 level in serum and cecum. In the immunohistochemical analysis, expression of ICAM-1 in the surgery group increased remarkably, berberine reduced ICAM-1 and ameliorated adhesion (Fig.4C-F). ICAM-1 is a key pro-adhesive protein that is markedly elevated in inflammatory diseases. The finding that berberine inhibited the expression of ICAM-1 in this study indicates that suppression of the ICAM-1 underlies the anti-adhesive action of berberine on post-surgery tissues. As ICAM-1 is involved in stabilizing cell-cell adhesions and participates in maintaining a pro-inflammatory environment conducive to leukocyte endothelial transmigration. In vitro, treatment of the intestinal HT29 cell line which are reported to have higher expression of ICAM-1 with berberine and ICAM-1 siRNA led to a decrease of ICAM-1 expression. Expression of ICAM-1 was significantly decreased in berberine high dose group and ICAM-1 siRNA group. The cell adhesion assay exhibited obvious reduction of relative adherent cells with berberine treatment and ICAM-1 siRNA transfection. Nevertheless, since inflammatory cytokines were inhibited by berberine, suppression of ICAM-1 may be an indirect effect of berberine by inhibition of inflammation. Until this is accomplished, the role of ICAM-1 in mediating berberine's anti-adhesion effects could be fully established. The IL-1 β , IL-6, TGF- β , TNF- α cascade has been shown to play a critical role in the pathogenesis of tissue inflammation. Recent study demonstrated that Cicaderma ointment, a mix of natural extracts, modulates inflammation, promotes re-epithelialization, and accelerates skin wound healing through the modulation of a few cytokines, including TNF- α , macrophage inflammatory

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protein-1 α , IL-12, IL-4, and macrophage–colony-stimulating factor (Morin et al., 2012). The increase of TNF- α could participate in the up-regulation of several inflammatory cytokines, i.e., IL-1, IL-6, IL-12, and IL-17, but also the synthesis of cell surface adhesion molecules involved in adhesion to endothelium (Mahdavian Delavary et al., 2011). Moreover, TAK1, a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family (Goldmann et al., 2013), is considered a key intermediate in a multitude of innate immune signaling pathways by directly regulating the JNK and extracellular signal-regulated protein kinase (ERK) pathways by activating SEK and MEK1/2 respectively (Gonzalez-Guerrero et al., 2013). TAK1 has also been demonstrated to be a major upstream activator of JNK, p38 mitogen-activated protein kinase (MAPK), and NF- κ B signaling in the obstructed kidney, and activation of JNK, p38, and the transcription factor NF- κ B drives renal inflammation and fibrosis (Ma et al., 2011). Therefore, we hypothesize that the suppression of TAK1 cascade may be one underlying mechanism of berberine’s beneficial effects on post-surgery intestinal inflammation. Our data indicate that treatment of abrasive surgery rats with berberine significantly inhibited the up-regulation of the active form of TAK1, phospho-TAK1, while having no effect on the total TAK1, the expression of which did not alter after abrasive surgery. JNK and its downstream transcription factor c-Jun have been associated with inflammation in several diseases, including asthma (Alrashdan et al., 2012), arthritis (Guma et al., 2011) and renal diseases, in association with renal inflammation, interstitial fibrosis, and decline of renal function (de Borst et al., 2009). In the present study, we found that the expression of phospho-JNK in the cecum of model rats was significantly

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up-regulated and this was attenuated by treatment with berberine. Another TAK1 cascade, NF- κ B, a protein responsible for cellular responses to stimuli such as cytokines, ultraviolet irradiation, free radicals, and viral or bacterial antigens and plays a crucial role in regulating the immune response to infection (Brasier, 2006; Perkins, 2007; Yadav et al., 2013; Dediego et al., 2014), was also implicated in the anti-inflammation effect of berberine. Our results showed that the activation of NF- κ B induced by abrasive surgery was significantly inhibited by berberine, as indicated by a decreased phospho-NF- κ B expression. These results strongly indicate that the inhibition of TAK1/JNK and TAK1/NF- κ B accounts for the anti-adhesion and anti-inflammation action of berberine.

Accumulating evidence implicated the utilization of mesh barrier materials or anti-inflammatory drugs to prevent the adhesion formation and directly interfere with the inflammatory process. Research to date has identified four main aspects of methods to prevent the formation of intestinal adhesions after abdominal surgery: 1) use of minimally invasive surgical procedures, such as laparoscopic surgery to reduce peritoneal trauma; 2) prevention of fibrin formation with pharmacological agents, such as heparin or tissue plasminogen activator; 3) reduction of the contact between organs and intra-abdominal contents by using biodegradable barriers such as Seprafilm; and 4) In addition to these solutions, a wide range of biologically active substances in the form of simple fluids, gels and solids, either combined or alone, have been investigated both clinically and experimentally to reduce or prevent abdominal adhesions (Tingstedt et al., 2007; Lauder et al., 2010; Takagi et al., 2013; Ten Broek et al., 2013a; ten Broek et al., 2013b). An ideal barrier should be biodegradable, biocompatible and surgically easy to

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handle and should act locally to avoid side effects. However, only certain materials meet these requirements, and no large prospective, randomized double-blind human studies have demonstrated their efficacy. Some of them are effective in the short-term, but the protective effects are lost in the long-term, some have no effect on inflammation and others have severe detrimental effect. Thus, little of these materials have been widely adopted by surgeons, indicating that the materials only decrease adhesion severity, not incidence (Lauder et al., 2010). Interceed (TC7) Absorbable Adhesion Barrier is a preformed sheet of oxidized regenerative cellulose and its safety and efficacy have been documented in several controlled clinical studies (Wiseman et al., 1999; Tsapanos et al., 2002). The data presented in our study demonstrated that administration of berberine decreased collagen synthesis, adhesion, and inflammation, and had no detrimental effect on the wound healing process. These results indicate that berberine may be an effective drug in the prophylaxis of adhesion formation with minimal side effects, which overcomes the shortage of current anti-adhesion methods.

We have in this study unraveled a novel pharmacological effect of berberine: effectively inhibiting adhesion and inflammation after abdominal surgery with little side-effect. Meanwhile, we have also deciphered the potential molecular mechanisms underlying the anti-adhesion properties of berberine. Our findings also strongly suggest that berberine is a promising candidate agent for clinical application to preventing adhesion after surgery. However, we are well aware that additional studies looking at the effects at a longer term are needed to obtain more definitive evidence for the sustained efficacy and the lack of toxic effects of berberine. Moreover, our results do not allow us

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to deduce a definite mechanism for the formation of adhesions and inflammations and for the anti-adhesion properties of berberine, which merits future investigations to advance and deepen our understanding of the issue.

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8. Authorship Contributions

Participated in research design: Y Zhang, B Yang.

Conducted experiments: Y Zhang, X Li, L Yang, Ju, Du, Chen, Cheng, Liu, Q Zhang, J Li, Bilal.

Performed data analysis: X Li, Xu, Wei.

Wrote or contributed to the writing of the manuscript: Y Zhang, X Li, Lu, B Yang.

Y Zhang and X Li contributed equally to this work.

References

- Alrashdan YA, Alkhouri H, Chen E, Lalor DJ, Poniris M, Hennes S, Brightling CE, Burgess JK, Armour CL, Ammit AJ and Hughes JM (2012) Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement. *Am J Physiol Lung Cell Mol Physiol* **302**:L1118-1127.
- Brasier AR (2006) The NF-kappaB regulatory network. *Cardiovasc Toxicol* **6**:111-130.
- Cao M, Wang P, Sun C, He W and Wang F (2013) Amelioration of IFN-gamma and TNF-alpha-induced intestinal epithelial barrier dysfunction by berberine via suppression of MLCK-MLC phosphorylation signaling pathway. *PLoS One* **8**:e61944.
- Chaturvedi AA, Lomme RM, Hendriks T and van Goor H (2013) Prevention of postsurgical adhesions using an ultrapure alginate-based gel. *Br J Surg* **100**:904-910.
- de Borst MH, Prakash J, Sandovici M, Klok PA, Hamming I, Kok RJ, Navis G and van Goor H (2009) c-Jun NH2-terminal kinase is crucially involved in renal tubulo-interstitial inflammation. *J Pharmacol Exp Ther* **331**:896-905.
- Dediego ML, Nieto-Torres JL, Regla-Nava JA, Jimenez-Guardeno JM, Fernandez-Delgado R, Fett C, Castano-Rodriguez C, Perlman S and Enjuanes L (2014) Inhibition of NF-kappaB-Mediated Inflammation in Severe Acute Respiratory Syndrome Coronavirus-Infected Mice Increases Survival. *J Virol* **88**:913-924.
- Dupre A, Lefranc A, Buc E, Delperro JR, Quenet F, Passot G, Evrard S and Rivoire M

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- (2013) Use of bioresorbable membranes to reduce abdominal and perihepatic adhesions in 2-stage hepatectomy of liver metastases from colorectal cancer: results of a prospective, randomized controlled phase II trial. *Ann Surg* **258**:30-36.
- Ellis H (1997) The clinical significance of adhesions: focus on intestinal obstruction. *Eur J Surg Suppl*:5-9.
- Emans PJ, Schreinemacher MH, Gijbels MJ, Beets GL, Greve JW, Koole LH and Bouvy ND (2009) Polypropylene meshes to prevent abdominal herniation. Can stable coatings prevent adhesions in the long term? *Ann Biomed Eng* **37**:410-418.
- Feng AW, Gao W, Zhou GR, Yu R, Li N, Huang XL, Li QR and Li JS (2012) Berberine ameliorates COX-2 expression in rat small intestinal mucosa partially through PPARgamma pathway during acute endotoxemia. *Int Immunopharmacol* **12**:182-188.
- Galicchio M, Rosa AC, Dianzani C, Brucato L, Benetti E, Collino M and Fantozzi R (2008) Celecoxib decreases expression of the adhesion molecules ICAM-1 and VCAM-1 in a colon cancer cell line (HT29). *Br J Pharmacol* **153**:870-878.
- Ghosheh B and Salameh JR (2007) Laparoscopic approach to acute small bowel obstruction: review of 1061 cases. *Surg Endosc* **21**:1945-1949.
- Goldmann T, Wieghofer P, Muller PF, Wolf Y, Varol D, Yona S, Brendecke SM, Kierdorf K, Staszewski O, Datta M, Luedde T, Heikenwalder M, Jung S and Prinz M (2013) A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nat Neurosci* **16**:1618-1626.
- Gonzalez-Guerrero C, Ocana-Salceda C, Berzal S, Carrasco S, Fernandez-Fernandez B,

JPET #212795

- Cannata-Ortiz P, Egido J, Ortiz A and Ramos AM (2013) Calcineurin inhibitors recruit protein kinases JAK2 and JNK, TLR signaling and the UPR to activate NF-kappaB-mediated inflammatory responses in kidney tubular cells. *Toxicol Appl Pharmacol* **272**:825-841.
- Gray PH and Lachance RA (1956) Assimilation of berberine by bacteria. *Nature* **177**:1182-1183.
- Guma M, Ronacher LM, Firestein GS, Karin M and Corr M (2011) JNK-1 deficiency limits macrophage-mediated antigen-induced arthritis. *Arthritis Rheum* **63**:1603-1612.
- Holmdahl L and Ivarsson ML (1999) The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. *Eur J Surg* **165**:1012-1019.
- Izumi Y, Yamamoto M, Kawamura M, Adachi T and Kobayashi K (2007) Cross-linked poly (gamma-glutamic acid) attenuates peritoneal adhesion in a rat model. *Surgery* **141**:678-681.
- Jeong HW, Hsu KC, Lee JW, Ham M, Huh JY, Shin HJ, Kim WS and Kim JB (2009) Berberine suppresses proinflammatory responses through AMPK activation in macrophages. *Am J Physiol Endocrinol Metab* **296**:E955-964.
- Jiang Q, Liu P, Wu X, Liu W, Shen X, Lan T, Xu S, Peng J, Xie X and Huang H (2011) Berberine attenuates lipopolysaccharide-induced extracellular matrix accumulation and inflammation in rat mesangial cells: involvement of NF-kappaB signaling pathway. *Mol Cell Endocrinol* **331**:34-40.
- Jung J, Ko SH, Yoo do Y, Lee JY, Kim YJ, Choi SM, Kang KK, Yoon HJ, Kim H, Youn J

JPET #212795

- and Kim JM (2012) 5,7-Dihydroxy-3,4,6-trimethoxyflavone inhibits intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 via the Akt and nuclear factor-kappaB-dependent pathway, leading to suppression of adhesion of monocytes and eosinophils to bronchial epithelial cells. *Immunology* **137**:98-113.
- Kim BH, Kim M, Yin CH, Jee JG, Sandoval C, Lee H, Bach EA, Hahm DH and Baeg GH (2011) Inhibition of the signalling kinase JAK3 alleviates inflammation in monoarthritic rats. *Br J Pharmacol* **164**:106-118.
- Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Li Z, Liu J and Jiang JD (2004) Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat Med* **10**:1344-1351.
- Kosaka H, Yoshimoto T, Fujimoto J and Nakanishi K (2008) Interferon-gamma is a therapeutic target molecule for prevention of postoperative adhesion formation. *Nat Med* **14**:437-441.
- Lauder CI, Garcea G, Strickland A and Maddern GJ (2010) Abdominal adhesion prevention: still a sticky subject? *Dig Surg* **27**:347-358.
- Ma FY, Tesch GH, Ozols E, Xie M, Schneider MD and Nikolic-Paterson DJ (2011) TGF-beta1-activated kinase-1 regulates inflammation and fibrosis in the obstructed kidney. *Am J Physiol Renal Physiol* **300**:F1410-1421.
- Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB and Beelen RH (2011) Macrophages in skin injury and repair. *Immunobiology* **216**:753-762.
- Meng S, Wang LS, Huang ZQ, Zhou Q, Sun YG, Cao JT, Li YG and Wang CQ (2012)

JPET #212795

Berberine ameliorates inflammation in patients with acute coronary syndrome following percutaneous coronary intervention. *Clin Exp Pharmacol Physiol* **39**:406-411.

Miller G, Boman J, Shrier I and Gordon PH (2000) Etiology of small bowel obstruction. *Am J Surg* **180**:33-36.

Mo C, Wang L, Zhang J, Numazawa S, Tang H, Tang X, Han X, Li J, Yang M, Wang Z, Wei D and Xiao H (2014) The Crosstalk Between Nrf2 and AMPK Signal Pathways Is Important for the Anti-Inflammatory Effect of Berberine in LPS-Stimulated Macrophages and Endotoxin-Shocked Mice. *Antioxid Redox Signal* **20**:574-588.

Morin C, Roumegous A, Carpentier G, Barbier-Chassefiere V, Garrigue-Antar L, Caredda S and Courty J (2012) Modulation of inflammation by Cicaderma ointment accelerates skin wound healing. *J Pharmacol Exp Ther* **343**:115-124.

Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* **8**:49-62.

Proud D and Leigh R (2011) Epithelial cells and airway diseases. *Immunol Rev* **242**:186-204.

Rothlein R, Dustin ML, Marlin SD and Springer TA (2011) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J Immunol*. 1986. 137: 1270-1274. *J Immunol* **186**:5034-5038.

Sahin M, Cakir M, Avsar FM, Tekin A, Kucukkartallar T and Akoz M (2007) The effects of anti-adhesion materials in preventing postoperative adhesion in abdominal

cavity (anti-adhesion materials for postoperative adhesions). *Inflammation* **30**:244-249.

Shan H, Zhang Y, Lu Y, Pan Z, Cai B, Wang N, Li X, Feng T, Hong Y and Yang B (2009) Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. *Cardiovasc Res* **83**:465-472.

Sigal A, Bleijs DA, Grabovsky V, van Vliet SJ, Dwir O, Figdor CG, van Kooyk Y and Alon R (2000) The LFA-1 integrin supports rolling adhesions on ICAM-1 under physiological shear flow in a permissive cellular environment. *J Immunol* **165**:442-452.

Takagi K, Araki M, Fukuoka H, Takeshita H, Hidaka S, Nanashima A, Sawai T, Nagayasu T, Hyon SH and Nakajima N (2013) Novel powdered anti-adhesion material: preventing postoperative intra-abdominal adhesions in a rat model. *Int J Med Sci* **10**:467-474.

Ten Broek RP, Kok-Krant N, Bakkum EA, Bleichrodt RP and van Goor H (2013a) Different surgical techniques to reduce post-operative adhesion formation: a systematic review and meta-analysis. *Hum Reprod Update* **19**:12-25.

ten Broek RP, van den Beukel BA and van Goor H (2013b) Comparison of operative notes with real-time observation of adhesiolysis-related complications during surgery. *Br J Surg* **100**:426-432.

Tingstedt B, Isaksson K, Andersson E and Andersson R (2007) Prevention of abdominal adhesions--present state and what's beyond the horizon? *Eur Surg Res* **39**:259-268.

- Townsend KL, Race A, Keane M, Miller W, Dishaw L, Fisher ER, Russell DS and Allen MJ (2011) A novel hydrogel-coated polyester mesh prevents postsurgical adhesions in a rat model. *J Surg Res* **167**:e117-124.
- Tsapanos VS, Stathopoulou LP, Papathanassopoulou VS and Tzingounis VA (2002) The role of Seprafilm bioresorbable membrane in the prevention and therapy of endometrial synechiae. *J Biomed Mater Res* **63**:10-14.
- van't RM, Vrijland WW, Lange JF, Hop WC, Jeekel J and Bonjer HJ (2002) Mesh repair of incisional hernia: comparison of laparoscopic and open repair. *Eur J Surg* **168**:684-689.
- van Goor H (2007) Consequences and complications of peritoneal adhesions. *Colorectal Dis* **9 Suppl 2**:25-34.
- Ward BC and Panitch A (2011) Abdominal adhesions: current and novel therapies. *J Surg Res* **165**:91-111.
- Werry EL, Liu GJ, Lovelace MD, Nagarajah R and Bennett MR (2012) Glutamate potentiates lipopolysaccharide-stimulated interleukin-10 release from neonatal rat spinal cord astrocytes. *Neuroscience* **207**:12-24.
- Wiseman DM, Trout JR, Franklin RR and Diamond MP (1999) Metaanalysis of the safety and efficacy of an adhesion barrier (Interceed TC7) in laparotomy. *J Reprod Med* **44**:325-331.
- Yadav VR, Sahoo K, Roberts PR and Awasthi V (2013) Pharmacologic suppression of inflammation by a diphenyldifluoroketone, EF24, in a rat model of fixed-volume hemorrhage improves survival. *J Pharmacol Exp Ther* **347**:346-356.

- Yao J, Kong W and Jiang J (2013) Learning from berberine: Treating chronic diseases through multiple targets. *Sci China Life Sci*.
- Yigitler C, Karakas DO, Kucukodaci Z, Cosar A, Gulec B and Akin ML (2012) Adhesion-preventing properties of 4% icodextrin and canola oil: a comparative experimental study. *Clinics (Sao Paulo)* **67**:1303-1308.
- You Z, Luo C, Zhang W, Chen Y, He J, Zhao Q, Zuo R and Wu Y (2011) Pro- and anti-inflammatory cytokines expression in rat's brain and spleen exposed to chronic mild stress: involvement in depression. *Behav Brain Res* **225**:135-141.
- Zhang Y, Sun L, Liang H, Li X, Cai R, Wang L, Du W, Zhang R, Li J, Wang Z, Ma N, Wang X, Du Z, Yang B, Gao X and Shan H (2013) Overexpression of microRNA-1 causes atrioventricular block in rodents. *Int J Biol Sci* **9**:455-462.
- Zhang Y, Zhang L, Chu W, Wang B, Zhang J, Zhao M, Li X, Li B, Lu Y, Yang B and Shan H (2010) Tanshinone IIA inhibits miR-1 expression through p38 MAPK signal pathway in post-infarction rat cardiomyocytes. *Cell Physiol Biochem* **26**:991-998.

Footnotes

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Figure Legends:

Fig. 1. Molecular structure of berberine.

Fig. 2. Histopathology assessment results of the 5 groups.

(A-C). The sham group; (D-F). The surgery group; (G-I).The berberine groups; (J-K). The Interceed group, n=3. “→” present vascular proliferation and congestion. “*” present giant cells with increased numbers of admixed lymphocytes, plasma cells, eosinophils and neutrophils and even numerous admixed inflammatory cells with microabscesses. “□” present the connective tissue and fibrosis is florid and dense. Scale bars: 10µm.

Fig. 3. Fibrosis and collagen deposition of the 5 groups.

(A-D). Masson trichrome stain (×400) indicating collagen production and deposition (blue) is located mainly in the mesenchymal, mucosa layer, submucosal areas, and muscularis propria in the sham, surgery, berberine and Interceed groups, n=3. (E). MRNA level of Col-1 in cecum in the 5 groups: sham group; surgery group; Berberine 1 (1.5mg/ml) group; Berberine 2 (0.75mg/ml) group and the Interceed group, n=8. (F). mRNA level of Col-3 in cecum in 5 groups. (G). Collagen content result indicating collagen deposition of the cecum tissues in 5 groups. *p, #p < 0.05 ; **p, ##p < 0.01; mean±SEM; *p: sham vs surgery; #p: surgery vs berberine.

Fig. 4. Results of the expression of ICAM-1 in serum and in mRNA level.

(A) Concentration of ICAM-1 in 5 groups using enzyme-linked immunosorbent assay;
(B) Real-time-PCR results showing ICAM-1 mRNA relative level in the 5 groups, sham group; surgery group; Berberine-1 (1.5mg/ml group); Berberine-2 (0.75mg/ml) group and the Intreceed group, n=5. *p, #p < 0.05 ; **p, ##p < 0.01; mean±SEM; *p: sham

vs surgery; #p: surgery vs berberine. (C-F). Immunohistochemistry results of ICAM-1.

Sham, surgery, berberine and Interceed group, respectively.

Fig.5. Effects of BBR on inflammatory cytokines secretion in serum.

(A) Concentration of IL-1 β in serum in the 5 groups. n=7; (B) Concentration of TGF- β in serum; (C) Concentration of TNF- α in serum; (D) Concentration of IL-6 in serum *p, #p < 0.05, **p, ##p < 0.01; mean \pm SEM. *p: sham vs surgery. #p: surgery vs berberine.

Fig. 6. MRNA expression of inflammatory cytokine genes in cecum.

(A) IL-1 β mRNA relative level in 5 groups. n=7; (B) TGF- β mRNA relative level; (C) TNF- α mRNA relative level; (D) IL-6 mRNA relative level. *p, #p < 0.05 **p, ##p < 0.01; mean \pm SEM. *p: sham vs surgery. #p: surgery vs berberine.

Fig. 7. Results of western blot on the TAK1/JNK and TAK1/NF- κ B pathway.

Activity of the TAK1/JNK and TAK1/NF- κ B signaling was performed by the western blot in the 5 groups, n=3. (A) Representative western blot bands; Relative protein expression of p-TAK1 (B), TAK1 (C), p-p65 NF- κ B (D), p65 NF- κ B (E), p-JNK (F) and JNK (G). *p, #p < 0.05, **p, ##p < 0.01; mean \pm SEM. *p: sham vs surgery. #p: surgery vs berberine.

Fig. 8. Schematic mechanisms of the essential role that BBR may play on the adhesion formation and inflammation response after abrasive abdominal operation.

Berberine hydrochloride is an effective strategy to prevent adhesion by downregulating ICAM-1 and reduce inflammation action by inhibiting the TAK1/JNK and TAK1/NF- κ B signaling after abdominal surgery.

Tables

TABLE 1.

Distribution of adhesion scores indicating the adhesion severity and adhesion area in the 5 different groups: con, surgery, Berberine-1(1.5mg/ml), Berberine-2 (0.75mg/ml) and Interceed group.

The adhesion on the 14 days post-operation were remarkably alleviated with berberine treatment at concentrations of 1.5mg/ml and 0.75mg/ml and Interceed group compared with the surgery group. Comparisons between different groups were applied using the Kruskal-Wallis H and the Mann-Whitney U test by the SPSS 19.0. **p, ##p < 0.01; *p: sham vs surgery. #p: surgery vs berberine and Interceed groups.

groups	Adhesion score					
	0	1	2	3	4	5
1 sham	10	0	0	0	0	0
2 Abrasion surgery**	0	1	2	0	3	4
3 Berberine-1##	5	2	2	1	0	0
4 Berberine-2##	4	2	1	2	1	0
5 Interceed ##	4	2	2	1	1	0

Figure 1

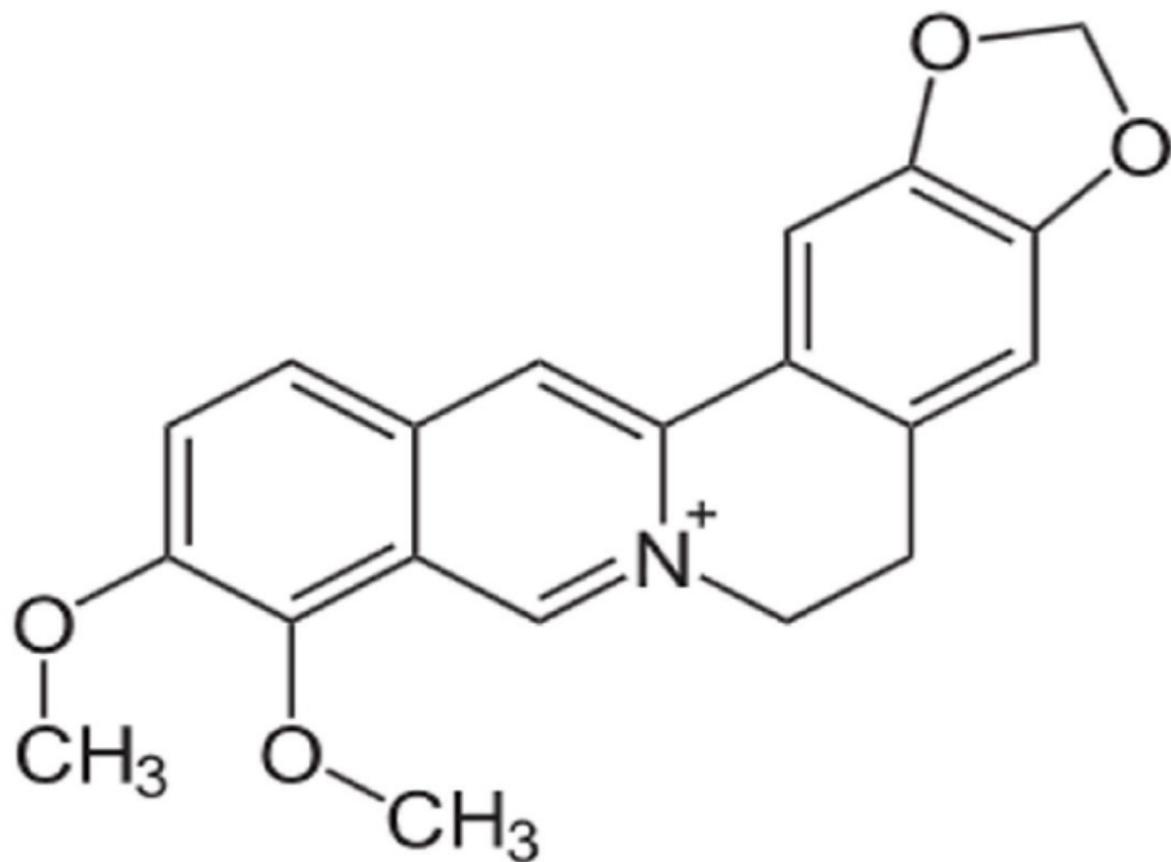


Figure 2

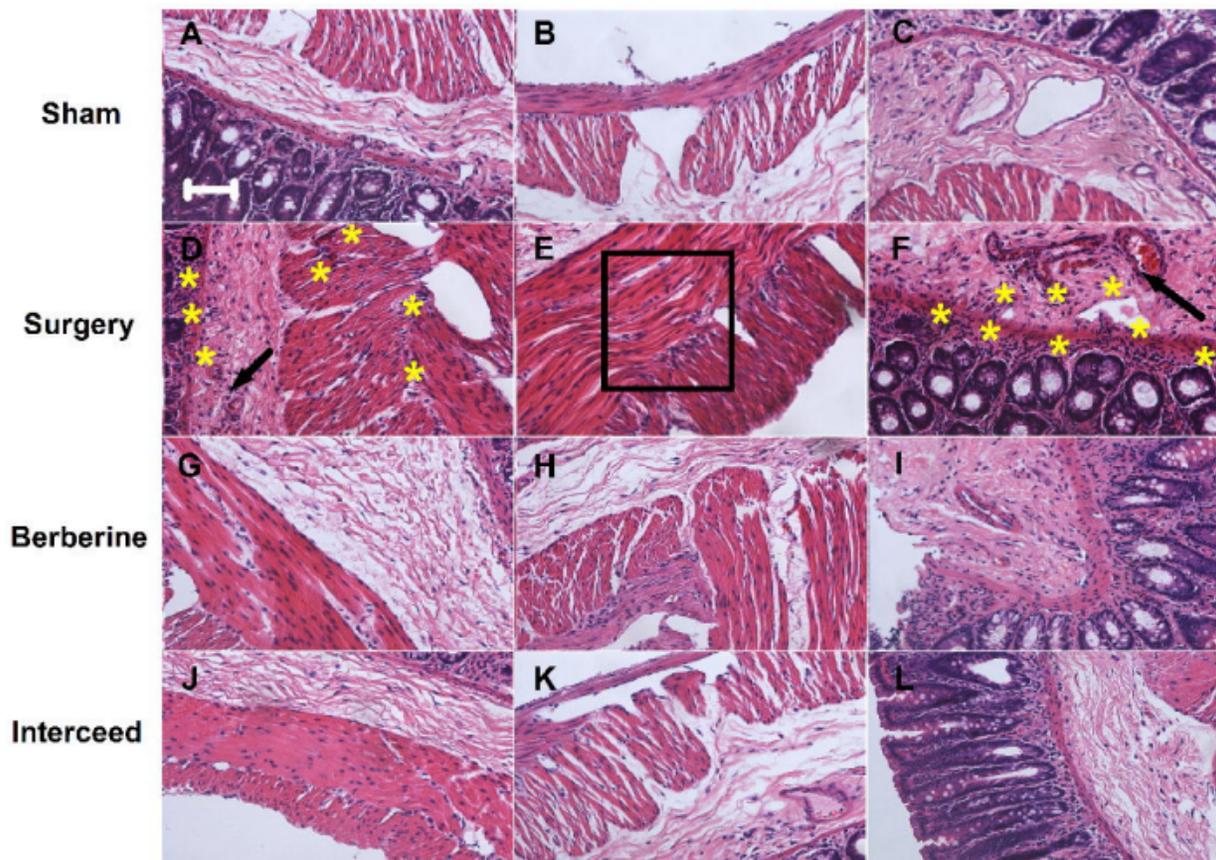


Figure 3

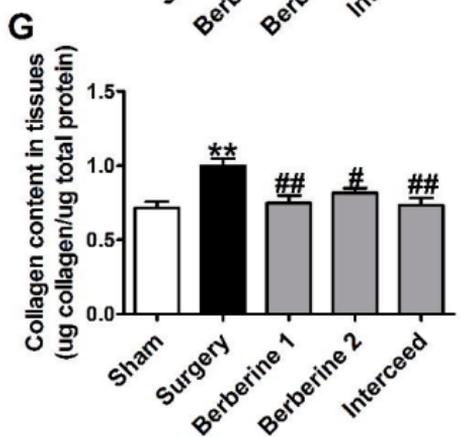
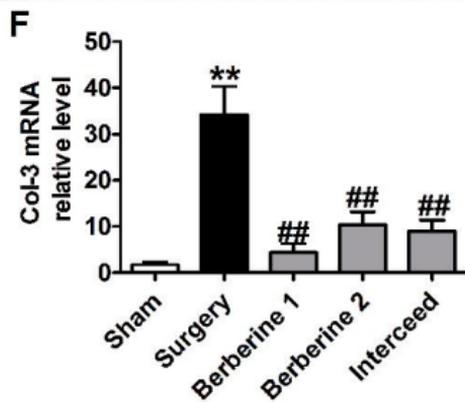
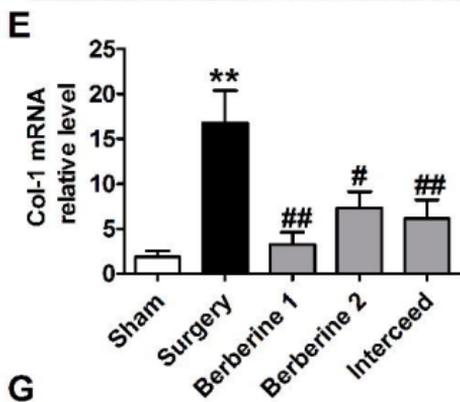
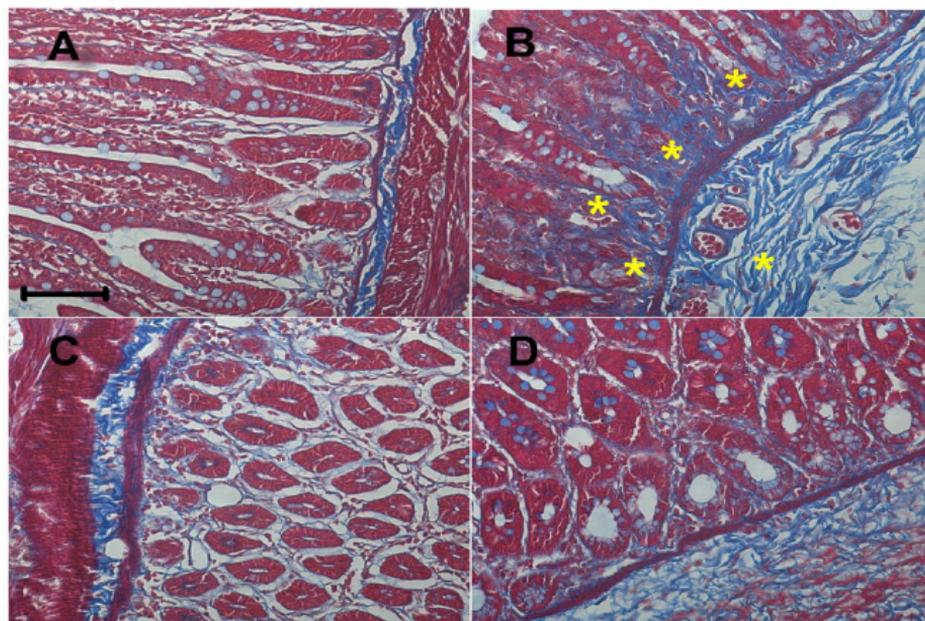


Figure 4

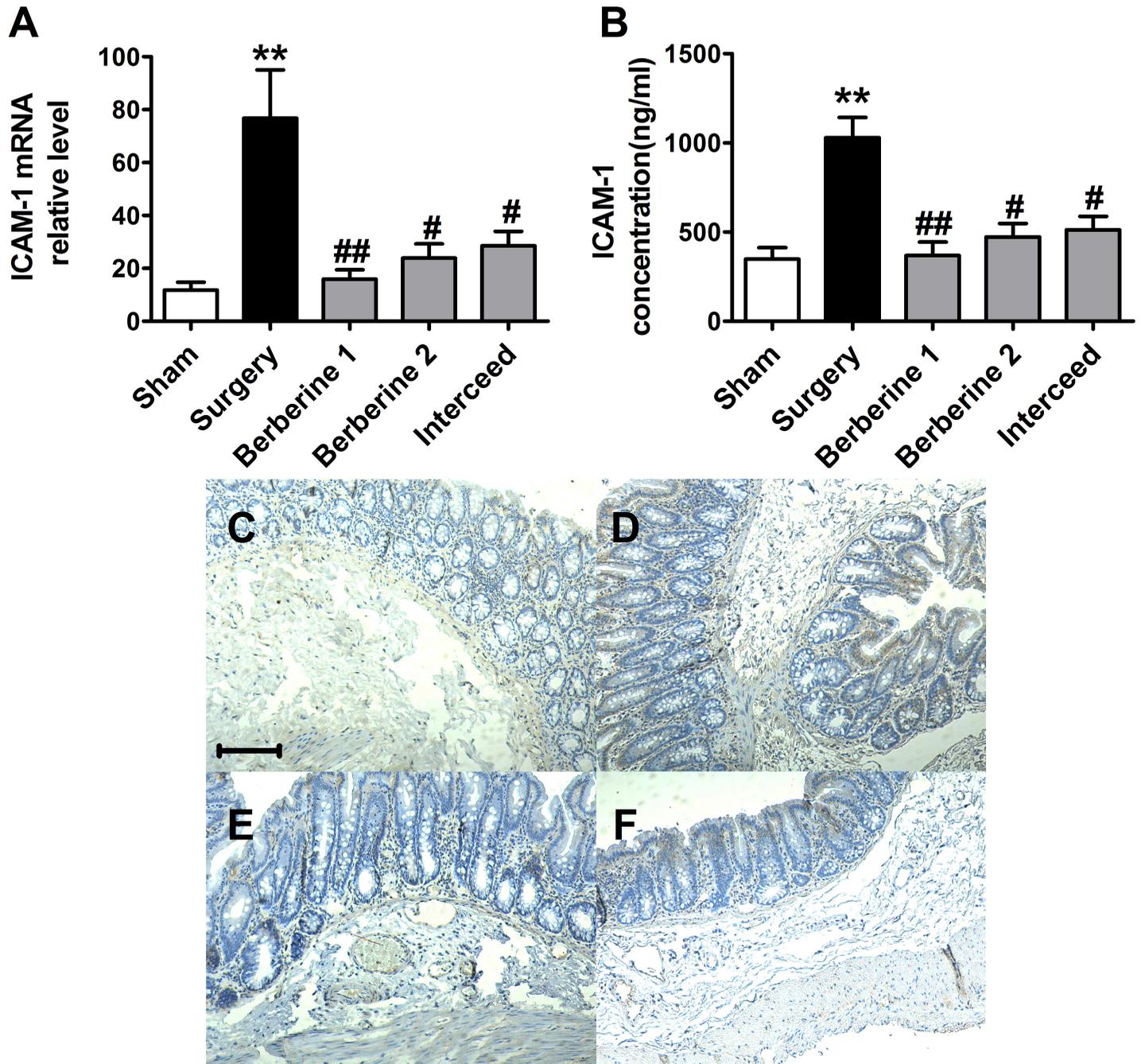


Figure 5

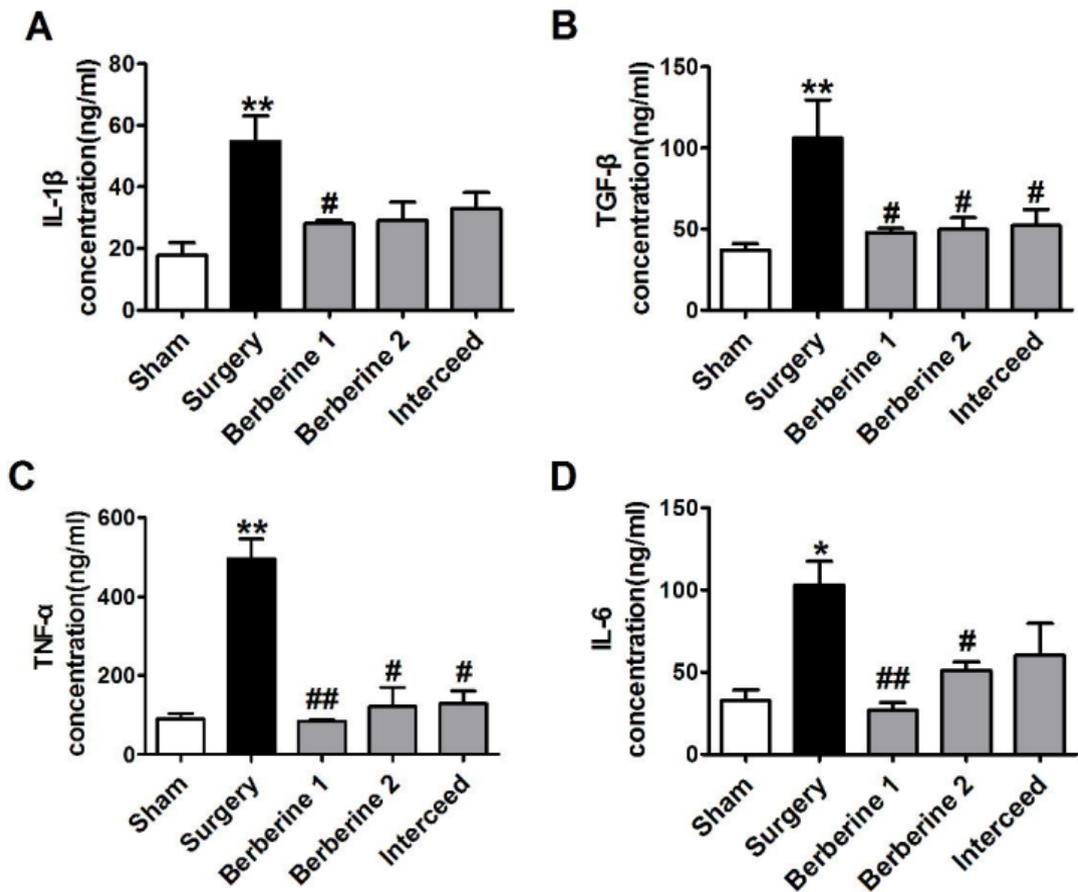
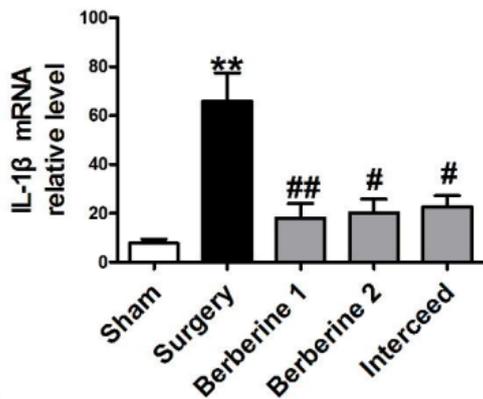
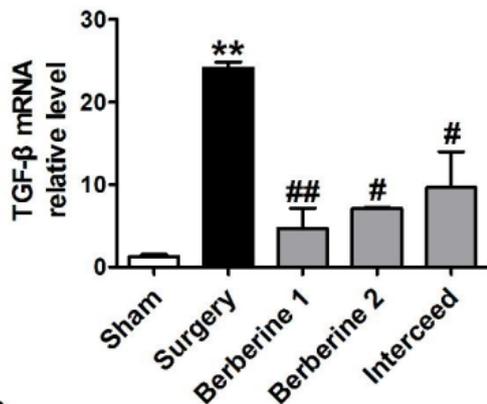


Figure 6

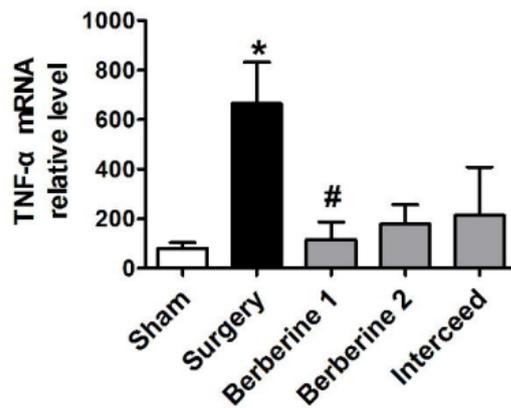
A



B



C



D

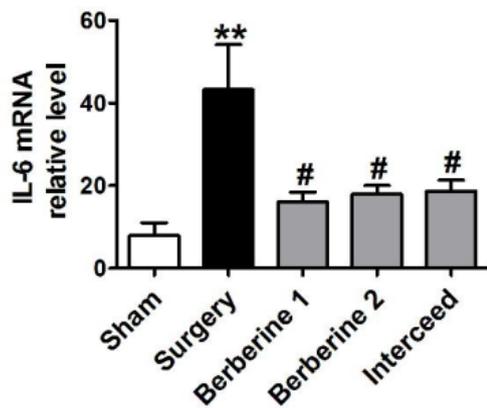
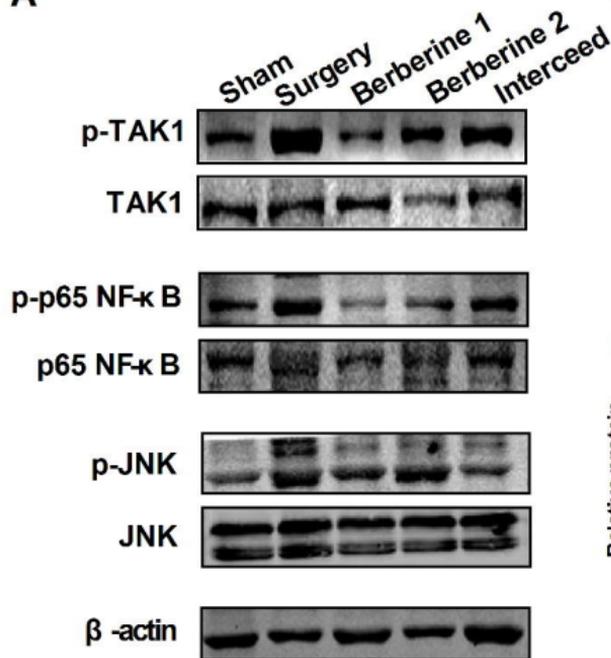
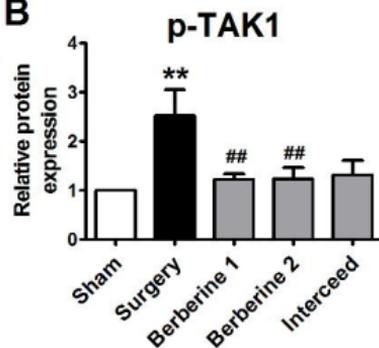


Figure 7

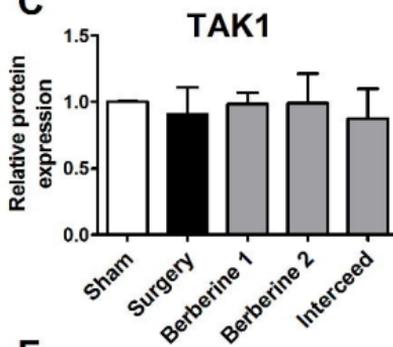
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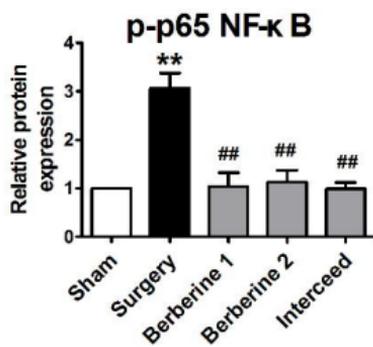
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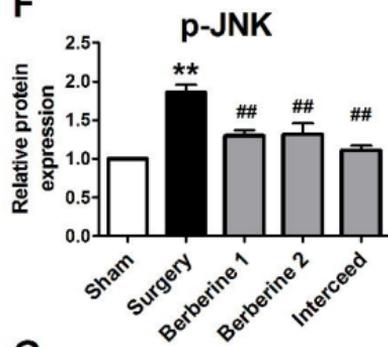
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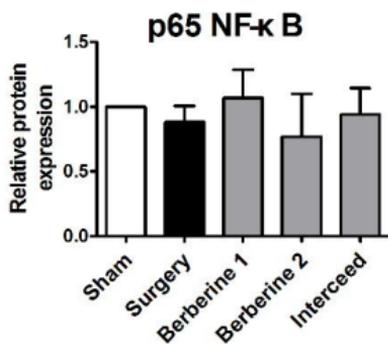
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F



E



G

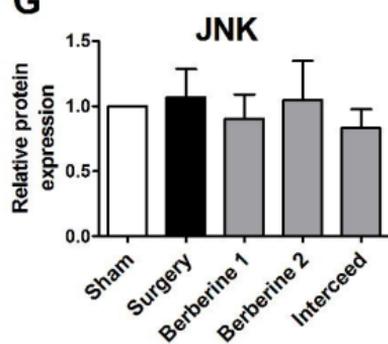
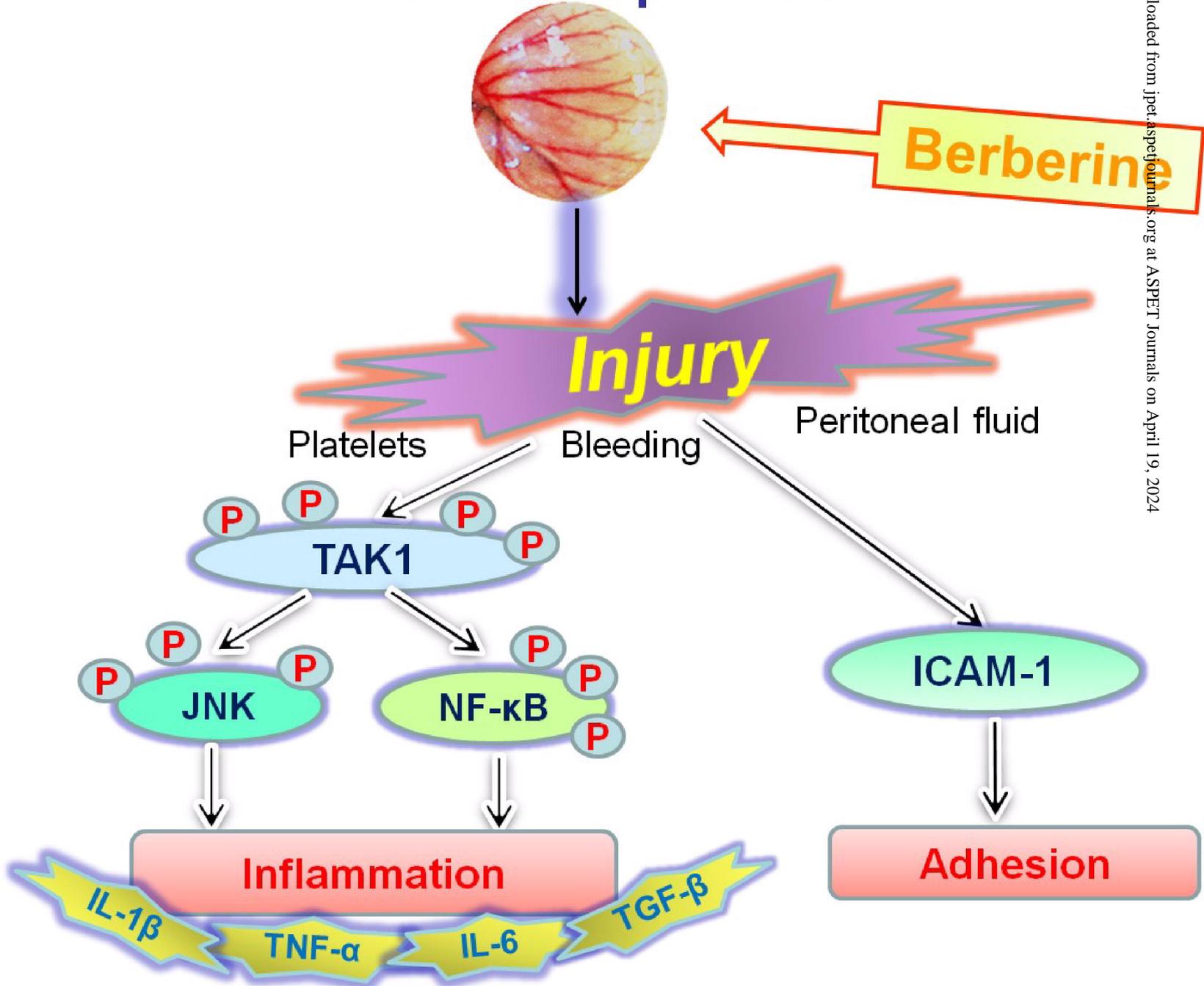


Figure 8

Abdominal Operation



Supplemental Data

Berberine Hydrochloride Prevents Post-Surgery Intestinal Adhesion and Inflammation in Rats

Yong Zhang, Xiaoguang Li, Qingwei Zhang, Jiamin Li, Jiaming Ju, Ning Du, Xin Liu, Xiaohui Chen, Feiran Cheng, Lei Yang, Chaoqian Xu, Muhammad U. Bilal, Yunwei Wei, Yanjie Lu, Baofeng Yang

Supplementary methods

HT29 cell culture and transfection. Intestinal HT29 Cells were seeded in DMEM containing 10% fetal bovine serum (FBS) and used for all experiments. Cells were transfected with optimem medium (invitrogen) and lipo2000 (invitrogen). Cells was divided into four groups. Negative control group was transfected with ICAM-1 siRNA negative control synthesized by RIbo Life Science Co.,Ltd, berberine high dose group: berberine 1 (100uM), berberine low dose group: berberine 2 (20uM), and siRNA group which was transfected with ICAM-1 siRNA synthesized by RIbo Life Science Co.,Ltd, cultured in 37 °C for 24 hours.

HT29 cell adhesion assay. The cells were incubated with 50uM fibronectin (BD™ Fibronectin, human) diluted in PBS for 1h and then blocked with BSA at 37°C for 2 h. The cell adhesion and viability was detected by measuring the absorbance of each well at 490 nm.

Table S1. The scoring system used for the macroscopic evaluation of the adhesion formation

Grade	Description
0	no adhesions
1	loose filmy adhesions that could be separated by blunt dissection
2	adhesions requiring <50% sharp dissection for separation
3	adhesions requiring >50% sharp dissection for separation
4	serosal injury
5	full-thickness injury

Table S2. Nucleotide sequence of the forward and reverse primers for the Real-Time PCR.

Gene	Primer Sequence	GenBank No.
rat-IL-1 β	Forward CCCTGCAGCTGGAGAGTGTGG	NM 031512.2
rat-IL-1 β	Reversed TGTGCTCTGCTTGAGAGGTGCT	
rat-IL-6	Forward TTCCTACCCCAACTTCCAATG	NM 012589.1
rat-IL-6	Reversed ATGAGTTGGATGGTCTTGGTC	
rat-TNF- α	Forward GACCCTCACACTCAGATCATCTTCT	NM 012675.3
rat-TNF- α	Reversed TGCTACGACGTGGGCTACG	
rat-TGF- β	Forward GCGAGCGAAGCGACGAGGAG	NM 031131.1
rat-TGF- β	Reversed TGGGCGGGATGGCATCAAGGTA	
rat-ICAM-1	Forward TCATGCCCGTGAAATTATGGT	NM_012967.1
rat-ICAM-1	Reversed AGGACCCTTCTAAGTGGTTGGAA	
rat-GAPDH	Forward AAGAAGGTGGTGAAGCAGGC	NM 017008.3
rat-GAPDH	Reversed TCCACCACCCAGTTGCTGTA	
rat-Col-1	Forward CAATGGCACGGCTGTGTGCG	NM_053304.1

Reversed CACTCGCCCTCCCGTCTTTGG

rat-Col-3 Forward TGAATGGTGGTTTTTCAGTTCAG NM_032085.1

Reversed GATCCCATCAGCTTCAGAGACT

Supplemental Figure 1

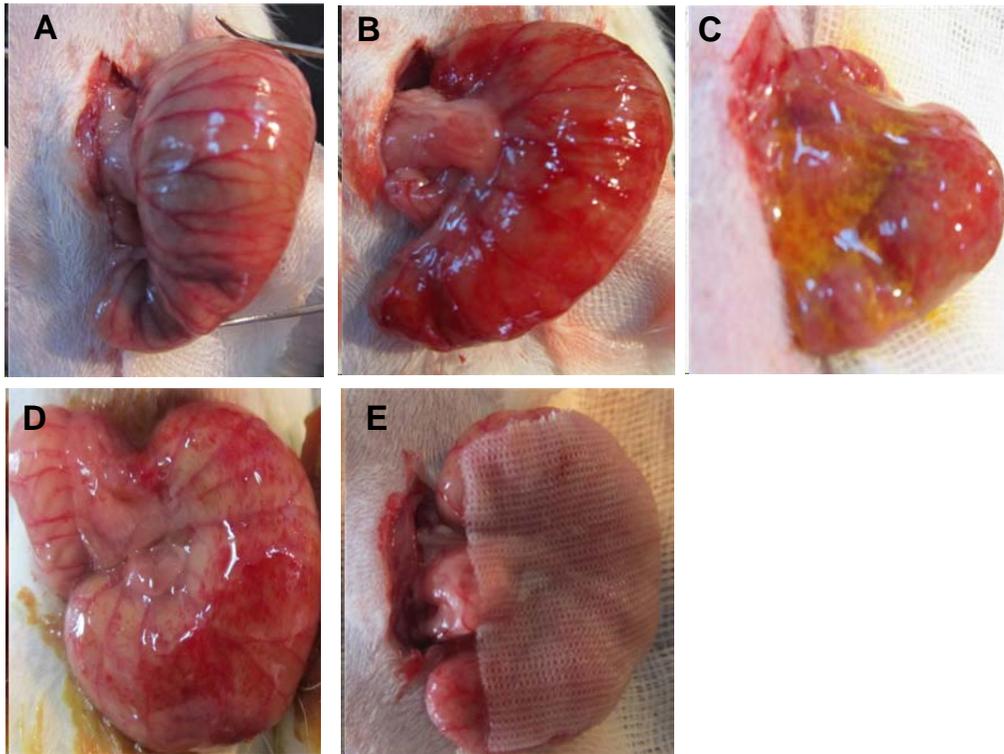


Fig. S1. Surgical Procedure: (A) Sham: approximately 2.0 ml of normal saline was dropped into the abdominal cavity without the surgery. (B) Approximately 2.0 ml of normal saline was dropped into the abdominal cavity immediately after the surgery of abrasion. (C) Berberine hydrochloride in normal saline (1.5mg/ml) dropped into the abdominal cavity immediately after the surgery of abrasion. (D) Berberine hydrochloride in normal saline (0.75mg/ml) dropped into the abdominal cavity immediately after the surgery of abrasion. (E) a 3.0×2.0-cm piece of Interceed was attached to the abdominal wall defect of abrasion

Supplemental Figure 2

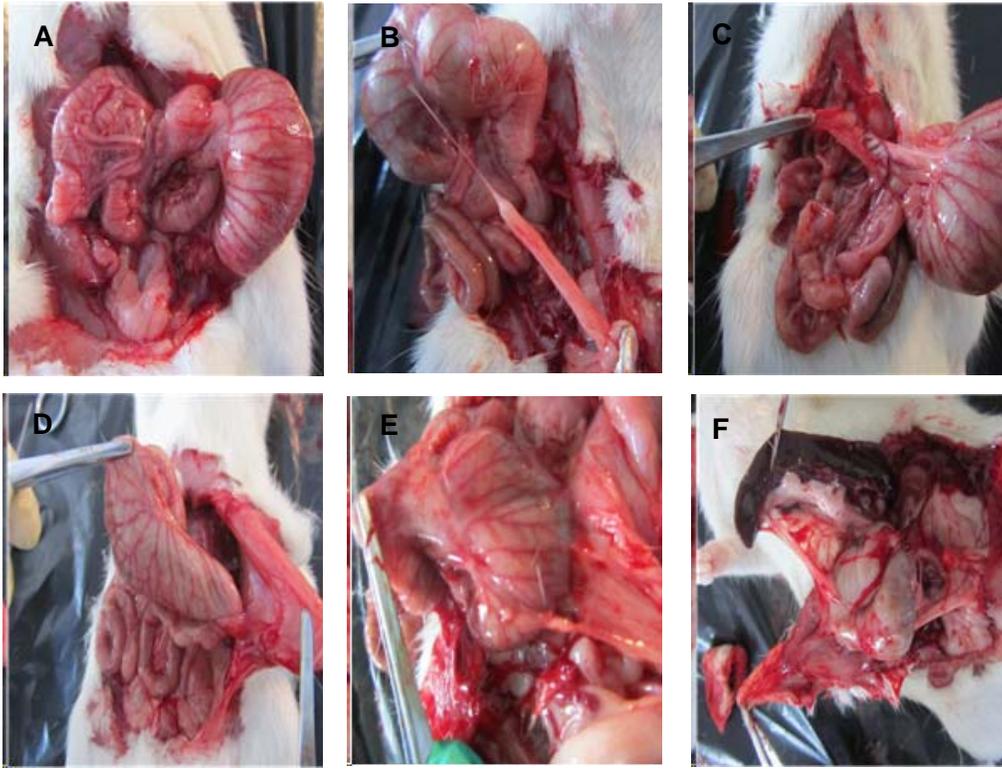


Fig. S2. Photographic images at post mortem evaluation of adhesions on 14 days postoperation. (A). Adhesion score 0 in a sham model. (B). Adhesion score 1 in a berberine-1 (1.5mg/ml) model. (C). Adhesion score 2 in a berberine-2 (0.75mg/ml) model. (D). Adhesion score 3 in an abrasion surgery model. (E). Adhesion score 4 in an abrasion surgery model. (F). Adhesion score 5 in an abrasion surgery model

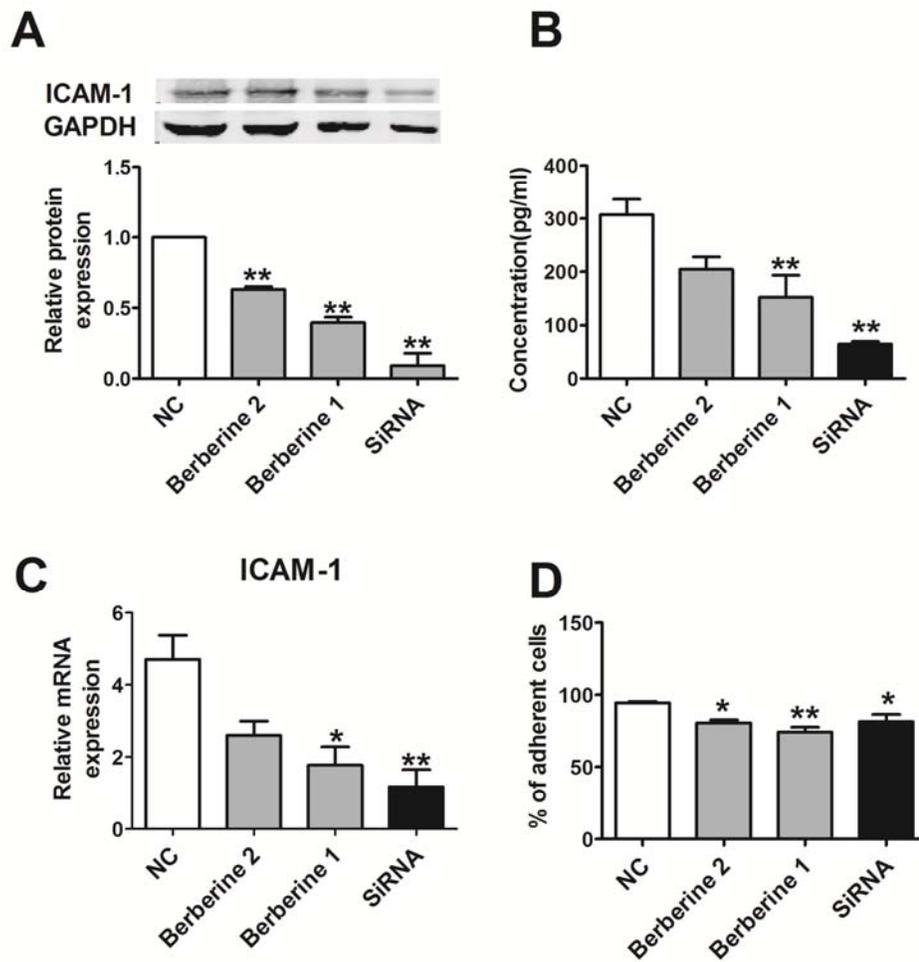


Fig.S3. Expression of ICAM-1 in HT29 cell line and results of cell adhesion assay.

(A). Relative protein expression of ICAM-1 in HT29 cell line in NC, berberine 1, berberine 2 and siRNA groups. (B). Concentration of ICAM-1 in cell supernatant in the 4 groups. (C). Relative mRNA expression in HT29 cell line. (D). Cell adhesion assay. n=3. *p, #p<0.05, **p, ##p<0.01; mean±SEM. *p: sham vs surgery. #p: surgery vs Berberine.